Trends in Microbiology

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Review

Ménage à trois: Virus, Host, and Microbiota in Experimental Infection Models†

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Infections of mammals with pathogenic viruses occur mostly in the polymicrobial environment of mucosal surfaces or the skin. In recent years our understanding of immune modulation by the commensal microbiota has increased dramatically. The microbiota is today accepted as the prime educator and maintainer of innate and adaptive immune functions. It became further apparent that some viral pathogens profit from the presence of commensal bacteria and their metabolites, especially in the intestinal tract. We further learned that the composition and abundance of the microbiota can change as a consequence of acute and chronic viral infections. Here we discuss recent developments in our understanding of the triangular relationship of virus, host, and microbiota under experimental infection settings.

Highlights

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The microbiota is today accepted as the prime educator and maintainer of innate and adaptive immune functions.

Some viral pathogens profit from the presence of commensal bacteria and their metabolites, especially in the intestinal tract.

The composition and abundance of the microbiota can change as a consequence of acute and chronic viral infections.

Virus Infections in a Polymicrobial Host Environment

Conservative estimates place the number of commensal bacterial cells colonizing the human body equal or slightly higher than the number of human cells [1]. In recent years it has been appreciated how deeply the coexistence of the commensal eubacterial, archaebacterial, viral, and fungal microbiota with the human host affects numerous physiological and pathological processes [2]. The implementation of culture-independent, 16S rRNA gene-targeted next-generation sequencing (NGS) techniques and, more recently, culturomics [3], combined with state-of-the-art bioinformatics analysis, has paved the way for our current understanding of commensal eubacterial communities in different body sites and under various physiological and pathological conditions [4]. In human patients, an imbalanced microbiota (a state commonly termed 'dysbiosis') was correlated with obesity [5,6], diabetes [7], chronic inflammatory disease [8–10], chronic infections [11], and neurodegenerative disease [12]. Animal models established causal relations between changes in the composition of the microbiota and pathological phenotypes for some of these conditions [13–16]. A major obstacle in our understanding of microbiota-driven processes in human patients is the high interindividual variation of the microbiota in humans [17], largely based on environmental factors [18]. At birth, mammals leave the quasi-sterile environment of the uterus and are instantly colonized by a variety of microbes. Colonization and establishment of a robust adult-like gut microbiota requires about 3–4 years in humans [19–21] and around 3–8 weeks in mice [22]. Disturbance of colonization in this critical phase, for example by extensive antibiotic therapy, permanently shapes the composition of the microbiota, potentially with effects lasting into adulthood [14,23].

Less is known about the interplay between the host microbiota and microbial pathogens, and the consequences of infection on the composition of the microbiota. Recently a number of studies correlated the composition of human patient bacterial microbiota with altered outcomes in virus infections or susceptibility to viral infections [24–26]. For ethical and technical reasons, human microbiome studies focus largely on easily accessible sampling sites, such as skin, the nasal or oral cavity (more rarely bronchoalveolar lavage, BAL), genital tract or

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fecal samples. In most cases, when investigating changes in microbiota in the context of infectious disease, causality is difficult to establish in studies on human subjects. It is consequently equally challenging to demonstrate, with certainty, whether microbiota with a given composition alters susceptibility to viral pathogens. Here we shed light on the complex relationship between host immunity, bacterial microbiota, and viral pathogens (summarized in Figure 1).

In this review we focus on three questions relating to the intricate triangular relationship of virus, host immune system, and bacterial microbiota within the human body. This includes how commensal bacteria directly affect viral pathogenesis. We also examine how commensal bacteria shape the innate and adaptive host responses to viral infection. Finally, we discuss how viral infections shape the composition of commensal bacteria in the microbiota, and the consequences of this process for the host. In order to establish causal relations, this review is largely restricted to literature based on experimental infection settings, either in humans or in mammalian model systems.

Commensal Bacteria Impact Viral Pathogenicity

Commensal bacteria colonize the skin and every mucosal surface of the body; this is largely tolerated by the host immune system. At the same time these surfaces are major entry ports for microbial pathogens. Hosts with low or no bacterial colonization are prone to infection with pathogenic bacteria, presumably due to inefficient occupation of ecological niches and an intrinsic immune deficiency as compared with fully colonized hosts (see also the section entitled 'The Commensal Microbiota Shapes the Innate and Adaptive Host Response to Viral Infections Locally and Systemically') [27,28].

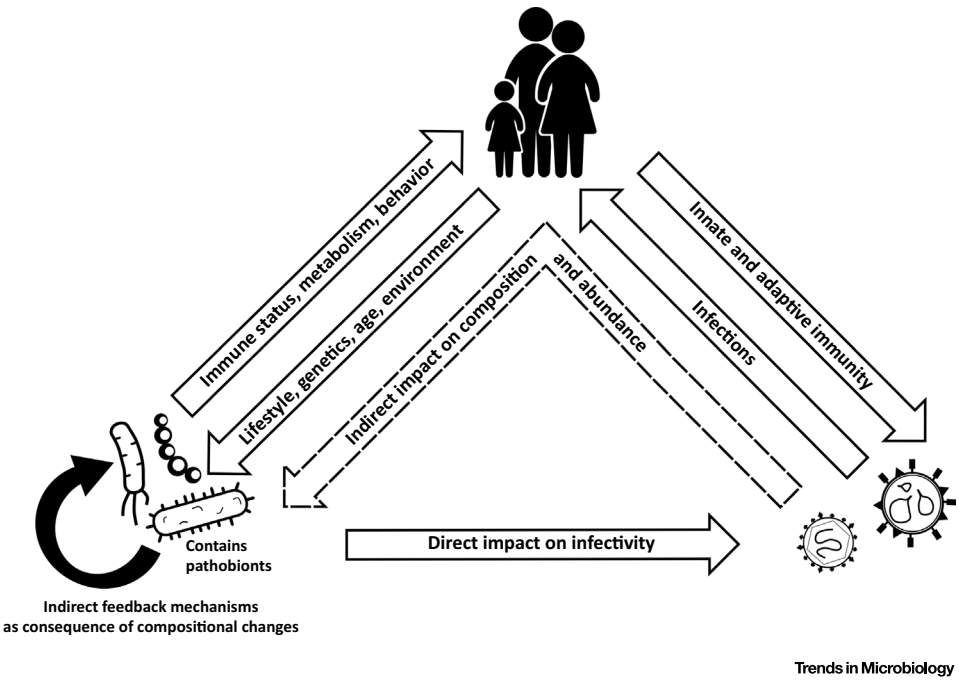

Figure 1. Triangular Relationship of Virus (Bottom Right), Host (Top), and Bacterial Microbiota (Bottom Left). Arrows indicate the effect(s) of each of these components on another component, or on itself.

Enteric viruses, which adapted to the bacteria-rich environment of the intestine, take advantage of the presence of bacterial products in their replication cycle. A landmark paper in 2011 by the team of Julie Pfeiffer [29] elegantly demonstrated the importance of bacterial cell wall components in the replication of poliovirus. The lipopolysaccharides (LPS) of Gram-negative bacteria and the peptidoglycans of Gram-positive bacteria enhanced the thermostability and host cell attachment of poliovirus particles, albeit to a different extent; LPS was the more potent enhancer. Consequently, antibiotic-treated (ABX) mice (with a 6 $\log_{10}$-fold reduced bacterial load in the intestine) show a twofold reduced mortality after polio challenge. In these animals, virus replication was delayed, and peak titers were lower when compared with untreated animals. The same group demonstrated that direct binding of LPS depends on residue 99 of the capsid protein [30]. Similarly, 5- to 10-fold decreased viral titers were observed in ABX mice infected with reovirus, when compared with control animals. The mechanism for this decrease in replication is currently unknown [29]. In 2014 Jones and colleagues [31] showed microbiota-dependent enhanced infection of B cells by human norovirus (NoV). They found 600-fold increased NoV genome copy numbers in a B cell/intestinal epithelial cell coculture system incubated with human stool samples as compared with cocultures incubated with filtered stool samples. Mechanistically, it was proposed that the norovirus capsid binds to histone-blood group antigens (HBGA)-like proteins expressed on enteric bacteria [31]. In accordance, ABX mice displayed reduced viral titers after NoV challenge. Addition of VP1 capsid-specific antibody ablated the increased infectivity, indicating that direct binding of capsid to bacteria is required. Baldridge and colleagues showed that interferon lambda (IFNλ) signaling is required for the ABX treatment effect [32,33]. However, the exact mechanism of action of IFNλ in this context remains elusive. A more indirect immune evasion mechanism was proposed for mouse mammary tumor virus (MMTV) infection [34]. MMTV is a murine retrovirus which is vertically transmitted via mother's milk to the offspring. Maternal MMTV was shown to be loaded with LPS in the stomach of suckling pups. The authors demonstrated that sufficient transmission of MMTV required Toll-like receptor 4 (TLR4)-dependent induction of IL-6, which, in turn, upregulates the immune suppressive cytokine IL-10. This induction cascade required the presence of commensal bacteria, independent of their composition [34]. In a follow-up study the same group showed incorporation of LPS receptors into the viral envelope, which again supports viral transmission. The authors propose a requirement for MMTV to concentrate LPS in order to stimulate TLR4 since the majority of mouse and human gut Gram-negative bacteria provide only weakly immune stimulatory LPS variants [35].

Viruses replicating in the intestinal tract do not always benefit from the presence of bacteria. A direct antiviral effect of probiotic bacteria was proposed for rotavirus, a major cause of pediatric enteric infections. A very recent study found 50% decreased host cell binding of rotavirus in the presence of *Escherichia coli* Nissle [36]. The authors then used a gnotobiotic piglet model to show reduced virus shedding in animals colonized with *E. coli* when compared with control animals. It needs to be addressed whether the *in vivo* protection also depends on reduced target cell binding as implicated. Nevertheless, certain members of the gut microbiota might actually be beneficial for the host to directly prevent enteric viral infections. However, in human patients, corroborating surveillance data showing a negative correlation between *E. coli* colonization and increased resistance to rotaviruses are so far missing.

Currently only enteric viruses were shown to directly profit from commensal bacteria or their metabolites. Presumably, the high bacterial burden of their natural habitat provides sufficient selective advantages for these viruses to adapt to their bacterial 'neighbors'. However, it cannot be excluded that similar mechanisms apply for other bacteria-rich environments, such as the oral or nasal mucosa.

The Commensal Microbiota Shape the Innate and Adaptive Host Response to Viral Infections Locally and Systemically

Initial colonization of mammals occurs during birth and continues through early childhood. At the age of 3 years the composition of microbiota of healthy children is similar to that in adults, while in the elderly the complexity of the microbiota decreases [20,21]. Strikingly, both young and elderly are considered less immune-competent and thus more susceptible to viral infections, potentially in part due to the reduced microbial colonization. There are ongoing efforts in the microbiota research community to identify common denominators or a 'core microbiome' among healthy individuals or patients with similar pathologies [37]. However, the high interindividual variation, even among healthy subjects, suggests that there is more than one healthy combination of commensal bacteria that supports equivalent physiological functions. Since only about 50% of healthy individuals carry these 'common' denominators [37], the concept of 'enterotypes' was introduced for the gut microbiota [38]. Enterotypes take into account bacterial abundance, but also the molecular functions of individual bacterial species and their composition. This concept was later expanded to other body niches, such as the lung [39].

Without doubt, mammals need commensal bacteria to fight viral pathogens. Since the 1960s, several lines of experimental evidence support that colonization density and complexity impacts sensitivity to viral infections. Figure 2 summarizes the relationship of microbiota complexity

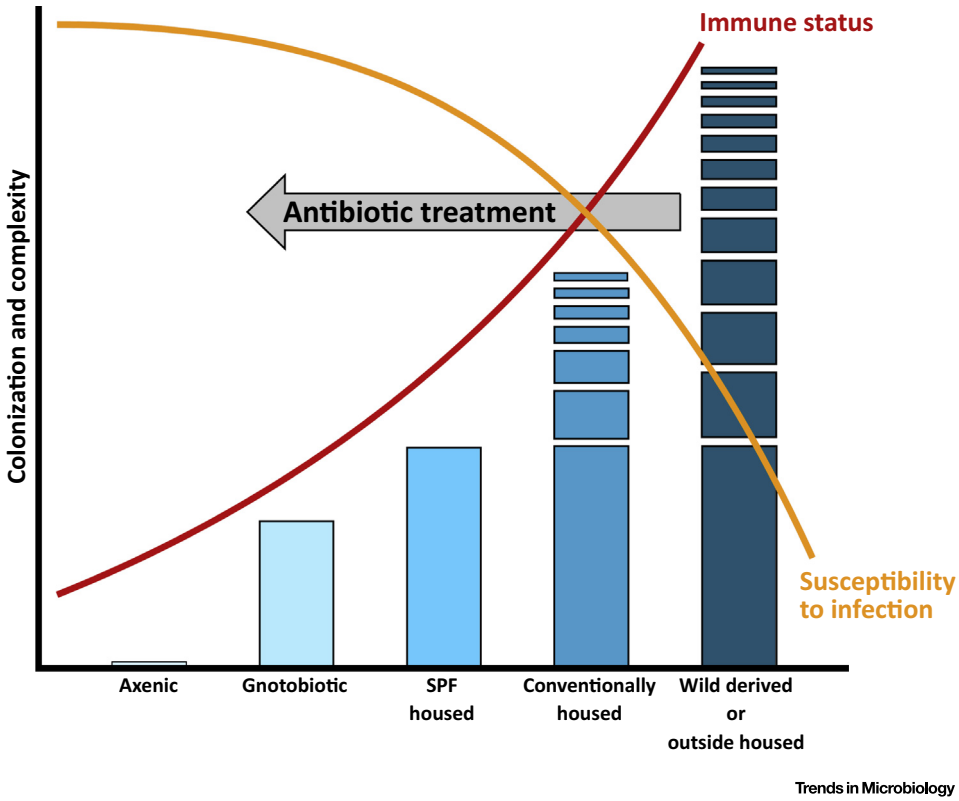

Figure 2. The Density/Complexity of the Microbiota Dictates Host Immune Status and Susceptibility to Infection. Columns represent colonization density and complexity under the housing conditions indicated. The interrupted parts represent higher variability in the composition of the microbiota in conventional and outside-housed mouse models. The horizontal arrow represents the effect of antibiotic treatment on either of the colonized mouse models. SPF, specific-pathogen-free.

(columns, interrupted columns indicate higher variability) with immune status of the host (red line) and susceptibility to infectious disease as well as severity of clinical signs during viral infection (yellow line). As an example, germ-free animals display more severe symptoms after influenza A virus (IAV) infection [40], while Wang and colleagues showed reduced sensitivity to IAV infection of conventionally housed mice when compared with specific-pathogen-free (SPF) mice [41]. Along the same lines, outside-housed SPF mice display enhanced vaccine responses to IAV when compared with indoor-housed SPF mice [42]. Complex microbiota, derived from wild mice, protect the host from the detrimental effects of IAV infection as well as from inflammatory tissue damage [43]. Similar resistance of 'wild colonized' mice was previously shown to infection by bacteria and parasites [44].

The use of mouse models colonized with 'wild-derived' microbiota, or outside-housed mice, was suggested to better reflect the natural human exposure to both nonpathogenic and pathogenic bacteria when compared with the reduced complexity altered Schaedler Flora based SPF models [43,44]. However, it should be taken into consideration, when using more complex colonized mouse models, that variability between animals could increase and facility-to-facility variations will become more problematic. This would make mechanistic approaches certainly more challenging.

Besides different levels of colonization, a growing number of studies used antibiotic treatment to address the effect of reduced colonization on susceptibility to viral infections (indicated by an arrow in Figure 2). Thackray and colleagues found enhanced sensitivity to infection with different flaviviruses in ABX mice [45]. They show reduced immune responses after oral ABX treatment against West Nile Virus, dengue Virus, and Zika Virus. Abt and colleagues showed decreased immune control of lymphocytic choriomeningitis virus (LCMV) in ABX mice [46]. Similarly, quantitative depletion of microbiota with an ABX cocktail renders initially colonized adult mice more vulnerable to IAV infection than control animals [47]. It should be taken into consideration that ABX treatment will affect the density of the microbiota systemically and does not allow conclusions to be reached on the local role of lung or intestinal microbiota. The same applies to models with different colonization status. Mechanistically, reduced viral pathology in colonized mice is based on an elevated cytotoxic T lymphocyte (CTL) response, which is primed by bacterial pathogen-associated molecular patterns (PAMPs) in a TLR-dependent fashion and requires production of IL-1β through NLRP3 inflammasomes [47]. Interestingly, exogenous application of different PAMPs complemented for the absence of commensal bacteria (Table 1). IL-1β is required for migration of lung dendritic cells to the draining lymph nodes. In line with these findings, induction of IL-33 (another NLRP3-dependent proinflammatory cytokine) by dysbiosis impairs antiviral responses in the vaginal mucosa [48]. Anti-HIV effects of cultivated vaginal microbiota were demonstrated in cell culture systems [26], further implicating the activity of commensal bacteria in an immune-stimulatory way on multiple mucosal surfaces besides the intestine. Changes in the gut microbiota were also shown to impact the inflammatory status of the liver, presumably by bacterial metabolites such as LPS transported in the blood (reviewed in [49]). These findings place inflammasome-dependent recognition of imbalanced or reduced gut microbiota at the center of systemic mucosal immunity against viral pathogens.

An important question remains. How are commensal bacteria sensed? Local priming of antigen-presenting cells by commensal bacteria was shown in the context of respiratory infection. The alveolar macrophages (AMs) of ABX mice respond more weakly to IFN treatment or IAV infection [46], which implicates direct action of lung commensals on the alert state of antigen-presenting cells. This result might also partially explain the findings by Ichinohe and

Table 1. Commensal Bacterial Pathogen-Associated Molecular Patterns (PAMPs) and Metabolites Alter Antiviral Immunity[a]

Pattern-recognition receptor (PRR)	Bacterial PAMP or chemical analog tested	Proposed mechanism of immune activation	Refs
Nod2	MDP	Recruitment of Ly6C^{high} monocytes to the lung	[104]
TLR2	Polysaccharide A (*Bacteroides fragilis*), peptidoglycan (*Bacillus subtilis*)	Lung: recruitment of and polarization of M2 AM (express anti-inflammatory cytokines and reduce virus-induced lung injury); gut: activation of NLRP3 inflammasome	[41,47]
TLR3	polyI:C	Activation of NLRP3 inflammasome	[47]
TLR4	LPS	IFNβ-dependent activation of innate antiviral responses, activation of NLRP3 inflammasome	[34,47,105]
TLR5	Flagellin	TLR5-dependent sensing of multiple commensal bacteria (not classified) affects early antibody response	[106]
TLR9	CpG DNA	Activation of NLRP3 inflammasome	[47]
n.d.	Desaminotyrosine	Upregulation of type I IFN	[107]

[a]Abbreviations: AM, alveolar macrophage; CpG, cytosine–phosphate–guanine; LPS, lipopolysaccharide; MDP, muramyl dipeptide; polyI:C, polyinosinic–polycytidylic acid; TLR, Toll-like receptor.

colleagues [47]. The direct impact of respiratory microbiota on respiratory monocytic cells against IAV infections was confirmed in a *Staphylococcus aureus* priming model. Priming of mice with *S. aureus* through the intranasal route promotes monocyte recruitment and M2 (alternatively activated macrophages) polarization of AM, which, in turn, suppress inflammatory cells of the lung during IAV infection [41]. Ultimately, this results in reduced immune pathology and enhanced survival of IAV-infected mice. In humans, microaspiration of the upper-airway microbiota into the lung primes TH17 responses and blunts TLR4-dependent signaling in alveolar macrophages. Interestingly, the more the lower-airway microbiota resembled the upper-airway microbiota, the greater the number of lymphocytes that were found in BAL samples [50]. In an airway epithelial cell culture model it was further demonstrated that antiviral responses could be primed by exposure to commensal respiratory bacteria [51].

Taken together, these observations suggest that the respiratory microbiota keeps cells of the innate and adaptive arm of the immune system on the tip of their toes, ready to respond rapidly to pathogenic threats when required. This 'pre-activation' of the host immune system might come with a price tag. Germ-free animals were shown to have a significantly longer life expectancy than colonized mice (either SPF or conventionally housed) [52,53]. While formal proof is missing, one could speculate that even low-level inflammation, caused by exposure to commensal microbiota, might harm the host tissue, resulting ultimately in faster aging of the organism. However, it should be taken into consideration that early studies were performed under conventional housing settings, which do not reflect current hygiene standards. Thus, a potential reduction in life expectancy in conventionally housed mice by pathogenic microbes cannot be excluded with certainty.

So far we have discussed only the effects of quantitative differences in host microbiota on innate and adaptive immune responses. Besides the abundance of bacteria in a given mucosal niche, their composition and complexity likely contribute to the observed increase in immunity to viral infections. In 2009, Dan Littman's team published a landmark study in which they compared immune responses of genetically identical mice from two commercial vendors, harboring slightly distinct microbiota. They identified segmented filamentous bacteria (SFB) as key inducers of intestinal TH17 cells [54], which, in turn, could affect antimicrobial responses and viral pathogenesis [55]. The significance of SFB in humans is still under debate. The commensal bacterium *Corynebacterium pseudodiphtheriticum* protects infant mice from respiratory syncytial virus (RSV)

infection, and from *Streptococcus pneumoniae* superinfection and systemic spreading, by promoting T cell and macrophage responses in the respiratory tract [56]. This protective effect depends on viable bacteria, implicating a requirement for colonization and active bacterial metabolism. Along the same lines, the use of probiotics reduces disease burden following viral infections of the respiratory tract in mice and humans [57–60]. Conversely, neomycin-dependent depletion of Gram-positive bacteria from the intestine, but not from the nasal cavity, is responsible for reduced adaptive immune responses against IAV [47]. This means that a certain subset of commensals is required to achieve immune homeostasis.

Commensal bacteria carry all the molecular signatures of their pathogenic cousins (PAMPs), and occasionally cohabitate the same body site with them without causing disease. Recognition of commensal bacteria and downstream priming of the host immune system occurs by, or can be mimicked by, agonists of a panel of pattern-recognition receptors (PRRs) (Table 1) to promote antiviral activity. An infection by pathogenic bacteria, or an expansion of pathobionts beyond a level of tolerance, might be additionally sensed by detection of danger signals and VITA-PAMP (signatures of microbial viability) [61].

There is little doubt that the composition, complexity, and abundance of the microbiota impact the antimicrobial host response continually, beyond induction of early-childhood peripheral tolerance. Recognition of commensal microbiota by various innate PRRs results in low-level inflammatory stimulation and keeps innate and adaptive immune cells in a finely balanced state (Table 1) ready to respond to incoming microbial threats. Since these metabolites could potentially be produced by different bacteria, this might explain the high variability of the microbiota in seemingly healthy, immune-competent individuals.

Viral Infections Shape the Composition of the Commensal Microbiota

Virus infection and the antiviral host response are influenced by commensal microbiota. The impact of acute and chronic viral infections on the composition of the microbiota, and the resulting short- and long-term consequences for the host, are probably the least studied aspects of the triangular relationship of virus, host, and microbiota. Table 2 summarizes compositional and quantitative changes observed in experimental viral infection systems.

Few studies have addressed the impact of chronic viral infection on the composition of the host microbiota. Subclinical cytomegalovirus (CMV) infection in mice enhances the prevalence of butyrate-producing bacteria in the gut microbiota [42]. Clinical associations of chronic viral infections with altered microbiota composition were established for HIV (reviewed in [62]). It remains to be proven whether the viral agent causes changes in the host microbiota or if it profits from an existing microbiota with altered composition and infects these individuals more readily. Evidence for HIV alteration of respiratory microbiota comes from a study of long-term simian/human immune deficiency virus (SHIV)-infected *Cynomolgus macaques* [63] (Table 2).

Frequently, viral respiratory tract infections increase the susceptibility of the host to secondary bacterial infections (Box 1); this indicates a direct or indirect impact on bacterial ecology. Some recent publications highlight the effects of experimental acute viral respiratory tract infections on local (respiratory tract) and systemic microbiota (intestinal or fecal) in animal models and in human volunteers. While experimental infection of human volunteers with rhinovirus did not result in detectable changes in the respiratory microbiota [64], Tracy Hussell's team first described altered bacterial composition in BAL of IAV-infected mice [65]. IAV infection was further shown to restructure the upper respiratory tract (URT) microbiota in an IL28R (IFNλ receptor)-dependent fashion, resulting in increased bacterial colonization of the URT [66]. It is

Table 2. Compositional Changes of Commensal Microbiota by Virus Infection Based on 16S rRNA Gene Next-Generation Sequencing or 16S rRNA Gene-Specific qPCR[a]

Host organism	Virus	Sample	Relative changes in the composition of the microbiota in infected animals	Refs
Macaca fascicularis	SHIV	BAL	Enrichment of *Fusobacterium*, *Prevotella*, *Veillonella*, *Neisseria*, and *Porphyromonas*, in BAL samples of SHIV-infected animals that developed COPD	[63]
Macaca mulatta	CMV	Rectal swabs	Increase in *Coprococcus, Faecalibacterium* and Clostridiaceae	[42]
Mus musculus	IAV	Feces	Increase in Proteobacteria (mainly Enterobacteriaceae), Decrease in SFB	[84]
Mus musculus	IAV	Lung, small intestine	Lung: decrease in alpha and beta Proteobacteria and Clostridia Small intestine: decrease in *S24-7* and increase in *Lactobacillus* by spec. qPCR revealed drastic quantitative reduction in both	[67]
Mus musculus	IAV	Feces	Increase in *S24-7* Porphyromonadaceae Decrease in Ruminococcaceae, Lactobacillaceae, Lachnospiraceae	[85]
Mus musculus	IAV	BAL	Increase in *Acinetobacter, Staphylococcus, Joquetella, Peptostreptococcus, Prevotella, Moraxella,* Decrease in *Brevundimonas and Stenotrophomonas*	[65]
Homo sapiens	IAV	Oropharyngeal swabs	Increase in Bacteroidetes	[68]

[a]Abbreviations: BAL, bronchoalveolar lavage; CMV, cytomegalovirus; COPD, Chronic obstructive pulmonary disease; IAV, influenza A virus; SFB, segmented filamentous bacteria; SHIV, simian/human immune deficiency virus.

currently uncertain if the differential colonization of the URT of wild-type or IL28R −/− mice is a direct consequence of the distinct antiviral responses or of a different baseline microbiota. It further remains to be solved which downstream effector molecules of the IFNλ response are responsible for the shift in the composition of the microbiota. The increased bacterial burden in the URT or lower respiratory tract (LRT) was not confirmed in a second study [67], which might indicate that the baseline microbiota in experimental animals is critical for the sensitivity to IAV infection-induced quantitative changes in the URT microbiota. Surprisingly, the LRT microbiota was only mildly affected by IAV infection [67], with an increase in Proteobacteria shown by 16S rRNA gene-specific NGS. Bacterial culture of lung homogenates confirmed the outgrowth of a member of the family Enterobacteriaceae in IAV-infected mice. In humans, an experimental challenge of healthy volunteers with an H3N2 IAV strain caused only minor changes at the phylum or genus level, and surprisingly did not promote outgrowth of pathobionts in the URT [68]. This contradicts correlative studies showing the enhanced presence of bacterial pathogens in the URT of influenza-infected patients [24]. It is possible that the good health conditions of experimentally infected volunteers at baseline prevented major microbiota changes.

Box 1. Secondary Bacterial Infections

Bacterial superinfection is a common complication of viral infections. The bulk of the literature concerns respiratory infection models, mainly performed with IAV (reviewed in [69]). Even the live-attenuated IAV vaccine (LAIV) was shown to promote colonization by *S. pneumoniae* and *S. aureus* in a mouse model [70]. In humans, these pathobionts are part of the natural commensal flora and are often the cause of secondary bacterial infections by gaining a growth advantage under virus-induced disease conditions. The reasons for enhanced colonization with bacterial pathogens or outgrowth of pathobionts are likely multifactorial, combining viral factors and host factors, and they depend on the type of bacterial pathogen.

Some IAV factors were shown to have a positive effect on bacterial growth. For example, influenza neuraminidase function is required to 'cut' through the respiratory mucus layer [71] and free sialic acids which can then be used by *S. pneumoniae* as an energy source [72]. Variants of the IAV accessory protein PB1-F2 were shown to enhance secondary bacterial infections [73].

On the host side, numerous mechanisms have been proposed to support bacterial superinfection following IAV infection. IAV was shown to impair natural killer (NK) cell function, thus increasing susceptibility to *S. aureus* [74], a mechanism which depends on IL-10 [75]. Different studies showed that *S. pneumoniae* adhesion and colonization were increased following virus-induced epithelial cell apoptosis enhances adhesion of *S. pneumoniae* [76] and lung damage caused by TNF-related apoptosis inducing ligand ($TRAIL^+$) monocytes [77]. Reduced tolerance towards tissue damage in consequence of IAV infection also promotes *Legionella pneumophila* [78]. Furthermore, influenza was shown to alter cytokine expression levels (i.e., IL-1β and IL-27), which, in turn, increases host susceptibility to bacterial infection [79–81]. In humans experimentally challenged with LAIV and superinfected with *S. pneumoniae*, CXCL10 was positively correlated with the severity of pneumonia [82], as previously shown in mice [83].

Density and composition of bacterial colonization of the host is a factor; as yet, this has not been causally linked to secondary bacterial infections in the lung (see Figure 2).

Interestingly, respiratory tract infections also remotely affect the intestinal tract microbiota [67,84,85]. In human patients, similar alterations of gut microbiota were observed in the context of H7N9 IAV infections [86]. This confirms the bidirectionality of the gut–lung axis [87]. Effects of the composition of the microbiota are quite diverse in the following three studies. Tregoning *et al.* observed a relative decline in Firmicutes and an increase in Bacteroidetes in fecal microbiota after infection with respiratory syncytial virus (RSV) and IAV. This depended on LRT infection since LAIV did not provoke comparable changes in the fecal microbiota [85]. Yildiz *et al.* showed that quantitative depletion of bacteria from the small intestine causes enhanced susceptibility to invasion by *Salmonella enterica* serovar Typhimurium [67], confirming a previous correlation by Deriu and colleagues [84]. This could implicate a competitive effect of gut microbiota and invading pathogens, as shown for ABX mice or gnotobiotic mice [88]. Interestingly, both streptomycin treatment and IAV infection mainly diminished Bacteroidetes [67], which might mean that these are directly or indirectly responsible for the host resistance to invasion by *S. enterica* serovar Typhimurium. Increased levels of gammaproteobacteria in the feces were correlated with increased intestinal tissue inflammation and higher susceptibility to infection by *S. enterica* serovar Typhimurium [84,89], but this could not be confirmed by a third study [67]. Since the application of the dsRNA analog polyI:C has a similar effect in mice [84], it could be assumed that components of the innate host response trigger systemic effects on the composition and abundance of the microbiota.

The signaling events, bridging isolated viral respiratory tract infection with alterations of gut microbiota, are still poorly understood. Lung-derived $CCR9^+$ $CD4^+$ T cells were shown to produce IFNγ in the small intestine after severe IAV infection, which increased proteobacteria in the intestinal microbiota [89]. Type I interferon-dependent changes in the gut microbiota occurred after IAV infection [84]; however, systemic application of IFNα did not mimic this phenotype [67]. A common denominator of virus-induced dysbiosis in mouse models is the outgrowth of Proteobacteria observed in different body niches [66,67,84,89]. It remains

currently elusive if Proteobacteria are solely flourishing better under immune-activation conditions, or if they actively contribute to the host immune pathology under virus infection. In humans, the blooming of proteobacteria was proposed to be a marker for numerous disease states, including chronic inflammatory lung diseases (reviewed in [90]).

Since there are no examples of mammalian pathogenic viruses which infect or eliminate prokaryotes, it is reasonable to assume that the virus-induced changes in the composition of the microbiota are largely an indirect consequence of an antimicrobial host response. In immune-competent hosts, viral infections are accompanied by a rapid inflammatory and antiviral response, eventually leading to clearance of the virus by the following adaptive immune response. Compared with the highly specific adaptive immune response, the first-responding, innate immune response is a rather nonspecific, broad-spectrum antimicrobial program. It ramps up PRR levels to an alert state in the infected tissue, and it encompasses a wide spectrum of signaling molecules to attract and prime innate and adaptive immune cells. Effector molecules of the innate response directly target a plethora of microorganisms. Given the close resemblance of commensal and pathogenic microorganisms, it is reasonable to assume that the innate response following viral infections would also affect commensal microorganisms. Regulation of commensal bacteria by the immune system depends on the composition of the mucus layer [91], antimicrobial peptides [92,93], commensal-specific IgA [94], and potentially other components of the adaptive immune system [95]. IAV and RSV infections cause an increase in mucin production (Muc5ac) in the LRT and intestine, which might contribute to changes in the composition of the microbiota [96]. Increased activity of Paneth cells, which are the main producers of alpha defensins (a major class of antibacterial peptides in mice) in the small intestine [97], was observed after IAV infection [67]. However, causality has not been determined for either of these effector molecules in the context of virus-induced alterations in the microbiota.

While we are still at an early point in investigations, it has becomes evident that there is high variability in the outcome of the above-cited studies. The innate host response is certainly a factor in shaping respiratory and gut microbiota in virus-infected individuals; however, much work lies ahead to define common signaling mediators and downstream effector molecules. Like most metagenomics approaches in animal systems, the choice of vendor and housing conditions severely impacts the baseline microbiome. Additionally, sampling techniques and downstream analysis impact the 16S rRNA NGS analysis. Standardization of protocols could improve the comparability and reproducibility of these studies [98].

Concluding Remarks

We are living in, and are part of, a highly interactive polymicrobial world. It has become increasingly evident that the majority of our body functions are influenced by commensal microbiota and their metabolites. Imbalances in this complex ecosystem have consequences for host physiology. Dysbiosis can cause chronic inflammatory states, as seen in inflammatory bowel disease (IBD), resulting in tissue damage [99]. Re-establishment of eubiosis after viral infections and preventive treatment with probiotics were shown to be beneficial for pediatric and geriatric patients, which are usually colonized by a less stable and less complex microbiota [100–103]. On the other hand, excessive use of antibiotics might deplete essential commensal bacteria and raise susceptibility to viral infections. An urgent diagnostic goal of microbiota research is thus the identification of individual bacterial species/strains or mixtures of these (e.g., proposed enterotypes or pneumotypes [38,39]) which promote or prevent disease states (see Outstanding Questions). For viral infections this correlation is still missing, partly because we lack the tools to eliminate individual commensals from complex bacterial mixtures in a given

Outstanding Questions

Which enteric bacteria make us more susceptible to enteric virus infection, and which commensals protect us from virus infection?

Are there synergistic effects of bacterial and viral enteric pathogens?

What is the impact of the microbiota on immunity, and what is the direct contribution of the microbiota to viral pathogenesis in the context of enteric viral infection?

Are similar effects taking place in body sites with a bacterial density lower than that in the gut (e.g., lower respiratory tract or skin)?

What is the role of commensal fungi, archaea, and viruses/phages in immune priming?

Does the core microbiota provide a 'healthy' qualitative and quantitative mix of PAMPs to prime the host immune system, at a low level and at all times, against potential invaders?

How do commensal PAMPs differ from the PAMPs of pathogens? Are they less immunogenic?

Are all viruses equally sensitive to microbiota-dependent stimulation, education, and maintenance of the host immune system?

What are the biological costs of maintaining a 'wild' bacterial microbiome causing permanent low-level inflammation (more extreme in IBD for example)?

Which commensal bacteria prevent secondary bacterial infection in eubiotic conditions, and which promote it in dysbiosis?

Can we use probiotics to correct dysbiotic states after acute viral infections?

How do acute infections shape the microbiome of individuals with less established complexity, for example, children, or the elderly?

body site, without affecting other colonized niches in the body. The development of oligo-colonized gnotobiotic mouse models [88] could help to establish causal relations of individual commensals with certain body functions. Obvious discrepancies in observational studies in humans will require further investigation – which is necessary for refinement of the currently used animal models. This will be the basis for translating the principle findings from mouse studies into clinical applications in humans. Commensal bacteria are, without doubt, an important factor in the immune defense against viral pathogens and a promising future target for diagnostics and treatment.

Which signaling and effector molecules of the host innate response cause systemic dysbiosis in virus-infected hosts?

Does the change in microbiota after viral infection, in turn, affect adaptive immune responses to the very same virus?

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