Interplay between respiratory viruses and cilia in the airways

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Shareable abstract (@ERSpublications)
A review of respiratory virus and ciliated cell interactions (host entry, viral replication and dissemination, deciliation, reduced ciliary beat frequency, and dyskinesia); understanding of these dynamics is required to develop new therapies and models. https://bit.ly/3WAgIct

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Abstract

The airway epithelium is the first point of contact for inhaled pathogens. The role of epithelial cells in clearance, infection and colonisation of bacteria is established. The interactions of respiratory viruses and cilia is less understood, but viruses are known to target ciliated epithelial cells for entry, replication and dissemination. Furthermore, some respiratory viruses impair and/or enhance ciliary activity. This review examines what is known about the interactions between cilia and viral infection and how respiratory viruses effect cilia function with subsequent consequences for human health. We discuss the models which can be used to investigate the relationship between respiratory viruses and the host airway.

Introduction

The airway epithelium is a first point of contact with pathogens and plays a critical role to protect the host from infection. Airway pathogens trigger epithelial and immune cell signalling pathways that initiate innate immune responses by cytokines, chemokines and interferons (IFNs) to contain the infection and activate adaptive immune responses. Mucus, secreted by epithelial goblet cells and submucosal glands, provides antimicrobial protection through molecules including defensins, immunoglobulin A and lysozymes. Mucus also physically entraps bacteria and debris, which can then be cleared from the airway by synchronous beating of motile cilia by the process of mucociliary clearance (MCC) (figure 1). Respiratory bacteria such as *Pseudomonas aeruginosa* attempt to overcome MCC by producing factors that impair ciliary activity [1]. Furthermore, many chronic respiratory conditions (*e.g.* primary ciliary dyskinesia, asthma, cystic fibrosis and bronchiectasis) are associated with impaired MCC and increased risk of bacterial infection. Whilst the importance of cilia and MCC to protect against bacterial infections is understood, the relationship between cilia and respiratory viral infections is less clear.

Strains of influenza A virus, influenza B virus, respiratory syncytial virus (RSV), rhinovirus (RV), adenovirus, coronavirus (CoV) and parainfluenza virus are frequent causes of respiratory viral infections, resulting in a spectrum of disease severity from asymptomatic or mild upper airway symptoms to severe pneumonia or life-threatening acute respiratory distress syndrome. Viral infections can also be involved in the pathogenesis of chronic lung disease [2–4] and can expediate a decline in individuals with existing conditions such as COPD [5] and idiopathic pulmonary fibrosis [6]. Ciliated cells are often primary sites of viral infection resulting in subsequent impairment of ciliary function and immunity, risking secondary bacterial infection [7]. Whether cilia protect against viral infection is unclear and intact MCC might even facilitate early distribution of viruses through the airway during infection [8].

Morbidity and mortality caused by respiratory viruses places significant burden on health and social care, workforces and the economy even during nonpandemic years. The coronavirus disease 2019 (COVID-19)

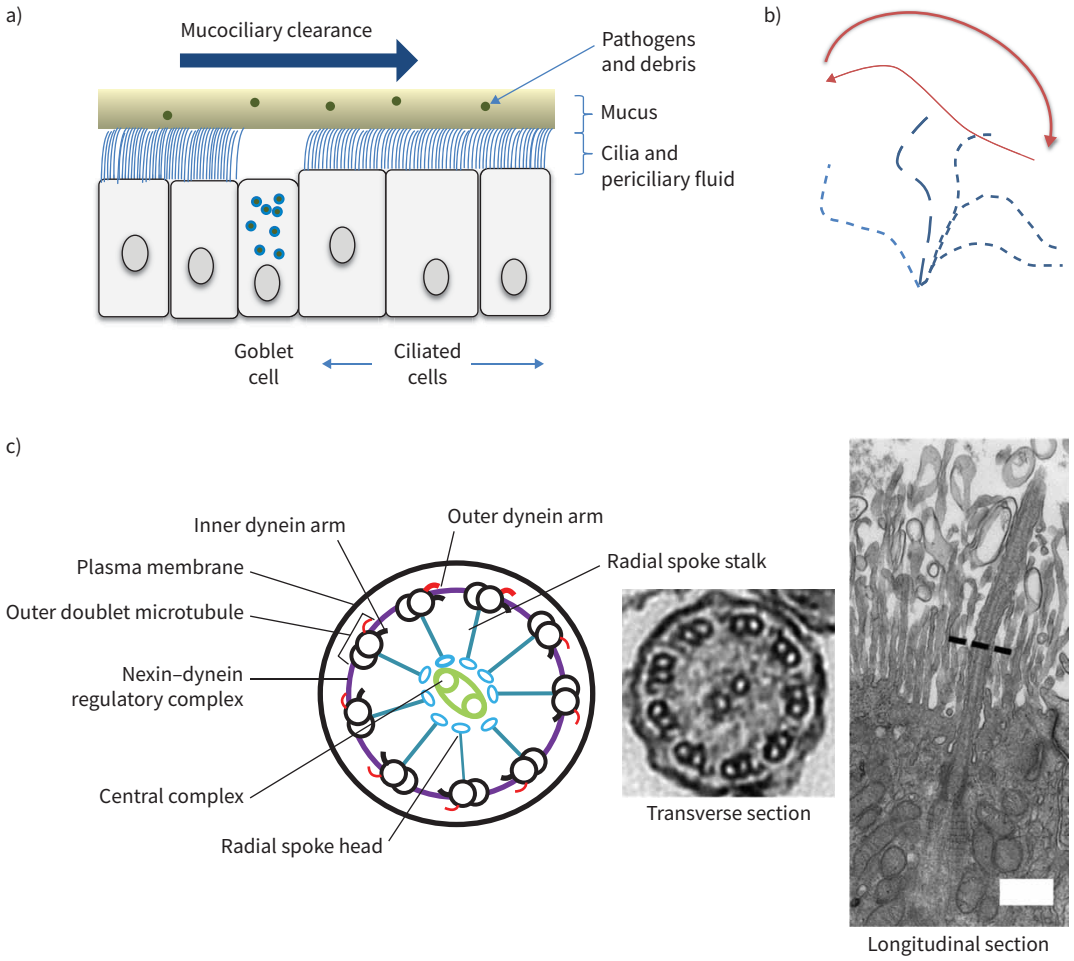

FIGURE 1 Respiratory cilia beat in a coordinated sweeping pattern, a) which moves mucus and debris, including pathogens towards the oropharynx for swallowing or expectorating. b) The cilia beat forwards in a metachronal wave to propel mucus forward, followed by a recovery stroke. c) Labelled diagram of a normal ciliary axonemal ultrastructure and transmission electron micrographs of normal ciliary ultrastructure in transverse and longitudinal sections (labelled). The white scale bar in longitudinal section image: 500 nm. A normal ciliary axoneme is approximately 200 nm in diameter.

pandemic highlighted the need for improved understanding of viral infections as a prerequisite to improved diagnoses, treatment and prognoses. In the recent pandemic, people with primary ciliary dyskinesis (PCD) who have inherent ciliary dysfunction and severely impaired MCC did not appear to fare better or worse than the general population or those with other respiratory diseases [9, 10]. In this review, we discuss cilia in the airway and then provide an overview of common respiratory viruses. We then explore what is known about the reciprocal relationship between motile cilia and respiratory viruses, highlighting how viruses interact with ciliary structures and functions and the repercussions of this interplay on the course of acute viral infections and on progression of chronic respiratory disease. We discuss models that might be used to investigate cilia–virus interactions and consider how these might facilitate development of novel therapies.

Literature search strategy

This narrative review was informed by a literature search. PubMed was searched by the authors for publications relating to cilia and respiratory virus interactions (December 2023 updated August 2024) using the term “cilia”, in conjunction with (“respiratory virus”; rhinovirus; influenza; coronavirus; respiratory syncytial virus). Titles and abstracts were used to identify primary publications for full-text review. Review articles were used to identify further primary research studies. We additionally included manuscripts known to the authors concerning cilia or respiratory viruses for the general background review.

Cilia

Cilia are microtubule-based organelles protruding from the apical surfaces of cell membranes [11]. They have an axonemal ultrastructure comprising ≈600 proteins, with protein expression and axonemal composition differing somewhat between tissue types [12–14]. Fundamentally, there are two main cilia categories, namely primary and motile cilia. Primary monocilia are present on most mammalian cells and have a sensory function, acting as an extracellular antenna to transduce "signals" to the nucleus. Motile cilia primarily serve to create dynamic fluid flow and MCC. Embryonic nodal cells have a single motile cilium and multiple cilia are found on columnar epithelial cells of the airways, fallopian tubes, Eustachian tubes and cerebral ventricles.

All cilia share a similar base, basal body, transition zone and elongated axonemal scaffold. The axonemal structure of all cilia contains an arrangement of nine peripheral doublet microtubules (a and b) adjoined by nexin links (figure 1).

Motile cilia in the airways

The human airway from the nasal cavity to the terminal bronchioles is lined by a pseudostratified columnar epithelium predominantly composed of ciliated and basal progenitor epithelial cells, mucus secreting cells and immune modulating cells. There are approximately 200 motile cilia on the apical surface of each ciliated epithelial cell, with the percentage of ciliated cells decreasing distally in the respiratory tree. Motile cilia in the airways share the axonemal structure outlined above, with an additional two central singlet microtubules surrounded by a protein sheath referred to collectively as the "central pair complex", creating a "9+2" microtubular arrangement (figure 1). Radial spoke structures coordinate central and peripheral microtubule dynamics. Associated with the peripheral doublets of motile cilia, axonemal dyneins are ATP-driven, mechano-chemically regulated, motor proteins responsible for cilia motility.

Mucociliary clearance in the airways

Airway mucus traps extracellular debris, microbial pathogens, environmental particulates and allergens. Motile cilia coordinate beating to produce a metachronal wave movement across the epithelial surface, propelling mucus with entrapped foreign matter towards the oropharynx where it can be swallowed or expectorated [15–17] (figure 1).

The consequences of severely impaired MCC are exemplified in people with PCD, a heterogenous syndrome effecting ≈1:7500 of the population [18, 19]. PCD is usually inherited as an autosomal recessive condition caused by bi-allelic variants in over 50 cilia-related genes. The resulting ciliary dysfunction leads to severely compromised MCC. Individuals with this condition have recurrent bacterial infections and chronic colonisation of their airways leading to suppurative lung disease, chronic rhinosinusitis, serous otitis media and bronchiectasis [20].

Airway inflammation and respiratory infection in otherwise healthy individuals can cause secondary ciliary dyskinesia, also impairing MCC, although the consequences may be short-lived once the acute condition has resolved. However, people with chronic airway diseases such as asthma, COPD, cystic fibrosis and idiopathic bronchiectasis often incur secondary ciliary dyskinesia and impaired MCC due to ongoing inflammation and infection [21–23]. Secondary, nonspecific cilia ultrastructural abnormalities are commonly seen by transmission electron microscopy in individuals with chronic airways disease and abnormalities of ciliary beat pattern are also observed [24]. Importantly, respiratory viruses, including influenza, RVs, CoVs and RSV, have evolved strategies to target ciliated airway cells, thereby impeding MCC [25–29]. Whatever the underlying cause of the dyskinesia, impaired MCC leads to accumulation of mucus, particles and infectious agents in the airways, exacerbating inflammation, tissue damage and creating a favourable environment for secondary bacterial infections [30, 31]. Furthermore, respiratory viruses cause hypersecretion of viscous mucus through common as well as distinctive mechanisms [32]. This mucus might be difficult to clear even if cilia are capable of functioning normally and impaired MCC is exacerbated if the infection also causes ciliary dysfunction. Eventually, the cycle of infection, inflammation, airway obstruction and airway damage can lead to irreversible bronchiectasis. These interactions are complex, with each pathophysiological step contributing to all the others, forming a "vortex" [33] (figure 2). Therefore, an acute viral infection might trigger a self-perpetuating cascade of events which if not stopped results in progressive and irreversible lung damage.

Respiratory viruses

Viruses that cause disease in the respiratory tract are primarily RNA viruses either containing positive or negative sense genomes. The biology of these two viral groups is quite distinct, reflecting the diverse

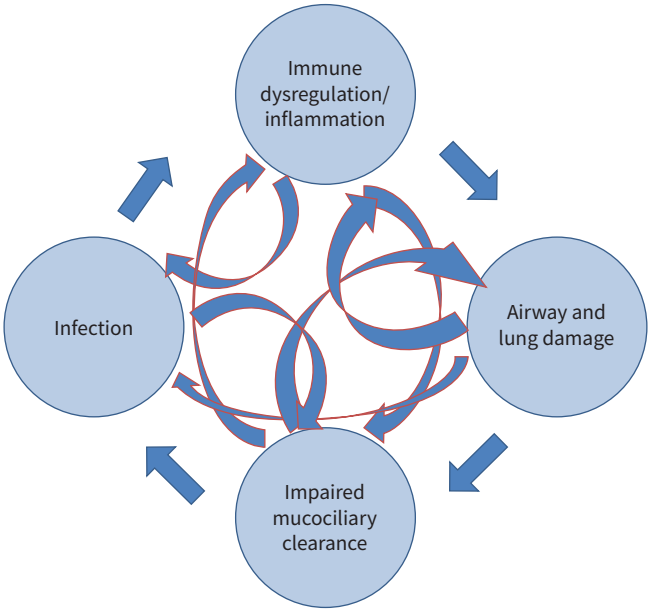

FIGURE 2 A triggering event (*e.g.* viral infection), or genetic condition affecting mucociliary clearance (*e.g.* primary ciliary dyskinesis), can lead to a persistent and progressive "vortex" of processes including inflammation and airway damage. Adapted from Flume *et al*. [33].

clinical outcomes these viruses cause. We outline the disease-causing respiratory viruses in terms of their biology and respiratory cell tropism.

Rhinoviruses

RVs are the most well recognised cause of the common cold and account for over 40% of all viral upper respiratory tract infections [34]. Symptoms are often mild in the immunocompetent, typically resulting in acute rhinosinusitis. However, RV infection can cause exacerbations of asthma and COPD, and in some cases, increase the risk of bacterial superinfection [35]. RV are members of the *Picornaviridae* family, which are nonenveloped, positive-sense RNA viruses, with a single-stranded genome ranging from 6.7 to 10.1 kilobases in length. Three RV species exist, namely A, B and C, each of which contain a wide range of genotypes, with typing based on the genetic diversity of the viral VP1 structural protein, a key subunit of the icosahedral viral capsid [36].

Coronaviruses

CoVs are a diverse family of enveloped viruses with the largest documented positive sense RNA genome. Their namesake is derived from the large multimeric spike protein that protrudes from the viral particle conferring a "crown-like" shape. Belonging to the *Nidovirales* order, the *Coronavirdae* are classified into four subgenera, Alpha-, Beta-, Delta- and Gamma-CoVs, all possessing a broad mammalian and avian host range. Seven CoVs are known to infect humans, NL63 and 229E from the alpha genus and OC43, HKU1, Middle East respiratory syndrome (MERS)-CoV, severe acute respiratory syndrome (SARS)-CoV-1 and SARS-CoV-2 from the beta genus. Until the 2003/4 outbreak of SARS-CoV-1, these viruses were not considered pathogenic, as circulating human CoVs caused only mild disease in immunocompetent individuals. However, people infected with SARS-CoV-1 develop systemic symptoms of myalgia, fever and lymphocytopenia, accompanied by severe respiratory distress, cough and viral pneumonia [37] The mortality during the 2003 outbreak was 9% and was much higher in the elderly population. Stringent public health measures, including testing symptomatic individuals, isolating suspected cases and restricting travel, brought the outbreak under control.

With the subsequent outbreaks of MERS-CoV and the SARS-CoV-2 pandemic, the perception that CoV infections are mostly inconsequential changed. MERS-CoV first emerged in humans in 2012, with isolated cases and sporadic outbreaks associated with an estimated mortality of 35%. Although human-to-human transmission can occur, most spread of MERS-CoV to humans is from animals, *e.g.* camels. More recently, the outbreak of SARS-CoV-2 that started in 2019 rapidly progressed to a global pandemic. Associated

with high human-to-human transmission and severe respiratory disease, it is estimated that deaths attributable to the virus now exceed 7 million [38].

When comparing the newly emergent SARS-CoV-2 with MERS and SARS-CoV, the clinical symptoms were noted to be broader and more variable, highlighting a potential discrepancy in viral shedding and/or replication efficiency reflecting an altered *in vivo* replication site [39–42]. The increased tropism of SARS-CoV-2 for ciliated cells of the upper respiratory tract compared to MERS and SARS-CoV is likely a key contributing factor accounting for the success of this pathogen as a pandemic-causing virus.

Negative-strand RNA viruses

Influenza

The influenza viruses are subdivided into three subtypes, influenza A (IAV), B (IBV) and C (ICV), with A and B causing the seasonal epidemics and C often resulting in a subclinical infection [43, 44]. The symptoms of influenza are comparatively more severe than the typical common cold, particularly in the elderly or those with underlying health issues. As members of the *Orthomyxoviridae*, these viruses are negative-strand, segmented RNA viruses with their genome uniquely organised into seven (ICV) or eight (IAV, IBV) segments encoding the viral proteins and replicase. The genome is the enclosed by a nucleocapsid surrounded by a lipid envelope to form the mature virion.

Respiratory syncytial virus

RSV is a pnuemovirus of the *Paramyxovirdae* typically characterised by a linear single-stranded negative-sense RNA genome contained within an enveloped nucleocapsid particle. Other notable members of this viral family include mumps, parainfluenza and measles viruses. RSV is the single most important cause of respiratory disease and hospitalisations in infants [45, 46] and is the most common cause of bronchiolitis and pneumonia in infants and young children worldwide [47–49]. Historically, RSV has been categorised into two distinct subtypes, namely RSV-A and RSV-B, based on antigenic variability in the G glycoprotein, although there is limited conclusive evidence for any significant distinctions in disease outcome with either subtype [50, 51]. Severe infection in infants can lead to long-term respiratory sequalae, including asthma [52], emphasising the importance of early intervention and preventative measures.

Motile cilia and respiratory virus infections

Host entry sites during viral infection are often on ciliated epithelial cells and viruses may impair ciliary function (figure 3). The strategic utilisation of ciliated cells as entry points, replication sites and for dissemination demonstrates their ability to exploit host cell features for their survival and propagation. However, understanding of interactions between cilia and respiratory viruses remains limited. Here we review what is known about ciliated epithelial interactions with key respiratory viruses.

Rhinoviruses and cilia

The host entry factors necessary for infection vary between RV genotypes, with RV-A and RV-B utilising the intracellular adhesion molecule (ICAM-1) [53], while RV-C uses cadherin-related family member 3 (CDHR3) as a receptor [28, 54, 55]. As ICAM-1 is expressed apically on both on ciliated and nonciliated epithelia cells, this enables RV-A and RV-B to infect both ciliated and secretory respiratory cells [53]. In contrast, RV-C exclusively infects ciliated epithelia, correlating with CDHR3 expression [28, 54, 55], highlighting how the distinction in receptor usage defines the cellular tropism of RV genotypes within the respiratory tract. RV does not rapidly induce cell damage and death, but does cause deciliation and decreased cilia beat frequency [28, 56, 57]. Recent work suggests that RV-C infection in particular is associated with more severe respiratory illness and increased exacerbation of asthma in children [58]. The predominance of RV-C to exclusively infect ciliated epithelia may contribute to the altered pathogenesis with this genotype and warrants further investigation. This is an example where immotile cilia (*e.g.* PCD caused by variants in *DNAH5*) or reduced numbers of cilia (*e.g.* PCD caused by variants in *CCNO*) from persons with PCD could be used to model hypotheses of these modes of interaction.

Coronaviruses and cilia

Host entry factors differ between the various CoVs, which dictates their dissemination throughout the respiratory tract. CD13, the receptor for 229E for example, is expressed on the apical surface of nonciliated epithelial cells, with recent studies showing productive infection primarily in alveolar macrophages and to a much lesser degree in type 1 pneumocytes [59]. OC43, however, infects ciliated nasal and bronchial airway epithelial cells as well as the alveolar epithelia, gaining entry *via* apically expressed human leukocyte antigen-class I and sialic acid [60, 61].

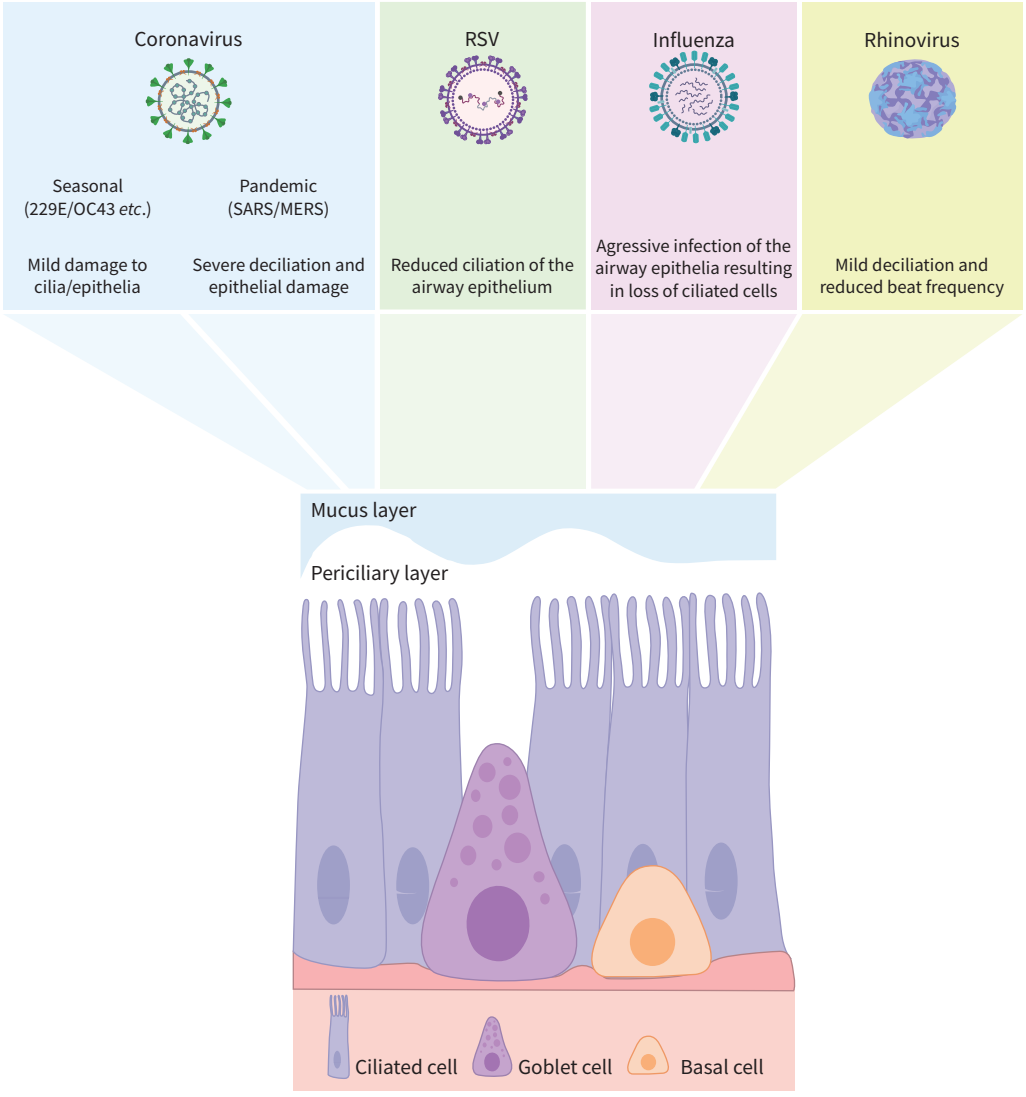

FIGURE 3 Respiratory viruses have differing impacts on the ciliated airway epithelia, ranging from mild deciliation (rhinovirus) to significant damage and loss of ciliated cells (severe acute respiratory syndrome (SARS) and influenza). This spectrum of viral effects on ciliated airway epithelia underscores the importance of understanding the specific mechanisms by which different respiratory pathogens interact with and potentially disrupt the mucociliary clearance system. MERS: Middle East respiratory syndrome; RSV: respiratory syncytial virus.

SARS-CoV and SARS-CoV-2 share the same receptor, namely angiotensin-converting enzyme 2 (ACE2) [62], which is highly expressed on the apical surface of the ciliated epithelium. Unlike the SARS-CoVs, MERS utilises dipeptidyl peptidase 4 (DPP4) to enter cells, enabling infection of a broad range of respiratory epithelial cells including the alveolar epithelia. When comparing the newly emergent SARS-CoV-2 with MERS and SARS-CoV, the clinical symptoms were noted to be broader and more variable, highlighting a potential discrepancy in viral shedding and/or replication efficiency reflecting an altered *in vivo* replication site [39–42]. Analysis of upper and lower respiratory tract tissues revealed a proximal to distal gradient of infection with SARS-CoV-2 reflective of a preferential replication in the upper airways correlating with viral receptor expression [63]. The increased tropism of SARS-CoV-2 for ciliated cells of the upper respiratory tract compared with MERS and SARS-CoV is likely a key contributing factor accounting for the success of this pathogen as a pandemic-causing virus.

The emergence of SARS-CoV-2 variants of concern, with altered respiratory tract tropism, was an important development in the evolutionary adaption of the virus to the host. Amongst these variants, it is

now clear that the Omicron lineage was optimised for replication in the upper respiratory tract with more limited replication in the lower airways, whilst Delta resulted in the most cytopathic infection in both the upper and lower areas of the lung, severely compromising both barrier integrity and ciliary beat function [64].

The critical role of airway cilia during infection and airway spread of SARS-CoV-2 has recently been demonstrated [41]. SARS-CoV-2 binds to ciliary ACE2 receptors to facilitate entry prior to trafficking along the cilium to the cell body and deletion of cilia inhibits infection. Nascent virus particles then locate to microvilli, triggering formation of highly branched microvilli that extend into the mucus layer for dispersal to infect other susceptible airway cells. The authors next investigated the importance of ciliary beating and MCC, using primary ciliated airway cultures from individuals with PCD. This confirmed reduced viral spread and replication, with the virus only infecting immediately surrounding cells compared with the large dissemination of viral infection in epithelial cell cultures from individuals with normal ciliary function [41]. Perhaps in contradiction, a separate study reported that pharmacological augmentation of MCC inhibited SARS-CoV-2 replication and that there was no apparent difference in SARS-CoV-2 infectivity at 72 h between nasal epithelia from healthy (n=2) and PCD (n=2) [65]. Clinical observations are that people with PCD do not have increased susceptibility to SARS-CoV-2 infection and that the clinical course is not severe, suggesting that severely impaired MCC does not result in worse SARS-CoV-2 outcomes [9].

Neither 229E nor OC43 cause notable cytopathology in *in vitro* models of infection [66], although some cilia damage is observed in patients with secondary inflammatory processes [67]. In contrast, destruction of ciliated epithelial cells in both the upper and lower respiratory tract is a key pathological feature of SARS-CoV-2 infection in both *in vitro* and *in vivo* models of infection [68–70]. In addition to cellular damage, SARS-CoV-2 replication leads to axoneme loss and misorientation of the basal bodies, with impaired MCC [71]. Downregulation of Foxj1, a regulator of ciliogenesis, occurs prior to cilia loss, implicating its role in this process.

In summary, a small number of studies confirm that cilia have an important role at various stages of CoV infection, with most data available for SARS-CoV-2. This virus exploits airway cilia and MCC for host entry and dissemination. Once the virus spreads to the deeper lung parenchyma, it is no longer dependent on ciliated epithelial cells, cilia or MCC, and we postulate that SARS-CoV-2 has an advantage by destroying these ciliated cells.

Influenza and cilia

The two viral glycoproteins hemagglutinin (HA) and neuraminidase (NA) govern entry and release of progeny virions, with HA binding α2,3- and α2,6-linked sialic acid (α2,3-SA; α2,6-SA) permitting entry though membrane fusion, while NA cleaves sialic acid to release nascent viral particles from the surface of infected cells [44]. In the human airways, α2,6-SA is vastly more abundant in nasal epithelium than α2,3-SA [72]. Similarly, α2,6-SA expression dominates the pharynx, trachea and bronchi, enabling infection of both ciliated and nonciliated secretory cells by influenza [73], resulting in an aggressive infection resulting in high levels of apoptosis and loss of ciliated epithelial cells [74]. Further, an in-depth analysis of respiratory epithelia subtypes infected by influenza demonstrated that ciliated cells are the dominant producer of infectious virions in the upper airway, whilst secretory cells exhibited a reduced viral burden attributed to differential baseline expression of IFN-stimulated genes [75].

IAV preferentially binds α2,6-SA as and IAV antigens are trafficked from the ciliary tips down the ciliary shaft to establish infection in epithelial cells [74]. Based on limited studies looking at different time-points, it appears that after a transient increase in ciliary beat frequency (CBF) [25], beat frequency is decreased between days 2 [76] and 3 [77], and then absent by day 6 [77]. Proposed mechanisms for alterations in CBF are extracellular ATP release in response to Toll-like receptor 3 (TLR3)-activation increasing ciliary activity [25] and specific downregulation of membrane-associated ring-CH-type finger 10, a microtubule-associated protein important for ciliary apparatus, the loss of which reduces ciliary activity [76]. In porcine airways, Fu *et al*. [78] demonstrated that IAV recovery increases when ciliary motility is pharmacologically inhibited within 24 h of infection, suggesting that functional motile cilia limit IAV infection. Additionally, epithelial cell apoptosis becomes apparent 24 h post-IAV infection [74, 79]. These observations align with reduced MCC in *in vitro* IAV infection models [77, 80, 81], indicating that early infection stages are characterised by a rapid decrease in multiciliated cells and impaired ciliary function. Further, a study on IAV infection in murine lung cells [82] identified distinct subpopulations of multiciliated cells with varying responses to infection, potentially explaining the range of disease severity observed. Dumm *et al*. [83] reported that IBV infection led to the emergence of a "ciliated-like survivor

cell population", which differs both phenotypically and transcriptionally from infected and uninfected neighbouring ciliated cells. While these findings are intriguing, further research is necessary to determine whether the observed effects are influenced by the timing of cell infection.

Respiratory syncytial virus and cilia

The primary cellular targets of RSV are airway epithelial cells, which include luminal columnar and ciliated epithelial cells [84–88]. Viral entry is mediated through the fusion (F) and attachment (G) glycoproteins *via* interaction with C-X3-C motif chemokine receptor 1 (CX3CR1) and nucleolin, which are highly expressed on cilia. Of note, during RSV infection, CX3CR1 is sequestered from the tips of the motile cilia to vesicles in the peri-nuclear region, suggesting that viral particles transit through the ciliary shaft *via* intra-flagella transport [89, 90]. This exploitation of the interior ciliary transport mechanisms highlights a novel target for therapeutic intervention, likely applicable to multiple viral families.

RSV progeny bud out from ciliated cells, but when in filamentous form, the virions are too large and are instead released from the apical side of the epithelium, independent of cilia [91, 92]. Compared with influenza viruses, reported cell cytotoxicity and damage to the epithelium by RSV is minimal [57, 85, 91–94]. Given the lack of overt cell damage or apoptosis coupled with observed reductions in cilia number, it is likely that multiciliated cells shed their cilia, dedifferentiate or transdifferentiate [91, 94, 95]. Furthermore, electron microscopy of ciliary ultrastructure post-RSV infection is suggestive of induced microtubular disorganisation and mitochondrial damage [94, 95]. Despite these reduced numbers of cilia and ultrastructural defects, CBF did not significantly alter over a 6-day RSV infection period in primary human bronchial epithelial cells [92], suggesting that ciliary defects could be localised to the infection foci.

Antiviral therapies

Antiviral strategies have been developed and implemented to combat respiratory viral infections. Compounds that directly target aspects of the virus lifecycle such as genome replication are referred to as direct-acting antivirals (DAAs), where the primary goal is to limit the formation of progeny virions to stop further spread throughout the tissue. In addition to pharmacological agents, various monoclonal antibodies are approved for the prevention or treatment of respiratory tract infections [96]. Despite the substantial impact of viral respiratory tract infections on global morbidity and mortality rates, there is a paucity of effective treatment options, which are available for only a limited subset of respiratory viruses. This has led to increased research efforts in developing novel antiviral strategies, including broad-spectrum antivirals and host-directed therapies. Additionally, there is growing interest in exploring the potential of immunomodulatory approaches to enhance the host's natural defence mechanisms against respiratory viral infections [97].

Amongst the clinically available antivirals, those targeting the influenza virus NA are perhaps the most well developed. These compounds exhibit an inhibitory effect on viral release by impeding the ability of the viral NA enzyme to cleave nascent virions from the infected cell surface [98]. Globally, oseltamivir (Tamiflu) and zanamivir are clinically available NA inhibitors for the treatment of influenza, as well as laninamivir and peramivir being available in the USA, China, Japan and South Korea [99]. A key caveat with anti-influenza drugs is the development of resistance amongst the circulating influenza strains, which has resulted in previously approved drugs such as amantadine and rimantadine no longer being recommended as antiviral treatments [100]. New promising targets are under development for influenza, with one such candidate baloxavir morboxil, targeting the cap-dependent endonuclease of the viral polymerase, which is essential for the stability of newly synthesised viral RNA transcripts [101]. Currently, this compound is approved for use in the USA and Japan with minimal adverse events noted, marking the first non-NA inhibitor approved for use against flu since 2014 [102]. A notable ongoing phase II study called the Adaptive ASessment of Treatments (AD ASTRA) for influenza is currently seeking to evaluate the antiviral effects of multiple influenza antivirals against uncomplicated disease with high viral burdens to determine the relative efficacy of the current treatment regimens (NCT05648448). The outcomes of this trial could potentially reshape current treatment protocols and improve patient outcomes in influenza management.

The first line antiviral for RSV is palivizumab, a neutralising antibody, targeting the antigenic site in the viral F protein with proven effectiveness for preventing infection [103]. There are few direct-acting therapeutic options for RSV; however, two inhibitors of RSV fusion protein mediated entry, rilematovir and presatovir, show promise and favourable safety profiles [104]. Perhaps the most notable development has been the recent approval of subunit and mRNA vaccines for RSV in adults over 60 and the maternal population [105]. The protection of newborns in the first 6 months of life is a substantial step to limit the

burden of disease, yet given the high burden of RSV in young children beyond 6 months of age, further development of vaccine options is warranted potentially in the form of a live attenuated form to protect these groups.

The recent COVID-19 pandemic led to the development of two antiviral compounds, nirmatrelvir (Paxlovid) and molnupiravir (Lageviro), each targeting distinct aspects of the viral lifecycle. Nirmatrelvir targets the SARS-CoV-2 main protease, limiting processing of the viral polyprotein precursors and preventing the formation of productive replicase complexes [106]. Of note, this drug is combined with ritonavir, which increases the pharmacokinetics of nirmatrelvir though inhibiting its metabolism by cytochrome P450 3A4 [107]. Molnupiravir is a cytidine ribonucleoside analogue which induces a high frequency of mutations in the viral genome though promoting lethal mutagenesis. Importantly, despite the proofreading activity of the CoV polymerase, the active form of molnupiravir forms stable base-pairs with either guanosine or adenosine in the active site of the viral RNA-dependent RNA polymerase, circumventing the proofreading activity of the non-structural protein 14 exonuclease [108]. Notably, whilst many antivirals for SARS-CoV-2 showed potent inhibitory activity in pre-clinical studies, few translated to significant clinical effects. Given the complexity of the coronaviral lifecycle and the substantial involvement of the cellular host factors in the viral lifecycle, a more targeted approach of conserved factors essential for replication may result in the development of more effective antivirals. Further, the assessment of compounds in more physiologically relevant model systems, such as respiratory organoid cultures, may provide more accurate data regarding their efficacy for clinical applicability.

An important consideration of DAA-based treatment approaches is that their relative effectiveness through conventional administration routes, such as oral or parenteral, can be hindered due to the site of infection often being at the terminal end of the airways. Delivery of antiviral compounds through inhalation has been utilised with success in the prophylactic and direct treatment of respiratory viral infections [109], achieving therapeutic concentrations not easily reached through conventional routes. At present, only inhaled ribavirin and the NA inhibitor zanamivir are approved for the treatment of influenza; however, this route of drug delivery warrants more detailed investigation. Whilst few studies have ascertained what the impact of antiviral drugs are on cilia function, intranasal administration of ribavirin has been shown to slow cilia beat frequency at high concentrations [110]. Investigating the long-term effects of inhaled antivirals on respiratory tract health and function, including ciliary activity, could provide valuable insights for optimising treatment strategies.

Primary monocilia and viral infections

Primary monocilia chemo-sense environmental stimuli to transduce cell signals to the nucleus. The role of airway primary monocilia during respiratory virus infection is scant and anecdotal and the impact of infection on these cilia is unknown. For example, in the instance of SARs-CoV2 infection and pathogenesis, cilia in different tissue types immunolabelled with anti-ACE2 antibodies and multiple infection sites were proposed [27, 111]. In olfactory epithelium, odorant receptors presented on primary monocilia, are purported to detect extracellular odorants and transduce signals to the olfactory cortex within the brain [112]. A frequently reported symptom of SARS-CoV-2 infection, as with other CoV infections, is anosmia with taste impairment possibly caused by primary cilia dysfunction [113–115].

Although the impact of respiratory viral infection on primary cilia is not understood, evidence from Zika virus (ZikV) infection demonstrates primary monocilia targeted effects. This single-stranded RNA flavivirus is transmitted by the bite of an *Aedes* mosquito, increasing the risk of microcephaly in babies born to infected mothers. In an experimental chick embryo model, ZikV-NS5 viral proteins accumulated at the basal bodies of primary monocilia, colocalised with centrosomal and rootletin protein markers, both required for ciliogenesis [116]. Electroporation of ZikV-NS5 constructs into neural progenitor cells, resulting in shorter primary monocilia development compared to empty vector controls, suggesting that viral-induced ciliopathy may in part explain the abnormal cerebral ventricles of affected newborns [116]. It may be postulated that respiratory viruses could harbour similar abilities to associate with and hijack primary ciliary functions in the airway.

Airway models for assessing virus infections

Various models have been used to understand the interactions and disease mechanisms. Although experimental studies using these models have documented the interactions of cilia and respiratory viruses, the clinical situation may differ depending on numerous factors, including the model system, method of virus delivery, time course, infection magnitude and methods used to analyse cilia.

Human *ex vivo* lung biopsies are scarce yet provide a whole tissue model that retains multicellular complexity. Physiological models for assessing airway–virus infections are critical for investigating the mode and propagation of infection, viral host immune evasion mechanisms and effectiveness of antiviral treatments [117]. Animal (*e.g.*, mouse, rabbit, pig, chicken) models are instrumental to aiding researchers' understanding of whole lung and systemic effects of respiratory infection; however, differences in the anatomy, physiology and cell biology does not fully recapitulate the human lung condition. Of note, two recent studies using primary porcine respiratory epithelial models have generated useful insights into the innate immune response and infection potential of the ciliated epithelia of both CoV and swine influenza (porcine hemagglutinating encephalomyelitis virus) viral models [118, 119]. Investigations of swine models are of particular relevance to influenza, given that pigs are a crucial intermediate host, linked to the generation of novel influenza viruses with the potential to infect the human population [120]. Studies also show that this swine model has high replicative ability towards the human pathogenic phenotype in the swine host [121].

Viral infections have been studied in various murine models. For example, the effect of RNA influenza A virus on MCC has been investigated in tracheal explant samples from wild-type and TLR3-knockout (KO) BALB/c mice. In short-term (>2 h) infected open tracheal explants, fluorescent beads were applied to the ciliated epithelia and the migration of the beads, including both distance and direction, were tracked over time. Whilst directional bead flow was not affected, small but statistically significant increases in CBF and mean bead velocity were seen in wild type explants. TLR3 KO explants retained baseline ciliary frequency and bead velocity, leading the authors to suggest that TLR3 is mechanistically required for CBF increases during initial IAV infection [25].

Two-dimensional monocultures and three-dimensional (3D) *in vitro* human airway epithelia models are most often used as experimental platforms in the absence of patient whole tissue explant models. 96-well culture formats have been used for both aerosolised and liquid media suspension delivery of viruses to assess IAV effect on CBF, cytokine and chemokine production and cilia and barrier integrity [74]. This high-throughput system has also been used with IAV and human CoVs [122]. A scarcity of primary samples has pushed researchers into using immortalised cell lines that do not retain primary cell behaviours after immortalisation, including their lack of ability to differentiate and/or ciliate, *e.g.* 16HBE, BEAS2B, A549 and NCI-H441 H292 adenocarcinoma derived cell lines. Others that are used due to ease of respiratory virus infection lack innate antiviral IFN signalling pathways or multiciliated cells, *e.g.* Madin–Darby canine kidney, Calu-3 and Vero E6 (derived from African green monkey kidney).

Advanced human-derived *in vitro* ciliated cell culture models, with new molecular approaches and high-resolution imaging techniques, permit investigation of molecular and structural changes during respiratory viral entry, replication and infection shedding. Viral replication rates between different strains have also been reported using airway culture models, as shown with emerging SARs-CoV-2 strains [64, 123]. Post-viral infection host response times can also differ dramatically depending on virus and strain. For example, transcriptomic data in human pluripotent stem cell derived airway cell cultures show an antiviral response within 8 h to H3N2 influenza and 72 h to SARS-CoV-2 infection [124]. Following H1N1 influenza A infection, cultured distal airway stem cells become rapidly proliferative and assemble into "alveoli-like" clusters expressing markers of alveoli differentiation [125]. In nasal epithelial air–liquid interface (ALI) cultures, RSV causes airway cell actin cytoskeleton remodelling, observed as "micropinocytosis-like actin projections" through the cilia and peri-ciliary layer. RSV uses the insulin-like growth factor-1 (IGF-1) receptor on cilia to infect the host cell but benefits from interaction with a second co-receptor, nucleolin. RSV F-protein initial engagement with IGF-1 promotes subsequent recruitment of nucleolin to the cell surface, enhancing entry of RSV as well as other viruses and bacteria [126]. Others have shown uneven ALI culture differentiation in response to RSV infection, driven by cytoskeletal remodelling and actin polymerisation and increased immunological (IFN-γ-induced protein 10/C-X-C motif chemokine ligand 10) responses and contend that these processes underpin an inflammatory phenotype that promotes bronchial wall thickening [92]. "Human nose organoid" ALI cultures provide pre-clinical therapy models and have shown divergent host cell responses to different viral infection. Nasal washes and swabs are enzymatically digested and suspended in Corning Matrigel® for cell expansion over approximately 1 week. Cells are then passaged to Corning Transwells® and grown until confluent, with a conditional basal cell reprogramming rho-kinase inhibitor added. The cells are then taken to ALI culture for 3 weeks. SARS-CoV-2 *versus* RSV infection showed similar virus shedding and loss of culture thickness, but SARS-CoV-2 caused a higher loss in the percentage of cilia coverage and RSV caused greater IFN-γ and mucus hypersecretion responses [127]. Basolateral application of palivizumab, which targets the fusion protein of RSV, inhibited apical RSV infection, IFN-γ expression and mucus hypersecretion [127]. Donor age also affects RSV infection responses, with infant-derived human nasal

organoids at ALI culture producing higher cytokine and mucus responses with increased epithelial cell damage compared to adult samples [128]. Surrogates of viral components can be used to mimic cellular immunity responses triggered by viral replication. A synthetic double-stranded RNA polyinosinic: polycytidylic acid (poly I:C) is an antiviral immunostimulant due to its cross-interaction with TLR3 and stimulation of NF-κB, IFN-α and IFN-β expression. Interestingly, in response to poly I:C, human nasal epithelial cells show a reduction in the expression of motile cilia genes and ciliogenesis markers with a concomitant reduction in the proportion of ciliated cells to increased goblet cell differentiation. Cilia length is reduced and these authors reported mis-localisation of the outer dynein arm marker DNAH5 [129].

Whilst culture models are powerful tools that enable researchers to standardise differentiation time points and predominant cell phenotypes, they can be limited by their simplicity. This includes lacking the multicellular *in vivo* state and, for human samples, inter-donor variability and differential cell responses, which can affect the reproducibility of results and increase the need for more donors and replicates. The composition of culture medium for *in vitro* testing can cause differential host cell responses. Redman *et al.* [130] in 2024 demonstrated with single-cell RNA sequencing that cell type distribution, gene profile, cell signalling, epithelium morphology and secretory cell fate could differ depending on the application of four differently composed culture mediums, which also caused differential expression of the SARS-CoV-2 entry factor ACE2. Hence, it is important to consider methodological approaches to facilitate the research question or to better replicate the human condition.

Researchers are adding multicellular complexity to models, particularly looking at immune cell and epithelial cell crosstalk. Organoids are self-organising 3Dl structures that recapitulate organ structure and function but are cultured from stem cells. Sachs *et al.* [131] developed pseudostratified airway organoids consisting of basal cells, multi-ciliated cells, goblet cells and club cells from broncho-alveolar resections or lavage fluid. This method enabled the assessment of cystic fibrosis transmembrane conductance regulator (CFTR) function in an organoid swelling assay that allowed these researchers to assess CFTR modulator drug efficacy in cystic fibrosis patient organoids. Additionally, in RSV-infected organoids co-cultured with neutrophils, neutrophil recruitment could be observed. Deng *et al.* [132] also showed that physiological concentrations of neutrophils added to RSV-infected nasal cultures caused greater epithelial cell detachment, reduced tight-junction protein expression, cilia loss and decreased ciliary motility compared to RSV infection alone. Reports also exist of reconstructed human lung-like models in a perfused bioreactor utilising rat lungs re-epithelialised with human bronchial epithelial cells and with added neutrophils [133]. The contention is that these multicellular 3D organ-like models can deliver better pre-clinical data. These authors demonstrated the efficacy of the broad-spectrum antiviral remdesivir as a proof-of concept for future drug screening [133].

Summary

The interplay between viruses and cilia highlights the complexity of biological systems as well as a real need for comprehensive research to better understand these interactions in healthy individuals and those with chronic lung conditions. Viruses, though nonliving entities, have the remarkable ability to interact with living cells and exploit their mechanisms for replication and survival. In the airways, it is often multiciliated cells that are targeted. A common theme with most respiratory viral infections is the targeting of key aspects of cilia biology, encompassing motility and structure, increasing the risk of secondary bacterial infection events. Yet, despite documentation of viral-induced disruption of ciliated cells, the pathogenesis remains ill-defined. Mechanistic work is needed to understand the time-dependent impact of respiratory viral infection on ciliary activity and MCC in human airway epithelia, looking at ciliary function and inducible responses at the infection foci and surrounding cells. Although reported cilia loss could be due to viral-induced damage, it is possible that there are ciliary adaptive mechanisms at play, attenuating viral ability to exploit the host cell. Furthermore, our growing realisation that in addition to their nonmotile counterparts, motile cilia may also function as sensory organelles vital for immune surveillance, inflammatory responses and barrier integrity emphasises their significance beyond their purely mechanical function in MCC. The impact of viral infection on these signalling pathways and the resulting functional consequences are currently unclear, highlighting a knowledge gap. The development of advanced *in vitro* ciliated cell culture models and high-resolution structural techniques to model viral infections provides a platform to explore these important questions.

Understanding the interplay between viruses and motile cilia holds promise for novel therapeutic interventions. Additionally, deciphering how viruses manipulate ciliary-mediated signalling pathways may offer insights into enhancing host immune responses against viral infections. Innovative therapeutic approaches that harness the dynamics between viruses and cilia could pave the way for more effective treatments and management strategies for viral infections.

Questions for future research

- What is the best laboratory model to recapitulate the human airway in order to obtain clinically relevant data?
- Are there human respiratory virus strain-dependent differences that preclude *in vitro* modelling research?
- What time-dependent host-specific receptor interactions and cellular mechanisms during viral infections of ciliated epithelium are clinically relevant?
- Is ciliated epithelium more or less susceptible to viral infection than other airway cells and does ciliary function facilitate or prevent viral infection, replication and spread?
- How do nonmotile primary cilia and motile cilia function as sensory organelles for viral surveillance and immune response?
- Can improved understanding of virus and motile cilia interactions lead to novel therapeutic interventions?

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