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Review

Extracellular vesicles: Vehicles of *en bloc* viral transmission

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ABSTRACT

En Bloc transmission of viruses allow multiple genomes to collectivelly penetrate and initiate infection in the same cell, often resulting in enhanced infectivity. Given the quasispecies (mutant cloud) nature of RNA viruses and many DNA viruses, en bloc transmission of multiple genomes provides different starting points in sequence space to initiate adaptive walks, and has implications for modulation of viral fitness and for the response of viral populations to lethal mutagenesis. Mechanisms that can enable multiple viral genomes to be transported *en bloc* among hosts has only recently been gaining attention. A growing body of research suggests that extracellular vesicles (EV) are highly prevalent and robust vehicles for *en bloc* delivery of viral particles and naked infectious genomes among organisms. Both RNA and DNA viruses appear to exploit these vesicles to increase their multiplicity of infection and enhance virulence.

1. Introduction

The number of infecting genomes that initiate replication in a recipient cell is relevant to the progression of the infection and fitness of the viral progeny. Single virus particles have long been viewed as sufficient for infectivity and optimally suited for intra- and inter-organismal transmission. Particles traveling independently from one another have access to a greater number of hosts, do not have to compete with each other for cellular resources and increase the chances of at least one or more particles escaping host immune responses. This single virus-centric view of transmission has also been the basis of many common laboratory methods such as the plaque or focus forming assays that measure viral titers and infectivity. Furthermore, electron micrographs of viruses released from infected cells or found in body fluids and excretions (e.g. feces, saliva, semen) have lent support to individual virus particles as the infectious units. However, inefficient replication is also a common feature of cells infected with single or few numbers of virus particles (i.e. low multiplicities of infection [MOI]), especially in the early stages of infection, when viral proteins are low in abundance and viruses are most vulnerable to host innate immune defenses and aborted infections. In contrast, with high MOIs, viral translation and replication kinetics are enhanced and innate immune defenses evaded faster (Gifford, 1963; Stitz and Schellekens, 1980; Luque et al., 2009; Stiefel et al., 2012; Chen et al., 2015a,b; Zaritsky et al., 2015; Andreu-Moreno and Sanjuán, 2018; Santiana et al., 2018). Indeed, even bacteriophages have been reported to exploit high MOIs to overcome bacterial CRISPR/Cas9 mediated anti-viral responses (Borges et al., 2018).

2. *En bloc* transmission: implications for viral quasispecies dynamics

One way to enhance MOI is by collective simultaneous viral infection such as when multiple viruses are transported in an extracellular vesicle (EV) to infect another cell, a mode of transmission we discovered and termed *en bloc* (Chen et al., 2015a,b; Altan-Bonnet, 2016; Santiana et al., 2018). Other mechanisms that can also be considered as en bloc transmission are short-range transfers boluses of free viruses between cells; movement of virus aggregates or clumps (Saif et al., 1977; Cuevas et al., 2017); and transfer through attachment of multiple

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viruses to a bacterial scaffold (Erickson et al., 2018).

An important feature in particular of RNA viruses, which may favor their *en bloc* transmission, is that as a result of largely of uncorrected replication errors (about one mutation introduced per round of copying of the entire genome or its complementary strand), their progeny are mutant, so-called quasispecies, collectives (Domingo et al., 2012; Domingo and Schuster, 2016; Domingo and Perales, 2018). A key issue related to quasispecies dynamics is that the mutant distributions in subsequent infections do *not* behave as mere aggregates of viral genomes that act independently of each other. For example, intra-population complementation within mutant spectra replicating under standard mutation rates has been reported by comparing the replicative fitness of an entire bacteriophage Qβ population with that of individual clones retrieved from the same population. Four individual Qβ clones exhibited growth rates that were 0.8 to 0.9 those of the uncloned parental mixed Qβ population (Domingo et al., 1978). In another more extensive study with vesicular stomatitis virus (VSV), it was determined that the fitness values for 98 subclones derived from a parental, clonal, high fitness VSV population were 0.82-fold of the fitness of the parental population (Duarte et al., 1994). Lastly, increased genomic recombination among polioviruses was observed, resulting in removal of deleterious mutations, when viruses were transmitted to cells *en bloc* via bacterial scaffolds (Erickson et al., 2018). These results point towards a *distinct fitness advantage of mutant virus collectives* relative to individual viruses separated from their surrounding mutant cloud.

One interpretation of these findings is that under basal mutation rates, multiple complementation events among components of the mutant spectrum, which likely include otherwise viable genomes with slightly deleterious mutations, enhance the replicative capacity of the ensemble. In some cases, the underlying biochemical mechanisms that mediate cooperation or complementation among specific variants have been characterized (Shirogane et al., 2012,2016,2019). An alternative, non-mutually exclusive second possibility, is that infection by multiple components of a mutant spectrum provide different initial points in sequence space from which to explore adaptive pathways (e.g. increased replication) in a newly infected cell. In either of these possible scenarios, the cloud nature of viral populations (with an average or at least one mutation per individual genome) plays a critical role (Domingo and Perales, 2018).

A related aspect that renders *en bloc* transmissions relevant to the progress of infections is that the number of viral particles required to initiate an infection without significant fitness loss depends on the fitness of the initial population (Novella et al., 1995). Measurements with VSV suggest that *en bloc* transmissions may serve to modulate fitness evolution in a fitness dependent manner. That is, joint transmission of multiple particles may contribute to maintain or increase fitness when the initial population has low fitness, and this may avoid excessive fitness increases when the parental viral population has already reached high fitness. *En bloc* transmission-mediated modulation of fitness evolution could also be particularly relevant for persistent viruses that may benefit of limited replication rates and viral loads, parameters which are directly related to fitness (Domingo et al., 2012).

An additional implication of *en bloc* transmission can be expected in the process of lethal mutagenesis of viruses, an antiviral strategy based on virus extinction by an excess of mutations evoked by nucleotide analogues during viral genome replication [reviewed in (Perales et al., 2019)]. Two major steps can be distinguished in the transition from a viable mutant distribution into its pre-extinction form: an initial increase of defective genomes when mutagenesis is either limited or at its initial stages, and a second phase in which the number of mutations per genome is such that infectious progeny can no longer be produced. The first step is termed lethal defection because its mechanism is the interference that some defective genomes (termed defectors) exert on the replication of standard, non-defective genomes present in the same mutant cloud (Grande-Pérez et al., 2005). The genomes endowed with a defector phenotype should be competent in replication of their RNA to exert their interfering activity, although they are defective regarding production of infectious progeny (Perales et al., 2007). The second stage, that we have termed overt lethality, is due to the collapse of viral functions due to an excessive average number of mutations per genome (Perales and Domingo, 2016).

The mechanisms of lethal mutagenesis have been investigated mainly in cell culture, and they are supported by several theoretical models (Domingo and Schuster, 2016). It is not known to which extent the same mechanisms operating in model systems apply to lethal mutagenesis in vivo. Increasing evidence supports lethal mutagenesis as a mechanism of antiviral activity of nucleoside analogues in vivo, associated with increases of mutant spectrum complexity and of minority mutations (Arias et al., 2014; Guedj et al., 2018). Increased mutagenesis is expected to affect the replication complexes within single infected cells both regarding lethal defection and overt lethality. Lethal defection may operate at earlier stages of lethal mutagenesis if a lethal defector phenotype is co-transported into a cell along with the standard fully infectious genomes, as the formers replication can interfere with the latter. Experimental studies to investigate which may be the effect of *en bloc* transmission on the efficacy of lethal mutagenesis are required. What we have learned of the implications of quasispecies dynamics on adaptation (fitness gain) or de-adaptation (fitness loss) suggests important effects of *en bloc* transmission of viruses on the progress of viral infections. Lethal mutagenesis is an example of de-adaptation due to excess of mutations. Thus, a clarification of the consequences of *en bloc* transmission for viral dynamics may contribute to the understanding of the molecular basis of lethal mutagenesis.

3. *En bloc* viral transmission with EVs

EVs have been found to collectively transmit viruses and other cargo in vitro, *in vivo* and among animals (Madison et al., 2015; Santiana et al., 2018). EVs are released by nearly all cells (prokaryotic and eukaryotic) and enable both close-and long-range modulation of cellular and organismal behavior (Tkach and Théry, 2016; Santiana et al., 2018). By being released as membrane cloaked packets with sizes ranging from a few nanometers to tens of microns, they can collectively transport and simultaneously transfer a mixture of proteins, lipids and nucleic acids, thereby targeting multiple cellular pathways at once in the receiving cell. In eukaryotes EVs are largely derived from three distinct cellular pathways (Fig. 1): direct budding from the plasma membrane to generate microvesicles which can have a wide distribution of sizes (100 nm to few microns in diameter); fusion of specialized endosomes called multivesicular bodies (MVB) with the plasma membrane, resulting in the release of small vesicles (50–200 nm in diameter) termed exosomes; and finally the fusion of (double-membrane) autophagosomes with the plasma membrane to release single-membrane vesicles with diameters 350–500 nm s (Ponpuak et al., 2015; Tkach and Théry, 2016; Mutsafi and Altan-Bonnet, 2018).

Non-enveloped viruses, such as poliovirus, hepatitis A virus, rotavirus, adenovirus, norovirus and numerous others, were thought to only be able to exit cells through lytic mechanisms. However, reports of vectorial release of poliovirus from polarized cells in culture (Tucker et al., 1993) and a role for autophagy in this exit pathway (Jackson et al., 2005) suggested that this view needed revision. In 2013, Lemon and colleagues blurred the separation between enveloped and non-enveloped viruses with their discovery of a second *enveloped* form of hepatitis A virus (Feng et al., 2013). Subsequently, multiple other studies reported poliovirus, Coxsackievirus and rhinovirus could exit cells in culture *prior to lysis,* inside EVs derived from *secretory autophagosomes* (Bird et al., 2014; Robinson et al., 2014; Chen et al., 2015a,b) (Fig. 1). Importantly, super-resolution light and electron microscopy revealed that such EVs carried *multiple virus particles collectively* (Chen et al., 2015a,b). Given the typical size of these vesicles (˜350 nm) and the typical size of one of these viruses (˜30 nm), each vesicle was conjectured to carry anywhere from tens to hundreds of virus particles

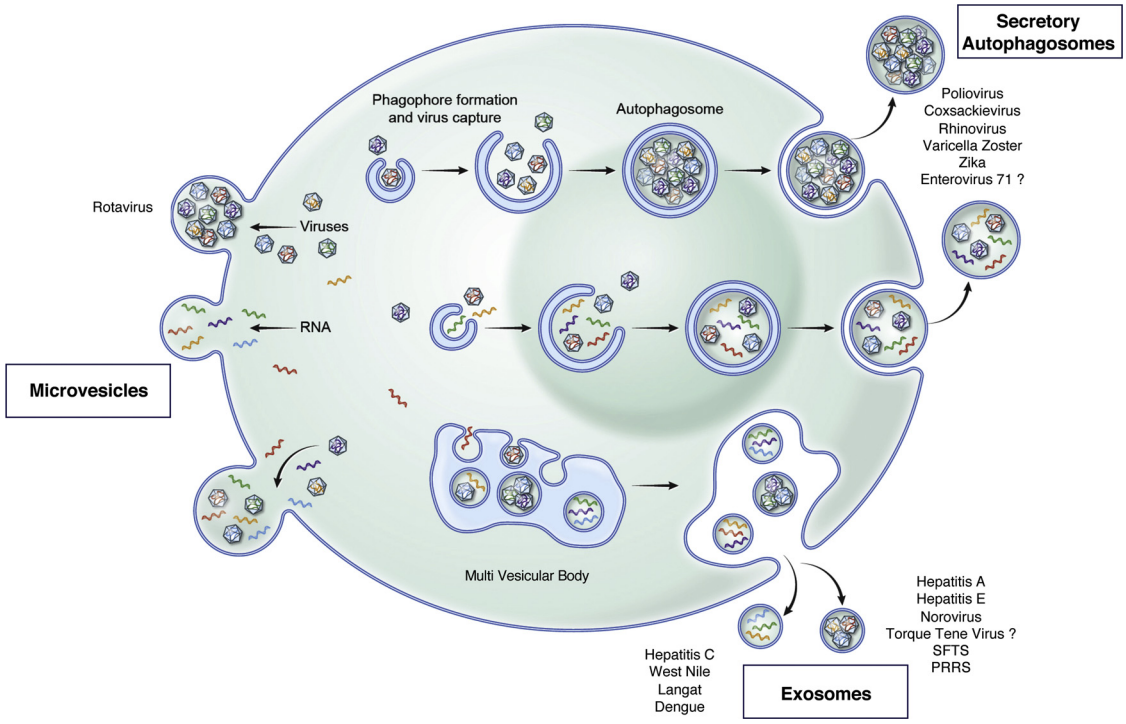

Fig. 1. Extracellular vesicles can be derived from cellular multivesicular bodies fusing with the plasma membrane; direct budding of the plasma membrane; and from autophagosomes fusing with the plasma membrane. A wide spectrum of RNA and DNA viruses have been found to exploit these different extracellular vesicle production pathways and they are indicated in the figure. Note that EVs shed from these virally infected cells may contain not only viral particles but also naked viral genomes as well as cellular and viral proteins and nucleic acids that can potentially modulate the infection in the next host.

depending on their packing. Since these first reports, the list of EVs implicated in *en* bloc viral transmission has been steadily growing (Table 1), including both RNA and DNA virus cargo (particles and infectious naked genomes) and has been implicated as a highly virulent instrument for *en bloc* transfer of viruses among the animal and human populations. Note that in cases where EVs were reported to transport few virus particles (< 5), whether they also co-transported multiple naked infectious genomes remains to be investigated.

While isolated EVs readily infect susceptible new cells (Feng et al., 2013; Robinson et al., 2014; Chen et al., 2015a,b) how they do so is still unclear. Imaging has revealed that EVs are frequently internalized by endocytic pathways. For EVs carrying poliovirus, Coxsackievirus, rhinovirus or murine norovirus, infection is still virus receptor dependent (Chen et al., 2015a,b; Santiana et al., 2018). This suggests that EV membranes get disrupted, possibly by endosomal lipases and lipid extractor proteins, following endocytosis (Yin et al., 2016). On the other hand, infectivity appears to be independent of known virus receptors for EVs carrying enveloped bunyaviruses (Silvas et al., 2015) or infectious naked HCV, Dengue or WNV genomes (Fig. 1) (Bukong et al., 2014; Zhou et al., 2018; Vora et al., 2018). While a yet to be discovered host receptor for bunyaviruses or naked HCV/Dengue/WNV genomes cannot be ruled out, this data suggests that EVs may also directly fuse with the cell membrane (plasma or endosomal). It is unclear if fusion can be with any cell type or whether there are specific protein/lipid cues on the EVs that direct, tether and enable fusion with specific cell types. Even if the former, intrinsic cellular barriers to replication such as the availability of a virus-specific replication factor or the strength of the innate immune response may also end up restricting viruses to certain cell types (Gifford, 1963; Stitz and Schellekens, 1980; Zaritsky et al., 2015; Andreu-Moreno and Sanjuán, 2018).

4. EVs enhance virulence

A key feature of *en bloc* transmission is the collective delivery of multiple viral genomes into a host cell. Single molecule RNA fluorescence in situ hybridization (FISH) studies on cells infected with poliovirus-containing EVs have demonstrated multiple viral genomes being delivered, in spatially close proximity, to the host cytosol (Chen et al., 2015a,b). With EV-mediated inoculums, even at low virus titers, cells infected with multiple viral genomes (pre-replication) could be readily found whereas similar cultures inoculated with equivalent free poliovirus titers had none. Notably, when total viral production in these cultures was measured and compared, EV inoculated ones produced many more infectious new virions than free virus inoculated ones (Chen et al., 2015a,b), thus indicating barriers to replication when low numbers of viral genomes enter individual cells (Altan-Bonnet, 2016). A similar phenomenon was recently reported with VSV aggregates (Andreu-Moreno and Sanjuán, 2018). The replication advantages of the aggregates were found to be cell type dependent suggesting variability in the replication barriers (to at least VSV) among cells and potentially due to differences in strength of the innate immune responses (Gifford, 1963; Stitz and Schellekens, 1980; Zaritsky et al., 2015; Andreu-Moreno and Sanjuán, 2018).

Remarkably the replicative advantage of EV mediated transmission was not limited to cell culture studies. EVs full of enteric viruses were found naturally shed in human and animal feces and such EVs could be ingested and efficiently bloc transmit their viral cargo to other hosts (Santiana et al., 2018). Rotavirus and norovirus are two pathogenic enteric viruses that are the major causes of mortality and morbidity from gastrointestinal diarrhea and are transmitted via the fecal-oral route to other hosts including humans. Both viruses are non-enveloped, replicate in the intestine and had been thought only until recently to be shed and transmitted to other organisms as freely dispersed viral particles in stool. But analysis of stools from rotavirus- or norovirus-infected humans, pigs and mice revealed significant pools, sometimes as high as 50%, of viruses embedded within EVs. In particular, each rotavirus-containing EV could collectively transport tens of rotavirus particles. These vesicles in stool could be ingested by other animals and

Table 1
Viral particles and infectious genomes transmitted in extracellular vesicles. ND: Not Determined.

Non-enveloped eukaryotic viruses	RNA or DNA virus	Extracellular vesicle size	Extracellular vesicle origin	PS lipids	Viruses per vesicle	Naked infectious genomes	Reference
Poliovirus	ss (+) RNA	~350 nm	Secretory Autophagosome	Yes	> 25	ND	Chen et al., 2015a,b
Coxsackievirus	ss (+) RNA	~350 nm	Secretory Autophagosome	Yes	> 25	ND	Chen et al., 2015a,b
Rhinovirus	ss (+) RNA	~350 nm	Secretory Autophagosome	Yes	> 25	ND	Chen et al., 2015a,b
Norovirus	ss (+) RNA	< 200 nm	Multi Vesicular Body	Yes	1-5	ND	Santiana et al., 2018
Rotavirus	ds RNA segmented	300-600nm	Plasma membrane	Yes	> 25	ND	Santiana et al., 2018
Hepatitis A	ss (+) RNA	50-100nm	Multi Vesicular Body	Yes	1-	ND	Feng et al., 2013
Hepatitis E	ss (+) RNA	50-100nm	Multi Vesicular Body	Yes	1	ND	Nagashima et al., 2014; Chapuy-Regaud et al., 2017
Enterovirus 71	ss (+) RNA	50-100nm	ND	ND	None	Yes	Mao et al., 2016
Torque Tene Virus	DNA	< 200 nm	MVB?	ND	ND	ND	Martelli et al., 2018
Enveloped eukaryotic viruses							
Hepatitis C	ss (+) RNA	< 200 nm	Multi Vesicular Body	ND	None	Yes	Ramakrishnaiah et al., 2013; Bukong et al., 2014; Longatti et al., 2015
Dengue	ss (+) RNA	< 200 nm	Multi Vesicular Body	ND	None	Yes	Vora et al., 2018
West Nile	ss (+) RNA	< 200 nm	Multi Vesicular Body	ND	None	Yes	Zhou et al., 2018
Langat	ss (+) RNA	< 200 nm	Multi Vesicular Body	ND	None	Yes	Zhou et al., 2018
Severe Fever with Thrombocytopenia Syndrome (SFTS)	ss (-) RNA	< 200 nm	Multi Vesicular Body	ND	1-5	ND	Silvas et al., 2015
Porcine Reproductive and Respiratory Syndrome (PRRS)	ss (+) RNA	~100nm	Multi Vesicular Body	ND	1-5	ND	Wang et al., 2018
Zika	ss (+) RNA	< 200 nm	Secretory Autophagosome	Yes	ND	ND	Peng et al., 2018; Cao et al., 2017
Pegivirus	ss (+) RNA	< 200 nm	Multi Vesicular Body	ND	ND	ND	Chivero et al., 2014
Varicella Zoster	DNA	~350 nm	Secretory Autophagosome	ND	1	ND	Buckingham et al., 2016
Marseillevirus	DNA	300nm-3.5μm	Endoplasmic Reticulum	ND	> 50	ND	Arantes et al., 2016

remarkably retained their infectious cargo as they passed through the hosts' gastrointestinal tract, ultimately bloc delivering their viral cargo to enterocytes in the upper intestine (Santiana et al., 2018). Experiments carried out on animals that were either orally inoculated with rotavirus-containing EVs or orally inoculated with equivalent numbers of free rotavirus particles revealed that EV-fed animals exhibited clinical symptoms of rotavirus infection far earlier and much more severely, compared to animals inoculated with the equivalent numbers of free virions. Only when animals were fed much higher quantities of free viruses could they reproduce the onset and severity of the disease kinetics of vesicle-fed animals, again indicating that there were replication barriers when viruses infected intestinal cells with low numbers (Santiana et al., 2018). Moreover, the EVs shed into stool selectively contained the activated infectious form of the virus where the outer VP4 capsid proteins had been proteolytically pre-cleaved into VP5 and VP8 (Santiana et al., 2018). In contrast, some freely dispersed rotavirus stool particles, lacking the protection of an EV membrane cloak, were found to be extensively degraded, likely by stool proteases. These data indicate that not only EVs can increase the MOI through bloc transmission to overcome replication barriers in intestinal cells, but also can further enhance virulence by facilitating the selective and protected transmission of only the most activated infectious viruses.

Lastly, superinfection exclusion is a phenomenon whereby a previously infected cell becomes resistant to a subsequent infection. It is often interpreted as a host defense mechanism that can decrease virulence. Different viral genomes delivered simultaneously into the same cell by EVs may avoid the superinfection exclusion mechanisms that would operate had the different genomes reached the cell sequentially (Nethe et al., 2005; Webster et al., 2013). However, to our knowledge, the effect of bloc transmission on superinfection exclusion has not yet been investigated.

5. Unique lipid composition of EV membranes and implications for enhanced virulence

In addition to their ability to bloc transmit multiple viral genomes, recent reports indicate that the EV membrane itself influences the course of infection by stimulating viral uptake and shielding viral cargo from the host immune system. For instance, phosphatidylserine (PS) lipids which are found on the outer membrane leaflet of all EVs transporting viruses (Table 1) appear to facilitate endocytosis of EVs by target cells (Amara and Mercer, 2015). Masking the PS with PS-binding proteins such as Annexin V or TIM4, prevents EV internalization and inhibits infection (Chen et al., 2015a,b; Santiana et al., 2018). PS lipids stimulate endocytic uptake by associating with PS receptors on opposing cells. PS receptors can be found on many different cell types including epithelial and endothelial cells, osteoclasts, oligodendrocytes, glial cells, neurons, platelets, dendritic cells and macrophages (Birge et al., 2016). The interactions of PS lipids with PS receptors can be direct or indirect, the latter through the soluble PS-binding proteins Gas and Pros1. The main PS receptors include the TAM (Axl, Mertk, Tyro3), TIM and CD300 families (Rothlin et al., 2015; Birge et al., 2016, Vitallé2018). PS binding to TAM receptors triggers Akt and PKC activation leading to cell survival, cell proliferation and induction of phagocytosis (Hoffmann et al., 2001). Notably the CD300lf receptor appears to play a dual role in the murine norovirus lifecycle: as the cognate receptor for capsids to bind to (Orchard et al., 2016) and possibly as a PS receptor to tether and facilitate internalization of EVs containing murine norovirus (Santiana et al., 2018).

PS lipids are also well-known suppressors of inflammation and trigger production and release of anti-inflammatory cytokines and chemokines (Fadok et al., 1992; Huynh et al., 2002; Hoffmann et al., 2005; Chan et al., 2016; Birge et al., 2016). For example, PS binding to MerTK receptor tyrosine kinases transmits signals that interfere with NF-KB activation and release of inflammatory molecules such as iNOS, TNF-α, IL-1β and IL-12 by macrophages. Instead it triggers release of

Relm-α, IL-10 and TGF-β, factors that are critical for tissue repair and promoting tolerance to self-antigens (Kimani et al., 2014; Birge et al., 2016; Akalu et al., 2017). Given the above, hiding within and transmitting through PS-rich EVs could certainly potentiate the infectivity of viral cargo by both promoting their uptake through stimulation of phagocytosis and by suppressing the activation of the innate and adaptive immune systems. Indeed, even many enveloped viruses that disseminate as free particles, including Vaccinia, Dengue, Ebola, Marburg, HIV, Lassa and Chikungunya utilize the PS lipids within their envelopes to potentiate infection (Amara and Mercer, 2015).

EV membranes around viral clusters can also physically obstruct the host antibody response. In vitro studies have shown that neutralizing antibodies cannot gain access to bind to viral particles when the latter are inside EVs (Feng et al., 2013) thus potentially impeding the host adaptive immune response. Consistent with this, animals inoculated orally with rotavirus-containing vesicles exhibit clinical signs of disease (e.g. diarrhea, weight loss) and shed virus long after the free virus fed animals have cleared the infection (Santiana et al., 2018). Note however that, at least in cell culture studies, the protection afforded by the EV membrane decreases post- endocytosis of the vesicles (Feng et al., 2013). This suggests that antibodies are either co-internalized in the same or parallel endosome (possibly Fc-mediated), meeting up with viral capsids once the vesicle membrane has been disrupted or antibodies neutralize viruses through unconventional processes such as TRIM21 (Bottermann and James, 2018). It is also likely that viral transmission by EVs does not entirely prevent the production and/or action of neutralizing antibodies but instead delays the host response to give viruses an upperhand in the initial stages of infection. Furthermore, viruses may eventually be exposed to antibodies as a result of stochastic vesicle lysis or lysis after internalization by antigen presenting phagocytes such as macrophages. Intriguingly, EVs transporting Porcine Reproductive and Respiratory Syndrome viruses have been shown to contain viral membrane proteins that trigger neutralizing antibodies (Wang et al., 2018). This is likely to be more wide spread as EV membranes of many viruses often derive from the very same organelles (endoplasmic reticulum, plasma membrane and endosomes) that are sites where viral non-structural and structural integral membrane proteins are found.

Finally, EVs (containing viral or other cargo) appear to be remarkably stable structures, retaining membrane integrity in stool, blood, urine and the low pH stomach environment. They have been isolated from bodily fluids and cell culture supernatants even after decades of frozen storage (Sokolova et al., 2011; Kalra et al., 2013; Boukouris and Mathivanan, 2015; Santiana et al., 2018; He et al., 2019) and can withstand repeated freeze-thaw cycles with little to no change in size or loss of lumenal contents (Sokolova et al., 2011). They are also partially resistant to treatment with non-ionic detergents such as Triton X-100 and NP40. Lipidomic analysis of EVs has revealed their membranes to be highly enriched (up to 3-fold) in cholesterol, sphingomyelin (SM) and phosphatidylserine (PS) lipids over cellular membranes (Skotland et al., 2017; Santiana et al., 2018). Cholesterol and SM are known to be critical modulators of membrane fluidity (Papahadjopoulos et al., 1973). Their ability to decrease membrane fluidity at high temperatures and increase membrane fluidity at low temperatures may explain why EVs can withstand large swings in temperature and not become disrupted by repeated freeze-thaw cycles. Indeed the cholesterol/SM rich feature of sperm facilitates their resistance to damage during long-term freezer storage and subsequent thaw (Combes et al., 2000); and of plasma membrane raft domains to disruption by non-ionic detergents (Harder et al., 1998).

6. Conclusion

EVs are effective vehicles for the intercellular and inter-organismal *en bloc* transmission of viral particles and naked infectious genomes. They enhance virulence and potentially viral fitness by increasing the multiplicity of infection and providing an opportunity for cooperative and complementary interactions to take place among quasispecies. They also protect cargo from antiviral host factors (antibodies, proteases, nucleases), facilitate increased virus uptake and suppression of immune responses.

Investigations into the role of EVs in viral transmission are still in their infancy. Many important questions remain unanswered. Firstly, there is a need for precise quantification of viral numbers and quasispecies diversity per EV; the distributions in virus quantities among EVs for each virus type; and whether cargo are particles, infectious naked genomes or both. Secondly, what other molecules are co-transported with viral cargo and do they modulate infection? Thirdly, by traveling *en bloc* in EVs, with the advantages of high multiplicity infection, invisibility to neutralizing antibodies and PS' immunosuppressive effects, are viruses able to replicate in tissues that normally would exclude the free particles? Fourthly what is the full impact on the host innate and adaptive immune responses when viruses are transmitted in EVs versus free viruses? Fifthly, does transmission by EVs further increase quasispecies diversity and possibly enhance the fitness of the viral collective in particular in relation to selection pressures such as anti-viral drugs and antibody responses? And lastly, how do EV mediated *en bloc* transmissions impact lethal mutagenesis: do EVs introduce defector genomes together with fully infectious genomes into the same cells? These and other questions are ripe for investigation and the findings will undoubtedly provide fresh insight into the dynamics of the virus-host interface.

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