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Review

To assemble or not to assemble: The changing rules of pneumovirus transmission

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ABSTRACT

Pneumoviruses represent a major public health burden across the world. Respiratory syncytial virus (RSV) and human metapneumovirus (HMPV), two of the most recognizable pediatric infectious agents, belong to this family. These viruses are enveloped with a non-segmented negative-sense RNA genome, and their replication occurs in specialized cytosolic organelles named inclusion bodies (IB). The critical role of IBs in replication of pneumoviruses has begun to be elucidated, and our current understanding suggests they are highly dynamic structures. From IBs, newly synthesized nucleocapsids are transported to assembly sites, potentially *via* the actin cytoskeleton, to be incorporated into nascent virions. Released virions, which generally contain one genome, can then diffuse in the extracellular environment to target new cells and reinitiate the process of infection. This is a challenging business for virions, which must face several risks including the extracellular immune responses. In addition, several recent studies suggest that successful infection may be achieved more rapidly by multiple, rather than single, genomic copies being deposited into a target cell. Interestingly, recent data indicate that pneumoviruses have several mechanisms that permit their transmission en bloc, *i.e.* transmission of multiple genomes at the same time. These mechanisms include the well-studied syncytia formation as well as the newly described formation of long actin-based intercellular extensions. These not only permit en bloc viral transmission, but also bypass assembly of complete virions. In this review we describe several aspects of en bloc viral transmission and how these mechanisms are reshaping our understanding of pneumovirus replication, assembly and spread.

1. Introduction

Pneumoviruses are a family of enveloped, non-segmented negative-sense RNA viruses (nsNSVs) (Afonso et al., 2016). Two of the most significant pediatric respiratory viruses, human metapneumovirus (HMPV) and respiratory syncytial virus (RSV) belong to this family. Both viruses can cause upper and lower respiratory tract infections, which can lead to bronchiolitis and pneumonia. According to data surveillance obtained for children under the age of five, RSV infections account for > 57,000 hospitalizations and over 2 million outpatient visits per year in the US (Hall et al., 2009). For the same pediatric population, HMPV associated infections are responsible for an estimated 1 in 1000 hospitalizations, similar to those associated with influenza (Edwards et al., 2013). However, no FDA approved vaccines are available against RSV or HMPV. A humanized monoclonal antibody that targets the RSV fusion protein (F), palivizumab, is available for use in premature or immunocompromised high-risk infant populations. No effective antivirals have been reported against HMPV, and the use *in vitro* of ribavirin, heparin, and the sulfated sialyl lipid NMSO3 have had only limited success (Wyde et al., 2003, 2004).

As with other enveloped viruses, the process of budding and release of pneumovirus virions involves the coalescence of viral components at specific locations on cellular membranes, followed by membrane deformation and pinching-off of a viral particle (El Najjar et al., 2014). Critical roles for the viral matrix protein (M) and the glycoproteins have been described in these processes, which implicate a high energetic cost. Though the release of particles into the extracellular environment is required for host-to-host viral spread, as a mechanism of spread within the host it does present several disadvantages. Examples of these disadvantages are the low stability of the particles in the extracellular environment and the susceptibility of virions to be targeted by immune cells. While the model of target cell infection resulting from primarily single particle entry is prevalent, this concept has been challenged by novel reports suggesting that infection of target cells may often be the

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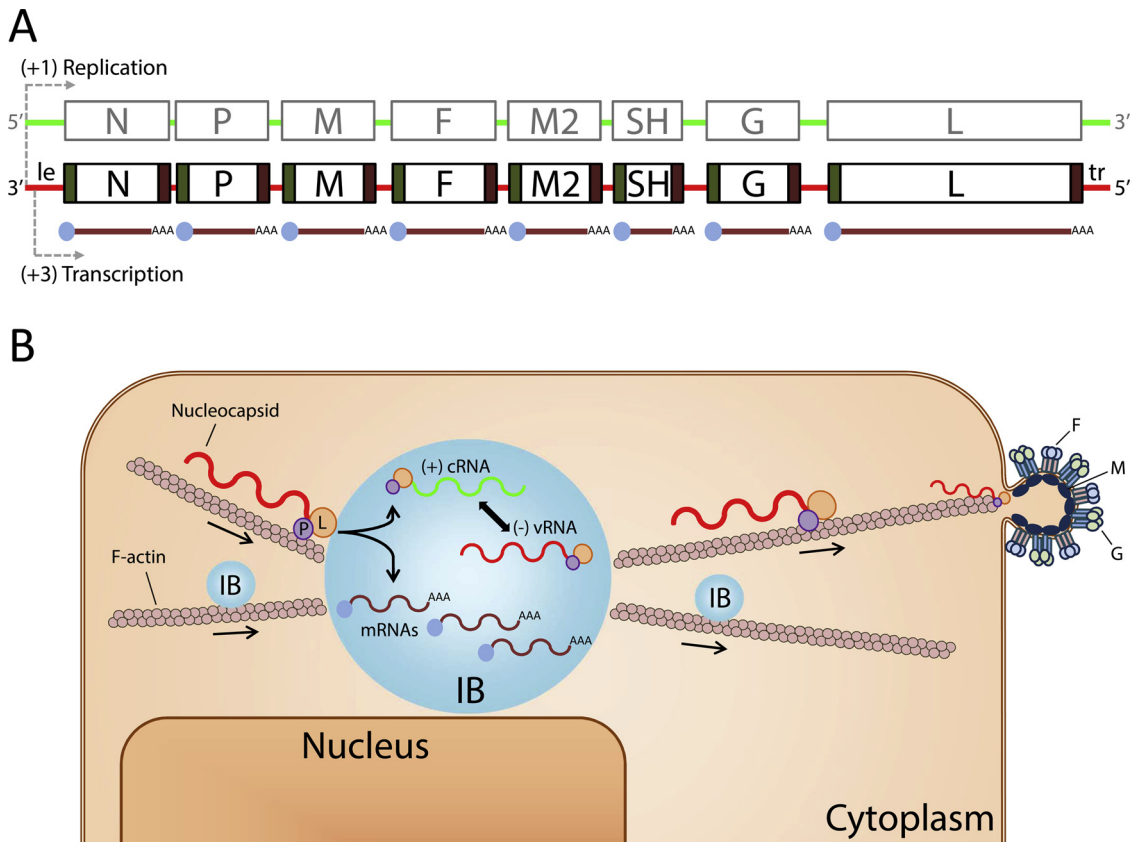

Fig. 1. Schematic representation of the pneumovirus genome and its replication. A) The HMPV genome (red) contains different elements including the 3′ leader (le) and 5′ trailer (tr) regions, and gene start (dark green) and gene end (maroon) signals within each of the individual genes. RSV possess a larger genome that includes two additional proteins, NS1 and NS2 (not depicted). B) Inclusion bodies (IB, light blue) are the central replication station for pneumoviruses, and they result from the coalescence of nucleocapsids and smaller IBs, a process dependent on actin polymerization. From IBs, nucleocapsids are transported to assembly sites were they will be incorporated into nascent virions. Smaller IBs can also be transported long distances from the cell body.

result of infection by multiple particles. In this context, pneumoviruses have developed alternative mechanisms of spread that potentially allow the virus to directly deposit multiple copies of the viral genome into target cells simultaneously. This review discusses some of the mechanisms for transport of multiple genome copies and their implications for virus spread.

2. Pneumovirus genome organization and replication

2.1. Pneumovirus nucleocapsids

Pneumoviruses possess a non-segmented, negative-sense single-stranded RNA genome that encodes between 9–11 proteins. Respiratory syncytial virus genomic RNA (vRNA) is about 15.2 Kb in size, while for HMPV a slightly smaller RNA molecule of about 13.2 Kb serves as the genome (Fig. 1a). Pneumovirus vRNA is entirely coated by the nucleoprotein (N), giving rise to long, flexible, left-handed helical nucleocapsids (Bakker et al., 2013; Ruigrok et al., 2011). Each nucleoprotein subunit binds to seven bases of the RNA (Renner et al., 2016; Tawar et al., 2009), in contrast to the paramyxovirus nucleoprotein subunits which bind to six RNA bases (Ruigrok et al., 2011). Pneumoviral nucleocapsids are additionally decorated by copies of the viral phosphoprotein (P), which interacts with N and serves as a polymerase cofactor (Garcia-Barreno et al., 1996). The viral phosphoprotein also recruits the large RNA-dependent RNA-polymerase (L) and the M2-1 protein onto the nucleocapsids. It is widely accepted that viral nucleocapsids not only protect the vRNA from degradation, but also serve as the template for viral replication and transcription (Fields et al., 2013). Sequences located at the 3′ (leader, le) and 5′ (trailer, tr) ends of the vRNA contain promoter signals that are important for viral genome replication and transcription (Noton and Fearns, 2015). Replication of the vRNA starts after the viral polymerase is recruited to the +1 site at the 3′ le region (Cressey et al., 2018) and occurs through synthesis of a full-length positive-sense copy of the genome, also known as antigenome (Fig. 1a). The antigenome and genome are simultaneously coated by the nucleoprotein (Fields et al., 2013). Transcription of the genome starts at the +3 site in the promoter of the 3′ le region (Cressey et al., 2018) and proceeds through a sequential termination-reinitiation mechanism (Noton and Fearns, 2015). This mechanism is controlled by the presence of gene start (GS) and gene end (GE) signals for each individual gene, which ultimately results in the synthesis of sub-genomic, capped and poly-adenylated viral mRNAs (Fig. 1a) (Fields et al., 2013). Unlike the vRNA or the antigenome, viral mRNAs are not coated by N, but a recent study suggested they might contain copies of the M2-1 protein (Rincheval et al., 2017).

2.2. Inclusion bodies: the viral genome factories

Reference to inclusion body (IB) formation upon infection of Vero cells by RSV was reported early in 1970 by using electron microscopy (Norrby et al., 1970). Inclusion bodies induced by RSV are described as cytosolic, compact, membraneless organelles that incorporate replicative viral proteins such as N, P, and L, while excluding others such as M or the glycoproteins G and F (Norrby et al., 1970; Garcia et al., 1993; Carromeu et al., 2007). The minimal components necessary for formation of RSV IBs were defined to be the RSV N and P proteins (Garcia et al., 1993). More recently, the HMPV N and P proteins were also shown to be the minimal components required for assembly of

inclusion bodies (Derdowski et al., 2008). Though initially IBs were believed to represent a station for accumulation of misfolded proteins, several recent reports have highlighted their role as the sites of active viral genome replication (Fig. 1b). Combining metabolic labelling with fluorescence *in situ* hybridization (FISH) and high resolution microscopy, viral genome replication was shown to occur within RSV IBs (Rincheval et al., 2017). Different compartments within IBs, denominated IB-associated granules (IBAGs) were shown to contain newly synthesized RNA, most likely viral mRNAs (Rincheval et al., 2017). HMPV IBs were also shown to contain vRNA as well as viral mRNAs, plus the N and P replicative proteins, while excluding assembly proteins such as M or F (Cifuentes-Munoz et al., 2017). Interestingly, HMPV IBs were shown to result from the coalescence of multiple smaller replicative spots during the early steps of HMPV infection, revealing an intrinsic plasticity for pneumovirus IBs (Fig. 1b) (Cifuentes-Munoz et al., 2017). Remarkably, formation of IBs represents a replication strategy used beyond the *Pneumoviridae*. Although quite different in their host range and symptoms, the role of IBs in replication of the viral genome has been recently reported for rhabdoviruses (Heinrich et al., 2010; Lahaye et al., 2009), filoviruses (Becker et al., 1998; Kolesnikova et al., 2000; Hoenen et al., 2012) and for some paramyxoviruses (Carlos et al., 2009; Zhang et al., 2017).

2.3. Transport of viral nucleocapsids inside the cells

Inclusion bodies are a station from where newly synthesized nucleocapsids must be transported to viral assembly sites at the plasma membrane. Though the exact mechanism of how this occurs has remained elusive for pneumoviruses, several reports point out an important role of the actin cytoskeleton in this process (Fig. 1b). In early times post-infection, when large inclusion bodies have not formed, incoming -as well as newly synthesized- HMPV nucleocapsids coalesce in a process that is dependent on actin polymerization (Cifuentes-Munoz et al., 2017). Coalescence of nucleocapsids into larger IBs was shown to be important for efficient replication and transcription of the HMPV genome, likely due to the concentration of replicative components within these organelles. At later times post infection, coalescence of large IBs still occurs (Fig. 1b) (Cifuentes-Munoz et al., 2017). Coalescence of IBs has been additionally observed for other nsNSVs including rabies virus (Nikolic et al., 2017), Zaire Ebola virus (ZEBOV) (Hoenen et al., 2012), and the paramyxovirus parainfluenza virus type 3 (PIV3) (Zhang et al., 2017). Interestingly, coalescence of PIV3 IBs was shown to be dependent on acetylated α-tubulin, through a direct interaction with the N–P complex (Zhang et al., 2017). Interaction of the RSV N–P complex was not found with acetylated α-tubulin (Zhang et al., 2017), most likely because the RSV N–P complex interacts mainly with components of the actin cytoskeleton (Santangelo and Bao, 2007). Purified RSV particles contain actin, but exclude other cytoskeletal components such as vimentin, cytokeratin and tubulin (Burke et al., 1998; Ulloa et al., 1998). Human metapneumovirus purified particles also contain actin (El Najjar et al., 2016), and a major role of the actin cytoskeleton, but not microtubules, in the replication and assembly of both HMPV and RSV has been well documented (Cifuentes-Munoz et al., 2017; Santangelo and Bao, 2007; Burke et al., 1998; Ulloa et al., 1998; El Najjar et al., 2016; Barik, 1992; Huang et al., 1993). In this context, pneumovirus nucleocapsid transport may operate in a similar way to filovirus nucleocapsid transport. Marburg virus (MARV) and EBOV nucleocapsid cytoplasmic transport was shown to be dependent on actin polymerization and the actin nucleating complex Arp2/3, but independent of microtubules (Schudt et al., 2015, 2013). Transport of nucleocapsids over long distances from IBs to the tip of filopodia, the budding sites for filoviruses, was detected (Schudt et al., 2015, 2013). Interestingly, HMPV IBs have been observed far from their usual perinuclear localization, which suggests their movement as an independent organelle over long distances (El Najjar et al., 2016). The mechanisms and cellular components involved in their movement remain to be addressed.

3. En bloc transmission of pneumoviruses

Recent evidence suggests that novel mechanisms of direct cell-to-cell spread might be an important avenue for pneumovirus transmission (Cifuentes-Munoz et al., 2017; Mehedi et al., 2016). These mechanisms, and others described previously, would allow pneumovirus spread to operate at least partially en bloc, defined here as spread of multiple genomes occurring at the same time. This notion challenges the more accepted concept of one infectious particle infecting one cell at the time. Based on evidence primarily obtained for pneumoviruses but also for other related viruses, we discuss several mechanisms for how en bloc viral transmission could occur.

3.1. Cell-to-cell fusion

Cell to cell fusion results in the appearance of large multinucleated cells, named syncytia. Syncytia formation has been reported for HMPV and RSV *in vitro* (Hamelin et al., 2004), but their presence *in vivo* is less clear (Zhang et al., 2002). However, lung autopsy specimens have revealed the presence of large syncytia from both HMPV and RSV patients, which strongly argues for their formation *in vivo* (Neilson and Yunis, 1990; Vargas et al., 2004). Mechanistically, syncytia formation is facilitated by the accumulation of the viral fusion protein (F) in the plasma membrane of infected cells. F binds to the membranes of adjacent cells, and through the aperture of a fusion pore that enlarges it completely fuses both membranes. As a result, cytosolic content mixing occurs, and consequently infection is spread directly from cell-to-cell (Sattentau, 2008). Once an infected cell has completely merged with an uninfected cell, a massive transmission of nucleocapsids and IBs would have occurred, therefore rapidly spreading infection (Fig. 2a). Interestingly, an RSV F with a hyperfusogenic phenotype has been associated with increased pathogenesis in mice (Hotard et al., 2015). Future studies will elucidate other essential aspects of this mechanism of en bloc viral transmission for pneumoviruses and how they impact viral pathogenesis.

3.2. Intercellular extensions

Recent reports have described a novel route of direct cell-to-cell spread by pneumoviruses that involves the formation of filamentous structures that extend towards distant cells. Respiratory syncytial virus induces formation of filopodia in lung epithelial A549 cells, which facilitate viral spread (Mehedi et al., 2016). Induction of filopodia was shown to be dependent on the RSV F protein and the cellular actin-related protein 2 (Arp2), an important actin-nucleation factor. At the surface of filopodia, clusters of filamentous structures most likely corresponding to RSV virions were shown to contact neighboring uninfected cells. This phenotype suggests a mechanism of spread through which multiple virions can be shuttled from one cell to another without the need for release. Additionally, formation of filopodia conferred increased motility to RSV infected A549 cells, which was shown to also facilitate cell-to-cell spread (Mehedi et al., 2016). Formation of filopodia was not as prominent in HMPV-infected A549 cells, but human bronchial epithelial BEAS2B cells infected with HMPV have been shown to result in conspicuous formation of intercellular extensions (El Najjar et al., 2016). Cellular factors including F-actin and the Rho GTPases Cdc42 and Rac1 were shown to be important for the induction of HMPV intercellular extensions. Similar to RSV-induced filopodia, the surface of HMPV-induced extensions is covered by clusters of branching filaments that contain viral proteins and RNA, which we hypothesize correspond to filamentous virions. Hence, the potential massive shuttle of HMPV virions directly from cell-to-cell arises as an efficient mechanism of spread (El Najjar et al., 2016). Interestingly, HMPV cell-to-cell spread was shown to be at least partially independent of the

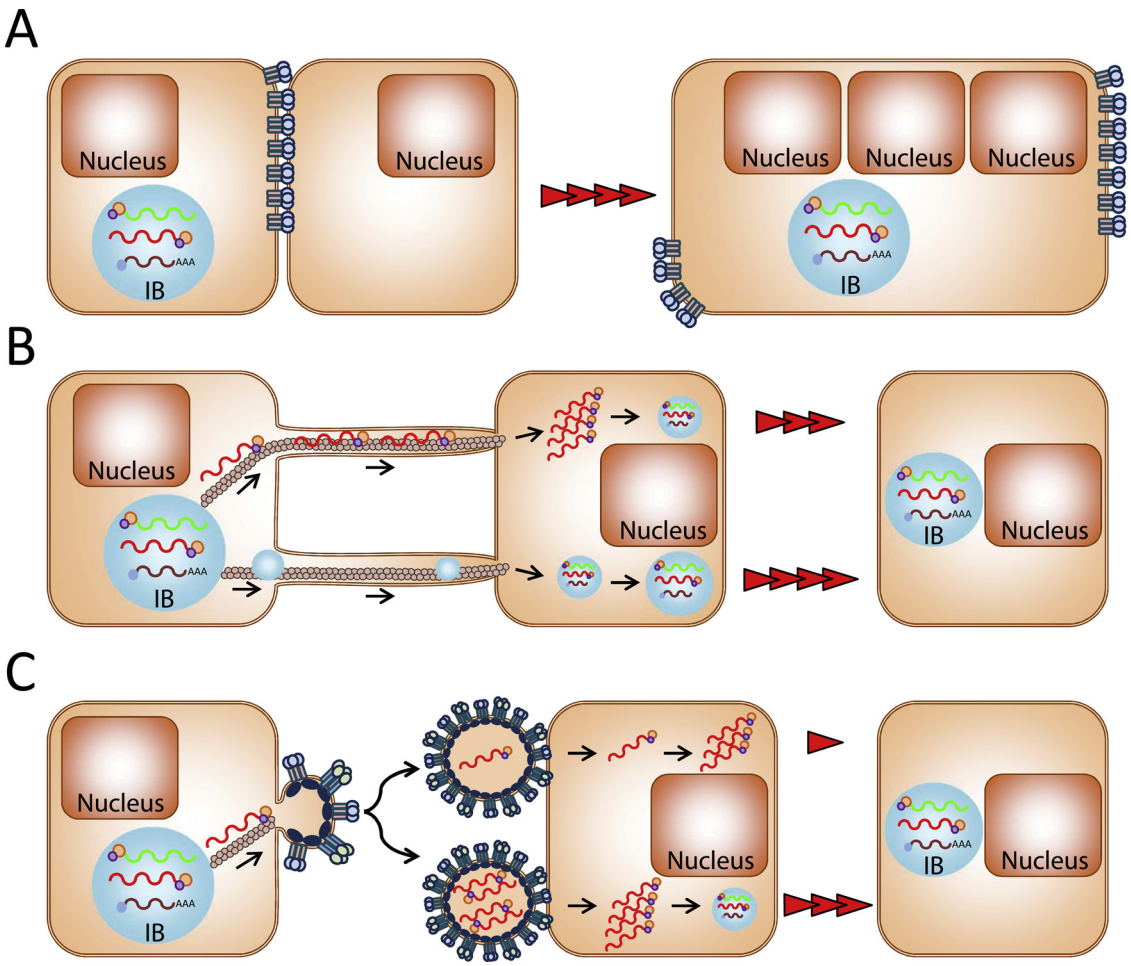

Fig. 2. En bloc mechanisms of spread proposed for pneumoviruses. A) Cell-to-cell fusion is mediated by the viral fusion protein and results in formation of syncytia. The cytosolic contents of the infected cell, including IBs and viral mRNAs, are then transferred to the newly formed giant cell. B) Direct cell-to-cell spread mediated by actin-based intercellular extensions results in mass transport of genomes between cells. We propose this could happen either by sequential transport of individual nucleocapsids, or by transport of IBs, which would result in a fast avenue to establish infection in the target cell. C) Newly assembled virions can contain one or multiple copies of the viral genome. The advantage of carrying multiple genomes is that after entry, these will act in a complementary way to establish rapidly infection by formation of IBs. Red arrowheads represent efficiency for establishment of large IBs.

appropriate attachment factors and of the presence of neutralizing antibodies. This suggests that cell-to-cell spread might occur through a direct connection between the cytoplasms of an HMPV infected cell and a target cell. In support of this, vRNA as well as IB-like structures were shown to be present in the HMPV-induced intercellular extensions, potentially being transported towards neighboring cells (Fig. 2b) (El Najjar et al., 2016). Transmission of nucleocapsids through long intercellular structures has been reported for influenza virus in lung epithelial cells (Roberts et al., 2015; Kumar et al., 2017). Though the open-ended nature of pneumovirus intercellular extensions remains to be addressed, this mechanism of en bloc viral transmission represents an advantageous way to rapidly spread infection.

3.3. Polyploid virions

Pneumovirus purified particles display a wide size variability, ranging from 100 nm up to 1000 nm, and from 150 nm up to 600 nm, for RSV and HMPV, respectively. Not only is the pneumovirus particle size quite heterogeneous, but also their shape, and usually two major forms, pleomorphic and filamentous, can be observed (Liljeroos et al., 2013; van den Hoogen et al., 2001; Bachi and Howe, 1973; Peret et al., 2002). Some of the filamentous RSV particles have been observed to grow up to 10 μm in length (Bachi and Howe, 1973). In the case of HMPV, which grows predominantly cell-associated *in vitro*, filaments budding from infected cells, potentially corresponding to viral particles, are usually above 2 μm in length (El Najjar et al., 2016; Loo et al., 2013). Though the number of pneumovirus nucleocapsids packaged within filamentous virions could not be determined from the above mentioned studies, it is possible, based on the size of the particles as well as their heterogeneity, that polyploid virions are present. Polyploid virions have been described for paramyxoviruses including Newcastle disease virus (NDV), measles virus and Sendai virus (SeV) (Rager et al., 2002; Loney et al., 2009; Dahlberg and Simon, 1969). More important, the presence of multiple genomes -up to 6 nucleocapsids in the case of SeV- within one viral particle has been shown not to be detrimental for viral infection (Rager et al., 2002). In the context of mass transport of genomes, the presence of multiple nucleocapsids within particles fits with the proposal that infection is more successfully initiated by multiple particles rather than by single particles (Fig. 2c).

3.4. En bloc transmission of pneumoviruses: A double edged sword

An important feature during replication of different RNA viruses *in vitro* is the synthesis of truncated forms of the vRNA denominated defective viral genomes (DVGs), initially named defective viral particles, as they can interfere with viral replication (Huang and Baltimore, 1970). Defective viral genomes exist in different conformations and, as they are truncated forms of the genome, in order to propagate they need

a helper virus (reviewed in (Lazzarini et al., 1981)). Early studies with measles virus DVGs demonstrated that their presence correlated with the establishment of persistent infections in cell lines (Rima et al., 1977). Moreover, other nsNSV DVGs have been described as strong inducers of the type-I IFN response (Lopez, 2014). When passaged at high titers in cell lines, RSV as well as HMPV infections generate defective interfering genomes (Treuhaft and Beem, 1982; van den Hoogen et al., 2014). Similar to measles, the presence of RSV DVGs has been associated with the establishment of viral persistence in cell lines (Valdovinos and Gomez, 2003). More recently, it was shown that RSV DVGs can stimulate the expression of antiviral genes in mice, leading to decreased viral titers and symptoms (Sun et al., 2015). A direct correlation was found in human lung tissues between the amount of DVGs and the quality of the antiviral response mounted, which suggests DVGs have a role *in vivo* (Sun et al., 2015). Considering the previous mechanisms described for en bloc pneumoviral transmission, and the large presence of DVGs upon infection, a valid question that arises is whether these mechanisms exist as a viral strategy for rapid spread or as a cellular antiviral strategy to counteract the infection. Future work will elucidate how both possibilities might operate simultaneously.

4. En bloc transmission of Pneumoviruses: Implications for viral dynamics

The mechanisms of en bloc viral transmission described here permit the potential transfer of many viral genomes into a target cell at the same time. In the context of pneumoviruses, this might represent a crucial mechanism for successful infection. Pneumoviruses are within the group of nsRNA viruses, and estimations indicate that mutation frequencies for these viruses can range from 10^{-5} to 10^{-6} per nucleotide per infectious cycle (Sanjuan et al., 2010). In the absence of proofreading mechanisms, the high mutation frequency for these viruses results in multiple variations of vRNA populations, which are usually referred to as quasispecies (Borderia et al., 2011). The infection of a cell with viral quasispecies could result in a complementation of a defect in one genome by the presence of additional genomes. This cooperativity would be less likely in the context of an individual viral particle carrying an attenuated genome. Additionally, the massive transfer of genomes into a target cell ensures a higher initial concentration of viral proteins and RNA, which we have previously shown to rapidly coalesce to form large IBs (Cifuentes-Munoz et al., 2017). A higher concentration of these replicative components would facilitate infection and would allow the virus to bypass the critical first hours of infection, potentially allowing high levels of viral replication before antiviral responses are in place. Though some of these hypotheses remain merely speculative for pneumoviruses, a deeper understanding of the virus spread mechanisms will help us improve the way we approach the design of antiviral strategies.

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