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Respiratory Viral Infection-Induced Microbiome Alterations and Secondary Bacterial Pneumonia

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Influenza and other respiratory viral infections are the most common type of acute respiratory infection. Viral infections predispose patients to secondary bacterial infections, which often have a more severe clinical course. The mechanisms underlying post-viral bacterial infections are complex, and include multifactorial processes mediated by interactions between viruses, bacteria, and the host immune system. Studies over the past 15 years have demonstrated that unique microbial communities reside on the mucosal surfaces of the gastrointestinal tract and the respiratory tract, which have both direct and indirect effects on host defense against viral infections. In addition, antiviral immune responses induced by acute respiratory infections such as influenza are associated with changes in microbial composition and function ("dysbiosis") in the respiratory and gastrointestinal tract, which in turn may alter subsequent immune function against secondary bacterial infection or alter the dynamics of inter-microbial interactions, thereby enhancing the proliferation of potentially pathogenic bacterial species. In this review, we summarize the literature on the interactions between host microbial communities and host defense, and how influenza, and other acute respiratory viral infections disrupt these interactions, thereby contributing to the pathogenesis of secondary bacterial infections.

Keywords: gut microbiome, respiratory viral infection, bacterial pneumonia, viral-bacterial interaction, influenza, host-microbe interaction

INTRODUCTION

Influenza and bacterial pneumonia are the leading cause of morbidity and mortality from infectious diseases worldwide. Influenza and other respiratory viral infections predispose patients to secondary bacterial super-infections, which are frequently associated with a more severe clinical course. It is estimated that the so-called "Spanish Flu" pandemic of H1N1 influenza A virus from 1918 to 1919 resulted in more than 50 million deaths, with many caused by bacterial super-infection leading to secondary pneumonia (1–7). Even in the antibiotic era, over half of patients with severe infections in the 1957 H2N2 and 1968 H3N2 pandemics had bacterial complications (8–10). Bacterial co-infection was also detected in ~30% of cases in the 2009 H1N1 pandemic, with high mortality rates despite administration of appropriate antibiotics (11–18). Thus, it is evident that a better understanding of the pathogenesis of secondary bacterial pneumonia following viral infections is needed in order to make therapeutic strides for this devastating complication.

The mechanisms of post-viral bacterial infection are complex, comprising multifactorial processes mediated by interactions between viruses, bacteria, and the host immune system.

The pathogenesis of super-infection has been attributed to direct mucosal/epithelial damage by influenza virus, increased bacterial colonization of the upper and lower respiratory tracts (URT and LRT, respectively), and dysregulation of immune responses, which all lead to increased susceptibility to secondary bacterial infections. However, emerging evidence suggests that our microbial communities residing on our mucosal surfaces likely shape the rigor of our immune responses and shape the ecological relationships between host and pathogens. Over the past 10 years, intense interest has focused on examining how the microbial communities which inhabit our bodies—which some consider to be a separate "organ system" given the sheer physical bulk, number of genes, and metabolic activities—govern the balance between health and susceptibility to diseases, including infections. This raises the possibility that disruptions in the normal microbial communities by an acute viral infection might contribute to the development of post-viral bacterial pneumonia.

The recent development of culture-independent methods of microbial identification has enabled the study of microbial communities on mucosal surfaces of the human body, referred to as "microbiota." The microbial communities of mammalian hosts are diverse, comprised of bacterial, viruses, archaea, parasites, and fungi. The Human Microbiome Project (HMP) and other similar large-scale sequencing projects worldwide have characterized the distinct microbial communities that have adapted to the unique environmental niches within our bodies, such as the gut, skin, airways, genitourinary tract, and oral cavity. The gut microbiome, in particular, has been shown to play an integral role in shaping the immune system starting early in life, with continued influence on priming the nature and robustness of immune responses throughout one's lifetime. The respiratory tract also harbors distinct communities of microbes, with multiple discrete ecological niches (e.g., nasal cavity, oropharynx, upper airways) that vary in terms of temperature, pH, oxygen tension, mucus production, and other factors.

The effects of viral infections on both the gut and respiratory microbiome have recently undergone examination. Surprisingly, influenza infection has been found to result in significant changes in the gut microbiome, despite the lack of detectable virions in the GI tract. By comparison, the effects of viral infection on the respiratory microbiome appear to be relatively modest, but detectable. While the effects of these alterations on risk of secondary bacterial pneumonia have not been studied, potential mechanisms by which these changes might modulate susceptibility to secondary bacterial infections include alterations in the nature and magnitude of the immune response in the host (microbiome on host effects) and facilitating growth of pathogens in the absence of normal commensals (inter-microbial effects). In this article, we review the current understanding of how alterations in the microbiome following viral infection might alter host immune responses and increase susceptibility to secondary bacterial infections. Although the term "microbiome" encompasses all microbial communities, there is currently a paucity of studies on how the mycobiome (fungal microbiome) and the virome (viral microbiome) affect host defense against respiratory infections and vice-versa; thus, this review will focus on the bacterial microbiome literature.

THE GUT MICROBIOME AND RESPIRATORY INFECTIONS

Of the niches in the body, the gut microbial community has been the most intensively studied, with over 20,000 publications to date. While the virome and mycobiome (fungi) are also being analyzed, the bulk of the literature has focused on the bacterial component of the microbiome, and thus most of our understanding of the relation of the gut microbiome to host immunity and pathogenesis of chronic diseases comes largely from studies of the bacterial community. During health, the human gut bacterial community is diverse, with each individual harboring over 100 trillion bacteria, comprised of over 150 different species. The gastrointestinal microbiota is dominated by Firmicutes (e.g., *Lactobacillus*, *Bacillus*, and *Clostridium*) and Bacteroidetes (e.g., *Bacteroides*), with lower abundances of Proteobacteria (e.g., *Escherichia*) and Actinobacteria (e.g., *Bifidobacterium*) (19, 20). Wild-living mice exhibit more diverse microbiomes, with significant abundance of Proteobacteria as well as Firmicutes and Bacteroidetes (21). The gut microbiome, in addition to its metabolic functions in the host, plays an integral role in the development, instruction, and priming of the immune system. Germ-free (GF) mice (which lack microbiota) have markedly underdeveloped gut-associated lymphoid tissues, decreased number and smaller-sized Peyer's patches and mesenteric lymph nodes, and defects in antibody production, compared to specific pathogen free (SPF) mice. Not surprisingly, germ-free animals exhibit increased susceptibility to multiple types of infections, including viruses, bacteria, and parasites (22–27). However, compared to free-living mice or laboratory animals exposed to gut flora from wild mice, SPF animals have a more limited microbial community and are also more susceptible to inflammatory diseases, with a reduced immune repertoire including deficits in memory responses (21, 28, 29).

Although an extensive discussion of the healthy gut microbiome and its impact on host immunity is beyond the scope of this review, we will highlight a few important aspects of how the intestinal bacterial community microbiome maintains a healthy host immune environment. First, bacterial metabolites generated by gut commensals contribute to the maintenance of intact epithelial integrity, regulatory T-cell development, and a relatively anti-inflammatory immune state. In particular, short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate are fermentation products of dietary fiber and carbohydrates by large intestinal bacteria (30). In addition to being a major energy source for intestinal epithelial cells, SCFAs promote the development of naive $CD4^+$ T cells into regulatory T cells (31, 32), induce "tolerogenic" dendritic cells in the intestinal mucosa (33), and limit autoimmity (34, 35). At the same time, microbial metabolites are integral for promoting immune responses in the gut against pathogens, including inducing secretion of IL-18 (36) and defensins (37, 38). Thus, the products of microbiome metabolism are integral to the appropriate regulation of mucosal barrier integrity and immune homeostasis. In addition, specific members of the bacterial community have been shown to foster the proper maturation and development of the immune system. While this is still an

area undergoing intense investigation, one notable example is the discovery that segmented filamentous bacteria are critical promoters of intestinal mucosal IgA production (39, 40)and Th17 cell induction (41, 42).

Dysbiosis, or an imbalance in the normal composition of the microbiome, is associated with a variety of chronic diseases, many of which are characterized by chronic inflammation or abnormal metabolism, including inflammatory bowel disease, cardiovascular disease, and diabetes. Thus, fostering appropriate levels of diversity and composition of the gut microbial community is critical for promoting health and immune homeostasis. During health, the composition of the microbiome is governed by a number of selective pressures unique to each anatomic niche, including temperature, nutrient availability, pH, oxygen tension, and the local immune environment. Short-term perturbations in the gut microenvironment caused by illness, antibiotic usage, or dietary changes (e.g., starvation) can alter the gut microbiome and subsequently lead to transient alterations in immune responses. Thus, investigating whether influenza and other respiratory viruses alter the gastrointestinal microbiome could have mechanistic implications for viral-mediated suppression of antibacterial immune responses.

Effects of Acute Respiratory Viral Infection on Gut Microbiome

Although the composition of the gastrointestinal microbiome is largely influenced by dietary patterns, respiratory viral infections could also contribute, along with other stress inducers such as broad-spectrum antibiotics exposure and chronic inflammation. Using animal models of pulmonary infections by influenza and respiratory syncytial virus (RSV), multiple groups have shown that the gut microbiome is clearly impacted by respiratory viral infections, despite the lack of detectable respiratory virus in the gut (43–47). In a murine model of influenza infection, the investigators found that although the total numbers of bacteria in the gut did not decrease, there was a reduction in the quantities of segmented filamentous bacteria (SFB) and *Lactobacillus/Lactococcus*, accompanied by increases in *Enterobacteriaceae*. Interestingly, although SFB have previously been shown to induce Th17 cells (41, 48), flu-infected mice had increased IL-17A levels and numbers of Th17 cells in the small intestine and colon, which appeared to contribute to intestinal injury (43). In this study, antibiotic treatment prior to influenza infection ameliorated the degree of intestinal injury, but not lung injury, suggesting that gut dysbiosis contributed to local but not systemic inflammation. Other groups have similarly reported increased Proteobacteria (the phylum of which *Enterobacteriaceae* are members) (44, 45), decreased Firmicutes (which include SFB, *Lactobacillus* and *Lactococcus* species), and increased Bacteroidetes (47) following infection by flu or RSVs but not after administration of live attenuated influenza vaccine (LAIV), indicating that live viral infection is required for these changes (47). The increase in Proteobacteria appears to be mediated by type I interferons (IFNs) (18), which not only depleted anaerobic bacteria but also increased susceptibility to secondary *Salmonella* colitis. However, caloric restriction also
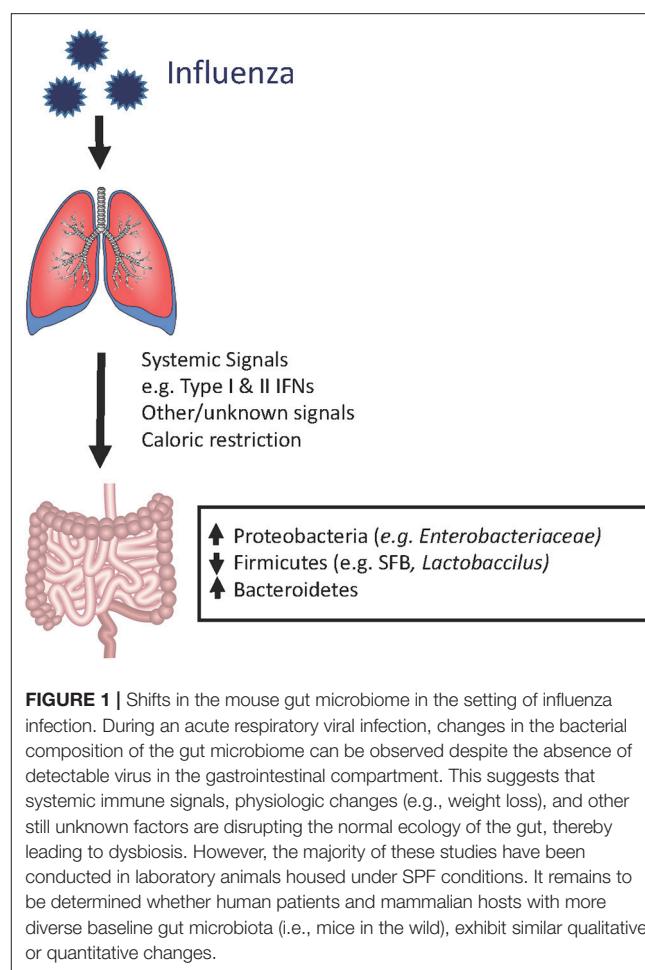

FIGURE 1 | Shifts in the mouse gut microbiome in the setting of influenza infection. During an acute respiratory viral infection, changes in the bacterial composition of the gut microbiome can be observed despite the absence of detectable virus in the gastrointestinal compartment. This suggests that systemic immune signals, physiologic changes (e.g., weight loss), and other still unknown factors are disrupting the normal ecology of the gut, thereby leading to dysbiosis. However, the majority of these studies have been conducted in laboratory animals housed under SPF conditions. It remains to be determined whether human patients and mammalian hosts with more diverse baseline gut microbiota (i.e., mice in the wild), exhibit similar qualitative or quantitative changes.

results in increased relative abundance of Proteobacteria and increased Bacteroidetes to Firmicutes ratio, raising the possibility that decreased oral intake during influenza may contribute to changes in the microbiome (45, 47, 49, 50). It has also been shown that influenza infection alters intestinal microbiota composition through type II IFN produced by lung-derived T cells recruited to the intestine (43). Thus, changes in the gut microbiome appear to result not from direct viral effects but from systemic inflammatory signals that travel from the lung and trigger local inflammatory responses in the gut (**Figure 1**).

Effects of Gut Microbiome on Host Immune Responses

Interactions between respiratory tract infections and the gut microbiome are bidirectional. While respiratory viral infections can change the gut microbiome, the gut microbiome also shapes the adaptive immune responses against respiratory pathogens. Mice pretreated with an antibiotic cocktail showed increased morbidity and mortality during influenza infection (51, 52). The severity of infection was associated with reductions in dendritic cell migration rate and the number of local T cells. Mice given a 4 week oral course of broad-spectrum antibiotics

before respiratory viral infection mounted an attenuated anti-PR8 antibody response, were incapable of inducing CD4$^+$ T cell-mediated IFN-γ response to PR8 antigen, and had fewer influenza-specific CD8$^+$T cells (51, 52). These mice also had higher viral titers in their lungs (51). Germ-free mice and antibiotic-treated mice also exhibit impaired antibody responses to seasonal influenza vaccination, which was restored by oral administration of flagellated *E. coli*, demonstrating a dependence on TLR5-mediated sensing of the host microbiota (53).

The gut microbiome is essential for priming innate immune responses against pulmonary infections as well. During viral infections, the degree of macrophage response to respiratory viruses depends on the presence of gut microbes. Macrophages from animals treated with antibiotics exhibited defective responses to type I and II IFNs and impaired capacity to limit viral replication, suggesting that intestinal microbiota provide immune stimulation that establishes an "activation threshold" for innate antiviral immune responses (52). A comparison of C57BL/6 mice from The Jackson Laboratory (which lack SFB in the stool) and Taconic Biosciences (which are SFB positive) revealed that SFB-deficient animals have increased lung bacterial burdens and more severe pneumonia when challenged with methicillin-resistant *Staphylococcus aureus* (MRSA) (54), which was associated with decreased IL-17-mediated responses in the lung. Another study using broad-spectrum antibiotic treatment followed by intranasal administration of *S. pneumoniae* in mice demonstrated that microbiome depletion led to decreased survival, increased lung bacterial burden, and increased systemic dissemination of bacteria (55). Antibiotic-pretreated animals displayed altered cytokine profiles in the lung compared to untreated controls following *S. pneumoniae* infection, including significantly decreased TNF-α levels at 6 and 24 h after infection. Additionally, in the microbiota-depleted group, alveolar macrophages and blood neutrophils exhibited decreased phagocytic activity, and decreased inflammatory cytokine production following *ex vivo* stimulation by Toll-like receptor (TLR) ligands such as lipoteichoic acid (LTA) (55). These effects might be mediated in part by decreased Nod1 sensing of meso-DAP (diaminopimelic acid)-containing peptidoglycan found in gut microbiota, which previously was shown to be essential for priming innate immune responses to *S. pneumoniae* (56). Thus, antibiotic-induced disruptions in the normal gut microbial community alter multiple aspects of normal host defense against acute respiratory pathogens (**Figure 2**).

Gut Microbiome: Therapeutic Avenues for Acute Respiratory Infections

Collectively, the studies above suggest that modulation of the gastrointestinal tract microbiome plays an important role in acute respiratory infections, but precisely how the microbiome should be manipulated to promote appropriate immune responses during acute respiratory infections is unclear. Currently, clinical studies have shown that although probiotics do not influence the incidence of respiratory tract infection, they do reduce the severity of symptoms and duration of the illness (57, 58). Pinpointing which members

of the gut microbial community are essential for proper immune priming is challenging, but necessary for guiding further microbiome-based therapies. *Clostridium orbiscindens*, a member of the human gut microbiome, has been found to produce desaminotyrosine (DAT) from metabolism of flavonoids and amino acids. Antibiotic-treated mice exhibited markedly decreased fecal and serum DAT levels, which was associated with attenuated type I IFN responses to influenza infection and increased mortality (59). Thus, identification of DAT-producing microbiota might serve as a modality for priming type I IFN responses against viral infections. Another group demonstrated that oral administration of *Lactobacillus plantarum* enhanced the type I IFN response and lowered viral titers in the lungs in a murine model of influenza infection (60). Other *Lactobacillus* strains are known to enhance TNF-α and IFN-γ production by nasal lymphocytes upon influenza infection (61). Oral administration of a probiotic cocktail containing *Lactobacillus* restored the immune response and enhanced the activation of signaling pathways associated with recognition of single-stranded RNA virus (62). An alternative approach to administering probiotics is to alter the local metabolic environment to regulate immune responses. A recent report demonstrated that animals fed a high fiber diet had increased generation of SCFAs, leading to enhanced antiviral CD8$^+$ T cell immune responses and attenuated neutrophil-mediated lung injury during influenza infection, resulting in improved survival (63). Thus, one strategy for decreasing the incidence of post-viral bacterial infections is to limit the severity of the primary viral infection.

However, activation of antiviral immune responses, including type I and type II IFNs, have been associated with increased susceptibility to secondary bacterial pneumonia (64, 65). Thus, another strategy is to enhance immune responses against common bacterial causes of pneumonia. One group re-colonized antibiotic-treated or germ-free mice with groups of cultivatable commensal bacteria, and found that administration of *Lactobacillus reuteri*, *Enterococcus faecalis*, *Lactobacillus crispatus*, and *Clostridium orbiscindens*, which are strong stimulators of NOD2 (i.e., cytosolic receptor for muramyl dipeptide, which is found in cell walls of certain bacteria), are able to protect against bacterial pneumonia by enhancing GM-CSF production (66). Whether viral-induced changes in the gut microbiome is associated with immune defects that promote secondary bacterial pneumonia, or whether the impaired antibacterial defenses observed in virally-infected hosts can be restored by augmenting certain components of the microbiome are important areas to be investigated.

THE RESPIRATORY TRACT MICROBIOME

The microbiome of the respiratory tract has also been investigated in the context of viral infections. Its role in the development of secondary bacterial pneumonia following influenza and other acute respiratory viral infections is unclear. The respiratory tract is the main site of continuous contact with
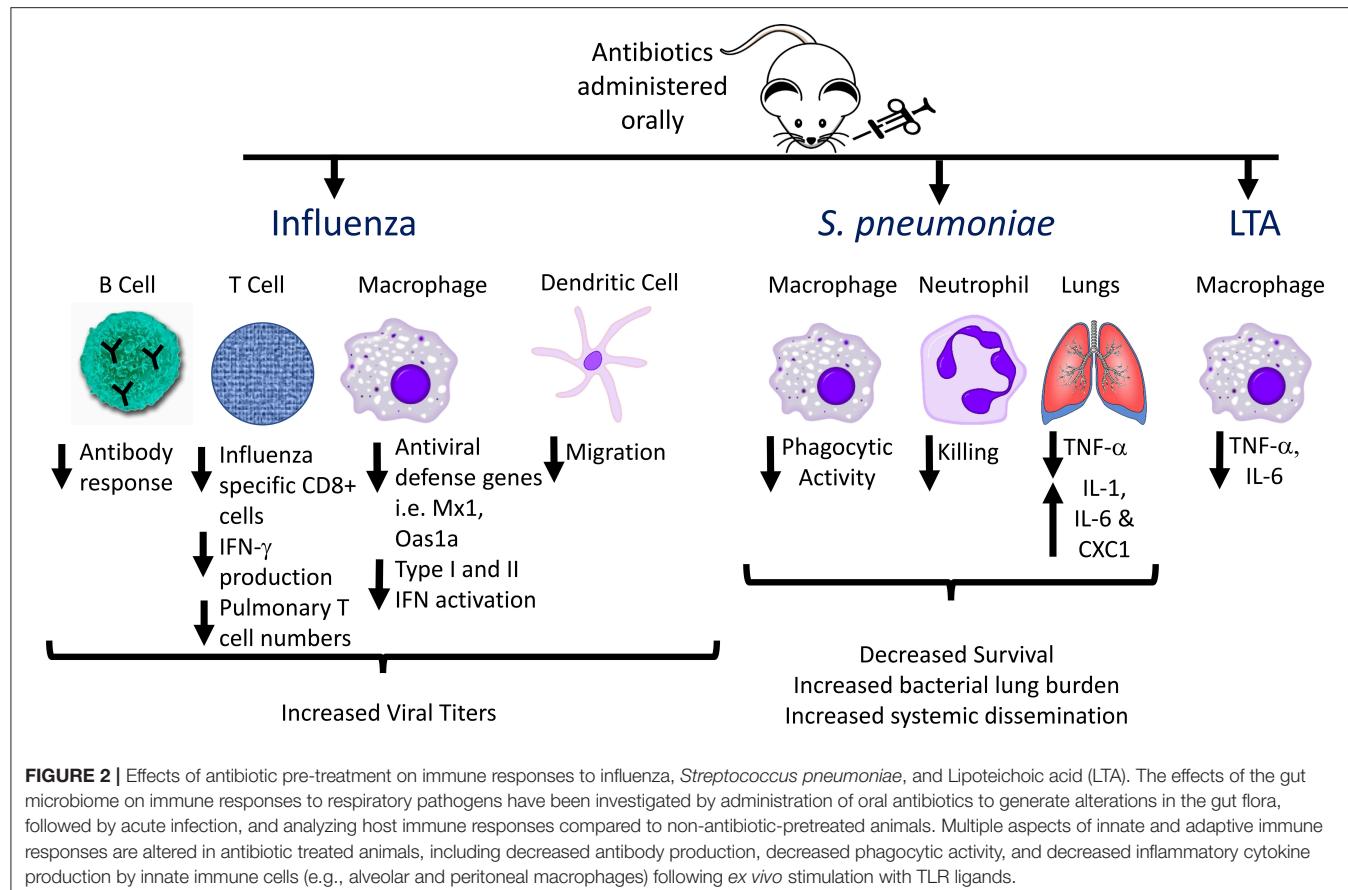
FIGURE 2 | Effects of antibiotic pre-treatment on immune responses to influenza, *Streptococcus pneumoniae*, and Lipoteichoic acid (LTA). The effects of the gut microbiome on immune responses to respiratory pathogens have been investigated by administration of oral antibiotics to generate alterations in the gut flora, followed by acute infection, and analyzing host immune responses compared to non-antibiotic-pretreated animals. Multiple aspects of innate and adaptive immune responses are altered in antibiotic treated animals, including decreased antibody production, decreased phagocytic activity, and decreased inflammatory cytokine production by innate immune cells (e.g., alveolar and peritoneal macrophages) following *ex vivo* stimulation with TLR ligands.

exogenous microbes. As is the case with the gut, immunity at the mucosal interface of the respiratory tract is a constant balance of tolerance of commensal and non-invasive microbes and immune activation against pathogens. The URT and LRT have similar microbial community compositions, although microbe densities are much higher in the former in healthy hosts. Several factors are known to influence airway microbiome composition including infection history, age, genetics, and structural lung disease.

The URT is an interconnected system consisting of the anterior nares, nasal cavity, nasopharynx, sinuses, Eustachian tube, middle ear cavity, oral cavity, oropharynx, and larynx, each of which serve as distinct niches with their own microbial communities. In healthy adults, bacteria present in the nasal cavity are typically those associated with skin, predominantly members of the Actinobacteria (e.g., *Corynebacterium* spp., *Propionibacterium* spp.), followed by Firmicutes (e.g., *Staphylococcus* spp.), and Proteobacteria (67–69). The oropharynx contains members of Firmicutes, Proteobacteria, and Bacteroidetes, including *Streptococcus*, *Neisseria*, *Haemophilus*, and *Lachnospira* spp. (68, 70, 71). Skin and oral cavity lineages are represented in the nasopharynx— e.g., *Streptococcus*, *Staphylococcus*, *Corynebacterium*, and *Prevotella* (70, 72, 73). A limited number of pathogens including *Streptococcus pneumoniae*, *Neisseria meningitides*,

and *Haemophilus influenzae* are commensal bacteria of the URT.

In healthy individuals, the microbial community richness (i.e., the total number of bacterial taxa) is lower in the LRT than that in the URT (70, 74–76). Contrary to dogma that normal healthy lungs are a sterile environment, a distinct, and somewhat dynamic lung microbiome can be identified using sequencing technology, with microaspiration serving as the primary route of microbial immigration from the URT to the LRT (76, 77). The major phyla in healthy lungs are Bacteroidetes and Firmicutes, which mainly include *Prevotella*, *Veillonella*, and *Streptococcus* (78–80). Individuals with chronic airway diseases (e.g., cystic fibrosis, COPD) have increased bacterial populations in the lungs (77) and differences in the relative abundance of certain species (81). Impaired airway clearance due to intrinsic or extrinsic factors leads to the proliferation of bacterial species that can exploit this growth opportunity (82). How respiratory viral infection affects the diversity of microbial communities and whether viral-induced dysbiosis influences immune functions is being examined. Nonetheless, bacterial colonization of the URT is generally considered as the first step in the development of invasive bacterial infections (83, 84), including secondary bacterial infections following respiratory viral infection. Bacterial abundance, species diversity, and factors that shape the immune

response to subsequent infections are discussed in greater detail below.

Studies of the URT Microbiome During Respiratory Viral Infection

Respiratory viruses enter the human body through the URT and are the most common type of acute infections of the respiratory tract. One possible mechanism by which influenza and other viral infections might predispose infected hosts to secondary bacterial pneumonia is by altering the microbial composition of the upper respiratory tract, fostering enhanced growth of pathogens, and facilitating the subsequent entry of large bacterial loads into the LRT (85). This section will examine recent literature on how acute respiratory viral infections have changed the URT microbiome.

Cross-Sectional Studies

Given the effects of viruses on enhancing bacterial adherence to the epithelium (86–88), it is perhaps not surprising that multiple studies of human subjects as well as in animal models have shown that viral infections are associated with increased colonization by potentially pathogenic bacteria (known as "pathobionts"). A comparative analysis using qPCR to detect specific bacteria in adult patients with or without influenza A infection showed that *Staphylococcus aureus*, *S. pneumoniae*, and *H. influenzae* were present in 12, 24, and 32% of infected patients, respectively as compared to 5, 11, and 10% of uninfected patients (89). In experimental *in vitro* models, viral infections increase the colonization rates of various bacteria in the URT (90–95), including *S. pneumoniae* and *H. influenzae* (86–88). In children, influenza is associated with a 15-fold increase in nasopharyngeal titer of *S. pneumoniae* (96). Animal models have similarly confirmed that viral infection, particularly influenza, increases bacterial colonization rates in the URT, enhancing the risk of secondary bacterial infections (97–99). Higher pneumococcal colonization density has been linked to respiratory virus co-infection and invasive pneumococcal pneumonia, after adjusting for age and sex (85). Another case-control study comparing nasopharyngeal bacteria with and without pneumonia also found an association between nasopharyngeal load of *S. pneumoniae*—but not of *H. influenza* and *M. catarrhalis*—and viral co-infection and pneumonia (96). In addition, viral infections potentially may enhance transmission of bacteria. In a study of mice colonized with *S. pneumoniae* and then infected with influenza A virus 3 days after, *S. pneumoniae* transmission occurred only when all mice were infected with influenza and was blocked by an influenza-neutralizing antibody (95). However, while specific bacteria might gain a competitive advantage during viral infections, this does not universally translate to all bacterial taxa. A recent study of subjects with and without respiratory viral infections demonstrated lower overall bacterial reads from nasopharyngeal samples in virally-infected subjects compared with uninfected controls (100).

The relationship between acute viral infections and bacterial colonization appears to be bidirectional. Bacterial carriage or their ligands can increase or decrease viral infectivity rate, thereby positively or negatively influencing the subsequent host immune response to viral infection. Viral replication in the respiratory tract can be enhanced by exposure to *S. pneumoniae* (101). Patients harboring *S. pneumoniae* are more likely to experience subsequent acute respiratory illness episodes than those without colonization (102). In addition, bacteria present in the airways can modulate host responses against viral infection. The presence of a nasopharyngeal commensal protected mice against RSV-induced airway hyperresponsiveness. RSV-infected mice who underwent antibiotic-mediated depletion of *Streptococcus viridans* in the nasopharynx exhibited increases in number of inflammatory lymphocytes and airway hyperresponsiveness, and decreases in regulatory T cell number and transforming growth factor-β production (103). Others have shown that colonization of the URT with *S. aureus* drastically reduced influenza-induced acute lung injury and mortality in mice by recruiting a C-C chemokine receptor type 2$^+$ cluster of differentiation (CD)11b$^+$ monocyte subset to the lungs and inducing an M2 macrophage phenotype (104).

With the availability of next-generation 16S rRNA sequencing, microbiome-based studies have attempted to discern global patterns of change in the bacterial community of each anatomic niche during viral infections, such as changes in diversity. Diversity can be assessed using a variety of indices, such as total number of unique species of the microbiome (i.e., richness) or other measures that account for both richness and the evenness of relative abundance of the members of the community (e.g., Shannon index). Results from microbiome analyses have not demonstrated consistent changes in diversity when comparing virally infected subjects with healthy controls. This is not surprising given the variability of the subjects sampled, differences in type and severity of viral infections, type and timing of sample collection, and analysis methodology. In some studies, increased bacterial diversity appeared to be associated with influenza severity. A French study of children admitted to the hospital with influenza revealed increased diversity of the nasopharyngeal microflora with increased influenza severity (105). Children with severe influenza showed decreased relative abundance of *S. aureus* and increased abundance of *Prevotella*, *Streptobacillus*, *Porphyromonas*, *Granulicatella*, *Veillonella*, *Fusobacterium*, and *Haemophilus*. A recent Chinese study in patients with H7N9 avian influenza demonstrated significantly increased diversity in the oropharyngeal microbiome of H7N9-infected patients compared to healthy controls, particularly H7N9 patients with secondary bacterial pneumonia (106). Conversely, a French study of nasopharyngeal samples and a South Korean study of oropharyngeal samples from patients with acute respiratory viral infections both displayed decreases in diversity indices during viral infections compared to healthy controls (71, 100). Both studies included subjects ranging from infants to adults >80 years of age, limiting conclusions about age-related effects. Longitudinal studies conducted in healthy volunteers who underwent experimental self-innoculation with rhinovirus also failed to demonstrate significant changes in diversity of the URT microbiome, while administration of LAIV vaccine to healthy adults led to increases in diversity measures following viral

challenge (73, 107). Thus, unlike other diseases where decreased diversity is considered deleterious to the host, the effects of viral infections on diversity *per se* are variable and not presently considered a good indicator of risk for complications, including secondary bacterial pneumonias.

Microbiome sequencing studies also enable investigators to identify changes in abundance among multiple bacterial taxa simultaneously, beyond just what can be cultured individually. This allows investigators to determine what groups of bacteria are changing in unison during viral infection and which are existing in competition with one another. This information may have implications for the development of probiotic therapies (as discussed below). A recent metagenomics-based study in France reported enrichment of *S. aureus*, *S. pneumoniae*, *H. influenzae*, *Moraxella catarrhalis* and *Klebsiella pneumoniae* in nasopharyngeal samples of subjects with confirmed respiratory viral infections compared to healthy controls (100). An examination of the oropharyngeal microbiome of pneumonia patients with and without 2009 influenza A H1N1 pandemic viral infection showed that Firmicutes (which include *Staphylococcus* and *Streptococcus* spp.) and Proteobacteria (mainly *Pseudomonas amygdali*, *P. fluorescens*, *Pseudomonas* sp. UK4, *Acinetobacter baumanii* and *A. junii*)—were significantly enriched in patients with influenza (108). Another study of patients with 2009 pandemic H1N1 influenza infection revealed that the predominant phyla of the upper respiratory tract (nasal and nasopharyngeal samples) in patients harboring pandemic H1N1 were Actinobacteria, Firmicutes, and Proteobacteria although normal controls were not included; however, the authors suggested that flu is associated with an expansion of Proteobacteria (109) which is generally less abundant in healthy hosts. These findings are supported by another group who found that *Moraxella* and *Enterobacter* spp. (which are classified as Proteobacteria) were the most highly represented bacteria in nasopharyngeal samples obtained from patients with pandemic H1N1 influenza (110). However, these studies demonstrated that there was considerable inter-subject variability, highlighting the need for longitudinal studies to decipher changes following viral infection.

Investigators have also sought to determine whether specific viruses are consistently linked to enrichment of certain bacterial taxa. In the nasopharyngeal compartment of Aboriginal and non-Aboriginal children in Australia, positive associations were detected between hRV and *S. pneumoniae*, *H. influenza,* and *Moraxella catarrhalis* carriage as well as between adenovirus and *M. catarrhalis* (111). Another study examining the presence of 20 respiratory viruses by PCR panel and prevalence of bacterial carriage in the nasopharynx of children found a strong positive association between *S. aureus* colonization and influenza virus (112). Moreover, *S. pneumoniae* colonization was positively associated with the presence of hRV and enteroviruses; *H. influenzae* was positively associated with hRV and RSV; and *M. catarrhalis* colonization was positively associated with coronaviruses and adenoviruses. A 16s rRNA sequencing-based study conducted in infants with acute RSV or hRV respiratory infections reported that infants with RSV had significantly higher abundance of *Staphylococcus* spp. compared to hRV-infected

infants (113). An analysis of the URT bacterial content of 57 healthy asymptomatic individuals and 59 patients with influenza virus, parainfluenza, hRV, RSV, coronavirus, adenovirus, or metapneumovirus by culture-independent pyrosequencing revealed six distinct bacterial profiles—i.e., *Streptococcus + Prevotella + Veillonella*, *Streptococcus + Haemophilus + Neisseria*, *Streptococcus*, *Moraxella*, *Haemophilus*, and *Klebsiella*. These profiles, however, were not associated with virus type but were linked to the age of subjects (71).

Given that many human studies are cross-sectional in nature, it remains unclear whether post-viral bacterial pneumonias might be the result of viral infections enhancing bacterial colonization or acquisition, colonizing bacteria influencing host susceptibility to respiratory viral infections, or a combination of both. Another complicating factor particularly in cross-sectional studies examining the microbiome during viral infections is that the groups are not well-controlled and the sample numbers are relatively small considering the number of variables that could affect the respiratory tract microbiome–such as age, gender, oral hygiene and nose-picking habits, healthcare-based employment status, smoking status, medication use, exposure to small children, etc. The underlying type of viral infection, sampling timepoint after onset of infection, severity of infection, and concomitant antimicrobial usage are other confounding factors. This may underlie the highly variable and sometimes discrepant observations from microbiome studies in patients with viral infections.
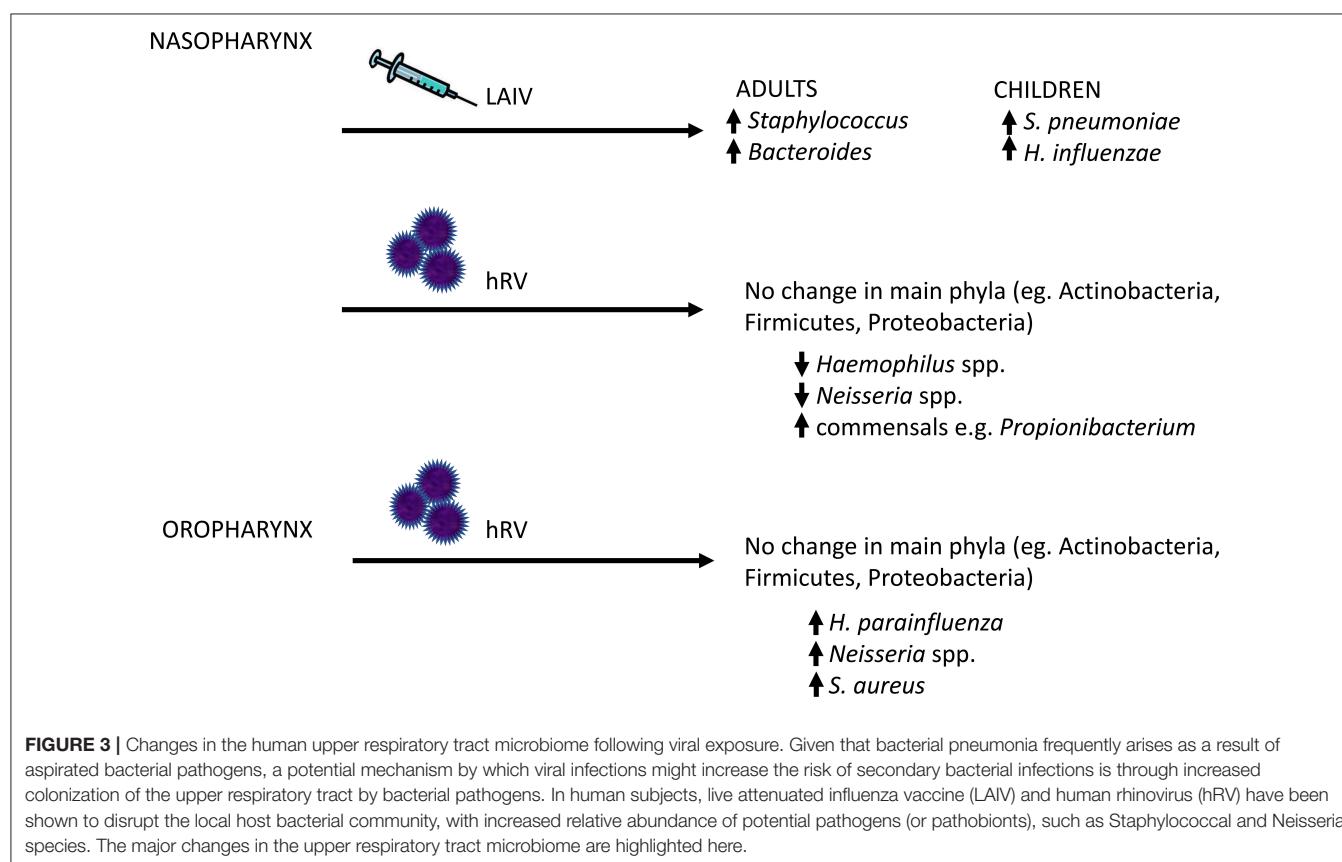
Longitudinal Studies

There have been few clinical studies comparing baseline pre- and post-infection microbiomes in otherwise healthy individuals with acute viral infections due to the difficulty of sampling before infection. However, the relatively few studies available provide insights into the dynamicity and stability of bacteria colonization patterns over time, and whether and how perturbations brought on by acute viral infections alter these patterns. In healthy children, the major phyla among nasopharyngeal microbiotas are Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, and Fusobacteria, with *Moraxella*, *Haemophilus*, *Streptococcus*, *Flavobacteria*, *Dolosigranulum*, *Corynebacterium*, and *Neisseria* as predominant genera. Changes in nasopharyngeal microbiome diversity were observed across seasons, with a predominance of *Proteobacteria* and *Fusobacteria* in fall-winter and *Bacteroidetes* and *Firmicutes* in spring; these differences were independent of recent antibiotics and viral co-infection (114). However, another analysis of two nasopharyngeal washes collected 5.5– 6.5 months apart from 40 children and adolescents with asthma showed no significant differences in nasopharyngeal microbiome diversity across seasons, although mean relative abundances of *Haemophilus*, *Moraxella*, *Staphylococcus*, and *Corynebacterium* varied significantly between summer and fall samples and between age groups. Moreover, in 87.5% of patients, operational taxonomic units (OTUs) in patients varied significantly between time points (115). An investigation of the frequency and seasonal variation in bacterial and viral load in asymptomatic healthcare professionals during the winter and summer months showed that of the 100 subjects

tested during the winter, 34 were colonized with at least one bacterial species and 11 tested positive for at least one virus. The most frequently detected pathogens were methicillin-resistant *Staphylococcus aureus* (MRSA), *M. catarrhalis*, and coronavirus. In contrast, of the 100 subjects tested during the summer, 37 harbored at least one bacterium (mainly MRSA and *K. pneumoniae*) and four tested positive for one virus (116).

Several larger scale surveillance studies of mainly pediatric populations have examined the natural temporal patterns in bacterial colonization during viral infections. One clinical investigation assessed the presence and density of *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* in the nasopharynx of children during URT infection and in the healthy state, and reported that the proportion of children colonized with these bacteria was higher during infection than during asymptomatic surveillance visits. Mean density of all bacterial species was significantly higher at each visit when a virus was detected. Interestingly, the percentage of colonized children and bacterial density were also higher at asymptomatic visits in which virus was detected than at those in which virus was not detected (117). Another study of 31 families with small children using longitudinal nasal swab sampling demonstrated that rhinovirus infection was associated with increased acquisition of *S. pneumoniae* from the community as well as increased transmission of *S. pneumoniae* within the family (118).

Other groups have examined the effects of experimental innoculation of hRV into the URT (nares) (**Figure 3**). These studies reported no significant changes in total read counts or of the main phyla (e.g., Actinobacteria, Firmicutes, and Proteobacteria) over time in nasopharyngeal samples (73) or throat swabs (119). In the oropharyngeal compartment, rhinovirus infection was associated with a strong trend toward transient increases in the relative abundances of *H. parainfluenzae*, *Neisseria subflava* and a weak trend toward an increase in *S. aureus* (119). By 60 days, abundance of these bacteria had returned to baseline. Nasopharyngeal sampling showed completely opposite results, with decreased relative abundance of *Haemophilus* and *Neisseria* spp., but an increase in the normal nasal commensal, *Propionibacterium*, in subjects following hRV infection (73). No differences in *Staphylococcus* were observed. However, the number of subjects were small in both studies, limiting the power to detect changes over time.

Nasopharyngeal microbiota composition has been shown to be altered by influenza vaccination (**Figure 3**). Administration of live attenuated influenza vaccine (LAIV), which is nasally instilled, to healthy children increased the nasal colonization density of *S. pneumoniae* in subjects who harbored this bacterium at the time of vaccination, and transiently increased rates of colonization by *H. influenza* (120). In healthy adult volunteers, it was demonstrated that intranasal LAIV administration induced an increase in the diversity of the nasopharyngeal microbiome,

FIGURE 3 | Changes in the human upper respiratory tract microbiome following viral exposure. Given that bacterial pneumonia frequently arises as a result of aspirated bacterial pathogens, a potential mechanism by which viral infections might increase the risk of secondary bacterial infections is through increased colonization of the upper respiratory tract by bacterial pathogens. In human subjects, live attenuated influenza vaccine (LAIV) and human rhinovirus (hRV) have been shown to disrupt the local host bacterial community, with increased relative abundance of potential pathogens (or pathobionts), such as Staphylococcal and Neisseria species. The major changes in the upper respiratory tract microbiome are highlighted here.

as well as relative abundances of *Staphylococcus* and *Bacteroides* (107). These changes were not observed in subjects given saline nasal spray. In a mouse model, bacterial density in the nasopharynx after LAIV administration was increased as much as 100,000 times compared to influenza-naive hosts, and the duration of carriage of *S. pneumoniae* or *S. aureus* was also increased 2 to 5-fold (121). However, systemic vaccination can also alter the URT microbiome. A longitudinal study of healthy subjects found a significant association between the presence of *Lactobacillus helveticus*, *Prevotella melaninogenica*, *Streptococcus infantis*, *Veillonella dispar*, and *Bacteroides ovatus* and influenza-specific H1 and H3 IgA antibody response (122). Thus, it is remarkable that a relatively mild viral stimulus such as flu vaccine can lead to detectable changes in the URT microbiome.

Although the data are still preliminary, animal studies have suggested that antiviral immune activation contributes to changes in the URT microbiome and facilitate colonization by potential pathogens, such as *S. aureus*. In a mouse model of *S. aureus* nasal colonization, the absence of type I IFN receptor was associated with decreased persistence of bacteria (107). Type III IFN, which is also induced during influenza infections, led to changes in the nasal microbiome, including increased numbers of culturable bacteria. Increased upper respiratory tract persistence of *S. aureus* as well as increased risk of *S. aureus* pneumonia was observed in flu-infected wildtype mice compared to mice lacking the type III IFN receptor (123). Currently, however, is it unclear to what extent viral-induced changes in the URT microbiome alter subsequent immune responses against secondary bacterial infections.

Studies of the LRT Microbiome During Respiratory Viral Infection

Compared to studies of the URT microbiome, studies of the LRT microbiome following viral infections are relatively scarce due to the difficulty of obtaining uncontaminated samples from the lung. Samples of convenience, such as sputum, suffer from oral contamination, but bronchoscopic samples are invasive and expensive to obtain on a regular basis. Moreover, it is unclear whether outside of patients with chronic lung disease (e.g., COPD), the lung microbial burden is of sufficient magnitude to exert robust effects on immune responses and risk of secondary bacterial infection during viral infection. Data from a mouse model of influenza infection seem to indicate that flu infection has only a modest effect on bacterial counts, diversity and composition of the lung microbiome (46). In subjects with chronic obstructive pulmonary disease (COPD) after hRV infection but not in healthy individuals, there was an increase in bacterial burden and growth of bacteria present at baseline, particularly *H. influenzae* (124). The researchers observed that the growth of bacteria seemed to arise from the existing community. *S. pneumoniae* intranasally inoculated into mice pre-infected with influenza virus first colonized the nose, followed by the trachea and lungs several days later with purulent inflammation. However, this effect was not observed in uninfected animals. This suggests that pneumococcal infection may sequentially develop from the URT to the LRT in influenza

virus-infected subjects (97). Thus, it is possible that some individuals with influenza infection might develop changes in their lung microbiome as a result of changes in their URT microbial communities.

Respiratory viruses not only alter the bacterial community in the URT, but also promote bacterial colonization of the LRT by a variety of mechanisms that impair bacterial clearance. First, mucus production in the respiratory tract is increased to facilitate viral clearance during infections. However, excessive mucus production can lead to airway obstruction by impeding mucociliary clearance (125). Second, viral infections can also reduce ciliary beat frequency and the number of ciliated cells, disrupt the coordinated movement of cilia, and impede the repair of respiratory epithelial cells, further leading to reduced mucociliary clearance (126, 127). Third, respiratory viral infections impair innate immune responses against bacteria (128–130). Innate immune cells including macrophages and neutrophils are recruited to the lung by cytokines and chemokines for phagocytosis and bactericidal activity. Prior viral infections dysregulate both alveolar macrophages (64, 131–136) and neutrophils (65, 128, 129), thereby inhibiting bactericidal activity. Thus, with multiple aspects of pulmonary host defense impaired, it would not be entirely surprising if a subset of influenza infected patients developed secondary bacterial pneumonia as a result of being unable to clear aspirated pathobionts from the URT.

INTERMICROBIAL INTERACTIONS AND POSTVIRAL SECONDARY INFECTIONS

In addition to enabling us to determine what is present during states of health, large-scale sequencing-based microbiome analyses have also revealed who is not present during disease. It has long been appreciated that mechanisms have evolved in bacteria that confer competitive advantages, permitting them to survive in an otherwise inhospitable host environment. However, interspecies competition also maintains homeostasis of the microbial community, either through their abilities to capture scare resources (e.g., iron), or targeted killing of other bacteria (e.g., bacteriocins), preventing one microbe from dominating the community. Thus, it is possible that the immune response incited by acute viral infections, changes in the host epithelial surface caused by the virus, or the virus itself might lead to elimination of a host commensal that is responsible for keeping pathobionts in check. For example, *S. epidermidis* and *Propionibacterium acnes* abundance in the nares has been shown to be negatively associated with *S. aureus* carriage (67). Understanding these interactions may create new avenues for therapeutic interventions aimed at reducing colonization by pathogenic bacteria during influenza epidemics or pandemics.

One group of commensals that has been examined for its role in inhibiting nasal carriage by *S. aureus* and *S. pneumoniae* is *Corynebacterium* spp. An early study in Japan reported on the effects of introducing a *Corynebacterium* strain into the nares of healthy adult hospital workers who were persistent carriers of *S. aureus*, with successful eradication in 71% of subjects
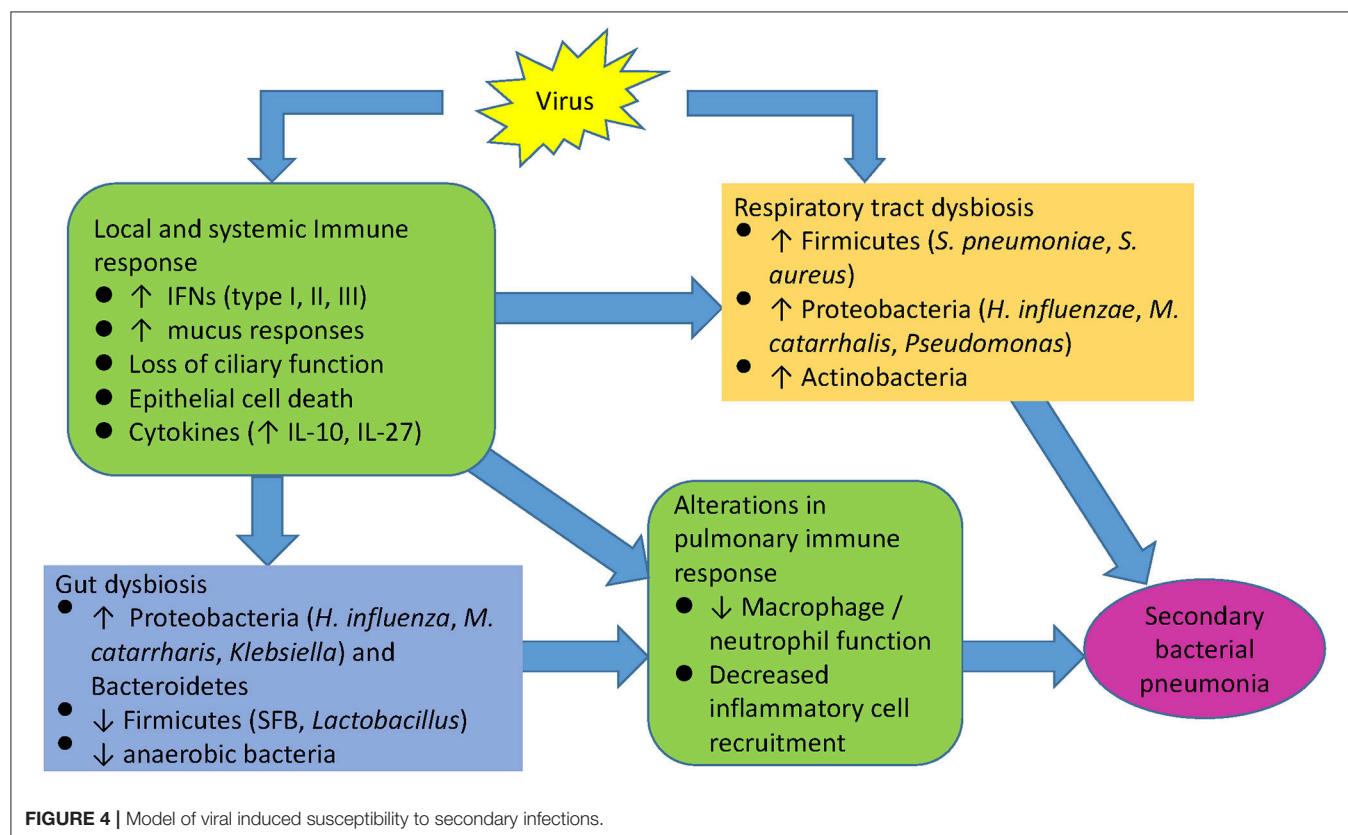
(137). The mechanism appeared to be bacteriocin-independent. In comparison, *S. epidermidis* implantation did not have an effect. Whether the *S. epidermidis* strain used expressed the serine protease Esp, which inhibits biofilm formation by *S. aureus* and nasal colonization (138), is unknown. Subsequent studies by another group reported that *C. pseudodiphtheriticum* inhibited *S. aureus* growth, whereas *C. accolens* and *S. aureus* appeared to support each other's growth (139). Conversely, other investigators observed that *Corynebacterium* spp. were enriched in children who were not nasally colonized with pneumococcus, and demonstrated that *C. accolens* inhibit *S. pneumoniae* growth *in vitro* by expressing a lipase that releases free fatty acids from skin surface triacylglycerols, which inhibit pneumococcal growth. Thus, painstaking identification and mechanistic interrogation of interspecies competition between commensals might lead to novel insights as to how viral infections might confer competitive advantage to pathobionts, and how to exploit natural strategies employed by commensals to restore homeostasis to the host microbial niche. Interestingly, a recent preclinical study using a murine model of RSV and *S. pneumoniae* superinfection employed nasal priming by a *C. pseudodiphtheriticum* strain to augment host defense against the viral infection, which enhanced clearance of secondary bacterial challenge and reduced lung injury measures (140).

Finally, direct effects of the infecting virus on bacteria that comprise the microbiome may facilitate the transition from pathobiont to pathogen. A metagenomic analysis showed that pH1N1-associated airway microbiotas were enriched in genes associated with cell motility, transcriptional regulation, metabolism, and response to chemotaxis compared to the same bacteria in non-infected patients (108). These data imply that influenza infection perturbs the respiratory microbiome, leading to the production of secondary metabolites including immune-modulating molecules. Viruses have also been found to impair bacterial biofilm formation and disrupt existing biofilm (141–144). Influenza has been shown to affect the *S. pneumoniae* transcriptome in terms of downregulating expression of genes associated with the colonizer state and upregulations of bacteriocins (142). Thus, direct effects of viruses on bacterial transcriptional patterns might be a mechanism by which colonizing bacteria acquire invasive potential, thereby leading to bacterial superinfections.

FUTURE DIRECTIONS

There are several areas that must be addressed by future respiratory microbiome research. First, it is necessary to standardize protocols used to analyze the respiratory microbiome, including sampling, processing, and bioinformatics methodologies. For example, sputum may be an appropriate material for investigations of respiratory diseases since it contains components of the LRT and can be obtained easily. However, more reliable information on the LRT requires invasive samples such as BAL or protected specimen brush

FIGURE 4 | Model of viral induced susceptibility to secondary infections.

or bronchial/lung biopsies. Second, most studies are limited to experiments conducted in animal models. Even in human studies, most analyses have been performed in a small number of patients and have described bacterial communities in the URT. The role of microbial communities outside of the lungs including gut, sinus, and skin should be considered in the context of airway diseases. Third, most studies on the microbiome have focused on the bacterial component, and have largely omitted fungi and viruses. The role of viruses—including the vast number of phages that infect bacteria—and fungi in respiratory diseases cannot be examined through 16S rRNA gene analyses, and there are no studies describing the composition and role of the respiratory virome due to the difficulty of comprehensive analyses for viruses. Fourth, it is not sufficient to study microbial communities based on species composition; a functional characterization through transcriptome and proteasome analyses is necessary to understand mechanistic role of microbiome on outcomes of infection. Finally, mucosal microbiome manipulations by vaccines, antibiotics, and probiotics in the gastrointestinal and respiratory tract niches represent novel approaches for the prevention, treatment, and management of acute and chronic lung diseases. However, given that antibiotic therapy could affect commensal bacteria and hasten the emergence of drug-resistant bacteria, more research is needed on the long-term effects of this therapy. Animal models should be developed to study the influence of the URT and LRT microbiomes on immune responses to respiratory viral infections; only then will it be possible to consider the clinical application of microbiome modulation strategies.

CONCLUSION

Respiratory viral infections can initiate a cascade of host immune responses that alter microbial growth conditions in the URT, LRT, and the gut (**Supplemental Table 1**). Activation of influenza-induced antiviral interferon pathways can lead to inadequate innate immune cell responses during host defense against secondary bacterial infections, resulting in the proliferation of

potentially pathogenic bacterial species. Concomitant changes in the gut microbiome caused by the initial viral infection may also alter immune cell priming against secondary bacterial challenge, although this has not been examined to date. Although the picture is incomplete, recent microbiome literature provides additional insights into the pathogenesis of dysregulated immune responses following acute viral infections, that may promote the development of secondary bacterial pneumonias (**Figure 4**). Clarifying the differences and dynamics of respiratory microbiota in healthy subjects and chronic lung diseases during acute respiratory viral infections can elucidate pathogenesis of viral-bacterial interactions and provide a basis for developing novel approaches for the prevention, treatment, or management of acute respiratory infection and exacerbation of chronic lung diseases.

AUTHOR CONTRIBUTIONS

SH and JD co-wrote the manuscript. SH, JD, and MP designed the figures and table. KC and MP edited and provided critical revisions of the manuscript. All authors approve the final version and agree to be accountable for the content of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02640/full#supplementary-material

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