Cell Host & Microbe
Review

The Interaction between Respiratory Pathogens and Mucus

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http://dx.doi.org/10.1016/j.chom.2016.01.001

The interaction between respiratory pathogens and their hosts is complex and incompletely understood. This is particularly true when pathogens encounter the mucus layer covering the respiratory tract. The mucus layer provides an essential first host barrier to inhaled pathogens that can prevent pathogen invasion and subsequent infection. Respiratory mucus has numerous functions and interactions, both with the host and with pathogens. This review summarizes the current understanding of respiratory mucus and its interactions with the respiratory pathogens *Pseudomonas aeruginosa*, respiratory syncytial virus and influenza viruses, with particular focus on influenza virus transmissibility and host-range specificity. Based on current findings we propose that respiratory mucus represents an understudied host-restriction factor for influenza virus.

Introduction

The classical roles of respiratory mucus are to maintain the hydration of the respiratory tract and to act as a protective barrier against the external environment by trapping particulate matter, including pathogens. Trapped matter can then be expelled from the airways by mucociliary clearance, the rhythmic beating of cilia bundles on the airway epithelium. It is now clear that this classical model is not complete and that mucus is a complicated, multicomponent secretion with numerous functions. The functions of respiratory mucus now include immune response regulation, the presentation of molecules that are inhibitory to pathogens, the regulation of cell differentiation and proliferation, and the maintenance of the barrier function of the epithelium.

Respiratory tract mucus is the first interaction that inhaled agents have with a potential new host. Accordingly, this mucus layer can determine the infectivity, and potentially the transmissibility, of respiratory pathogens including influenza viruses, respiratory syncytial virus (RSV) and rhinoviruses as well as pathogenic lung colonization by the opportunistic bacterial pathogen *Pseudomonas aeruginosa* (PA), particularly in patients with cystic fibrosis (CF). Here, we review the current understanding of respiratory tract mucus and its interactions with respiratory pathogens. We pay particular attention to studies investigating the interactions between influenza viruses and mucus and their importance in understanding influenza virus pathogenesis and host-range restriction.

Respiratory Tract Mucus

The epithelium of the respiratory tract is coated with mucus, a multicomponent secretion that has the properties of a viscous fluid or a soft, elastic solid depending on shear stress (reviewed in Lai et al., 2009). This secretion exists in two layers: a more viscous mucus gel layer on top of a periciliary liquid layer (PCL), where the cilia lie. The PCL is generally as deep as the cilia bundles are high, and the reduced viscosity compared to the top layer facilitates the beating of cilia bundles. While the PCL layer is less viscous than the gel layer above, diffusion of molecules into the PCL layer is impeded by membrane-spanning mucins and mucopolysaccharides associated with the cilia and the epithelium. These molecules form a type of molecular "brush" that helps maintain the boundary between the mucus and PCL layers. This is the "gel-on-brush" model of the mucus barrier (Button et al., 2012) (Figure 1A).

Mucins comprise a significant portion of airway mucus and contribute to the barrier function of mucus. Mucins are among the largest macromolecules encoded in the mammalian genome, being 200 kDa to 200 MDa in size. The majority of this weight, approximately 80%, is comprised of carbohydrate chains. Due to their size, the initial polymerization of mucins to form homodimers, which occurs after their translation, can expose mucin-producing cells to endoplasmic reticulum and Golgi stress (Martino et al., 2013). The size of mucins after their secretion exceeds the average size of the secretory vesicles in which they are packaged. This necessitates that mucins be packaged in a condensed and dehydrated state, which is thought to be mediated by calcium-dependent crosslinking within the molecule and by charge shielding the sialic acids and sulfate groups with calcium ions (Ridley et al., 2014; Verdugo et al., 1987a, 1987b). The release of mucins from secretory granules follows a rise in pH and a fall in calcium ion concentration, which mediates mucin unfolding and rapid hydration and leads to expansion and the formation of higher-order oligomers (Ambort et al., 2012b; Ridley et al., 2014). Mucins must adsorb in excess of 1,000 times their mass in water to achieve a viscoelasticity that facilitates mucociliary clearance (Button et al., 2012).

Mucins share a general multidomain structure (Figures 1B and 1C). The centers of these molecules are rich in serine and threonine residues interspersed with proline residues, termed PTS sequences. Mucins contain greater than 100 PTS sequences, which are important, as glycan chains are anchored onto the serine and threonine residues (Ambort et al., 2012a). This region is flanked by domains with sequence homology to von Willebrand domains, similar to those found on von Willebrand factors in the blood, which facilitate clotting in the event of vascular injury. These domains are involved in polymerization via disulfide linkages between mucin molecules (reviewed in Thornton et al., 2008).

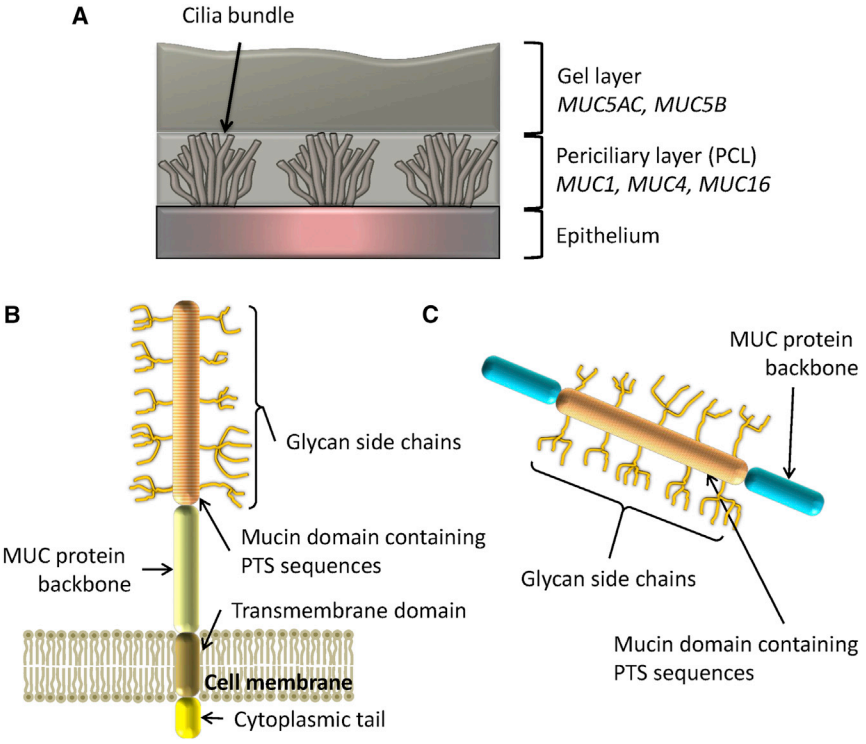

Figure 1. The "Gel-on-Brush" Model of Respiratory Mucus and the Structure of Mucins

(A) The gel-on-brush model of respiratory mucus describes mucus existing in two discreet layers, a more viscous gel layer on top and a periciliary layer (PCL) below. The gel layer contains the secreted mucins MUC5AC and MUC5B, while the PCL contains the membrane-tethered mucins MUC1, MUC4, and MUC16. The reduced viscosity of the PCL in comparison to the gel layer facilitates the beating of cilia and mucociliary clearance.

(B) Generic structure of a membrane-bound mucin.

(C) Generic structure of a secreted mucin.

Mucins are heavily glycosylated, such that there are 25–30 carbohydrate chains per 100 amino acids comprising 80% of the dry weight of the molecule, and this is important for their function (Brockhausen et al., 2009; Stanley et al., 2009). Mucins contain complex glycan chains consisting mainly of *O*-glycans to which *N*-acetylgalactosamine (GalNAc) is added, followed by the additional glycan moieties galactose, *N*-acetylglucosamine (GlcNAc), fucose, and sialic acids (Brockhausen and Schachter, 1997). The complexity and diversity of mucins offers a high degree of resistance to microbial proteases, as a number of diverse enzymes including proteases, glycosidases, and sialidases are needed to totally degrade the various bonds present in mucins (Corfield et al., 1992; Hoskins and Boulding, 1981). Most mucins have a high sialic acid content which, along with the high sulfate content, results in a strongly negative surface charge, increasing the rigidity of the polymer via charge repulsion (Shogren et al., 1989). As such, the sialic acid content of mucins is important, as it is thought to be an important determinant of the viscosity and elasticity of mucus (Puchelle et al., 1973).

There are at least 15 mucins in the human lung; MUC1, MUC2, MUC3, MUC4, MUC5AC, MUC5B, MUC7, MUC13, MUC15, MUC16, MUC18, MUC19, MUC20, MUC21, and MUC22 (reviewed in Lillehoj et al., 2013). The major airway mucins are MUC1, MUC4, MUC5AC, MUC5B, and MUC16. MUC1, MUC4, and MUC16 are membrane-tethered mucins, while MUC5AC and MUC5B are the major secreted mucins, secreted by goblet cells and the submucosal glands, respectively (Hovenberg et al., 1996; Wickström et al., 1998). Secreted mucins are evolutionarily old, as they can be traced back to early metazoan evolution, while tethered mucins are much younger, appearing in vertebrates (Lang et al., 2007). This history could reflect the functionality of tethered mucins being specific for vertebrates.

Experiments with knockout mice have demonstrated that Muc5b is particularly important for normal airway function, while Muc5ac is beneficial but not essential. Overexpression of *Muc5ac* in mice was protective against influenza virus infection, as discussed later, and *Muc5ac*$^{-/-}$ mice were viable and were capable of mucociliary clearance (Ehre et al., 2012; Roy et al., 2014) (Table 1). In contrast, *Muc5b*$^{-/-}$ mice showed a number of severe deficiencies in airway function and succumbed to bacterial infections (Roy et al., 2014). Mucociliary clearance was severely reduced in *Muc5b*$^{-/-}$ mice, despite the presence of functional ciliated cells in the airway, such that breathing was impaired due to obstruction of the upper airways. Inflammatory infiltrates and bacteria were also present in the lower airways of *Muc5b*$^{-/-}$ mice, particularly *Staphylococcus aureus*, which was an important cause of mortality in *Muc5b*$^{-/-}$ mice (Roy et al., 2014). There were also aberrations in the immune response in the lungs of *Muc5b*$^{-/-}$ mice, which were particularly evident by the accumulation of macrophages with impaired phagocytic functions. IL-23, a mediator of antimicrobial inflammatory responses, was also dramatically decreased in *Muc5b*$^{-/-}$ mice (Roy et al., 2014) (Table 1). Therefore, there appears to be a link between mucins and the innate immune response, two important aspects of the "first line of defense" against pathogens.

In addition to secreted mucins there are also membrane-tethered mucins that exist in the PCL, these being MUC1, MUC4, MUC16, and MUC20. Tethered mucins play a number of roles, including the activation of intracellular signal transduction pathways, regulation of the immune response, and cell differentiation and proliferation (reviewed in Lillehoj et al., 2013). MUC1 appears to be more strongly associated with microvilli, while MUC4 is more strongly associated with cilia (Sheehan et al., 2006). A PA challenge model using *Muc1*$^{-/-}$ mice revealed a role for MUC1 in regulating the inflammatory response. Postchallenge, these mice showed greater numbers of macrophages and greater amounts of the cytokines tumor necrosis factor alpha (TNFα) and keratinocyte chemoattractant (KC) in bronchoalveolar

Table 1. Summary of Mouse Experiments Studying the Roles of Mucins during Infection

Mouse Strain	Phenotype	References
Muc1$^{-/-}$	● Increased number of macrophages and expression of tumor necrosis factor alpha (TNFα) and keratinocyte chemoattractant (KC), both markers of inflammation, post-*Pseudomonas aeruginosa* challenge. ● Increased susceptibility to *Pseudomonas aeruginosa*-mediated lung injury.	Lu et al., 2006; Umehara et al., 2012
Muc5ac$^{+/+}$	● Reduced influenza virus replication in lung. ● No evidence of airway obstruction or lung inflammation.	Ehre et al., 2012
Muc5ac$^{-/-}$	● Mucociliary clearance is not affected, and mice are viable and healthy.	Roy et al., 2014
Muc5b$^{-/-}$	● Severe impairment of mucociliary clearance. ● Upper airway obstruction resulting in impairment of breathing. ● Inflammatory infiltrates and bacteria present in lower lung. ● Decreased IL-23, a mediator of antimicrobial inflammatory responses, in lung. ● Mortality due to bacterial lung infections.	Roy et al., 2014

lavage fluid (BALF), both of which are markers of inflammation, which again suggests a link between mucins and the innate immune response (Lu et al., 2006; Umehara et al., 2012). *Muc1*$^{-/-}$ mice were also more susceptible to greater lung injury due to inflammation following PA challenge (Lu et al., 2006; Umehara et al., 2012). The anti-inflammatory role of MUC1 was also observed in a RSV in vitro challenge model, where a negative feedback loop was observed (Li et al., 2010).

Respiratory mucus also contains numerous other substances secreted by epithelial cells with inhibitory activity to inhaled pathogens. These substances can closely associate with mucins, suggesting that mucins act as a "scaffold" to present and organize these proteins. These substances include lysozyme, lactoferrin, proteases, protease inhibitors like leukoprotease inhibitor and antichymotrypsin, nitric oxide, and hydrogen peroxide (reviewed in Ganesan et al., 2013). Lactoferrin, a bi-globular iron-binding glycoprotein that acts as an iron chelator, demonstrates antiviral and antibacterial activities and also displays other functions, including immunomodulation, modulation of cell growth, and antioxidant activity (Wakabayashi et al., 2014). Lactoferrin also has multifaceted inhibitory actions against a number of viruses, including influenza virus (Pietrantoni et al., 2012). Oxidants, such as nitric oxide that is produced following the induction of nitric oxide synthase-2 (NOS-2), have also been shown to inhibit viral infections. The role of nitric oxide is particularly evident in patients with CF, who are more susceptible to viral infections and show an impaired NOS-2 pathway, resulting in decreased nitric oxide in the airways (Zheng et al., 2004).

Pseudomonas aeruginosa

PA is an opportunistic Gram-negative flagellated bacterial pathogen that is the primary cause of pneumonia in immunocompromised and CF patients (Gibson et al., 2003). PA expresses several adhesins that appear to be physiologically relevant to lung colonization, such as pili on the bacterial surface that mediate epithelial cell adhesion. The bacterial flagellum is also important in this respect, as it binds the ectodomain of MUC1 (Lillehoj et al., 2002). The sialylation of MUC1 affects PA adhesion by reducing the accessibility of β-D-galactose sites on MUC1, which are necessary for PA adhesion via flagellin (Lillehoj et al., 2012; Pastoriza Gallego and Hulen, 2006). Sialyl-Lewis^x moieties on mucins in particular appear to be preferential binding sites for PA via flagellin (Colomb et al., 2014; Scharfman et al., 1999). Sialyl-Lewis^X moieties are tetrasaccharide-terminating glycan chains on *N*- and *O*-linked glycans that are important blood type antigens. They are located on mucins and the surface of leukocytes, where they are involved in immune cell recruitment. There is evidence that PA can increase the Sialyl-Lewis^X content on mucins via pyocyanin, a redox-active tricyclic toxin secreted by PA, thereby making the host environment more favorable for attachment and colonization (Jeffries et al., 2015). Pyocyanin has also been shown to stimulate the release of pro-inflammatory cytokines and MUC5AC and MUC2, which was mediated by the epidermal growth factor receptor (EGFR) pathway (Rada et al., 2011).

PA is the most important bacterial infection associated with CF, a genetic disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel protein. CF is characterized by mucus hypersecretion that leads to airway blockages and chronic bacterial lung infections predominated by PA that result in inflammation and progressive pulmonary damage (Hartl et al., 2012). There are indications that the lung environment in CF is proinflammatory, a state further exacerbated by the chronic bacterial infections common in CF (Berger, 2002). Inflammation exacerbates these infections as proinflammatory cytokines such as TNFα, IL-6, and IL-8 increase Sialyl-Lewis^x expression on mucins, which facilitates PA attachment (Colomb et al., 2014; Groux-Degroote et al., 2008) (Figures 2A and 2B). Mucins obtained from CF airways are enriched with Sialyl-Lewis^x, and this has been shown to correlate to the severity of infection in CF (Davril et al., 1999).

In the CF lung, PA is very difficult to eradicate, despite host immune responses and adequate concentrations of antibiotics in the airways during treatment. This is thought to result from PA forming a biofilm within the thick mucus of the CF lung. In this setting, PA adapts by losing motility, forming aggregates and relying on anaerobic respiration, which contributes to resistance against multiple front-tier antibiotics (Staudinger et al., 2014) (Figure 2C). PA strains isolated from CF patients with chronic infections are often nonmotile, lacking flagellin and pilin, while environmental isolates and isolates obtained during early colonization are motile (Luzar et al., 1985). These nonmotile strains are also resistant to phagocytosis by macrophages (Lovewell et al., 2014; Luzar et al., 1985). In contrast, non-CF mucus has been

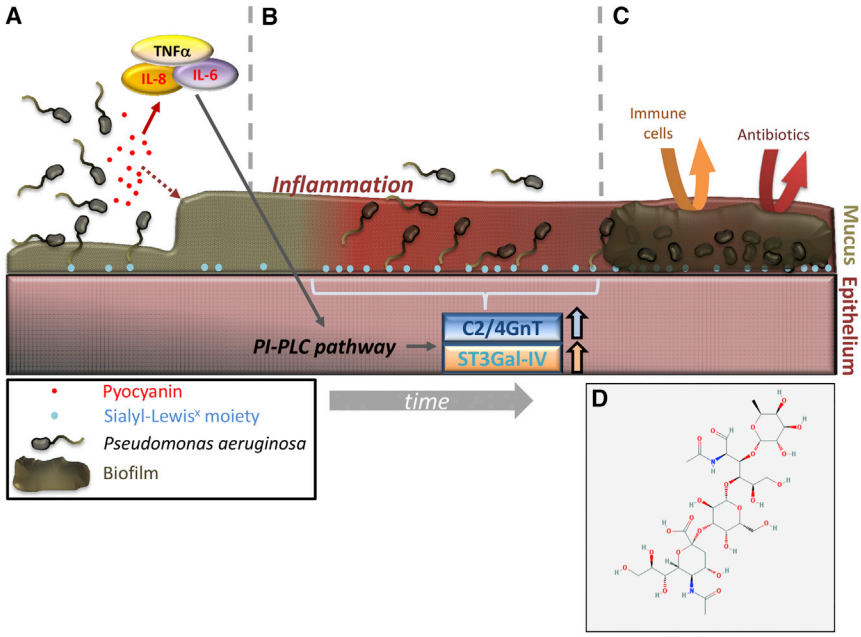

Figure 2. *Pseudomonas aeruginosa* Colonization of the Cystic Fibrosis Lung

(A) *Pseudomonas aeruginosa* (PA) binds preferentially to the sialyl-Lewis^x carbohydrate moieties (blue dots) present on mucins via the flagellar cap. The PA toxin pyocyanin (red dots) stimulates mucus hypersecretion by goblet cells (dashed arrow) and induces the production and release of the proinflammatory cytokines tumor necrosis factor alpha (TNFα) and interleukins 6 (IL-6) and 8 (IL-8) (solid arrow).

(B) TNFα, IL-6, and IL-8 lead to inflammation and upregulate the sialyl-Lewis^x biosynthesis glycosyltransferases core 2/core 4 beta-1,6-N-acetylglucosaminyltransferase (C2/4GnT) and α2,3-sialyltransferase IV (ST3Gal-IV) via the phosphoinositol-specific phospholipase C (PI-PLC) pathway (gray arrow), leading to an increase in sialyl-Lewis^x content and increased PA binding.

(C) PA is commonly found in a biofilm in the CF lung. In this setting, PA aggregate, lose their motility, and switch to anaerobic respiration, facilitating resistance to cells of the immune system and to multiple front-tier antibiotics.

(D) The structure of sialyl-Lewis^x ($C_{31}H_{52}N_2O_{23}$). Image was obtained from PubChem, CID number 643990.

shown in vitro to inhibit adhesion and the subsequent loss of motility (Yeung et al., 2012).

PA has also been shown to impact mucin secretion via lipopolysaccharide (LPS), a component of the bacterial cell wall. LPS can upregulate *MUC2* and *MUC5AC* gene expression, contributing to the excessive mucus production and airway blockage seen in CF. PA LPS upregulates *MUC2* expression via a c-Src-Ras-MEK1/MAPK signaling pathway, leading to the activation of nuclear factor (NF)-κB, which in turn triggers *MUC2* gene transcription (Li et al., 1997). PA LPS has also been shown to generate reactive oxygen species (ROS) via the PKC-NADPH oxidase signaling pathway in epithelial cells, which leads to upregulation of *MUC5AC* transcription (Li et al., 2013; Yan et al., 2008). The induction of CFTR-dependent airway surface liquid secretion following exposure to bacteria has also been observed in swine tracheal explants (Luan et al., 2014). It is hypothesized that PA may be responsible, via these modes of actions described, for the excessive mucus production seen in CF patients, rather than the CF lung environment itself.

Respiratory Syncytial Virus

RSV is the most common cause of bronchiolitis and viral pneumonia, such that by the age of three, all children will have been infected by RSV at least once (reviewed in Pickles and DeVincenzo, 2015). The epithelium of the upper respiratory tract is the major target for RSV during the early stages of infection. Later stages of infection are characterized by inflammation and obstruction of the airways due to the formation of mucus plugs containing mucus, fibrin, cellular debris, and lymphocytes (Aherne et al., 1970). RSV is an enveloped virus of the family *Paramyxoviridae* that contains a single-stranded, nonsegmented, negative-sense RNA genome. Among the 11 separate proteins encoded by RSV, three are transmembrane surface glycoproteins labeled glycoprotein (G), fusion (F), and small hydrophobic (SH). The function of SH is largely unknown (reviewed in Collins and Crowe, 2013).

The major attachment protein of RSV is the G protein, and most of the ectodomain of the G protein has a mucin-like structure, with a high content of PTS sequences and abundant glycosylation. The F protein facilitates viral fusion with host cell membranes to initiate infection, including cell-to-cell fusion leading to syncytia formation. Being major neutralizing antigens, both the F and G proteins can influence the immune response and mucus production in the lung (Connors et al., 1991).

The F protein has also been linked to RSV pathogenicity in the lower respiratory tract. Increased F-mediated RSV fusogenicity has been correlated to increased viral load early during infection in mice, resulting in severe lung damage (Hotard et al., 2015). This was linked to increased neutrophil infiltration of the lung, increased amounts of TNF-α, a neutrophil recruitment factor that stimulates the production of MUC5AC and MUC1, and increased numbers of IL-13-producing CD4$^+$ T cells in the lung (Stokes et al., 2013). Neutrophil numbers correlated to mucus production in the lung and associated with the formation of mucus plugs, mediated by TNF-α, in more severe cases (Bataki et al., 2005; Lora et al., 2005). The RSV G protein has also been associated with disease severity and mucus secretion in mouse models of RSV infection, independent of its role in viral replication (Boyoglu-Barnum et al., 2013). This may be mediated by type 2 cytokine expression, particularly IL-13, IL-4, and IL-5 and the chemokine MCP-1, as type 2 cytokines can induce mucin secretion (Temann et al., 1997; Zuhdi Alimam et al., 2000).

Signaling via the chemokine receptor CXCR2 has also been shown to contribute to increased mucus production via increased Muc5ac expression in a mouse model of RSV infection following allergic sensitization (Miller et al., 2003). Increased mucus production was thought to be due to increased IL-17 in the lung, a proinflammatory cytokine secreted by activated T cells (Chen et al., 2003; Hashimoto et al., 2004). The importance of IL-17 was shown in vivo using a knockout mouse model (Hashimoto et al., 2005).

Recently it has been shown that RSV and human metapneumovirus (hMPV), two RNA viruses of the *Paramyxoviridae* family, induce significantly different mucin expressions in A549 cells, an epithelial cell line (Baños-Lara Mdel et al., 2015). Although these two viruses are similar in structure and pathogenicity, it is interesting to see the differential expression of MUC2, MUC5AC, and MUC5B as well as membrane-bound mucins. RSV infection can change the composition of infected airway epithelium by increasing MUC5AC-secreting cells, indicating the important role of mucus secretion in pathogenicity (Persson et al., 2014).

Influenza Viruses

Influenza viruses are important respiratory pathogens that place a significant burden on society, such that influenza ranks in the top ten causes of death in the United States (Hoyert and Xu, 2012). Seasonal influenza viruses are responsible for a significant amount of mortality and morbidity during the influenza season, and influenza pandemics, which occur sporadically, can be associated with much greater disease severity and higher mortality rates.

The Influenza Virus Replication Cycle and the Roles of Hemagglutinin and Neuraminidase

Influenza viruses are enveloped and contain protein spikes protruding from the viral membrane consisting of the hemagglutinin (HA) and neuraminidase (NA) proteins, which play critical roles in the viral life cycle. HA exists as a trimer on the surface of influenza viruses and mediates cell attachment and viral entry. HA binds to cells via *N*-acetylneuraminic acids (sialic acids) located on the distal ends of oligosaccharide chains on the cell surface (reviewed in Palese and Shaw, 2013). The dense, diverse coating of glycoconjugates, glycoproteins, proteoglycans, glycolipids, and polysaccharides, such as hyaluronic acid, on the surface of cells is termed the glycocalyx. The glycocalyx has numerous functions, including structural roles in proteins, protein folding, and mediation of self-recognition by the immune system (reviewed in Springer and Gagneux, 2013). The structure of these chains and the linkages they form with the terminal sialic acid resides are important determinants of influenza virus host range, which is further described below. Following sialic acid-HA binding, the virus is internalized into the cell by endocytosis or macropinocytosis and trafficked to the endosome, where a pH-dependent conformational change in HA results in the release of the viral ribonucleoproteins and subsequent viral replication (reviewed in Palese and Shaw, 2013).

Following replication and assembly, progeny viruses are attached to the surface of the infected cell via the HA-sialic acid interaction. At this point, NA cleaves the sialic acid residues bound to the HA, releasing the progeny viruses. Therefore, the interaction of influenza viruses with the glycocalyx is very important in the viral life cycle. However, respiratory mucus also contains sialic acids and glycoproteins, and the influenza virus interaction with these is also important.

The Interactions between Influenza Virus and Respiratory Mucus and the Importance of Neuraminidase

Influenza viruses are currently classified in the *Orthomyxoviridae* family but were initially classified as myxo viruses, from the Greek *myxa* (mucus), based on their reactivity for certain mucins (Andrewes, 1954). The interaction between influenza viruses and mucins was first identified in studies conducted in the 1940s and 1950s (Anderson et al., 1948; Burnet, 1951). The observation that receptor-destroying enzyme from *Vibrio cholerae* could reduce the inhibitory action of mucoproteins over time in a similar fashion to influenza viruses was suggestive of an influenza virus sialidase (Stone, 1949). This sialidase was presented by Gottschalk, in which the authors "propose the name 'neuraminidase' for the enzyme and define its action as the hydrolytic cleavage of the glycosidic bond joining the keto group of neuraminic acid to D-galactose or D-galactosamine and possibly to other sugars" (Gottschalk, 1957).

Since these studies, NA has been shown to also be important in determining whether viruses can penetrate the mucus barrier to infect underlying cells. The NA inhibitor oseltamivir was shown to inhibit the infection of mucus-producing normal human bronchial epithelial (NHBE) cells (Matrosovich et al., 2004), and additional studies achieved similar results using A549 cells and Madin-Darby canine kidney (MDCK) cells (Ohuchi et al., 2006) (Figure 3A). However, when oseltamivir was added to erythrocytes, which express sialic acids on their surface, influenza virions became embedded in the erythrocyte membrane, while viruses remained attached to the cell surface in the absence of oseltamivir, indicating that NA plays a role in virus entry (Ohuchi et al., 2006).

It was hypothesized that NA may facilitate infection by cleaving sialic acids on mucins to prevent viruses from becoming trapped before they infect the epithelium. This was investigated in vitro by adding influenza viruses to a layer of porcine respiratory mucus. Over time, viruses penetrated the mucus layer and the extent of penetration could be facilitated or impaired by the addition of exogenous NA or oseltamivir, respectively, indicating the importance of NA in mucus penetration (Yang et al., 2014). This is likely achieved via cleavage of sialic acids, thus preventing or reversing viral attachment to mucus. Consistent with this hypothesis, sialidase pretreatment of human trachea and bronchial tissues containing a mucus layer reduced viral binding to mucus (Burnet, 1947; Cohen et al., 2013). Influenza virus has also been shown to be inhibited by exosomes, which are secreted cellular microvesicles present in mucus (Kesimer et al., 2009). Exosomes are rich in α2,6 sialic acids and interact with MUC1, MUC4, and MUC16. Cultures of human tracheobronchial epithelial cells secrete exosomes, and these are inhibitory to influenza virus infection of MDCK cells in vitro, via virus binding to sialic acids on the exosome (Kesimer et al., 2009). Mucins have been shown to be protective against influenza virus as mice overexpressing Muc5ac (Muc5ac-Tg) exhibited 9.3-fold less viral replication in the lung compared to wild-type (Ehre et al., 2012). While Muc5ac expression was approximately 20-fold greater in the large and small airways in Muc5ac-tg mice compared to wild-type, there was no evidence of airway obstruction or lung inflammation. Further, BALF from Muc5ac-Tg mice showed greater inhibition of viral infection of MDCK cells compared to wild-type BALF (Ehre et al., 2012) (Table 1).

Respiratory mucus may be a species barrier for influenza viruses. Experiments in vitro demonstrated that an overlay of human mucus can inhibit the infection of underlying MDCK cells by both swine- and human-origin viruses in vitro, while swine mucus does not appear to inhibit infection (Cohen et al., 2013; Zanin et al., 2015) (Figures 3A and 3B). The interaction between influenza viruses and mucus may also be a factor in viral

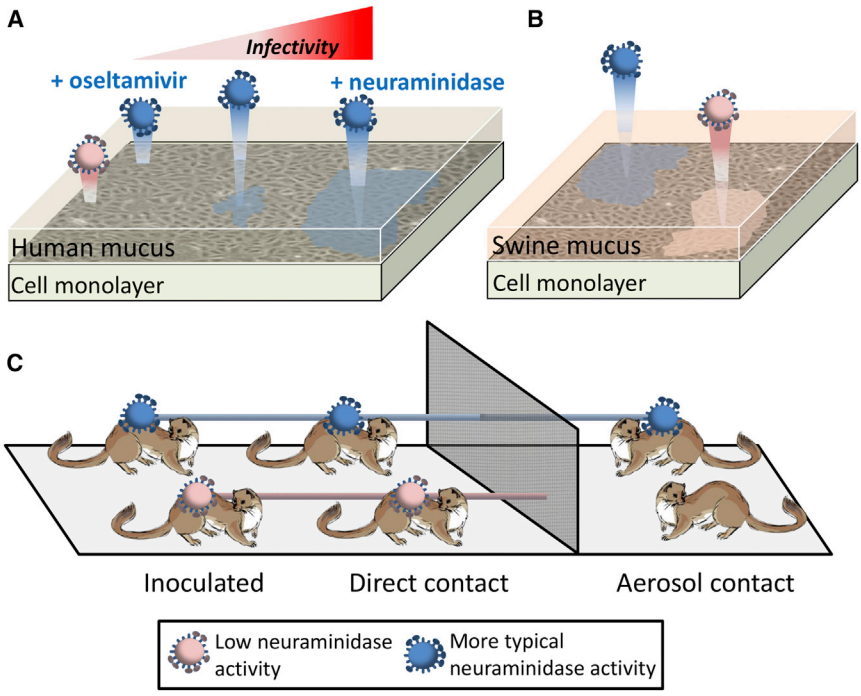

Figure 3. Influenza Viruses, Neuraminidase, Respiratory Mucus, Viral Transmissibility, and Host Range

Neuraminidase (NA), one of the surface glycoproteins on influenza virus, cleaves sialic acids to prevent the virus becoming trapped in the heavily sialydated mucins in respiratory mucus, thus facilitating infection of the underlying cells (represented here by shading on the cell monolayer).

(A) The antiviral drug oseltamivir inhibits the enzymatic activity of NA, which inhibits the viral penetration of mucus, decreasing the infectivity of the virus for the cells underlying the mucus layer. Viruses with low NA enzymatic activity are also inhibited by mucus. Conversely, addition of exogenous NA facilitates viral penetration of mucus and thus increases viral infectivity.

(B) Compared to human mucus, swine mucus appears to be less inhibitory to influenza viruses with low neuraminidase activity, for reasons not fully understood.

(C) Influenza viruses that express NA proteins with low enzymatic activity do not transmit by aerosol droplets efficiently in the ferret model of transmission, despite being shed from infected animals and transmitted to direct contact animals. The mechanism behind this is not fully understood.

transmissibility, particularly in the context of NA enzymatic activity. Pandemic H1N1 viruses isolated in swine with almost undetectable NA enzymatic activity were significantly inhibited by mucus in NHBE cell cultures in vitro (Zanin et al., 2015). These viruses did not transmit via aerosols in ferrets, and direct contact transmission was severely attenuated (Zanin et al., 2015). Similarly, viruses expressing NA proteins with short stalks that have decreased enzymatic activity against multivalent substrates such as those found on cells are inhibited by mucus in vitro (Castrucci and Kawaoka, 1993; Matsuoka et al., 2009). Replacing the NA of an efficiently transmitted H5N1 virus with an avian short-stalk NA protein abrogated aerosol transmission of the virus and reduced the efficiency of direct contact transmission in ferrets (Blumenkrantz et al., 2013). These studies strongly suggest a link between mammalian transmission and the interaction between mucus and NA (Figure 3C).

Sialic Acids and Host Restriction of Influenza Viruses

While host restriction of influenza viruses is a multifactorial phenomenon that is not fully understood, the HA binding preference for different sialic acids is involved. The types of sialic acids expressed are species dependent, but *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc) are common. Neu5Gc is converted from cytidine monophosphate-*N*-acetylneuraminic acid by the enzyme cytidine monophosphate-*N*-acetylneuraminic acid hydroxylase (Irie et al., 1998). Neu5Gc is absent in humans and sauropsids (birds and reptiles), as this enzyme is mutated and nonfunctional, although pigs and mice produce this enzyme and thus express Neu5Gc (Chou et al., 1998; Scharfman et al., 1999; Schauer et al., 2009). Old World monkeys also express Neu5Gc, while New World monkeys and humans do not. This gene was lost approximately 3 million and 30 million years ago, respectively, in ancestral hominids and New World monkeys (Springer et al., 2014).

Ferrets, like humans, also lack a functioning cytidine monophosphate-*N*-acetylneuraminic acid hydroxylase and therefore also do not express Neu5Gc (Ng et al., 2014). This is significant, as ferrets are widely used to study influenza virus infection and transmission, as the expression and distribution of sialic acids in the ferret respiratory tract is similar to humans, although not identical (Jia et al., 2014; van Riel et al., 2006). Neu5Gc may act as a decoy receptor for influenza viruses rather than a functional receptor, as shown using a Neu5Gc-expressing human lung cell line. The infectivity of a virus that binds to Neu5Gc was decreased in cells expressing Neu5Gc compared to cells only expressing Neu5Ac, and Neu5Gc-binding viruses accumulated on the surface of cells expressing Neu5Gc (Takahashi et al., 2014). In contrast, the infectivity of a virus that cannot bind to Neu5Gc was similar in cells either expressing or not expressing Neu5Gc (Takahashi et al., 2014).

There are also species differences in the types of linkages that exist between sialic acid, at the extremity of the glycan chain, and the underlying galactose molecule. The upper respiratory tract of humans primarily contains sialic acids bound to galactose by an α2,6 linkage, although α2,3 linkages are also present, while in the bronchus and alveoli, α2,3 linkages predominate (Gagneux et al., 2003; Nicholls et al., 2007; Shinya et al., 2006). The significance of these linkages with respect to influenza virus host restriction has been extensively studied. These studies showed that influenza viruses of avian and equine origin tend to bind better to sialic acids with α2,3 linkages, while human influenza viruses bind better to sialic acids with α2,6 linkages (Suzuki et al., 2000). The sialic acid binding preferences of avian and mammalian viruses correlates somewhat to the sialic acid distribution in human and avian hosts. In avian hosts, α2,3-linked sialic acids predominate in the gastrointestinal tract, while α2,6-linked sialic acids are more abundant in the upper respiratory

tract of humans (Gambaryan et al., 2002; Nicholls et al., 2007; Shinya et al., 2006). The porcine respiratory tract has been shown to express both α2,3 and α2,6-linked sialic acids, and for this reason swine have been referred to as "mixing vessels" that foster reassortment of influenza viruses (Ito et al., 1998). The relative lack of α2,3-linked sialic acids in the upper respiratory tract of humans is thought to be a host barrier to avian influenza viruses. This is evident in the strains of influenza viruses that have caused pandemics. These viruses contain HA proteins originating from nonhuman species yet exhibit specificity for human-type receptors (Childs et al., 2009; Matrosovich et al., 2000; Stevens et al., 2006). Further, viruses that preferentially bind to α2,3-linked sialic acids are not transmitted via the airborne route in mammals, while viruses with mixed binding preferences have an intermediate transmission phenotype (Matrosovich et al., 2000; Tumpey et al., 2007). The relatively low expression of α2,3-linked sialic acids in the mammalian upper respiratory tract is likely a major contributor to this restriction.

While the sialic acid linkages are important for host range specificity of influenza viruses, this is an oversimplification. There are numerous different species of α2,3- and α2,6-linked sialic acids present in the glycocalyx with varying length, topology, and complexity. The location of these sialic acids in the glycocalyx may also come to bear. For example, sialic acids located on glycoproteins such as mucins may show differential importance compared to those on gangliosides, which are glycolipids with sialic acid content. This is owing to their locations in the glycocalyx, as gangliosides generally lie close to epithelium and underneath larger glycoproteins that can display many glycan chains farther away from the epithelial layer. To attest to this complexity, the topology of sialic acids has been shown to impact virus host range. Human-adapted HA proteins have been shown to bind preferentially to α2,6-linked sialic acids on long glycan branches, which can assume an "umbrella-like" topology, while avian-adapted HAs do not bind to these structures. In the case of H5N1 viruses, those viruses that were efficiently transmitted in the ferret model showed binding preferences to long α2,6-linked sialic acids, while those that did not transmit efficiently bound preferentially to α2,3-linked or short α2,6-linked sialic acids, both of which assume a "cone-like" topology (Chandrasekaran et al., 2008; Stevens et al., 2006).

There is some experimental evidence that glycan structures play a role in influenza virus infection, although more work is needed to fully elucidate their importance. Experiments in cells lacking complex *N*-linked glycosylation showed influenza virus attachment, but not endocytosis. This indicates that sialic acids alone are not sufficient for infection and that *N*-linked glycoproteins are required for viral entry (Chu and Whittaker, 2004). However, other cell lines lacking complex *N*-linked glycosylation were highly susceptible to influenza virus infection, although serum interfered with infectivity, indicating that sialylated *N*-glycans are required for viral entry in the presence of serum (de Vries et al., 2012). This apparent importance of *N*-linked glycans may indicate that influenza viruses can evolve away from inhibition by mucus, which is mostly *O*-glycosylated.

NA displays preferences for both sialic acid types and linkages, which was studied in human N2 viruses isolated from 1957 to 1987. These N2s originally showed enzymatic activity only against α2,3-linked sialic acids; however, activity against α2,6-linked sialic acids became apparent over time, suggesting a selective advantage was obtained (Kobasa et al., 1999). N2 NAs of avian, swine, and human viruses all showed high specificity for NeuAc; however, there were differences in NeuGc specificity, as avian viruses showed low to moderate specificity for NeuGc, while swine viruses showed high specificity for NeuGc linkages (Kobasa et al., 1999). The specificity of N2s from the human viruses tested for NeuGc linkages appeared to change from low to moderate specificity between 1957 and 1962 to high in 1968, then returning to low to moderate by 1972, for reasons which are not fully understood (Kobasa et al., 1999). Some NA proteins, such as those from H3N2 viruses, can also bind to sialic acids and NAs from avian viruses also contain a hemadsorbing site away from the enzymatic active site (Laver et al., 1984; Lin et al., 2010; Zhu et al., 2012). The biological significance of these binding attributes of NA is not clear.

Perspective

The current understanding of respiratory mucus has revealed a complexity and functionality far beyond the classical role ascribed to it. Much of this knowledge has come from studying mucins, the large, heavily glycosylated, multidomain proteins that give mucus many of its physical properties. The interaction between mucins and respiratory pathogens is also more complicated than mere entrapment. Mucins have been revealed to play an integrated role in the host response to pathogens, both before and during the immune response, and the absence or disturbance of this role can be highly detrimental to the host. Despite the importance of mucins, current understanding of their functions is limited, which is partly due to the complexity of mucus and its interactions.

Mucus appears to be an important host barrier to influenza viruses. The NA protein of influenza has been shown to be important in evading this barrier, such that viruses with low NA activity, or viruses treated with NA inhibitors, are severely impeded by mucus in vitro. Interestingly, these viruses can still infect animals and be transmitted; however, this transmission is severely attenuated, pointing to a role of respiratory mucus in viral transmission, which is not understood. Also not understood is the potential role of mucus as a host-restriction factor for influenza viruses. In vitro data suggest that respiratory mucus could be a host restriction factor between swine and human hosts, but is this also true in vivo, and is it also true between other hosts of influenza viruses? If so, is this restriction merely due to the sialic acid content of mucus, or are there other components involved? Could mucus be an important selective pressure for transmissible viruses? Answering these questions is made more difficult by the complexity of mucus and the incomplete characterization of it. Therefore, further understanding of both mucus and the mucus-influenza virus interaction is likely to continue to provide insights into influenza virus transmission, host restriction, and viral adaptation to new hosts that could be invaluable in understating the genesis of new strains of influenza virus.

ACKNOWLEDGMENTS

We thank Dr. Sook-San Wong and Dr. Jason Rosch for critical review of this manuscript. This work was funded by the National Institute of Allergy and Infectious Diseases, the National Institutes of Health, under contract number HHSN272201400006C.

REFERENCES

Aherne, W., Bird, T., Court, S.D., Gardner, P.S., and McQuillin, J. (1970). Pathological changes in virus infections of the lower respiratory tract in children. J. Clin. Pathol. *23*, 7–18.

Ambort, D., Johansson, M.E., Gustafsson, J.K., Ermund, A., and Hansson, G.C. (2012a). Perspectives on mucus properties and formation—lessons from the biochemical world. Cold Spring Harb. Perspect. Med. *2*, a014159.

Ambort, D., Johansson, M.E., Gustafsson, J.K., Nilsson, H.E., Ermund, A., Johansson, B.R., Koeck, P.J., Hebert, H., and Hansson, G.C. (2012b). Calcium and pH-dependent packing and release of the gel-forming MUC2 mucin. Proc. Natl. Acad. Sci. USA *109*, 5645–5650.

Anderson, S.G., Burnet, F.M., et al. (1948). Mucins and mucoids in relation to influenza virus action; general discussion. Aust. J. Exp. Biol. Med. Sci. *26*, 403–411.

Andrewes, C.H. (1954). NOMENCLATURE of viruses. Nature *173*, 620–621.

Baños-Lara Mdel, R., Piao, B., and Guerrero-Plata, A. (2015). Differential mucin expression by respiratory syncytial virus and human metapneumovirus infection in human epithelial cells. Mediators Inflamm. *2015*, 347292.

Bataki, E.L., Evans, G.S., and Everard, M.L. (2005). Respiratory syncytial virus and neutrophil activation. Clin. Exp. Immunol. *140*, 470–477.

Berger, M. (2002). Lung inflammation early in cystic fibrosis: bugs are indicted, but the defense is guilty. Am. J. Respir. Crit. Care Med. *165*, 857–858.

Blumenkrantz, D., Roberts, K.L., Shelton, H., Lycett, S., and Barclay, W.S. (2013). The short stalk length of highly pathogenic avian influenza H5N1 virus neuraminidase limits transmission of pandemic H1N1 virus in ferrets. J. Virol. *87*, 10539–10551.

Boyoglu-Barnum, S., Gaston, K.A., Todd, S.O., Boyoglu, C., Chirkova, T., Barnum, T.R., Jorquera, P., Haynes, L.M., Tripp, R.A., Moore, M.L., and Anderson, L.J. (2013). A respiratory syncytial virus (RSV) anti-G protein F(ab')2 monoclonal antibody suppresses mucous production and breathing effort in RSV rA2-line19F-infected BALB/c mice. J. Virol. *87*, 10955–10967.

Brockhausen, I., and Schachter, H. (1997). Glycosyltransferases involved in N- and O-glycan biosynthesis. In Glycosciences: Status and Perspective, H. Gabius and S. Gabius, eds. (Weinheim, Germany: Wiley-VCH), pp. 79–100.

Brockhausen, I., Schachter, H., and Stanley, P. (2009). O-GalNAc glycans. In Essentials of Glycobiology, Second Edition, A. Varki, R.D. Cummings, J.D. Esko, H.H. Freeze, P. Stanley, C.R. Bertozzi, G.W. Hart, and M.E. Etzler, eds. (New York: Cold Spring Harbor Laboratory Press).

Burnet, F.M. (1947). Enzymic action of influenza viruses on glandular mucin and on purified blood group substances. Aust. J. Sci. *10*, 21.

Burnet, F.M. (1951). Mucoproteins in relation to virus action. Physiol. Rev. *31*, 131–150.

Button, B., Cai, L.H., Ehre, C., Kesimer, M., Hill, D.B., Sheehan, J.K., Boucher, R.C., and Rubinstein, M. (2012). A periciliary brush promotes the lung health by separating the mucus layer from airway epithelia. Science *337*, 937–941.

Castrucci, M.R., and Kawaoka, Y. (1993). Biologic importance of neuraminidase stalk length in influenza A virus. J. Virol. *67*, 759–764.

Chandrasekaran, A., Srinivasan, A., Raman, R., Viswanathan, K., Raguram, S., Tumpey, T.M., Sasisekharan, V., and Sasisekharan, R. (2008). Glycan topology determines human adaptation of avian H5N1 virus hemagglutinin. Nat. Biotechnol. *26*, 107–113.

Chen, Y., Thai, P., Zhao, Y.H., Ho, Y.S., DeSouza, M.M., and Wu, R. (2003). Stimulation of airway mucin gene expression by interleukin (IL)-17 through IL-6 paracrine/autocrine loop. J. Biol. Chem. *278*, 17036–17043.

Childs, R.A., Palma, A.S., Wharton, S., Matrosovich, T., Liu, Y., Chai, W., Campanero-Rhodes, M.A., Zhang, Y., Eickmann, M., Kiso, M., et al. (2009). Receptor-binding specificity of pandemic influenza A (H1N1) 2009 virus determined by carbohydrate microarray. Nat. Biotechnol. *27*, 797–799.

Chou, H.H., Takematsu, H., Diaz, S., Iber, J., Nickerson, E., Wright, K.L., Muchmore, E.A., Nelson, D.L., Warren, S.T., and Varki, A. (1998). A mutation in human CMP-sialic acid hydroxylase occurred after the Homo-Pan divergence. Proc. Natl. Acad. Sci. USA *95*, 11751–11756.

Chu, V.C., and Whittaker, G.R. (2004). Influenza virus entry and infection require host cell N-linked glycoprotein. Proc. Natl. Acad. Sci. USA *101*, 18153–18158.

Cohen, M., Zhang, X.Q., Senaati, H.P., Chen, H.W., Varki, N.M., Schooley, R.T., and Gagneux, P. (2013). Influenza A penetrates host mucus by cleaving sialic acids with neuraminidase. Virol. J. *10*, 321.

Collins, P.L., and Crowe, J.E., Jr. (2013). Respiratory syncytial virus and metapneumovirus. In Fields Virology, D.M. Knipe and P.M. Howley, eds. (Philadelphia: Lippincott Williams & Wilkins), pp. 1601–1646.

Colomb, F., Vidal, O., Bobowski, M., Krzewinski-Recchi, M.A., Harduin-Lepers, A., Mensier, E., Jaillard, S., Lafitte, J.J., Delannoy, P., and Groux-Degroote, S. (2014). TNF induces the expression of the sialyltransferase ST3Gal IV in human bronchial mucosa via MSK1/2 protein kinases and increases FliD/sialyl-Lewis(x)-mediated adhesion of Pseudomonas aeruginosa. Biochem. J. *457*, 79–87.

Connors, M., Collins, P.L., Firestone, C.Y., and Murphy, B.R. (1991). Respiratory syncytial virus (RSV) F, G, M2 (22K), and N proteins each induce resistance to RSV challenge, but resistance induced by M2 and N proteins is relatively short-lived. J. Virol. *65*, 1634–1637.

Corfield, A.P., Wagner, S.A., Clamp, J.R., Kriaris, M.S., and Hoskins, L.C. (1992). Mucin degradation in the human colon: production of sialidase, sialate O-acetylesterase, N-acetylneuraminate lyase, arylesterase, and glycosulfatase activities by strains of fecal bacteria. Infect. Immun. *60*, 3971–3978.

Davril, M., Degroote, S., Humbert, P., Galabert, C., Dumur, V., Lafitte, J.J., Lamblin, G., and Roussel, P. (1999). The sialylation of bronchial mucins secreted by patients suffering from cystic fibrosis or from chronic bronchitis is related to the severity of airway infection. Glycobiology *9*, 311–321.

de Vries, E., de Vries, R.P., Wienholts, M.J., Floris, C.E., Jacobs, M.S., van den Heuvel, A., Rottier, P.J., and de Haan, C.A. (2012). Influenza A virus entry into cells lacking sialylated N-glycans. Proc. Natl. Acad. Sci. USA *109*, 7457–7462.

Ehre, C., Worthington, E.N., Liesman, R.M., Grubb, B.R., Barbier, D., O'Neal, W.K., Sallenave, J.M., Pickles, R.J., and Boucher, R.C. (2012). Overexpressing mouse model demonstrates the protective role of Muc5ac in the lungs. Proc. Natl. Acad. Sci. USA *109*, 16528–16533.

Gagneux, P., Cheriyan, M., Hurtado-Ziola, N., van der Linden, E.C., Anderson, D., McClure, H., Varki, A., and Varki, N.M. (2003). Human-specific regulation of alpha 2-6-linked sialic acids. J. Biol. Chem. *278*, 48245–48250.

Gambaryan, A., Webster, R., and Matrosovich, M. (2002). Differences between influenza virus receptors on target cells of duck and chicken. Arch. Virol. *147*, 1197–1208.

Ganesan, S., Comstock, A.T., and Sajjan, U.S. (2013). Barrier function of airway tract epithelium. Tissue Barriers *1*, e24997.

Gibson, R.L., Burns, J.L., and Ramsey, B.W. (2003). Pathophysiology and management of pulmonary infections in cystic fibrosis. Am. J. Respir. Crit. Care Med. *168*, 918–951.

Gottschalk, A. (1957). Neuraminidase: the specific enzyme of influenza virus and Vibrio cholerae. Biochim. Biophys. Acta *23*, 645–646.

Groux-Degroote, S., Krzewinski-Recchi, M.A., Cazet, A., Vincent, A., Lehoux, S., Lafitte, J.J., Van Seuningen, I., and Delannoy, P. (2008). IL-6 and IL-8 increase the expression of glycosyltransferases and sulfotransferases involved in the biosynthesis of sialylated and/or sulfated Lewisx epitopes in the human bronchial mucosa. Biochem. J. *410*, 213–223.

Hartl, D., Gaggar, A., Bruscia, E., Hector, A., Marcos, V., Jung, A., Greene, C., McElvaney, G., Mall, M., and Döring, G. (2012). Innate immunity in cystic fibrosis lung disease. J. Cyst. Fibros. *11*, 363–382.

Hashimoto, K., Graham, B.S., Ho, S.B., Adler, K.B., Collins, R.D., Olson, S.J., Zhou, W., Suzutani, T., Jones, P.W., Goleniewska, K., et al. (2004). Respiratory syncytial virus in allergic lung inflammation increases Muc5ac and gob-5. Am. J. Respir. Crit. Care Med. *170*, 306–312.

Hashimoto, K., Durbin, J.E., Zhou, W., Collins, R.D., Ho, S.B., Kolls, J.K., Dubin, P.J., Sheller, J.R., Goleniewska, K., O'Neal, J.F., et al. (2005). Respiratory syncytial virus infection in the absence of STAT 1 results in airway dysfunction, airway mucus, and augmented IL-17 levels. J. Allergy Clin. Immunol. *116*, 550–557.

Hoskins, L.C., and Boulding, E.T. (1981). Mucin degradation in human colon ecosystems. Evidence for the existence and role of bacterial subpopulations producing glycosidases as extracellular enzymes. J. Clin. Invest. *67*, 163–172.

Hotard, A.L., Lee, S., Currier, M.G., Crowe, J.E., Jr., Sakamoto, K., Newcomb, D.C., Peebles, R.S., Jr., Plemper, R.K., and Moore, M.L. (2015). Identification of residues in the human respiratory syncytial virus fusion protein that modulate fusion activity and pathogenesis. J. Virol. *89*, 512–522.

Hovenberg, H.W., Davies, J.R., and Carlstedt, I. (1996). Different mucins are produced by the surface epithelium and the submucosa in human trachea: identification of MUC5AC as a major mucin from the goblet cells. Biochem. J. *318*, 319–324.

Hoyert, D.L., and Xu, J. (2012). Deaths: preliminary data for 2011. Natl. Vital Stat. Rep. *61*, 1–51.

Irie, A., Koyama, S., Kozutsumi, Y., Kawasaki, T., and Suzuki, A. (1998). The molecular basis for the absence of N-glycolylneuraminic acid in humans. J. Biol. Chem. *273*, 15866–15871.

Ito, T., Couceiro, J.N., Kelm, S., Baum, L.G., Krauss, S., Castrucci, M.R., Donatelli, I., Kida, H., Paulson, J.C., Webster, R.G., and Kawaoka, Y. (1998). Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. J. Virol. *72*, 7367–7373.

Jeffries, J.L., Jia, J., Choi, W., Choe, S., Miao, J., Xu, Y., Powell, R., Lin, J., Kuang, Z., Gaskins, H.R., et al. (2015). Pseudomonas aeruginosa pyocyanin modulates mucin glycosylation with sialyl-Lewis^{x} to increase binding to airway epithelial cells. Mucosal Immunol. Published online November 11, 2015. http://dx.doi.org/10.1038/mi.2015.119.

Jia, N., Barclay, W.S., Roberts, K., Yen, H.L., Chan, R.W., Lam, A.K., Air, G., Peiris, J.S., Dell, A., Nicholls, J.M., and Haslam, S.M. (2014). Glycomic characterization of respiratory tract tissues of ferrets: implications for its use in influenza virus infection studies. J. Biol. Chem. *289*, 28489–28504.

Kesimer, M., Scull, M., Brighton, B., DeMaria, G., Burns, K., O'Neal, W., Pickles, R.J., and Sheehan, J.K. (2009). Characterization of exosome-like vesicles released from human tracheobronchial ciliated epithelium: a possible role in innate defense. FASEB J. *23*, 1858–1868.

Kobasa, D., Kodihalli, S., Luo, M., Castrucci, M.R., Donatelli, I., Suzuki, Y., Suzuki, T., and Kawaoka, Y. (1999). Amino acid residues contributing to the substrate specificity of the influenza A virus neuraminidase. J. Virol. *73*, 6743–6751.

Lai, S.K., Wang, Y.Y., Wirtz, D., and Hanes, J. (2009). Micro- and macrorheology of mucus. Adv. Drug Deliv. Rev. *61*, 86–100.

Lang, T., Hansson, G.C., and Samuelsson, T. (2007). Gel-forming mucins appeared early in metazoan evolution. Proc. Natl. Acad. Sci. USA *104*, 16209–16214.

Laver, W.G., Colman, P.M., Webster, R.G., Hinshaw, V.S., and Air, G.M. (1984). Influenza virus neuraminidase with hemagglutinin activity. Virology *137*, 314–323.

Li, J.D., Dohrman, A.F., Gallup, M., Miyata, S., Gum, J.R., Kim, Y.S., Nadel, J.A., Prince, A., and Basbaum, C.B. (1997). Transcriptional activation of mucin by Pseudomonas aeruginosa lipopolysaccharide in the pathogenesis of cystic fibrosis lung disease. Proc. Natl. Acad. Sci. USA *94*, 967–972.

Li, Y., Dinwiddie, D.L., Harrod, K.S., Jiang, Y., and Kim, K.C. (2010). Anti-inflammatory effect of MUC1 during respiratory syncytial virus infection of lung epithelial cells in vitro. Am. J. Physiol. Lung Cell. Mol. Physiol. *298*, L558–L563.

Li, W., Yan, F., Zhou, H., Lin, X., Wu, Y., Chen, C., Zhou, N., Chen, Z., Li, J.D., and Shen, H. (2013). P. aeruginosa lipopolysaccharide-induced MUC5AC and CLCA3 expression is partly through Duox1 in vitro and in vivo. PLoS ONE *8*, e63945.

Lillehoj, E.P., Kim, B.T., and Kim, K.C. (2002). Identification of Pseudomonas aeruginosa flagellin as an adhesin for Muc1 mucin. Am. J. Physiol. Lung Cell. Mol. Physiol. *282*, L751–L756.

Lillehoj, E.P., Hyun, S.W., Feng, C., Zhang, L., Liu, A., Guang, W., Nguyen, C., Luzina, I.G., Atamas, S.P., Passaniti, A., et al. (2012). NEU1 sialidase expressed in human airway epithelia regulates epidermal growth factor receptor (EGFR) and MUC1 protein signaling. J. Biol. Chem. *287*, 8214–8231.

Lillehoj, E.P., Kato, K., Lu, W., and Kim, K.C. (2013). Cellular and molecular biology of airway mucins. Int. Rev. Cell Mol. Biol. *303*, 139–202.

Lin, Y.P., Gregory, V., Collins, P., Kloess, J., Wharton, S., Cattle, N., Lackenby, A., Daniels, R., and Hay, A. (2010). Neuraminidase receptor binding variants of human influenza A(H3N2) viruses resulting from substitution of aspartic acid 151 in the catalytic site: a role in virus attachment? J. Virol. *84*, 6769–6781.

Lora, J.M., Zhang, D.M., Liao, S.M., Burwell, T., King, A.M., Barker, P.A., Singh, L., Keaveney, M., Morgenstern, J., Gutiérrez-Ramos, J.C., et al. (2005). Tumor necrosis factor-alpha triggers mucus production in airway epithelium through an IkappaB kinase beta-dependent mechanism. J. Biol. Chem. *280*, 36510–36517.

Lovewell, R.R., Hayes, S.M., O'Toole, G.A., and Berwin, B. (2014). Pseudomonas aeruginosa flagellar motility activates the phagocyte PI3K/Akt pathway to induce phagocytic engulfment. Am. J. Physiol. Lung Cell. Mol. Physiol. *306*, L698–L707.

Lu, W., Hisatsune, A., Koga, T., Kato, K., Kuwahara, I., Lillehoj, E.P., Chen, W., Cross, A.S., Gendler, S.J., Gewirtz, A.T., and Kim, K.C. (2006). Cutting edge: enhanced pulmonary clearance of Pseudomonas aeruginosa by Muc1 knockout mice. J. Immunol. *176*, 3890–3894.

Luan, X., Campanucci, V.A., Nair, M., Yilmaz, O., Belev, G., Machen, T.E., Chapman, D., and Ianowski, J.P. (2014). Pseudomonas aeruginosa triggers CFTR-mediated airway surface liquid secretion in swine trachea. Proc. Natl. Acad. Sci. USA *111*, 12930–12935.

Luzar, M.A., Thomassen, M.J., and Montie, T.C. (1985). Flagella and motility alterations in Pseudomonas aeruginosa strains from patients with cystic fibrosis: relationship to patient clinical condition. Infect. Immun. *50*, 577–582.

Martino, M.B., Jones, L., Brighton, B., Ehre, C., Abdulah, L., Davis, C.W., Ron, D., O'Neal, W.K., and Ribeiro, C.M. (2013). The ER stress transducer IRE1β is required for airway epithelial mucin production. Mucosal Immunol. *6*, 639–654.

Matrosovich, M., Tuzikov, A., Bovin, N., Gambaryan, A., Klimov, A., Castrucci, M.R., Donatelli, I., and Kawaoka, Y. (2000). Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals. J. Virol. *74*, 8502–8512.

Matrosovich, M.N., Matrosovich, T.Y., Gray, T., Roberts, N.A., and Klenk, H.D. (2004). Neuraminidase is important for the initiation of influenza virus infection in human airway epithelium. J. Virol. *78*, 12665–12667.

Matsuoka, Y., Swayne, D.E., Thomas, C., Rameix-Welti, M.A., Naffakh, N., Warnes, C., Altholtz, M., Donis, R., and Subbarao, K. (2009). Neuraminidase stalk length and additional glycosylation of the hemagglutinin influence the virulence of influenza H5N1 viruses for mice. J. Virol. *83*, 4704–4708.

Miller, A.L., Strieter, R.M., Gruber, A.D., Ho, S.B., and Lukacs, N.W. (2003). CXCR2 regulates respiratory syncytial virus-induced airway hyperreactivity and mucus overproduction. J. Immunol. *170*, 3348–3356.

Ng, P.S., Böhm, R., Hartley-Tassell, L.E., Steen, J.A., Wang, H., Lukowski, S.W., Hawthorne, P.L., Trezise, A.E., Coloe, P.J., Grimmond, S.M., et al. (2014). Ferrets exclusively synthesize Neu5Ac and express naturally humanized influenza A virus receptors. Nat. Commun. *5*, 5750.

Nicholls, J.M., Bourne, A.J., Chen, H., Guan, Y., and Peiris, J.S. (2007). Sialic acid receptor detection in the human respiratory tract: evidence for widespread distribution of potential binding sites for human and avian influenza viruses. Respir. Res. *8*, 73.

Ohuchi, M., Asaoka, N., Sakai, T., and Ohuchi, R. (2006). Roles of neuraminidase in the initial stage of influenza virus infection. Microbes Infect. *8*, 1287–1293.

Palese, P., and Shaw, M.L. (2013). Orthomyxoviridae: the viruses and their replication. In Fields Virology, D.M. Knipe and P.M. Howley, eds. (Philadelphia: Lippincott Williams & Wilkins), pp. 1647–1689.

Pastoriza Gallego, M., and Hulen, C. (2006). Influence of sialic acid and bacterial sialidase on differential adhesion of Pseudomonas aeruginosa to epithelial cells. Colloids Surf. B Biointerfaces *52*, 154–156.

Persson, B.D., Jaffe, A.B., Fearns, R., and Danahay, H. (2014). Respiratory syncytial virus can infect basal cells and alter human airway epithelial differentiation. PLoS ONE *9*, e102368.

Pickles, R.J., and DeVincenzo, J.P. (2015). Respiratory syncytial virus (RSV) and its propensity for causing bronchiolitis. J. Pathol. *235*, 266–276.

Pietrantoni, A., Ammendolia, M.G., and Superti, F. (2012). Bovine lactoferrin: involvement of metal saturation and carbohydrates in the inhibition of influenza virus infection. Biochem. Cell Biol. *90*, 442–448.

Puchelle, E., Zahm, J.M., and Havez, R. (1973). [Biochemical and rheological data in sputum. 3. Relationship between the biochemical constituents and the rheological properties of sputum (author's transl)]. Bull. Physiopathol. Respir. (Nancy) *9*, 237–257.

Rada, B., Gardina, P., Myers, T.G., and Leto, T.L. (2011). Reactive oxygen species mediate inflammatory cytokine release and EGFR-dependent mucin secretion in airway epithelial cells exposed to Pseudomonas pyocyanin. Mucosal Immunol. *4*, 158–171.

Ridley, C., Kouvatsos, N., Raynal, B.D., Howard, M., Collins, R.F., Desseyn, J.L., Jowitt, T.A., Baldock, C., Davis, C.W., Hardingham, T.E., and Thornton, D.J. (2014). Assembly of the respiratory mucin MUC5B: a new model for a gel-forming mucin. J. Biol. Chem. *289*, 16409–16420.

Roy, M.G., Livraghi-Butrico, A., Fletcher, A.A., McElwee, M.M., Evans, S.E., Boerner, R.M., Alexander, S.N., Bellinghausen, L.K., Song, A.S., Petrova, Y.M., et al. (2014). Muc5b is required for airway defence. Nature *505*, 412–416.

Scharfman, A., Degroote, S., Beau, J., Lamblin, G., Roussel, P., and Mazurier, J. (1999). Pseudomonas aeruginosa binds to neoglycoconjugates bearing mucin carbohydrate determinants and predominantly to sialyl-Lewis x conjugates. Glycobiology *9*, 757–764.

Schauer, R., Srinivasan, G.V., Coddeville, B., Zanetta, J.P., and Guérardel, Y. (2009). Low incidence of N-glycolylneuraminic acid in birds and reptiles and its absence in the platypus. Carbohydr. Res. *344*, 1494–1500.

Sheehan, J.K., Kesimer, M., and Pickles, R. (2006). Innate immunity and mucus structure and function. Novartis Found. Symp. *279*, 155–166, discussion 167–159, 216–159.

Shinya, K., Ebina, M., Yamada, S., Ono, M., Kasai, N., and Kawaoka, Y. (2006). Avian flu: influenza virus receptors in the human airway. Nature *440*, 435–436.

Shogren, R., Gerken, T.A., and Jentoft, N. (1989). Role of glycosylation on the conformation and chain dimensions of O-linked glycoproteins: light-scattering studies of ovine submaxillary mucin. Biochemistry *28*, 5525–5536.

Springer, S.A., and Gagneux, P. (2013). Glycan evolution in response to collaboration, conflict, and constraint. J. Biol. Chem. *288*, 6904–6911.

Springer, S.A., Diaz, S.L., and Gagneux, P. (2014). Parallel evolution of a self-signal: humans and new world monkeys independently lost the cell surface sugar Neu5Gc. Immunogenetics *66*, 671–674.

Stanley, P., Schachter, H., and Taniguchi, N. (2009). N-glycans. In Essentials of Glycobiology, Second Edition, A. Varki, R.D. Cummings, J.D. Esko, H.H. Freeze, P. Stanley, C.R. Bertozzi, G.W. Hart, and M.E. Etzler, eds. (New York: Cold Spring Harbor Laboratory Press).

Staudinger, B.J., Muller, J.F., Halldórsson, S., Boles, B., Angermeyer, A., Nguyen, D., Rosen, H., Baldursson, O., Gottfreðsson, M., Guðmundsson, G.H., and Singh, P.K. (2014). Conditions associated with the cystic fibrosis defect promote chronic Pseudomonas aeruginosa infection. Am. J. Respir. Crit. Care Med. *189*, 812–824.

Stevens, J., Blixt, O., Glaser, L., Taubenberger, J.K., Palese, P., Paulson, J.C., and Wilson, I.A. (2006). Glycan microarray analysis of the hemagglutinins from modern and pandemic influenza viruses reveals different receptor specificities. J. Mol. Biol. *355*, 1143–1155.

Stokes, K.L., Currier, M.G., Sakamoto, K., Lee, S., Collins, P.L., Plemper, R.K., and Moore, M.L. (2013). The respiratory syncytial virus fusion protein and neutrophils mediate the airway mucin response to pathogenic respiratory syncytial virus infection. J. Virol. *87*, 10070–10082.

Stone, J.D. (1949). Tryptic inactivation of the receptor-destroying enzeme of V. cholerae and of the enzymic activity of influenza virus. Aust. J. Exp. Biol. Med. Sci. *27*, 229–244.

Suzuki, Y., Ito, T., Suzuki, T., Holland, R.E., Jr., Chambers, T.M., Kiso, M., Ishida, H., and Kawaoka, Y. (2000). Sialic acid species as a determinant of the host range of influenza A viruses. J. Virol. *74*, 11825–11831.

Takahashi, T., Takano, M., Kurebayashi, Y., Masuda, M., Kawagishi, S., Takaguchi, M., Yamanaka, T., Minami, A., Otsubo, T., Ikeda, K., and Suzuki, T. (2014). N-glycolylneuraminic acid on human epithelial cells prevents entry of influenza A viruses that possess N-glycolylneuraminic acid binding ability. J. Virol. *88*, 8445–8456.

Temann, U.A., Prasad, B., Gallup, M.W., Basbaum, C., Ho, S.B., Flavell, R.A., and Rankin, J.A. (1997). A novel role for murine IL-4 in vivo: induction of MUC5AC gene expression and mucin hypersecretion. Am. J. Respir. Cell Mol. Biol. *16*, 471–478.

Thornton, D.J., Rousseau, K., and McGuckin, M.A. (2008). Structure and function of the polymeric mucins in airways mucus. Annu. Rev. Physiol. *70*, 459–486.

Tumpey, T.M., Maines, T.R., Van Hoeven, N., Glaser, L., Solórzano, A., Pappas, C., Cox, N.J., Swayne, D.E., Palese, P., Katz, J.M., and García-Sastre, A. (2007). A two-amino acid change in the hemagglutinin of the 1918 influenza virus abolishes transmission. Science *315*, 655–659.

Umehara, T., Kato, K., Park, Y.S., Lillehoj, E.P., Kawauchi, H., and Kim, K.C. (2012). Prevention of lung injury by Muc1 mucin in a mouse model of repetitive Pseudomonas aeruginosa infection. Inflamm. Res. *61*, 1013–1020.

van Riel, D., Munster, V.J., de Wit, E., Rimmelzwaan, G.F., Fouchier, R.A., Osterhaus, A.D., and Kuiken, T. (2006). H5N1 virus attachment to lower respiratory tract. Science *312*, 399.

Verdugo, P., Aitken, M., Langley, L., and Villalon, M.J. (1987a). Molecular mechanism of product storage and release in mucin secretion. II. The role of extracellular Ca++. Biorheology *24*, 625–633.

Verdugo, P., Deyrup-Olsen, I., Aitken, M., Villalon, M., and Johnson, D. (1987b). Molecular mechanism of mucin secretion: I. The role of intragranular charge shielding. J. Dent. Res. *66*, 506–508.

Wakabayashi, H., Oda, H., Yamauchi, K., and Abe, F. (2014). Lactoferrin for prevention of common viral infections. J. Infect. Chemother. *20*, 666–671.

Wickström, C., Davies, J.R., Eriksen, G.V., Veerman, E.C., and Carlstedt, I. (1998). MUC5B is a major gel-forming, oligomeric mucin from human salivary gland, respiratory tract and endocervix: identification of glycoforms and C-terminal cleavage. Biochem. J. *334*, 685–693.

Yan, F., Li, W., Jono, H., Li, Q., Zhang, S., Li, J.D., and Shen, H. (2008). Reactive oxygen species regulate Pseudomonas aeruginosa lipopolysaccharide-induced MUC5AC mucin expression via PKC-NADPH oxidase-ROS-TGF-alpha signaling pathways in human airway epithelial cells. Biochem. Biophys. Res. Commun. *366*, 513–519.

Yang, X., Steukers, L., Forier, K., Xiong, R., Braeckmans, K., Van Reeth, K., and Nauwynck, H. (2014). A beneficiary role for neuraminidase in influenza virus penetration through the respiratory mucus. PLoS ONE *9*, e110026.

Yeung, A.T., Parayno, A., and Hancock, R.E. (2012). Mucin promotes rapid surface motility in Pseudomonas aeruginosa. MBio *3*, e00073-12.

Zanin, M., Marathe, B., Wong, S.S., Yoon, S.W., Collin, E., Oshansky, C., Jones, J., Hause, B., and Webby, R. (2015). Pandemic swine H1N1 influenza viruses with almost undetectable neuraminidase activity do not transmit via aerosols in ferrets and are inhibited by human mucus, but not swine mucus. J. Virol. *89*, 5935–5948.

Zheng, S., Xu, W., Bose, S., Banerjee, A.K., Haque, S.J., and Erzurum, S.C. (2004). Impaired nitric oxide synthase-2 signaling pathway in cystic fibrosis airway epithelium. Am. J. Physiol. Lung Cell. Mol. Physiol. *287*, L374–L381.

Zhu, X., McBride, R., Nycholat, C.M., Yu, W., Paulson, J.C., and Wilson, I.A. (2012). Influenza virus neuraminidases with reduced enzymatic activity that avidly bind sialic Acid receptors. J. Virol. *86*, 13371–13383.

Zuhdi Alimam, M., Piazza, F.M., Selby, D.M., Letwin, N., Huang, L., and Rose, M.C. (2000). Muc-5/5ac mucin messenger RNA and protein expression is a marker of goblet cell metaplasia in murine airways. Am. J. Respir. Cell Mol. Biol. *22*, 253–260.