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## Viral effects on the content and function of extracellular vesicles

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### Abstract

The release of membrane-bound vesicles from cells has been increasingly recognized as a mechanism for intercellular communication. Extracellular vesicles (EVs) are also produced by virus-infected cells and are thought to be involved in intercellular communication between infected and uninfected cells. Viruses, in particular oncogenic viruses and viruses that establish chronic infections, have been shown to modulate the production and content of EVs. Viral microRNAs, protein and even entire virions can be incorporated into EVs, which can impact immune recognition of viruses or modulate neighboring cells. In this Review, we will discuss the roles that EVs have during virus infection to either promote or restrict viral infection in target cells. We will also discuss our current understanding of the molecular mechanisms that underlie these effects, the potential consequences for the infected host, and possible future diagnostic applications.

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The release of membrane-bound vesicles from cells has been increasingly recognized as a mechanism for intercellular communication. In this Review, Raab-Traub and Dittmer discuss the roles that extracellular vesicles have during virus infection.

### Introduction

Extracellular vesicles (EVs) are secreted from healthy, malignant and virus-infected cells. EVs are either released directly from the plasma membrane or during fusion between multivesicular bodies (MVB) and the plasma membrane<sup>1,2</sup>. EVs released from the MVB are termed exosomes. Similar to EVs, viruses can be released through multiple pathways including the plasma membrane and/or via the MVB route (reviewed in <sup>3</sup>). For example, some retroviruses such as human immunodeficiency virus type 1 (HIV-1) assemble at the inner leaflet of the plasma membrane whereas other retroviruses, such as Mason-Pfizer monkey virus (MPMV) assemble in the cytoplasm first before trafficking to the cell surface (reviewed in <sup>4</sup>). Some viruses are non-enveloped and do not require an envelope for

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#### Conflict of interest

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infectivity, but nevertheless can be incorporated into EVs. For example, hepatitis A virus (HAV) was recently shown to be secreted within EVs that can potentially transmit to uninfected cells within an infected individual<sup>5,6</sup>. Other enteroviruses may package up to ~20 particles within a single membrane vesicle and bud without destroying the cell<sup>7</sup>. Autophagy, which is usually thought of as a regulated mechanism to provide nutrients through digestion of intracellular organelles, is utilized in different ways in the egress of multiple viruses. Not only do some enteroviruses exit infected cells in packages wrapped into autophagic membranes, autophagic membranes form part of the envelope for herpesviruses, para and orthomyxoviruses<sup>8</sup>. Lipidated LC3, an essential mark of autophagosomes, has been detected in extracellular microvesicles containing coxsackie virus. Additionally, the exosome marker flotillin-1 was also found in these vesicles suggesting that picornaviruses utilize autophagy related EV release as one pathway for their exocytosis<sup>9</sup>

EVs are thought to have an important role in virus infection and a number of interactions between viral components and cellular components that are required for the biogenesis of EVs have been reported. Therefore, a critical comparison of virus particles with EVs may lead to a greater understanding of both viral life cycles and the function of EVs.

Due to their small size and similar biochemical composition, viruses and EVs can have similar biophysical properties. The term exosome is used if EVs are 100 nm in diameter and originate from the MVB, microvesicle if the diameter is 100–1,000 nm or apoptotic body if the diameter is >1,000 nm. Similarly, viruses range in diameter from 30 nm for poliovirus, 120–140 nm for herpesviruses and 200–300 nm for poxviruses (reviewed in<sup>10</sup>). This similarity in biophysical properties increases the difficulty in obtaining pure populations of EVs that are not contaminated with viruses and vice versa, which makes it difficult to determine the precise composition of EVs and virions (Box 1). The identification and characterization of virion-associated proteins has been the subject of intense study over many years<sup>11</sup>, whereas the identification of proteins that are associated with EVs has been more recent. Recent studies have identified some key components of EVs that can be used as markers to identify and assess the purity EVs (Table 1); however, it is important to recognize that not all EVs carry all of these markers<sup>12</sup>. In the context of virus infection, viral RNAs and proteins have been found in EVs<sup>13–16</sup>, which could be the result of selective incorporation of specific RNAs and these proteins or alternatively, reflect the total intracellular constituents. Further work is required to determine the precise composition of EVs. The development of mass spectrometers with enhanced specificities and sensitivities compared to existing instruments will undoubtedly advance our understanding of the composition of EVs. Further work is also required to optimize the purification of EVs and therefore careful consideration is required when attributing functional phenotypes to EVs in the context of virus infection.

### Box 1

#### Differentiating EVs from virions

The purity of any preparation of EVs is determined by measuring light-scatter and Brownian motion and by electron microscopy (Figure 2). More recently, flow cytometry-based methods have been introduced, but the small size of EVs makes it difficult to detect

EVs using conventional instruments. EVs can be isolated by size-exclusion chromatography, differential ultracentrifugation, density flotation, crowding agents, flow-cytometry or affinity purification (Table 2). Each method has specific advantages<sup>158</sup>. Size-exclusion chromatography is the best method for preserving the structure of EVs<sup>159–161</sup>, though it does not separate virions from EVs<sup>11,151,162</sup>. The use of crowding agents, such as PEG3000 followed by precipitation is the fastest way to isolate and concentrate EVs for subsequent applications, however, this approach also enriches soluble proteins and contaminants that are not part of EVs. When profiling miRNAs in EVs it is important to consider that Ago-associated miRNAs are present in serum<sup>163</sup>. These Ago-miRNA complexes also co-purify with EVs when only crowding agents are used, however, this problem can be circumvented by using affinity-based purification methods. Affinity purification using magnetic beads enables the high-throughput purification of EVs on robot platforms and it is able to separate EVs from viruses<sup>13</sup>. For example, EVs from B cell lymphomas are enriched for B cell surface antigens, including CD81 and CD63, which can be used to affinity purify EVs using antibodies that bind these proteins<sup>164</sup>. However, this approach will exclude populations of EVs which do not have the particular surface marker used for affinity purification, but which may nevertheless contribute biological functions of EVs<sup>12</sup>.

Table 2

<b>Method</b>	<b>Mechanism</b>	<b>Input volume</b>	<b>Virion co-purification</b>
<b>Differential ultracentrifugation</b>	Density and size	35 ml	Yes
<b>ExoQuick (SBI Biotech Inc.)</b>	Precipitation	250 µl	Yes
<b>Total EV (Invitrogen Inc.)</b>	Precipitation	250 µl	Yes
<b>PEG-2000</b>	Precipitation	250 µl – 250 ml	Yes
<b>CD63 magnetic beads</b>	Bead-based surface marker	1 – 35 ml	No
<b>Composite magnetic beads</b>	Bead-based surface markers (n=5 markers)	100 µl – 1ml	No
<b>Size exclusion chromatography</b>	Size-bases isolation	100 µl – 35 ml	Yes
<b>Density flotation (for example, Iodixanol)</b>	Density	35 ml	Yes

Research into understanding the role of EVs in viral infections is driven to a large extent by commercial interests in biomarker development. For example, miR-122 that is incorporated into EVs during acute liver injury could be used as a biomarker to determine the extent of damage to the liver<sup>17–19</sup>. The miR-122 is the most abundant miRNA in liver cells and perhaps reflecting this abundance it is incorporated into EVs. The correlation between the levels of miR-122 and alanine aminotransferase, an enzyme that is also released when the liver is damaged, has been established in the clinic. Therefore, miR-122 could be used as an alternative, more specific biomarker to alanine aminotransferase in the clinic. Notably, miR-122 is also required for HCV replication and therefore it is an attractive drug target for the development of new therapies against HCV<sup>20–22</sup>. The success of such therapies could be determined by minimally invasive profiling of EVs and plasma miR-122 levels since

miR-122 would add information about liver cell status beyond viral load. The use of highly multiplexed assays that are able to detect multiple miRNAs and viruses, next-generation sequencing and mass spectrometry will help drive the research of EVs for diagnostic applications

Studying EVs in the context of virus infection has been crucial in demonstrating the potential contribution of EVs to viral pathogenesis<sup>23</sup>, as EVs from virus-infected cells often transfer viral components to uninfected cells, for example, Epstein-Barr virus (EBV) LMP1 protein and viral miRNAs<sup>16,24</sup>. This intercellular transfer of viral cargo occurs in the absence of cell-to-cell fusion, cellular synapses or **membrane nanotubes**<sup>25–27</sup> and represents the existence of a host transfer mechanism that occurs in the absence of virus spread. Roles for intercellular transport by EVs have been described, for example, in mediating cross presentation for T cells<sup>28–30</sup> and in mediating synaptic transmissions<sup>31</sup>. Many of the vesicles that are used during these processes share biogenesis features and fusion mechanisms that are similar to EV and viruses. In the context of cross-priming, however, the vesicles tend to stay in the immediate microenvironment, such as the lymphnodes, and are not found circulating systemically in body fluids.

In this Review we will focus on the identified molecular and biological properties of EVs released from virally infected cells and consider how the virally modified EVs may either facilitate viral infection or promote resistance to immune recognition by antibodies or inhibition of innate immunity activation within recipient cells. In particular we focus on human viral infections with HIV, HAV, and HCV, and the two herpesviruses EBV and Kaposi Sarcoma-associated herpesviruses (KSHV). It is in the context of these chronic, persistent, and latent infections that EVs have been most thoroughly explored.

## Biological roles for EVs in viral infections

Oncogenic viruses and viruses that are able to establish long-term persistent infections have been shown to alter the content of EVs, which has been hypothesized to facilitate infection and contribute to persistence and pathogenesis. Persistent or chronic infections are characterized by low levels of viral replication and circulating virus particles ( $\sim 10^1$ – $10^4$  particles/ml)<sup>3</sup>. By contrast, during **latent infections** viruses cannot be detected in circulation. Latency has a defining role in herpesvirus and lentivirus infections. In the case of latent herpesvirus infections, viral miRNAs can be detected within EVs at times when conventional viral load assays are negative<sup>13,16,24</sup>. In the case of HIV-1 the viral protein Nef has been found in EVs that circulate in infected individuals<sup>32–34</sup>. Systemic circulation of viral proteins in EVs enables these viruses to modulate host cells without exposing viral proteins or virions to the immune system. By contrast, rapidly replicating viruses, such as Ebola virus, influenza A virus or Zika virus accumulate to high titers ( $10^6$ – $10^{11}$  particles/ml) in blood within days of primary infection. For these viruses, the viral titer is similar to the number of circulating EVs ( $10^{10}$ – $10^{12}$  particles/ml)<sup>35</sup>. Despite these high viral titers, it is possible that EVs that carry viral proteins or nucleic acids could modulate host cells, for example by determining their permissiveness to infection.

The biological function of EVs in the context of viral infections can affect viral infection in two opposing ways (reviewed in <sup>23,36–38</sup>). On the one hand, EVs can either modulate recipient cells by promoting viral replication or, on the other hand, EVs can restrict viral replication through triggering host immune responses.

## Properties of EVs

### Biogenesis

EVs are small membrane-bound carriers of intracellular cargo that are derived from MVBs or from the plasma membrane (Figure 1). Unlike virus particles, the membranes of EVs do not enclose a structured core, such as a **capsid**. Specific properties of EVs define and distinguish EVs from other types of microvesicles<sup>10</sup>. The assembly of EVs is an active, energy dependent and regulated process<sup>39,40</sup>. This process has been shown to specifically require sphingomyelinase<sup>41</sup> and components of the endosomal sorting complex required for transport (ESCRT) machinery. The content of EVs is determined by the protein and RNA composition of the cells from which they are derived. The composition of EVs frequently reflects the relative abundance of contents within the EV-producing cell. Thus EVs that are derived from virus-infected cells contain highly expressed viral miRNAs<sup>13,16,24</sup>. In addition, several recent studies have provided evidence for differential loading, where the relative abundance of the EV miRNAs is distinct from that within the producing cell<sup>16,24,42–45</sup>.

The budding of EVs into MVBs requires the ESCRT proteins tumor susceptibility gene 101 protein (TSG101) and Alix (also known as programmed cell death 6-interacting protein), which are also known markers of EVs<sup>2</sup>. TSG101 and Alix are also required for ESCRT-dependent budding of enveloped viruses from the plasma membrane, such as HSV-1<sup>46</sup>, therefore it is difficult to determine whether these are specific EVs as thus they do not conclusively establish EV originating from MVBs or the plasma membrane using these proteins as markers. Other membrane proteins that localize to lipid rafts in the plasma membrane, including tetraspanins such as CD63 and CD81, are also enriched in EVs. This process involves palmitoylation<sup>47</sup> which has been shown to be required for the incorporation of the EBV protein LMP1. LMP1 is enriched in lipid rafts and in EVs<sup>48</sup>. Depending on the experimental approach and cell line, other proteins such as Sortilin (SORT1), Synthenin-1 (SDCBP), or Syndecan-1 (SDC1) have also been found to have a role in the biogenesis of EVs<sup>49–51</sup>.

The Rab family of small GTPases regulates multiple steps in the trafficking of vesicles to distinct endocytic compartments and they also function in docking of the MVB to the plasma membrane (Figure 1). For example, Ras-related protein Rab-11A and Rab35 function during the recycling and sorting endosomes, whereas Rab27A and Rab27B are essential for the secretion of EVs<sup>52</sup>. It is likely that some viruses modulate this complex process to facilitate viral entry, trafficking, and egress. Rab6 and Rab7 also affect the flux between lysosomes and autophagosomes and could contribute to determining the content of EVs<sup>53–55</sup>. Multiple viruses affect the expression of ESCRT and Rab GTPases to promote viral entry and egress and are therefore likely to modulate the content of EVs<sup>56</sup>.

Additionally, it is known that members of the Rab family of GTPases also interact with members of the Ral family of GTPases and function during intracellular trafficking. It has been shown in *Caenorhabditis elegans* that Ral1 (the homologue of human RALA and RALB) regulates both MVB biogenesis and the secretion of EVs<sup>57</sup>. Activated Ral1 associates with syntaxin 5 (SYX5), a soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) complex protein, at the plasma membrane and is required for the secretion of EVs. Interestingly, SYX5, is also required for the release of human cytomegalovirus (HCMV) and syntaxin-4, another SNARE complex protein has been found to regulate HCV release<sup>58,59</sup>. Additional SNARE complex proteins have also been shown to interact with herpesvirus glycoproteins to promote release<sup>60</sup>. These examples demonstrate that viruses co-opt the cellular vesicular transport system during egress and therefore, they are also likely to modulate the content and secretion of EVs through similar interactions.

### EV Uptake

Viruses use cell surface receptors to initiate fusion with the plasma membrane and are known to use specific receptors to target specific cell types. This receptor-specificity determines their cellular tropism and is a distinguishing feature of EVs, which have the ability to enter a greater variety of cell types than viruses. Using fluorescent dyes that are incorporated into EVs, it was demonstrated that membranes of EVs can fuse with cell membranes<sup>16,24,61,62</sup>. Most cell types that have been tested can fuse with EVs and therefore EVs can be used to deliver cargo to a wide variety of cell types. This process is analogous to cationic lipid-mediated transfection approaches. In some cases, EVs use specific receptors for entry<sup>12,63</sup> and therefore the fusion of EVs with cellular membranes can be specific. For example, EVs from EBV-infected cells that express the viral glycoprotein gp350, or EVs that are engineered to express gp350, specifically target B cells that express the viral entry receptor CD21 and can block EBV infection of naïve B cells<sup>61,64</sup>.

Heparin is involved in the initiation of host cell entry for many viruses, including retroviruses and herpesviruses<sup>65</sup>. It is a glycosaminoglycan that binds almost all viral envelopes. Thus, exogenous heparin or heparin beads can inhibit virion attachment through competitive binding. Cell surface-bound heparin is thought to concentrate virions prior to specific receptor and co-receptor engagement. It is also thought to have a role in the entry of EVs<sup>66</sup>, though the fusion of EVs with the plasma membrane can occur at heparin concentrations that block herpes simplex virus 1 (HSV-1) entry. This provides an experimental tool to separate virion effects from EV phenotypes, as virus entry, even of non-infectious or defective HSV-1 particles, can be blocked by heparin. It suggests that EVs use different mechanisms for entry to cells compared to most viruses.

Similarly, annexin A5 (and potentially other annexin family members) mediates the fusion of EVs to the plasma membrane through binding to phosphatidyl-serine<sup>67</sup>. Importantly, Annexin A5 does not antagonize virus entry and thus can also be used to distinguish EV-mediated phenotypes from virus particle or soluble molecule-mediated phenotypes. Although the biogenesis of virions and EVs are similar, EVs and virions likely use different mechanisms to enter cells.



Integrins and integrin-binding matrix proteins, many of which contain a signature motif of arginine, glycine, and aspartic acid (RGD), have an important role in virus entry and their potential role in the entry of EVs is also beginning to be understood<sup>68,69</sup>. They are thought to act as attachment factors or co-receptors that synergize with the primary receptor for virus entry. Both EBV and KSHV utilize integrins as a co-receptor for viral entry<sup>70</sup>. RGD peptides and anti-integrin antibodies can interfere with the attachment of virions and EVs to cells<sup>68,70</sup> and affect signaling through the integrin homodimer and heterodimer and thus have pleotropic effects on cell physiology. For example, the anti-integrin antibody Etaracizumab is in clinical trials as anti-angiogenesis agent in non-virus associated cancers, because blocking integrin signaling can induce cell death.

Viruses have a strict requirement for cell-type specific receptors and co-receptors. These engage specific envelope glycoproteins and force large molecular rearrangements to expose the components of the viral fusion complex. Hence, hyper immune serum that was raised against virion components has clinical utility in blocking infection, for example, in treating patients that have been exposed to Ebola virus<sup>71</sup>. By contrast, entry of EVs is more promiscuous than viruses and clathrin-dependent, caveolae-dependent, macropinocytosis<sup>56,62</sup>, phagocytosis, and lipid raft-mediated uptake<sup>72</sup> have all been shown to contribute to the entry of EVs (reviewed in<sup>63</sup>).

### EV-mediated functions

Much of what we know about the physiological function of systemically circulating EVs derives from studies that have analyzed their contribution to cancer metastasis<sup>73–76</sup>. It was found that EVs released from tumor cells can modulate cells in the surrounding microenvironment and drive distant metastasis by modulating stromal cell growth, cell migration, induce growth factor secretion, and vascular permeability. A supportive microenvironment, for instance by providing growth factors and extracellular matrix attachment opportunities and increased endothelial cell permeability, are also essential for systemic virus spread. In the following sections we will review how EVs contribute to viral infection

**HCV**—HCV is a member of the *Flaviviridae*. It is distinct from the arbovirus members of this virus family as it is transmitted by blood-to-blood contact or through sexual intercourse, rather than by an insect vector. HCV virions are enveloped and are smaller than EVs (~50 nm in diameter in contrast to ~ 100 nm of EVs)<sup>77</sup>. EVs isolated from HCV-infected human hepatoma cell lines have been shown to contain HCV<sup>78,79</sup>. Some of the subgenomic HCV RNA co-localizes with CD81 and CD63 (markers of EVs). The HCV E2 protein was also found to co-localize with CD81 in EVs and EVs were found to transmit viral RNA to uninfected cells<sup>79–81</sup>. CD81 is the dominant HCV co-receptor<sup>82–84</sup>, therefore it is likely that HCV virions are incorporated into EVs by their interaction with CD81. HCV RNA that is transmitted by EVs induces an innate interferon (IFN)-alpha response in neighboring dendritic cells (DCs). This is in contrast to natural HCV infection, which also delivers viral RNA to cells, but down-regulates TLR and RLR signaling through the action of the viral NS3/4 protease. Therefore by specifically transmitting viral RNA, but not viral proteins, which antagonize innate immunity, EVs may provide a protective function for uninfected

cells in the immediate microenvironment during infection. Systemically circulating EVs that contain HCV virions may have a pro-viral role, as HCV has been shown to spread in the presence of neutralizing antibodies<sup>78,85</sup>. This spread may be facilitated by the masking of viral proteins in the EVs (Figure 2, **panel A**). Such a strategy of host evasion in which persistent viruses, such as HCV and more prominently HAV (see below), to escape the evolutionary selection pressure of neutralizing antibodies by being incorporated in EVs may be widespread. The carriage of HCV virions in EVs also suggests that HCV could enter a range of different cell types, in addition to hepatocytes, through EV-mediated fusion in which infection would not be dependent on the expression of a specific viral receptor.

**HAV**—HAV, a non-enveloped picornavirus, is the cause of acute enterically transmitted hepatitis and replicates efficiently in the liver. HAV rapidly replicates in susceptible cells and viral particles are released from these cells, however, no cytopathic effect has been observed. RNA-containing proteinaceous particles (density of 1.22 – 1.28 g/ml) represent the major species of HAV in feces<sup>6</sup>. HAV can be neutralized by antibodies that are elicited by the current HAV vaccines. It has been shown that HAV can be released from cells in host-derived membranes at a density of 1.06–1.10 g/ml<sup>5</sup>. These EVs that contain HAV are infectious and circulate in the blood of infected humans, whereas non-enveloped virus has only been found in feces (Figure 2, **panel B**)<sup>6</sup>. Coxsackie B virus and Enterovirus 71 (EV71), two other picornaviruses, have also been found in EVs<sup>7,86,87</sup>.

Two hypotheses have been made to explain importance of EV-encapsulated picornaviruses as a biological mechanism rather than a side product of cellular inefficiencies<sup>6</sup>. Firstly, the envelopment of HAV by host membranes may expand the tropism of the viruses as now EV surface proteins rather than the viral surface proteins engage the target cells. This may provide an additional route for HAV to spread within the liver and systemically to distant organs, such as the spleen, and lymph nodes, which function to filter and survey systemic fluids<sup>3</sup>. Second, the acquisition of a host membrane by HAV demonstrates that some non-enveloped viruses can acquire an envelope that is devoid of viral transmembrane proteins and thus provides evidence for a second, alternative egress pathway. This suggests that the egress that is mediated by EVs would be distinct from the normal egress route. The acquisition of a host-derived membrane could also affect antibody recognition of HAV capsid proteins and may allow persistence and spread in the presence of neutralizing antibodies.

**HIV**—HIV is a human retrovirus that contains an RNA genome and acquires its envelope from the cellular plasma membrane, where it buds from areas that are enriched in the viral Gag protein. After a month-long period of acute replication ( $10^5$ – $10^7$  viral particles/ml), HIV establishes a latent infection, which slowly progresses to acquired immune deficiency syndrome (AIDS) over a period of years in untreated individuals as the virus depletes CD4+ T cells. It is believed that during viral latency ( $5 * 10^1$  viral particles/ml in plasma), HIV directly and indirectly modulates the immune system, leading to chronic pathology. Many of these indirect effects on the immune system are observed even in patients on anti-retroviral therapy. Various hypotheses for this phenomenon have been proposed, including a role for EVs. In addition, soluble viral proteins, such as HIV Tat as well as secondary events to



immune compromise, such as extended LPS translocation may contribute to this phenotype<sup>88,89</sup> (reviewed in<sup>36</sup>). HIV assembly and the biogenesis of EVs have many similarities<sup>90–92</sup>. These similarities impair the biochemical and physical separation of EVs away from virions, which include mature, immature, and even defective particles. The molecular pathogenesis of HIV suggests potential EV-mediated effects on neighboring cells (Figure 2, **panel C**), because HIV kills more abortively infected or uninfected T cells than infected cells<sup>93–96</sup>. HIV infected cells may also release EVs with trapped HIV virions<sup>94–97</sup>.

More importantly, HIV encodes a number of accessory proteins, such as Vif<sup>98</sup>, which interfere with the cellular antiviral response. These pro-viral proteins may also be carried by EVs to prime neighboring cells to promote infection, in what is called the ‘Trojan Horse’ hypothesis<sup>23,36</sup>. Conversely, cellular anti-viral factors that are carried via EVs could facilitate an antiviral response in neighboring cells<sup>99</sup>. Additionally, EVs packaged with viral proteins may function to introduce viral proteins and would activate B cells and T cells through endosomal presentation of proteins, a process termed cross priming, rather than endogenous synthesis and presentation within MHC molecules. Thus these immune cells would become specifically activated without actual viral infection<sup>100,101</sup>.

It is known that HIV infection changes the repertoire of miRNAs within infected cells<sup>102–104</sup> and considering that miRNAs can be packaged into EVs<sup>13</sup>, the EVs from HIV-infected cells may contain distinct miRNAs compared to EVs from uninfected cells. In the context of AIDS-defining malignancies, such as Kaposi’s sarcoma, EVs isolated from HIV-infected patients have distinct miRNAs profiles prior to and concurrent with lesion development<sup>13,105</sup>. Initially, HIV was thought to encode miRNAs<sup>106</sup>, but these observations have since been questioned<sup>107,108</sup>. It is becoming clear that miRNAs with Drosha-dependent hairpin-looped precursors are not the only small RNAs that can be transcribed from RNA virus (including retroviruses) genomes<sup>109–111</sup> and some of these RNA species have been found in EVs<sup>112</sup>. The potential roles of miRNAs (as defined by their biogenesis and structural composition) or other small RNAs that do not fit the classical definition of miRNAs to EV function is a new area of investigation that is likely to reveal new properties that affect viral infection and pathogenesis.<sup>107,108,113</sup>

EVs from HIV-infected cells contain the viral protein Nef<sup>32–34</sup>. In addition, soluble HIV Tat protein circulates in interstitial spaces, blood, and mucosal barriers and can perform biological activities, such as promoting angiogenesis and endothelial cell reprogramming<sup>89,114–116</sup>. Soluble Tat has a fusogenic peptide sequence that enables its efficient uptake into cells<sup>117,118</sup>. Since the HIV Tat protein would not need to be incorporated into EVs to enter cells, the biological relevance of the incorporation of Tat into EVs remains unclear. HIV Nef associates with membranes and with members of the vesicular trafficking system, such as Alix and others<sup>119,120</sup>. Nef can be incorporated into EVs and may modulate the contents of EVs including miRNAs<sup>32–34,121</sup>; however, others have contested these findings as the level of Nef in EVs is at the limit of detection<sup>122</sup>. In sum, HIV provides an example of how latent viruses may use EVs to maintain a susceptible host environment over long periods of time during which no virions are detectable.

**EBV**—EBV, a gamma herpesvirus, was the first human tumor virus to be identified and a major human pathogen<sup>3</sup>. EVs containing viral proteins were first shown to be produced from B cells that were infected with EBV. The major EBV oncoprotein, latent membrane protein 1 (LMP1), was identified in EVs secreted from EBV infected cell lines<sup>123</sup>. LMP1 is required for B lymphocyte transformation (reviewed in<sup>124</sup>). The incorporation of this protein into EVs has not only been demonstrated in B cells and epithelial cells that were cultured *in vitro* but has also been detected in exosomes in the serum of patients with EBV-associated tumors and in the serum from mice carrying nasopharyngeal carcinomas (NPCs)<sup>16,125</sup>. The interaction of LMP1 with the tetraspannin CD63 may contribute to the selective incorporation of LMP1 into EVs<sup>48,126</sup> as does selective palmitoylation of LMP1<sup>47</sup>. Additionally, LMP1 is known to localize to lipid rafts within membranes and lipid rafts are present within the membranes of EVs<sup>127</sup>. It is possible that the presence of LMP1 in lipid rafts may contribute to enrichment of LMP1 within MVBs and subsequently enriched within exosomes.

EVs secreted from B cells containing LMP1 inhibit T cell proliferation and NK cell cytotoxicity<sup>123,128</sup>. EVs that are secreted from EBV-infected NPC cells also contain galectin-9, which is thought to contribute to these immunosuppressive effects<sup>128–130</sup>. It has long been known that EBV-infected NPC tumors are infiltrated with T cells that are apparently nonfunctional as they do not kill tumor cells or impair tumor growth. This lack of activity may reflect the abundant secretion of EVs during EBV infection, thus representing another viral immune evasion strategy.

Importantly, LMP1-containing EVs have been shown to deliver activated signaling proteins into uninfected cells<sup>131</sup>. This potentially important feature of EVs was revealed in studies that showed that the epidermal growth factor receptor (EGFR), which is highly induced by LMP1, was also abundant in LMP1-containing EVs<sup>16</sup>. LMP1 has also been shown to increase PI3CA levels within lipid rafts and within EVs. The delivery of LMP1 and EGFR and PI3CA through EVs induced growth stimulating signaling pathways in recipient cells, including the activation of the PI3kinase target, Akt1, and ERK1<sup>131</sup>. An early study revealed increased incorporation of fibroblast growth factor 2 (FGF2) from LMP1 expressing cells into EVs which could potentially affect the tumor environment through the direct growth stimulation of infected cells or supporting stromal cells<sup>132</sup>. Additionally, it has been demonstrated that LMP1 activates HIF1-alpha, which is also transferred by EVs into recipient cells and can activate HIF1-alpha targets<sup>133</sup>. HIF1-alpha is the major transcriptional regulator in hypoxic conditions that are characteristic of many tumors and could promote survival of tumor cells in an anoxic environment. An important target of HIF1-alpha is the vascular endothelial growth factor, which induces angiogenesis. Thus, through the transfer of EVs, EBV can affect the growth of neighboring cells. It is also known that within tumors that are caused by EBV infection, not all cells express LMP1. Therefore the secretion and uptake of LMP1 into cells that do not express LMP1 could affect the growth of additional tumor cells. This may be particularly important in the pathogenesis of NPC where not all cells express detectable levels of LMP1.

LMP1 may also modulate the selective sorting of proteins into the exosomal pathway, suggesting that EBV manipulates these pathways for intercellular communication. The

possibility of LMP1-mediated specific effects on the content of EVs was revealed from quantitative proteomics and 2-dimensional gel analysis of EVs that were purified from B-cell lines that were uninfected, infected with EBV, KSHV, or with both viruses<sup>131</sup>. Analysis of LMP1-positive versus LMP1-negative cell lines revealed 217 protein spots with significantly different expression ( $P < 0.05$ ). **Principal component analysis** to identify the distinguishing features among the EVs from these different cell lines revealed that LMP1 was a major determinant for the variance between samples. This strongly suggested that LMP1 had an effect on the exosomal protein content and provided additional evidence for specific viral effects on this process.

Spectral counting analysis also indicated that both KSHV and EBV had distinct effects on the content of EVs and that these effects reflected cellular changes that occur in infected cells<sup>131</sup>. Gene ontology pathway analyses of proteins that were identified in EVs that were derived from infected cells predicted that EVs from EBV and KSHV infected cells likely modulate cell death and survival, ribosome function, and protein synthesis. Analyses of the content of EVs from infected cells also indicated that KSHV EVs could affect cellular metabolism and that EVs from EBV infected cells could activate cellular signaling mediated by integrins, actin, interferon, and NF- $\kappa$ B through the transfer of critical regulatory proteins in these pathways.

An additional, novel finding was that EBV-encoded miRNAs were also detected in EVs that were secreted from EBV infected cells and that these viral miRNAs could then be transferred to uninfected recipient cells<sup>16,24</sup>. Importantly, the viral miRNAs were shown to specifically decrease previously identified viral miRNA targets, thus providing evidence of functional delivery of miRNAs through EVs<sup>24</sup>. This transfer also likely occurs *in vivo* as uninfected B cells that were isolated from patients with NPC contain viral miRNAs. Interestingly, EVs from NPCs have distinct patterns of EBV miRNA abundance compared to the intracellular levels in the producing cells<sup>16</sup>. This observation supports the hypothesis that there is selective sorting of specific miRNAs into EVs.

In addition to viral proteins and miRNAs, 5'pppEBER1, a small non-coding viral RNA, has also been found in EVs that are secreted from EBV infected cells<sup>134</sup>. EBER1 is the most abundant viral RNA in infected cells and 5'pppEBER1 enhances the immune function of dendritic cells. This unusual finding may indicate that EVs contribute to autoimmune diseases, such as lupus, that have been linked to EBV infection<sup>3</sup>. Overall, these findings suggest that EBV modulates EVs to specifically secrete viral and cellular proteins and miRNAs that likely contribute to intercellular communication and affect the function of uninfected cells. Modulating the content of EVs could be important for affecting the tumor environment by inducing cell growth, promoting angiogenesis, and inhibiting immune cell function, and also for potentiating metastasis.

**KSHV**—KSHV causes Kaposi's sarcoma and a variety of hyperplastic and neoplastic B cell disorders, such as primary effusion lymphoma (PEL). PEL usually present with liquid effusions without tumor masses in serous body cavities. Cell-free, primary PEL fluid is highly enriched in tumor-derived EVs<sup>13</sup>. Primary KSHV infection is asymptomatic in the healthy host and results in lifelong latency. In rare cases, immune reconstitution

inflammatory syndrome follows, which is associated with severe disease flares and clinical symptoms consistent with infection and inflammation. Common to all Kaposi's sarcoma pathology is neo-angiogenesis and the infiltration of the environment around the tumor cells with uninfected, non-transformed host cells, such as endothelial cells and macrophages. After hemangioma, Kaposi sarcoma is the most angiogenic cancer and therefore the study of KSHV can be useful in understanding the interaction between EVs and endothelial cells. KSHV angiogenesis is driven by paracrine effectors such as soluble cytokines and the growth factors vascular endothelial growth factor 1 (VEGF-1) and platelet derived growth factors PDGF (1–4)<sup>135</sup>. Initially, the paracrine drivers of Kaposi's sarcoma were thought to be only soluble cytokines (for example, vascular endothelial growth factor and platelet derived growth factor or IL6). More recently EVs have been shown to mediate some of these phenotypes such endothelial cell remodeling, migration and proliferation independent of IL-6<sup>13</sup>. PEL and Kaposi's sarcoma tumor cells release large quantities of EVs, which drive endothelial cell proliferation and invasion in the presence of neutralizing antibodies to IL-6<sup>13</sup>.

KSHV encodes multiple viral miRNAs<sup>136,137</sup> and these viral miRNAs constitute up to 50% of all miRNAs in infected cells<sup>138</sup>. Viral miRNAs and mRNAs have been detected in virion preparations of almost all herpesviruses<sup>14,15,139</sup>. Viral miRNAs are also readily detected in Kaposi's sarcoma-tumor derived EVs<sup>140</sup>. The levels of viral miRNAs in EVs are 10–100-fold higher than what has been reported for virion-associated miRNAs. Owing to the similar biophysical characteristics of virions and EVs, it is unclear if reports of virion-associated RNAs reflect contamination by EVs.

Several lines of evidence have attributed phenotypes that had been attributed to miRNAs incorporated into virions to miRNAs incorporated into EVs. These include the biochemical separation of EVs by positive selection on anti-CD63 beads (a marker of EVs) (Figure 4), as well as genetic approaches, such as the detection of KSHV miRNA-containing EVs in KSHV-microRNA transgenic mice, which cannot produce virus<sup>13</sup>. This suggests that during KSHV latency viral miRNAs can be incorporated into EVs via the same host cellular pathways that load host miRNAs into EVs. Similar to KSHV virions that contain a large proportion of the total pool of intracellular miRNAs, a large proportion of KSHV miRNAs is also incorporated into systemically circulating EVs<sup>13</sup>. The identity and abundance of miRNAs in EVs thus represents a snapshot of their cellular origin. A more active model hypothesizes that viral miRNAs and proteins are specifically and differentially loaded into EVs<sup>16,24,42–45</sup>.

KSHV infection changes many aspects of the physiology of the infected cell, including lipid metabolism and presumably vesicle biogenesis. The protein composition of EVs and their secretion is modulated during viral reactivation from PEL cell lines<sup>16</sup>, though KSHV proteins have not been detected in EVs<sup>131</sup>. The replication and maturation of KSHV is much slower compared to other viral infections<sup>3</sup>. In culture models of KSHV, the number of infectious virions is approximately 10<sup>5</sup> copies/ml, compared to 10<sup>7</sup> copies/ml for EBV or 10<sup>9</sup> copies/ml for flaviviruses<sup>141</sup>. In Kaposi's sarcoma patients, the viral titer ranges from 10<sup>3</sup>–10<sup>5</sup> copies/ml, whereas EBV or HCMV titers exceed 10<sup>6</sup> copies/ml in patients and viral titers during hepatitis virus infections range from 10<sup>5</sup>–10<sup>9</sup> copies/ml. We speculate that for

KSHV, preventing the accumulation highly immunogenic virions, and instead using host EVs to distribute viral miRNAs to neighboring cells represents a novel strategy to persist in the host (Figure 2, **panel E**). In this model, RNAs in EVs offer an evolutionary advantage for virus spread by priming neighboring cells for infection. KSHV infects and replicates primarily in endothelial cells. These need to be attracted and re-programmed to migrate towards the initially infected cell. PEL derived EVs that are secreted by PELs confer this property onto uninfected endothelial cells in culture through the transfer of viral miRNAs or even and protein coding RNAs<sup>142,143</sup>. In sum, KSHV represents another example, by which the virus modulates the host local environment by releasing EVs through EV reprogramming. Interfering with EV the release of EVs during KSHV infection may therefore have the potential to limit virus spread or virus pathology.

**Alphaherpesviruses and betaherpesviruses**—Alphaherpesviruses, such as herpes simplex virus type-1 (HSV-1), and betaherpesviruses, such as HCMV, also influence the biogenesis pathway of EVs and use these pathways for egress<sup>144–146</sup>. Similar to the biogenesis of EVs, HCMV virion maturation is dependent on the ESCRT machinery and they contain cellular markers that are associated with EVs<sup>147,148</sup>. Some of the observed systemic and biological phenotypes that are associated with HSV-1 and HCMV infection, may be result of massive re-programming of MHC-I and MHC-II trafficking from the secretory pathway. HCMV replicates in a variety of cell types including endothelial cells that line the blood and lymphatic vasculature and can cause graft rejection in organ transplantation. EVs that are released from HCMV-infected endothelial cells can exacerbate allogeneic graft rejection<sup>149</sup>.

More recently, it was shown that the immune sensor stimulator of interferon genes protein (STING) is incorporated into EVs that are secreted from HSV-1 infected cells and that the STING ligand cyclic GMP-AMP synthase (cGAMP) is present in EVs that are released from cells that are infected with murine cytomegalovirus<sup>150,151</sup>. The cGAMP nucleotide triggers the recognition of foreign molecules via STING and also augments RIG-I and TLR signaling, leading to a strong interferon response. It is possible that introducing cGAMP into cells via EVs may trigger an antiviral response in neighboring cells<sup>152</sup>. This represents perhaps the most direct demonstration that cellular EVs can play an anti-viral and pro-host role.

HSV-1 viral miRNAs miR-H28 and miR-H29 are also incorporated into EVs. These have been shown to have a pro-viral role in infection and facilitate infection in neighboring cells<sup>153</sup>, perhaps by weakening innate immune defenses. Further studies are needed to fully understand this observation, but it seems counterintuitive that the same infected cell would secrete pro-viral and antiviral EVs at the same time. More likely, these distinct effects identified in cultured cell lines represent different steps in natural infection perhaps by altering initial infection of different cell types, enhancing or limiting systemic spread throughout the host, or by altering viral virulence to facilitate persistence or establishment of latency. In sum, herpesviruses, by virtue of their large genomes (encoding > 100 proteins, as well as miRNAs and lncRNAs<sup>154</sup>) modulate EV biogenesis and EV function through multiple independent mechanisms.

## Summary and outlook

In this Review, we have discussed the multiple ways in which different viruses manipulate EVs for their benefit in order to increase their persistence, pathogenesis, and transmission. In recent years EVs have emerged as specific carriers of cellular and viral components including miRNAs, proteins, and viral genomes. This can happen during active viral replication or during viral latency. The majority of experiments that have been performed explore how EVs can deliver cytoplasmic content from one cell to the cells in the surrounding environment in the absence of cell-cell fusion. However, the role of EVs can also be more far-reaching than the local environment in which they are released into (Figure 4); the presence of EVs in blood and the lymphatic system suggests that EVs are able to transmit cargo over long distances.

The role of EVs in viral pathogenesis is similar to the role of EVs in cancer metastasis, where they are known to prime distant sites (soil) for reception of metastatic cells (seed) (**See Box**). EVs and their effects on recipient cells are mediated by individual EVs and the recipient cells do not produce and amplify these EVs. This is in contrast to virally infected cell which can produce thousands of viruses from a single infected cell (Figure 1) However, during chronic or latent viral infection a single infected cell may actually release EVs orders of magnitude greater than infectious viral particles, therefore the utilization of EVs to prime and enhance systemic viral infection is likely.

Experimental data that supports a role for EVs in priming the innate immune response has been reported. Cargo in EVs can elicit a TLR-dependent immune response in mice that have tumors<sup>155</sup> or prime neighboring cells, including DCs, to respond to viral infection by priming the adaptive response or by releasing interferon<sup>134,156</sup>. During this scenario, EVs may have evolved a role in the protection from pathogens, perhaps through the activation of innate immune responses in neighboring cells. A role for EVs in adaptive immunity has already been established, in which EVs (or some specialized membrane encapsulated vesicles) can efficiently cross-prime innate immunity and adaptive immune memory responses<sup>27,28,30,101,157</sup>.

Importantly, it is likely that the basic research in EV biogenesis and fusion will be enhanced by previous, similar studies that characterized virion egress and entry. Many steps in the biogenesis of EVs are also used by viruses for entry and egress, for example, the ESCRT family of proteins. Further study of viral effects on the pathways that involve EVs is likely to identify the critical regulators of endosomal and exosomal trafficking in host cell physiology as well as new mechanisms that modulate the infection of a complex organism through enhancing and inhibitory effects on the infecting virus and resultant viability of the infected host cell. The further study of EV biology provides a rich area to enhance our understanding of the complexities of viral redirection of cellular processes and the determination of how viral effects on EV production and content contribute to both viral pathogenesis and persistence.



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## Glossary

### Capsid

Proteins that encapsulate viral genomes. Capsids are rigid, highly structured and are similar to crystals with a defined symmetry. The size, shape and symmetry of the capsid can be determined by electron microscopy and is sometimes used to classify viruses into taxa.

### Endosomal sorting complex required for transport (ESCRT) machinery

A multi-protein complex that is involved in membrane vesicle biogenesis. Viruses use the ESCRT machinery to assemble virions and bud

### Nanotubes

Membranous protrusions that connect adjacent cells over extended distances (up to 100  $\mu$ M) and can transfer cellular components and viruses.

### Cross-priming

Transfer of antigens from one cell to another, often to a professional antigen presenting cell, that does not make the antigen; the phrase was originally coined to explain counter-intuitive aspects of T cell responses.

### Latent infection

Long-term presence of viral genomes (DNA or RNA) in a cell without evidence of virion production, for example, HIV integrated into the host genome or herpesvirus plasmids located in the nucleus. To differentiate latent from abortive or non-productive infection events, reactivation and subsequent release of virion particles is required for latent infection. Viral genomic material (RNA) that is transmitted by EVs can only lead to abortive infection.

### Paracrine

Affecting the physiology of neighboring cells without cell-to-cell contact, typically by cytokines or growth factors. If the growth factors act on the same cell, that they originate from the process is called autocrine loop.

### Persistent infection or chronic infection

Long-term presence of viral genomic information (DNA or RNA) in a cell or person with evidence of virus production and circulating infectious particles, but at a much lower level than observed during initial (primary) infection.

### Autophagy

Autophagy is a regulated mechanism to provide nutrients through digestion of intracellular organelles, is utilized in different ways in the egress of multiple viruses.

### Biomarker

Protein, mRNA or other small molecule that can be measured and that is associated with disease outcome either independent of treatment (prognostic) or in relation to treatment (predictive).

**Multivesicular bodies (MVB)**

Are structures below the plasma membrane that serve as the central hub for sorting of molecules into other specialized compartments, as well as in and out of the cell.

**Sphingomyelinase (EC 3.1.4.12)**

Enzyme involved in the synthesized of sphingomyelin.

**endosomal sorting complex required for transport (ESCRT)**

A structure below the plasma membrane that serves to remodel the plasma membrane for the purpose for exporting macromolecules and membrane proteins.

**RGD motif**

The tripeptide Arg-Gly-Asp (RGD) is recognized by many integrins either as part of a short peptide (blocking peptide) or as repeat region in extracellular matrix proteins.

**Hyperimmune serum**

Serum obtained from one or many infected, convalescent animals that contains high levels of blocking antibodies to the target virus.

**Cytopathic effect**

Measure of infectious virus on monolayer cells.

**Trojan Horse hypothesis**

Initially introduced by Gould<sup>23</sup> this hypothesis posits (a) an evolutionary relationship between retrovirus particle biogenesis and EV biogenesis and (b) the utility of this relationship as an alternative means to transfer viruses, or virus proteins and RNAs to neighboring cells independently of virion maturation and thereby unseen by the host immune response.

**Interstitial spaces**

Small, narrow spaces between tissues, which are typically filled with interstitial liquid.

**Angiogenesis**

Process of vessel growth into an organ carrying either blood or lymph, involving the migration, growth, and differentiation of endothelial cells

**Principal component analysis**

Statistical method to uncover relationships defined by 10–1000 or more correlated variables, which identifies those factors that contribute to variability. Typically the first 3–5 principal components are composed of those variables which have the greatest explanatory power.

**Hierarchical cluster analysis**

Statistical method to visualize relationships among variables and samples. Samples that on the basis of multiple variables are most similar are arranged together.

**Spectral counting**

Method that determines the relative presence or absence of a peptide in a pair of samples analyzed by mass spectroscopy methods

**Gene ontology**

Fixed vocabulary of cellular processes and molecules that defines concepts/classes used to describe gene function, and relationships between these concepts.

**Primary effusion lymphoma**

Diffuse large B cell lymphoma that is caused by Kaposi's sarcoma associated herpesvirus.

**Crowding agents**

Chemicals such as polyethylene glycol or acetone that change the availability of free solvent for proteins and other macromolecular structures with the result of causing aggregation.

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## Biographies

Dr. Raab-Traub received her Ph.D. from the University of Chicago and post-doctoral training at the University of North Carolina. She has studied contribution of the Epstein Barr Virus to the development of malignancy with particular focus on nasopharyngeal carcinoma. She identified the viral genes expressed in cancer and the properties of the viral oncogenes and miRNAs.

Dr. Dittmer obtained his PhD from Princeton University, followed by postdoctoral studies in virology at Stanford University and the University of California San Francisco. His research is focused on AIDS-defining cancers, particularly Kaposi Sarcoma and the role of EVs in viral pathogenesis. He directs the UNC Lineberger Vironomics core.

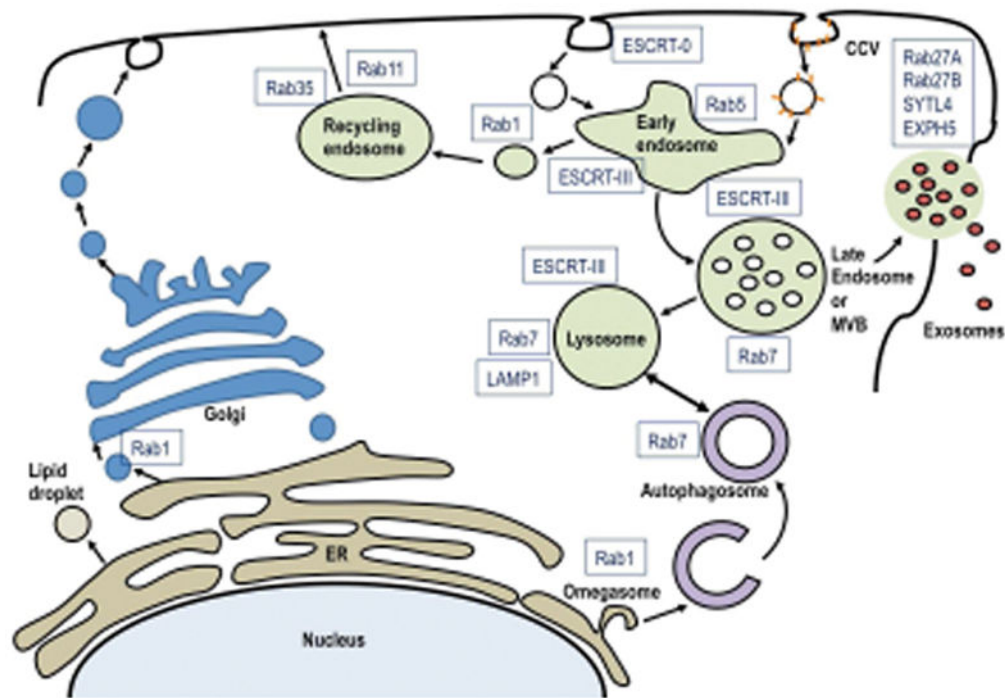


**Box 2****EVs in cancer**

Approximately 20%-30% of all cancers are associated with viral infections and many features of cancer are also features of viral infection, such as dissemination (metastasis in cancer), uncontrolled DNA replication and metabolic perturbation (for example, glycolysis and nucleotide biogenesis). Hence, insights that are gained from studying the physiological phenotypes of EVs in the context of cancer and metastasis could also be relevant to understanding the role of EVs in viral persistence<sup>165</sup>. EVs have been extensively studied in cancer research as they are released in high levels from tumor cells<sup>73-76</sup>. The contents of EVs that are released from tumor cells mirror the contents within the producing cells and therefore, these EVs are useful biomarkers. Therefore, the identification and characterization of EVs within body fluids from 'liquid biopsies' provides a noninvasive diagnostic and prognostic indicator for cancer development and progression<sup>13,131</sup>. The identification of proteins such as p53, epidermal growth factor (EGF), and fibroblastic growth factor (FGF) in EVs suggests a contributing role for EVs in oncogenesis. EVs are able to transfer proteins from malignant cells to neighboring or to distant cells, which can promote cancer growth through potential effects on the microenvironment, inhibition of the anti-cancer immune responses, and induction of angiogenesis. Interestingly, EVs have also been shown to facilitate metastasis by inducing epithelial to mesenchymal transition (EMT) and other changes within the target microenvironment<sup>73-75</sup>.

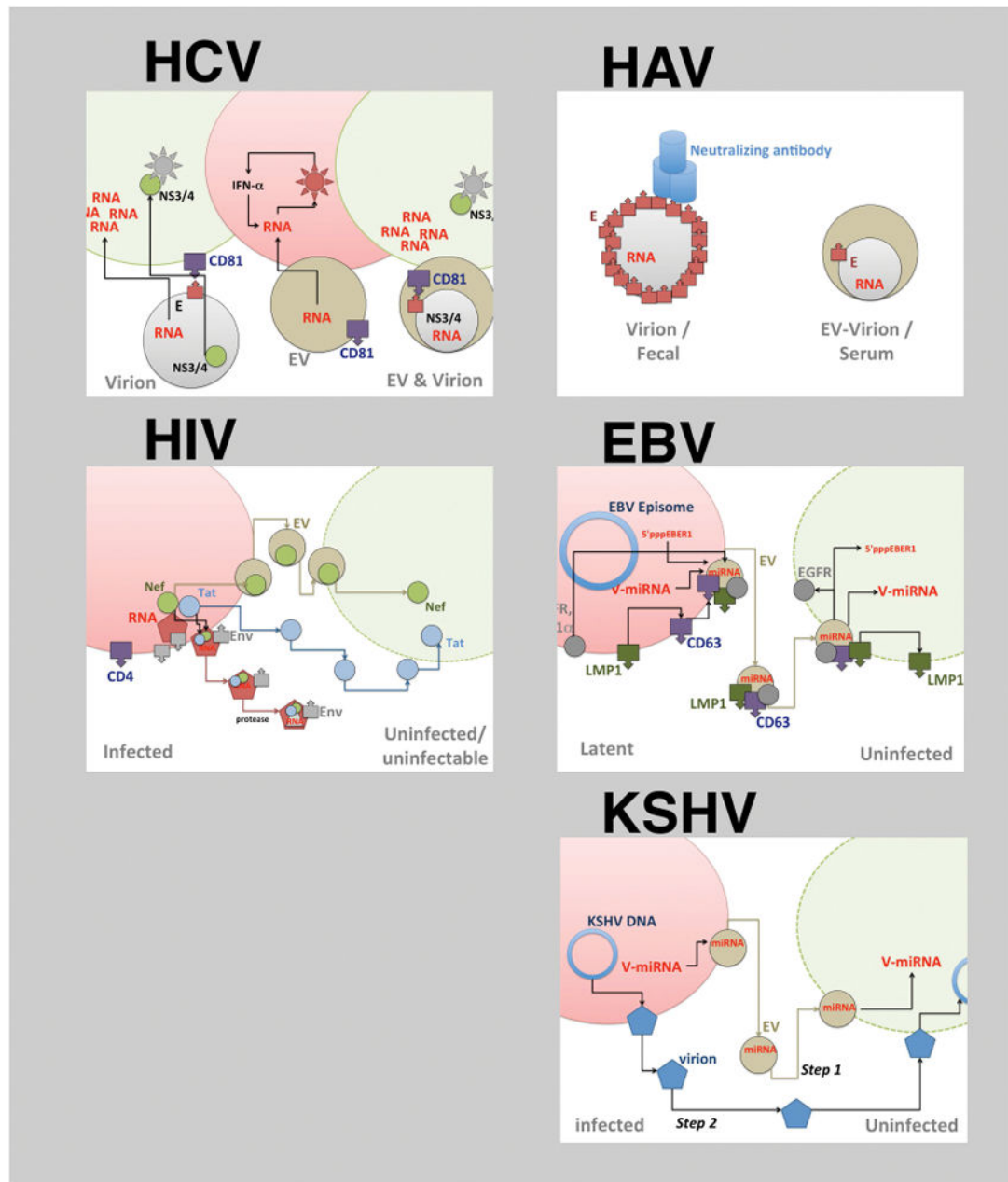
### Key points

- The considerable biochemical and physical similarity between viruses and exosomes complicates exosome purification. Both exosomes and viruses utilize the host vesicle trafficking machinery and therefore contain multiple proteins enriched in extracellular vesicles and viruses.
- Viruses have specific receptors and therefore usually have a more restricted repertoire of cells that permit viral entry. In contrast, exosome uptake is almost universal and can occur through several cellular endocytosis mechanisms in addition to direct fusion, a property that enables systemic delivery of exosome content.
- The differences in viral vs. exosome receptor usage can be used to separate and purify exosomes. This enables the identification of specific exosome-mediated effects and provides a mechanism to test novel compounds to inhibit exosome delivery and function.
- Viruses that establish chronic, persistent infections within the infected host likely utilize exosomes to enhance establishment and maintenance of infection. The exosomes produced from virus-infected cells may also restrict virus infection perhaps to enable continued host viability and persistent viral infection.
- Exosomes are also abundantly produced by malignant cells and have been shown to facilitate tumorigenesis and metastasis through effects on the tumor environment. Similarly, tumor viruses such as KSHV and EBV manipulate exosome content to enhance viral transformation and maintenance of latency.
- The non-enveloped small RNA viruses including picornaviruses and Hepatitis A virus may acquire a membrane envelope through exosome secretion. This would inhibit recognition of viral proteins by the immune system and facilitate spread within the host.



**Figure 1.**

Protein complexes in the EV maturation pathway that are modulated during viral infection. Shown are the principal egress pathways of a cell starting with the nucleus. The late endosome or MVB sorts contents from the early endosome into either the lysosome or EVs for egress. The early endosome is the first step in vesicle uptake and recycling. The individual molecules identified here have demonstrated functions in EVs or virus maturation.



**Figure 2.**

Modes of virus-EV interaction. The different panels representing different viruses (HCV, HAV, HIV, EBV and KSHV). For each virus a virion-dependent transfer and the EVs dependent transfer step is shown. Only in the case of HCV and HAV have entire virions been identified within EVs. For the other viruses, only individual RNAs or proteins, but never the whole genome is seen in EVs. In case of EBV many different moieties are transmitted via EVs from infected cells: cellular proteins, viral proteins cellular miRNAs, viral miRNAs as well as other small viral RNAs and RNA breakdown products. In case of KSHV (E) the cartoon exemplifies a temporal model, whereby viral miRNAs transmitted by

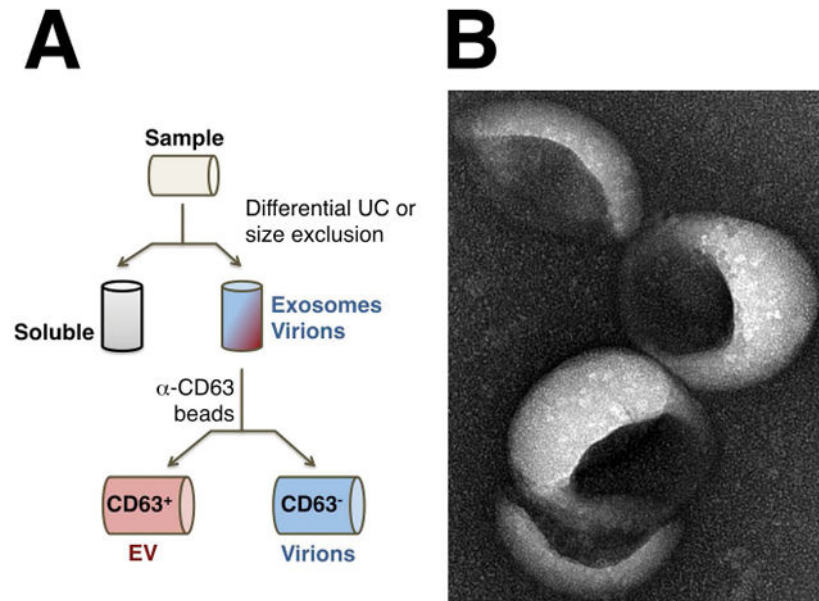
EVs precede infection by a viral particle (Step 1) and thus may prepare the recipient cells for infection (Step 2)

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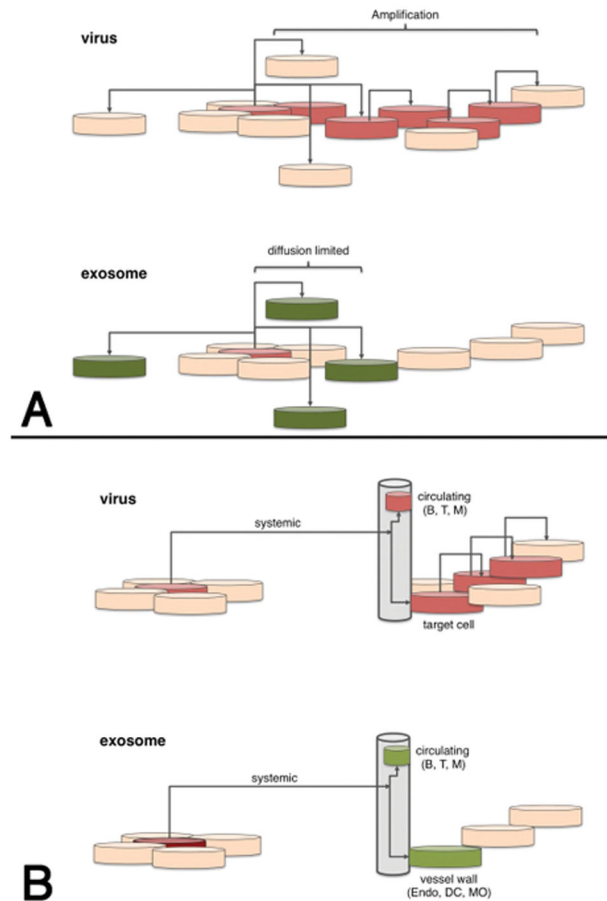
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**Figure 3.**

**A.** Separation of EVs from virions by positive selection. First, particles are concentrated on the basis of biophysical properties such as density, size and/or weight. Most virions co-purify with EVs under these conditions. Next, EVs are positively selected using anti-EV surface antibodies and virions, which do not contain these markers, are in the column flow-through. The reverse experiment (not shown) is possible as well, namely were virions are selected positively and EVs are present in the flow-through. P, pellet after filtration and differential ultracentrifugation (UC); S, supernatant, E, pellet of EVs and virions. **B.** Electron micrograph (EM) of EV. Shown is a surface EM picture of tumor cell line derived EVs after isolation and silver staining (courtesy of P.Chugh, S.Ozgun and J.Griffith).





**Figure 4.**

Conceptual difference between (A) viruses and (B) EVs. Cells are indicated as disks. Spread is indicated by black connecting arrows. Red indicates amplification of input material (virus), green indicates delivery (EV) without subsequent amplification. The upper two panels refer to local spread, i.e. within a tissue or microenvironment and the lower panels refer to systemic spread via blood, lymph or interstitial fluids. Upon virus infection the signal (i.e. virus derived miRNAs and proteins) is amplified in each permissive cell and over time the number of altered cells increases. Upon EVs absorption no amplification takes place and any phenotype caused by EVs is diffusion-limited. Only cells directly exposed to EVs in the circulation, which are mostly endothelial cells, can be modulated by EVs.

**Table 1**

Prominent EV markers and EV associated viral proteins.

Location	Protein* [Au: * OK? above refers to protein?]	Structural Class	Function
Surface exposed on EVs	CD9	Tetraspanin	Cell adhesion
	CD63	Tetraspanin	Cell signaling
	CD81	Tetraspanin	Cell signaling, proliferation marker
	MHC-I	Histocompatibility antigen, class I	Antigen presentation
	MHC-II	Histocompatibility antigen, class II	Antigen presentation
	CD86	Type I membrane protein, IgG superfamily	CTLA-4 Counter-Receptor B7.2
	FLOT1	Integral membrane component of caveolae	Scaffolding protein for vesicle formation
	ANXA5	calcium-dependent phospholipid binding protein (Annexin)	Phospholipid binding
Internal to EVs	HSP70	heat shock protein	Mediates folding
	HSP90	heat shock protein	Mediates folding
	ALIX	PDCD6-Interacting Protein	ESCRT pathway
	TSG101	-	ESCRT pathway, tumor suppressor
Virus	EBV LMP1	Membrane protein	Cell signaling/CD40 analog
	EBV LMP2a	Membrane protein	Cell signaling
	EBV gp350	Membrane/virion protein	Receptor binding
	HIV Nef	Membrane protein	CD4, MHC-I downregulation
	HSV-1 gB	Membrane/virion protein	Receptor binding
	Vaccina virus glycoproteins	Membrane/virion protein	Receptor binding
	HCV (whole virus)	virus (flavivirus), enveloped	infection
	HAV (whole virus)	Virus (picornavirus), non-enveloped	infection
	Poliovirus, Coxsackievirus, Rhinovirus	Virus (picornavirus), non-enveloped	infection

\* Based on information in 10,166 and [www.exocarta.org](http://www.exocarta.org). Note that not all markers are present in all EVs as demonstrated by comprehensive MS/MS analyses<sup>12</sup>