In the respiratory tract, viruses and bacteria can interact on multiple levels. It is well known that respiratory viruses, particularly influenza viruses, increase the susceptibility to secondary bacterial infections. Numerous mechanisms, including compromised physical and immunological barriers, and changes in the microenvironment have hereby been shown to contribute to the development of secondary bacterial infections. In contrast, our understanding of how bacteria shape a response to subsequent viral infection is still limited. There is emerging evidence that persistent infection (or colonization) of the lower respiratory tract (LRT) with potential pathogenic bacteria, as observed in diseases like chronic obstructive pulmonary disease or cystic fibrosis, modulates subsequent viral infections by increasing viral entry receptors and modulating the inflammatory response. Moreover, recent studies suggest that even healthy lungs are not, as had long been assumed, sterile. The composition of the lung microbiome may thus modulate responses to viral infections. Here we summarize the current knowledge on the co-pathogenesis between viruses and bacteria in LRT infections.

Epidemiology and relevance of respiratory co-infections

Co-infections of the lower respiratory tract (LRT) with viruses and bacterial pathogens are commonly observed during severe acute infections and in the course of chronic respiratory diseases. In acute conditions, such as community-acquired pneumonia (CAP), mixed infections were detected in up to 27% of all cases in which a pathogen could be identified (Bello et al., 2014). During exacerbations of chronic obstructive pulmonary disease (COPD), potential pathogenic bacteria and viruses have been found simultaneously in 12–25% of the cases (Bafadhel et al., 2011; Papi et al., 2006). Since many studies used conventional culturing techniques for detecting bacterial pathogens, rather than more sensitive molecular methods, the true prevalence of mixed infections might even be underreported.

It is increasingly recognized that the simultaneous presence of bacteria and viruses can affect the course and severity of infections. Mixed infections have been found to be associated with increased levels of inflammatory biomarkers such as procalcitonin and C-reactive protein during CAP (Bello et al., 2014) and have moreover been linked to more severe exacerbations of COPD (MacDonald et al., 2013; Papi et al., 2006; Wilkinson et al., 2006). Probably the most devastating example for a lethal viral/bacterial synergism is the 1918/1919 influenza pandemic, known as the 'Spanish flu'. Most deaths during this largest influenza pandemic of the 20th century are now believed to have been a consequence of complications arising from secondary bacterial infections, for example with *Streptococcus pneumoniae* or *Staphylococcus aureus*, rather than from the virus alone (Morrens et al., 2008).

The mechanisms leading to secondary bacterial infections following antecedent viral infections have been extensively investigated. In this context, particularly the co-pathogenesis of influenza viruses with bacterial infections has been extensively investigated and has recently been reviewed by McCullers (2014). In contrast, the role of other viruses, as well the possible consequences of a preceding bacterial exposure, e.g. in the form of acute and chronic infections or as part of the microbiome, for secondary viral infections are less well understood. This review gives an overview of the current knowledge on the mechanisms underlying bacterial–viral co-infections of the respiratory tract, in either order of infection.
Susceptibility and response to bacterial infections following primary viral infections

Viral infections have been implied to affect the risk and outcome of bacterial infections via a number of mechanisms. Broadly, these concern the epithelial barrier function and binding of bacteria to the epithelium, innate and adaptive immune response and the microenvironment of the LRT. These aspects will be discussed in more detail below and are summarized in Fig. 1.

Impairment of mucociliary clearance

The LRT is constantly exposed to small numbers of microbes from the environment and the upper respiratory tract, yet these bacteria usually do not persist in the LRT in large numbers. Under normal conditions, inhaled particles and infectious agents are trapped in mucus produced by goblet cells and cleared from the airways by the coordinated movement of cilia on epithelial cells.

During viral infections of the LRT, the production of mucus is increased in order to facilitate viral clearance; excessive mucus production, however, may contribute to airway obstruction and impede mucociliary clearance (Vareille et al., 2011). Viral infection can moreover cause a reduction of ciliary beat frequency, uncoordinated ciliary movement and a reduction in the number of ciliated cells (Chilvers et al., 2001; Smith et al., 2014a; Tristram et al., 1998). Given the importance of the mucociliary escalator for bacterial clearance, it is likely that reduced ciliary function hampers bacterial clearance from the LRT.

Enhancement of bacterial binding

Viral infections can augment bacterial attachment and thus help to establish bacterial infections. Increased attachment of bacteria to virus-infected cells and tissue has been observed for numerous combinations of respiratory pathogens, some of which will be discussed in more detail below (Avadhanula et al., 2006; Golda et al., 2011; Hakansson et al., 1994; Hament et al., 2004; Jiang et al., 1999; Raza et al., 1993; Sanford & Ramsay, 1987; Wang et al., 2009). Generally, bacterial attachment can be enhanced by (1) increased expression of host receptors after viral infection or (2) by viral structures serving as coupling agents for bacteria to host tissues.

Receptor expression.

Among the host molecules upregulated on airway epithelial cells upon viral infection, the platelet activating factor receptor (PAFR) has been of particular interest. PAFR can serve as an attachment molecule for S. pneumoniae, one of the pathogens complicating influenza infection, as well as for other phosphorylcholine-positive bacteria (Suri et al., 2014). Moreover, PAFR expression is increased by...
Inflammatory cytokines like TNF-α (Hinojosa et al., 2009), in COPD lungs and after exposure to cigarette smoke (Shukla et al., 2016; Suri et al., 2014). Virus-induced upregulation of PAFR and concomitantly increased binding of pneumococci and/or non-typeable Haemophilus influenzae has been reported after infection with influenza virus (van der Sluijs et al., 2006), human rhinovirus (HRV) (Ishizuka et al., 2003), respiratory syncytial virus (RSV) and parainfluenzavirus (Avadhanaula et al., 2006; Yokota et al., 2010). While Avadhanaula et al. (2006) found neutralizing PAFR binding sites to reduce bacterial binding to RSV-infected lung epithelial cells in vitro, the receptor’s relevance in vivo during other viral infections is debated. Although pharmacological inhibition of PAFR did not reduce mortality in a mouse model of co-infection with influenza and pneumococci, it modestly delayed mortality and clinical onset (McCullers & Rehg, 2002). A study from the same group, this time employing PAFR knockout mice, even found increased mortality in co-infected PAFR−/− mice compared with wild-type animals (McCullers et al., 2008). In contrast, others reported reduced bacterial dissemination into the circulation and decreased mortality in PAFR−/− mice sequentially infected with influenza virus and S. pneumoniae (van der Sluijs et al., 2006). Yet, the low survival rate and the severity of the pneumococcal infection model make a potential synergism of influenza virus and pneumococci in this study difficult to appreciate. These diverging findings, added to the fact that inhibition or deletion of PAFR cannot entirely rescue the increased susceptibility to secondary pneumococcal infection, indicate that co-pathogenesis of influenza viruses and pneumococci is multifaceted.

Similar to PAFR, viral infection also changes the expression of other transmembrane and extracellular matrix proteins such as intercellular adhesion molecule (ICAM-1) or fibronectin. Influenza neuraminidase can activate latent transforming growth factor (TGF-β), which then mediates an increase in the expression of α-integrins and fibronectin (Li et al., 2015). Bacteria that bind the α-integrin/fibronectin complex, such as H. influenzae, S. aureus and S. pneumonieae, can then increasingly attach to the respiratory epithelium. As shown by Passariello et al. (2006), the effects of viral infection on receptor-mediated binding to epithelial cells can be even more nuanced: although HRV infection did not increase the adherence of S. aureus to lung epithelial cells, it increased the efficiency with which bacteria were internalized.

**Binding of bacteria to viral proteins displayed on the host cell.** Next to increased expression of host receptors, viral proteins displayed on the cell surface can enhance adherence and internalization of bacteria. The RSV attachment glycoprotein (G) protein, present on the surface of either RSV virions or infected cells, can serve as a binding structure for non-typeable H. influenzae (Avadhanaula et al., 2007), S. pneumoniae (Avadhanaula et al., 2007; Hament et al., 2005) and Pseudomonas aeruginosa (Van Ewijk et al., 2007). Similarly, integration of influenza virus haemagglutinin into the cell membrane of infected cells facilitates attachment and invasive disease of group A streptococci in mice (Okamoto et al., 2003). On top of this, the binding of fibrinogen to cells infected with influenza A, and the modulation of several surface molecules in influenza-infected cells is thought to aid the binding and invasion of group A streptococci (Hafez et al., 2010; Sanford et al., 1982).

**Compromising the epithelial barrier function**

Death of airway epithelial cells due to viral replication, excessive inflammation and/or loss of repair functions can damage the integrity of the airway epithelium (Herold et al., 2008; Hinshaw et al., 1994; Kash et al., 2011; Schultz-Cherry et al., 2001; Zeng et al., 2013). One consequence of this compromised natural barrier can be invasive bacterial infection.

Additionally, viruses can compromise the integrity of the epithelial layer even in the absence of virus-induced cell death. Sajjan et al. (2008) showed that infection of polarized airway epithelial cells with HRV can lead to redistribution of the tight junction protein zona occludens 1 (ZO-1) from the membrane to the cytoplasm. Furthermore, HRV was shown to impair the repolarization of airway epithelium regenerating after injury (Faris et al., 2016). Integrity of the epithelial barrier likewise is reduced after RSV infection (Kilani et al., 2004). Increased release of vascular endothelial growth factor (VEGF) during infection hereby causes gap formation between cells and thus decreased transepithelial resistance, a surrogate marker for epithelial barrier function in vitro. Although the authors of this study did not investigate to what extent this reduced barrier function affects secondary bacterial infections, it is likely that bacterial transmigration is facilitated.

**Immunological aberrances during post-viral secondary bacterial infection**

Increased bacterial binding and reduced barrier function are important steps in initiating a secondary bacterial infection. In combination with aberrant immune responses due to viral infections, these can have devastating consequences. Immunologically, viruses alter the susceptibility and responsiveness to bacteria on several levels of innate and adaptive immunity.

**Interference with pathogen recognition receptor (PRR) expression and signalling**

Sensing of conserved microbial structures by PRRs, such as TLRs, is required for quick and efficient mounting of the innate immune response. PRRs recognize diverse structures, yet the pathways triggered upon receptor activation overlap and culminate in the activation of pro-inflammatory transcription factors (Kawai & Akira, 2011). To prevent over-activation, several feedback mechanisms are in place, which can induce a refractory state to successive
stimuli (Nahid et al., 2011; Neagos et al., 2015; de Vos et al., 2009). If cells encounter multiple pathogens, this can lead to a lack or delay in the response to secondary infection. Macrophages from influenza- or RSV-infected mice, for example, are hypo-responsive to subsequent stimulation with bacteria or bacterial mimics (Didierlaurent et al., 2008). Astonishingly, this desensitization can be sustained for weeks to months after the virus has been cleared. As a consequence, neutrophil recruitment and bacterial clearance were found to be severely impaired in this study.

Similarly, HRV infection can blunt the TLR-dependent response to bacterial challenge by causing degradation of IRAK-1 (interleukin 1 receptor associated kinase), an adapter kinase required for MyD88-dependent signaling (Unger et al., 2012). This process renders pulmonary epithelial cells less responsive to subsequent infection with non-typeable H. influenzae and delays the innate response and bacterial clearance. Because of the prolonged presence of bacteria, the inflammatory response – once initiated – persists for a longer period.

**Immune cell function**

As detailed below, viral infection can profoundly alter the number and function of immune cells in the lung, further delaying or hampering bacterial clearance.

**Alveolar macrophages (AMs).** AMs constantly sample the alveolar lumen for foreign particles and not only serve in bacterial clearance, but also secrete numerous cytokines and chemoattractants to recruit immune cells to the site of infection (Werner & Steele, 2014). In a murine model of post-influenza pneumococcal pneumonia, Ghoneim et al. (2013) observed an almost complete depletion of alveolar, but not interstitial, macrophages following influenza infection. This effect was temporal, and interestingly, the highest susceptibility to pneumococcal infection was in the time frame of lowest numbers of AMs. Once the AM population was restored, the susceptibility to pneumococcal infection was indistinguishable to that of mice not infected with influenza. Next to a depletion of AMs, influenza virus infection might also impair the cells' phagocytic capacity (Jakab et al., 1980; Warshauer et al., 1977). However, other studies found no such impairment (Nugent & Pesanti, 1979) or only impaired uptake of zymosan particles, but not of bacteria (Wang et al., 2012).

Unlike influenza, RSV and HRV do not or only limitedly infect macrophages (Franke-Ullmann et al., 1995; Gern et al., 1996), yet they can significantly influence number and function of AMs. Impaired phagocytic capacity and/or cytokine secretion after stimulation with bacterial products have been reported for AMs and monocyte-derived macrophages following exposure to HRV and RSV (Arre villaga et al., 2012; Franke-Ullmann et al., 1995; Oliver et al., 2008; Raza et al., 2000).

**Neutrophils.** In contrast to AMs, neutrophils are not tissue resident and need to be recruited from the bloodstream to the site of infection (Werner & Steele, 2014). This recruitment requires adequate recognition of pathogens and chemokine secretion by epithelial cells and macrophages. A desensitization of AMs to pathogen-associated molecular patterns (PAMPs) after viral infection (discussed above) might therefore also reduce neutrophil recruitment. Mouse models of secondary bacterial infections, however, point to a functional impairment of neutrophils rather than solely a lack of recruitment. Impaired bacterial clearance in influenza- or RSV-infected mice appeared to be related to decreased activity of the antimicrobial enzyme myeloperoxidase and decreased production of reactive oxygen species (LeVine et al., 2001; McNamie & Harmsen, 2006; Stark et al., 2006). Additionally, increased levels of IL-10, as observed during post-influenza pneumococcal pneumonia, are thought to negatively affect neutrophil function (van der Sluijs et al., 2004). Moreover, increased rates of neutrophil apoptosis have been observed after co-infection of neutrophils with influenza and pneumococci in vitro (Colamussi et al., 1999; Engelich et al., 2001).

Another antimicrobial property of neutrophils is their ability to sequester pathogens in neutrophil extracellular traps (NETs), which consist primarily of histones and DNA, but also contain antimicrobial peptides (AMPs) (Kaplan & Radic, 2012). Infection of neutrophils with RSV or influenza was found to enhance NET formation (Cortjens et al., 2016; Narasaraju et al., 2011). But whereas superinfection of influenza-infected mice with S. pneumoniae has been shown to further increase the formation of NETs, these did not confer protection against bacteria, due to partial degradation and loss of antibacterial activity (Narayana Moorthy et al., 2013). Prominent respiratory pathogens like S. pneumoniae and S. aureus have furthermore developed strategies to escape NET function (Beiter et al., 2006; Berends et al., 2010), and in the context of co-infection, increased NET formation might even contribute to airway obstruction, hamper clearance mechanisms and aggravate tissue damage, ultimately worsening the progression of secondary bacterial infections.

**NK cells.** NK cells can recognize viral structures on infected cells and respond to stress signals released by the infected host. They then contribute to antiviral immunity by killing infected host cells, regulating T-cell function and secreting interferon (IFN-γ) (Hesker & Krupnick, 2013). NK cells additionally contribute to clearance of pneumococcal infection through interaction with infected macrophages and dendritic cells (Elhaik-Goldman et al., 2011; Hesker & Krupnick, 2013; Mandelboim et al., 2001). Decreased numbers of NK cells in the lung and impaired NK cell function after influenza infection of mice have been shown to impair innate responses against S. aureus infection by contributing to a lack of activation of AMs (Small et al., 2010). Impairment of NK cell function has been observed for numerous other viruses (Ma et al., 2016), although it is
unclear to what degree this has consequences during sequential infections of the respiratory tract.

**Dendritic cells (DCs).** As professional antigen presenting cells, dendritic cells (DCs) are at the interface between innate and adaptive immunity in the lung, where different DC subsets have distinct functions (Guilliams et al., 2013; Hasenberg et al., 2013).

Co-infection of mice with influenza virus and *Mycobacterium bovis* has been found to reduce the expression of MHC class I and II on DCs (Flórido et al., 2013). This, in turn, impaired the generation of CD4+ and CD8+ T-cell responses against the bacterium. Alternatively, in human monocyte-derived DCs (moDCs), influenza virus has been shown to increase TLR3 expression (Spelmink et al., 2016). Although TLR3 is classically associated with the recognition of viral structures (dsRNA), the authors of this study showed that DCs also use TLR3 to recognize pneumococcal RNA. In addition, the authors reported increased expression of IL-12p70 (Spelmink et al., 2016). Since IL-12 is an important driver of T-helper (TH1) cell differentiation, skewing of the TH cell population might contribute to the outcome of co-infections.

Interference with DC function is not unique to infection with influenza virus. RSV has been reported to render moDCs less efficient in inducing CD4+ T-cell proliferation and cytokine secretion by inducing the release of a soluble mediator that is yet to be identified (de Graaff et al., 2005). Although co-infections were not studied here, the secretion of a soluble mediator affecting T-cell responses could have implications for heterologous infections as well.

**Impairment of T-cell response.** During viral infection, T-cells have numerous functions, including the killing of virus-infected cells by cytotoxic T-cells, and the activation and control of various immune cells by TH cells. The role of T-cells during respiratory infections has been extensively reviewed by Chiu & Openshaw (2015). Several T-cell subsets have been implicated in aberrant immune responses during post-influenza bacterial pneumonia. Blevins et al. (2014) found that co-infection of influenza-infected mice with *S. pneumoniae* affects the ongoing T-cell response to the virus not only by reducing the number of virus-specific CD8+ T-cells in the lungs, but also by reducing their cytokine production. Likewise, Wu et al. (2015) showed the CD4+ T-cell population to be reduced during post-influenza pneumococcal infection. Moreover, influenza virus infection has been demonstrated to impair the release of IL-17 by TH17 cells and IL-17-producing γδ T-cells during secondary infection of mice with bacteria (Kudva et al., 2011; Li et al., 2012). This aberrant production has been attributed to deficient IL-23 production by DCs (Kudva et al., 2011) and to suppressive effects of type I IFNs released during primary viral infection (Li et al., 2012).

CD4+ and CD8+ T-cells can additionally contribute to impaired initial clearance of bacteria by AMs by releasing type II IFN (IFN-γ). AMs respond to IFN-γ with decreased levels of the scavenger receptor MARCO, which is required for uptake of non-opsonized pneumococci (Sun & Metzger, 2008). Since IFN-γ is a common response to viral infection, its inhibitory effects on antibacterial pathways are probably not restricted to influenza virus infection, even though confirmation for other combinations of viruses and bacteria is pending.

Taken together, there is compelling evidence for virus-induced impairments of the number and/or function of the most important immune cell subsets in the lung. These deficiencies take place on multiple levels and span cells of both the innate and adaptive immune system, as well as interactions between both.

**Impairment of antibody-mediated immunity**

The involvement of antibody-mediated immunity during heterologous infections of the respiratory tract is largely unknown, and outcomes of experimental studies are divergent. Whereas Wolf et al. (2014) described increased levels of virus-specific IgG in a rather mild murine model of post-influenza bacterial pneumonia, others found a severe impairment of IgG, IgM and IgA production in co-infected mice (Wu et al., 2015). These contrasting findings have mainly been explained with differences in the experimental models (sub-lethal vs lethal co-infection). The discrepancies of these studies also highlight the influence of the choice of experimental protocols and the need for further investigation to elucidate the precise contribution of antibody-mediated immunity.

**Antimicrobial peptides**

Antimicrobial peptides (AMPs) can be produced by most cell types of the lungs, including epithelial cells, macrophages and neutrophils, and are effective against bacteria, fungi and viruses (Lecaillé et al., 2016). Expression of some AMPs can be induced by infectious and inflammatory stimuli, which prompted researchers to investigate their role in secondary infections. Experimental infection of COPD patients with rhinovirus significantly increased the incidence of bacterial infections (Mallia et al., 2012). In those COPD patients who also developed a secondary bacterial infection, sputum levels of the protease inhibitors SLPI (serine leukocyte peptidase inhibitor) and elafin were decreased. Interestingly, this decrease preceded peak bacterial loads, suggesting an involvement in the emergence of secondary bacterial infections. Co-infection models of the upper and lower respiratory tract with bacteria and, e.g. RSV or influenza, support a role for the deregulation of AMP production in facilitating secondary bacterial infection, yet the relative contribution of different AMPs and cell types may vary per pathogen (Lee et al., 2015; McGillivary et al., 2009; Robinson et al., 2014).
Cytokines and other secreted mediators

During viral infection, production of type I and III IFNs leads to expression of IFN-stimulated genes, which in turn establish an antiviral state. While crucial for viral clearance, the release of (particularly type I) IFNs appears to blunt several antibacterial responses by suppressing the expression of AMPs, macrophage and/or neutrophil recruitment and TH17 responses (Kudva et al., 2011; Lee et al., 2015; Nakamura et al., 2011; Shahangian et al., 2009). In contrast, exogenous IFN-β was found to inhibit bacterial transmigration and thus to protect mice from developing bacteremia after instillation of S. pneumoniae, suggesting an ambiguous role for type I IFNs during (secondary) bacterial infection (LeMessurier et al., 2013).

Increased release of the anti-inflammatory cytokine IL-10 (along with a number of pro-inflammatory cytokines) during co-infection of mice with influenza virus and pneumococci has been implicated in an increased susceptibility to pneumococcal pneumonia. Neutralization of IL-10 reduced lethality in co-infected mice, suggesting a prominent role for this cytokine in modulating anti-pneumococcal responses (van der Sluijs et al., 2004).

In mice, increased levels of glucocorticoids following influenza infection have been linked to decreased control of Listeria monocytogenes during systemic bacterial infection (Jamieson et al., 2010). Although surgical removal of the adrenal gland could initially reverse the increased bacterial burden in co-infected animals, these glucocorticoid-deficient mice, in contrast to sham-treated mice, eventually succumbed to co-infections, revealing an ambiguous role for glucocorticoids during co-infections.

Overall, viral infections can influence the immune response to subsequent bacterial infection on virtually all stages. These immunomodulatory effects span pathogen recognition, innate as well as adaptive immune responses, and cellular functions and secretion of soluble mediators alike.

Other mechanisms

Immunosuppressive strategies of pathogens. Many pathogens have evolved strategies to avoid the recognition and clearance by their host's immune system. Such immune evasion largely interferes with recognition and the early, i.e. innate, immune response. Considering that innate signaling responses to viruses and bacteria overlap, it is not surprising that immune evasion strategies not only affect immunity against the primary pathogen, but also extend to secondary infections.

Known suppressors of innate immunity are the RSV G protein or non-structural (NS) proteins of influenza, RSV and other respiratory viruses (Arnold et al., 2004; Liu et al., 2016; Polack et al., 2005; Zheng et al., 2015). In vitro, the RSV G protein suppresses the inflammatory response of several cell types to components of the virus itself, but also dampens monocyteic release of IL-6 and IL-1β upon stimulation with endotoxin. Influenza NS1 suppresses caspase-1 activity, which is required for the release of IL-1β and IL-18 (Stasakova et al., 2005). Although the authors of this study did not investigate the consequences of virus-induced caspase-1 inhibition on a heterologous infection, previous studies have demonstrated a vital role of the caspase-1 system during bacterial infection (reviewed, for example, by Netea et al., 2010), which suggests relevance for viral bacterial co-pathogenesis. Overall, the immunosuppressive effect of NS1 extend to the expression of multiple cytokines, likely contributing not only to impaired viral clearance, but also to impaired responses to unrelated pathogens (Fernandez-Sesma et al., 2006). Additionally, the genome of most influenza A isolates codes for the accessory protein PB1-F2, a cytoxin that worsens the outcome of secondary bacterial infections with S. aureus and S. pneumoniae (Iverson et al., 2011; McAuley et al., 2007).

Availability of nutrients and change of the microenvironment. By inducing changes in the microenvironment, viruses can change bacterial growth patterns. Influenza neuraminidase cleaves sialic acid from sialylated airway mucins enabling pneumococcal strains that are able to metabolize these sugars to increase their rates of division (Siegel et al., 2014). Moreover, elevated temperature and extracellular ATP occurring during viral infection can trigger the release of pneumococci from biofilms and induce changes in the bacterial transcriptome, associated with improved bacterial stress responses, altered metabolism and increased virulence (Marks et al., 2013; Pettigrew et al., 2014). Viral infections differentially impact the formation and maintenance of P. aeruginosa biofilms: while the release of extracellular iron and transferrin stimulates biofilm formation after RSV infection, the release of hydrogen peroxide during HRV infection triggers the release of P. aeruginosa and facilitates transmigration of bacteria through the epithelial layer and might thus contribute to the dissemination of infection (Chattoraj et al., 2011b; Hendricks et al., 2016).

Influence of the bacterial microbiome and bacterial exposures on viral infection

The presence of bacteria in the LRT was long considered to be an abnormality associated with underlying chronic lung diseases such as COPD, asthma or cystic fibrosis (CF). As molecular techniques evolved, it became clear that conventional, culture-based techniques missed a substantial part of the picture. Although bacterial loads decrease progressively from upper to lower respiratory tract (Charlson et al., 2011), bacterial genetic material has also been recovered from the lungs of healthy individuals, leading to a reassessment of the paradigm of 'sterile' lungs.

Beneficial effects of a healthy respiratory microbiome

The notion of a beneficial, healthy bacterial microbiome is well established for the resident microbial community in the

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gastrointestinal tract, where resident bacteria aid in establishing a balanced immunological phenotype, compete with ‘invading’ (and potentially harmful) micro-organisms and synthesize a variety of beneficial biomolecules, such as vitamins (reviewed, for example, by Kamada et al., 2013). Although the bacterial load and species diversity in the respiratory tract is substantially lower, similar beneficial effects of a healthy pulmonary microbiome are under investigation, and first studies suggest a role for colonizing bacteria in shaping the immune response to subsequent infection (Fig. 2).

Excessive inflammation following viral infection significantly contributes to airway pathology, thus a tolerance-inducing microbiome might aid in limiting tissue damage. Colonization of the upper respiratory tract with commensal bacteria has been shown to drastically reduce influenza-induced acute lung injury and mortality in mice by recruiting a CCR2⁺CD11b⁺ monocyte subset into the lungs and inducing a M2 macrophage phenotype (Wang et al., 2013). Strikingly, an intact microbiome has also been shown to be required for the formation of an adaptive response against influenza virus in mice (Ichinohe et al., 2011). Mice treated with antibiotics before influenza virus infection displayed lower antibody and T-cell responses to the virus, thereby impairing virus clearance. Of note, this effect was not due to a general immune suppression, but specifically impaired adaptive immune responses that require priming by an inflammasome – such as those to infection with influenza viruses. Since the effects of antibiotic treatment on bacterial communities are systemic and not restricted to the respiratory tract, the relative contribution of the respiratory microbiota to these findings is unclear.

**Bacterial pathogens influencing the response to viral infection**

The few clinical studies that investigated the interplay between bacterial colonization with potentially pathogenic micro-organisms (PPMs) and viral infections of the respiratory tract, however, mainly relied on samples of the nasopharynx to determine microbiota composition, which are not only significantly easier to obtain but also represent the

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**Fig. 2.** Effects of primary bacterial exposure on the response to respiratory viral infection.
main entry route for respiratory viruses. The most comprehensive studies on nasopharyngeal colonization and the risk and severity of viral infections have been performed in infants with RSV infection (de Steenhuijsen Piters et al., 2016; Suárez-Arrabal et al., 2015). Children suffering from RSV bronchiolitis were found more likely to be colonized with PPMs. Moreover, the predominance of certain bacterial clusters could be linked to the severity of RSV bronchiolitis and to the host response, particularly to inflammatory factors that makes patients more susceptible to both types of infections. If a causality exists, a shift in the nasopharyngeal microbiota caused by vaccinations against respiratory pathogens would have implications for unrelated infections as well (Biesbrock et al., 2014; Tarabichi et al., 2015). Several studies have reported a persistent presence of PPMs in the LRT of individuals with chronic pulmonary disease, most prominently COPD (Cabello et al., 1997; Monsó et al., 1999; Zalacain et al., 1999). Likewise, microbiome analyses have revealed a change in microbial communities in the LRT including a reduction in microbiome diversity and an increased abundance of PPMs in subjects with COPD (Erb-Downward et al., 2011; García-Nuñez et al., 2014). Clinical studies suggest an association between the presence of PPMs in the stable state of COPD and chronic inflammation, a more rapid decline in lung function and an increased risk for the development of acute exacerbations (reviewed, for example, by Mohan & Sethi, 2015). Whether chronic inflammation is a consequence of a afflicted microbiome or vice versa remains to be investigated. Next to morbidity associated directly with chronic bacterial infections, microbiome composition might also be linked to an altered risk for (or outcome of) viral infections, which themselves are considered important triggers of acute exacerbations (Fig. 2). Exacerbations during which PPMs and viruses were detected simultaneously were found to be on average more severe (MacDonald et al., 2013; Papi et al., 2006; Wilkinson et al., 2006). Due to the design of these studies, it cannot be said with certainty whether these events represent a viral super-infection on top of bacterial colonization of the LRT, yet this scenario appears to be more likely than a simultaneous acquisition of two acute infections.

The co-pathogenesis of chronic bacterial and acute viral infections might be explained by (1) a common underlying factor that makes patients more susceptible to both types of pathogens, e.g. compromised immune functions or increased exposure, (2) an increased risk to acquire a viral infection, e.g. due to the up-regulation of viral entry receptors, or (3) an altered response to viruses in patients with chronic exposure to pathogenic bacteria, e.g. by synergistic effects on inflammation or tissue damage. However, mechanistically, the question of how bacterial colonization or chronic bacterial infection of the lungs affects the susceptibility to (and outcome of) subsequent viral infections has rarely been addressed. The interplay between bacteria classically associated with chronic lung diseases, such as H. influenzae or P. aeruginosa, and respiratory viruses hereby served as models.

H. influenzae is the most commonly found bacterial species in the lungs of COPD patients, being cultured from samples of about 25% of all patients during stable disease (Cabello et al., 1997; Monsó et al., 1999; Zalacain et al., 1999). Exposure of respiratory epithelial cells to H. influenzae increases the expression of ICAM-1, the main receptor for major group HRV. By doing so, the bacteria increase viral binding and replication of this type of virus in vitro (Gulraiz et al., 2015; Sajan et al., 2006). Moreover, we have recently shown that H. influenzae and RSV can synergize in inducing the release of pro-inflammatory cytokines by respiratory epithelial cells (Bellinghausen et al., 2016). On the other hand, TLR3 expression, and consequently recognition of viral structures and antiviral type I/III IFN production, was shown to be impaired in bronchial epithelial cells exposed to Moraxella catarrhalis, another PPM commonly found in the LRT of COPD patients (Heinrich et al., 2016). These findings might, along with other factors, explain the increased susceptibility of COPD patients to viral infections.

CF patients are frequently colonized with P. aeruginosa (Hector et al., 2016) and, like COPD patients, are prone to develop exacerbations upon viral infection. Chatteraj et al. (2011a) showed decreased production of type I and III IFNs in CF cells exposed to P. aeruginosa and HRV compared with cells exposed to HRV alone. This attenuation of the antiviral response was reflected in higher viral titres. Infection with P. aeruginosa, however, did not affect the antiviral response in healthy bronchial epithelial cells. Further analysis revealed that impaired production of antiviral mediators was linked to increased levels of oxidative stress in the CF cells. Overall, however, the effects of preceding bacterial exposures on subsequent viral infections remain insufficiently understood.

**Direct interactions of bacteria and viruses: altered virulence and structural modifications**

Although significant evidence for a direct interaction between bacteria and viruses outside of laboratory conditions is still pending, there are intriguing experimental studies showing that the co-pathogenesis of bacteria and viruses might even start before infection (Fig. 3). Direct binding of the RSV G protein to pneumococci induces changes to the bacterial transcriptome and renders bacterial strains more virulent by increasing the expression of virulence genes such as pneumolysin (Smith et al., 2014b). Additionally, structural modifications might...
influence pathogenicity of bacteria. Influenza virus neuraminidase, usually required for cleaving sialic acid groups on host glycoproteins, can enzymically alter the structure of sialic acid bearing capsules of certain meningococcal serotypes (Rameix-Welzin et al., 2009). These structural modifications lead to enhanced bacterial adherence, which may contribute to invasiveness.

**Potential for clinical applications: innate imprinting, prophylactic activation of TLR signalling and manipulation of the microbiome**

Although inflammation-associated tissue damage contributes significantly to the pathology of infections, a temporary augmentation of airway inflammation by administration of aerosolized bacterial products has been suggested as potential prophylactic treatment during influenza epidemics. Mice pre-treated with aerosolized bacterial lysates were shown to mount an inflammatory response more rapidly after influenza challenge, while at later stages of the infection local inflammation (and associated tissue damage) was significantly decreased, as was mortality (Tuvim et al., 2009). In a similar approach, activation of TLR2/6 and TLR9 by pre-treatment with synthetic agonists reduced parainfluenza virus titres in guinea pigs; however, this regimen was not able to prevent virus-induced airway hyper-reactivity (Drake et al., 2013). Similar to the immunostimulatory effect of individual TLR agonists, innate immune imprinting by local application of modified heat-labile Escherichia coli toxins has been shown to confer protection against respiratory viral infection in murine models (Norton et al., 2010; Williams et al., 2004). The need for local application might even be circumvented by dietary intake of probiotics, specifically those bacterial strains that trigger type I IFN production by plasmacytoid DCs (Jouanai et al., 2015). Eventually, manipulation of the pulmonary microbiome may evolve as an alternative to the application of isolated microbial structures. However, for this approach to succeed, a better understanding of what constitutes a ‘healthy’ microbiome is needed.

Next to the use of bacterial or viral structures to evoke beneficial effects on the immune response to subsequent infections, viral–bacterial interactions also have implications for treatment approaches using, e.g. adenoviral vectors, which might yield unwanted interactions between the viral vector and subsequent infections (Brown et al., 2014).

**General remarks**

For the purpose of this review, we conceptually distinguished between secondary bacterial and secondary viral infection. Indeed, several studies point to distinct mechanisms of copathogenesis depending on the order of infection (Lee et al., 2010; McCullers & Rehg, 2002). In the host, however, the impact of sequential infections most likely does not stop there, as acute exposures to pathogens have been shown to trigger alterations in the local bacterial microbiome (Molyneaux et al., 2013; Tarabichi et al., 2015), which may impact on subsequent rounds of infection. Moreover, the pulmonary microbiome encompasses not only bacteria, but also fungi and viruses (Marsland & Gollwitzer, 2014). Eventually, it will not be sufficient to study microbial communities based on their species composition, but rather on the level of a functional characterization.

We restricted this review to interactions of bacteria and viruses during mixed infections, but cross-reactivity and copathogenesis is also observed during heterologous bacterial infections (Ratner et al., 2005). Likewise, sequential infections with several viruses can negatively affect the host’s immune response, whereas viral interference may prevent or delay infection with a second virus (Casalegno et al., 2010; Laurie et al., 2015; Nie et al., 2010; Welsh et al., 2010). Moreover, interactions between fungi and viruses on airway epithelial immunity have been reported, extending this concept even further (Zhu et al., 2014). Finally, interactions are not restricted locally, but can be linked to systemic or distal causes (e.g. general immunosuppression due to human immunodeficiency virus (HIV)/AIDS and susceptibility to tuberculosis (Nunn et al., 2005), or immunomodulatory effects of helminths (Scheer et al., 2014)).

**Conclusions**

Heterologous secondary infections can be facilitated by various mechanisms, including a breach of barrier function, profound immunological alterations, direct interactions of pathogens and changes of the microenvironment. While some of these mechanisms seem to apply to whole classes of pathogens, others are highly pathogen specific. All these aspects have primarily been investigated for viruses facilitating secondary bacterial infections, whereas the influence of the respiratory microbiome on subsequent viral infection is a relatively new, and hitherto neglected, concept.

To reveal functional consequences of an altered microbiome composition during viral infections, a better understanding of what constitutes a ‘healthy’ microbiome is

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**Fig. 3.** Direct viral–bacterial interactions of respiratory pathogens.
needed, which is currently hampered by the lack of large, longitudinal studies. Ultimately, larger studies will also be needed to identify interactions between different combinations of specific pathogens and to clarify whether chronic bacterial exposures have clinically significant effects on the outcome of viral infections.

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