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Extracellular Vesicles in Virus Infection and Pathogenesis

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Abstract

Viruses are obligate intracellular parasites that usurp cellular signaling networks to promote pathogen spread and disease progression. Signaling through extracellular vesicles (EVs) is an emerging field of study in the virus-host interaction network. EVs relay information both locally and distally through incorporated contents, typically without tripping innate immune sensors. Therefore, this extracellular signaling axis presents itself as a tantalizing target for promoting a favorable niche for the pathogen(s) takeover of the host, particularly for chronic infections. From the incorporation of virus-encoded molecules such as micro RNAs and proteins/enzymes to the envelopment of entire infectious particles, evolutionary distinct viruses have shown a remarkable ability to converge on this means of communication. In this review, we will cover the recent advances in this field and explore how EV can be used as potential biomarkers for chronic, persistent, or latent virus infections.

Graphical abstract

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Declarations of Interests

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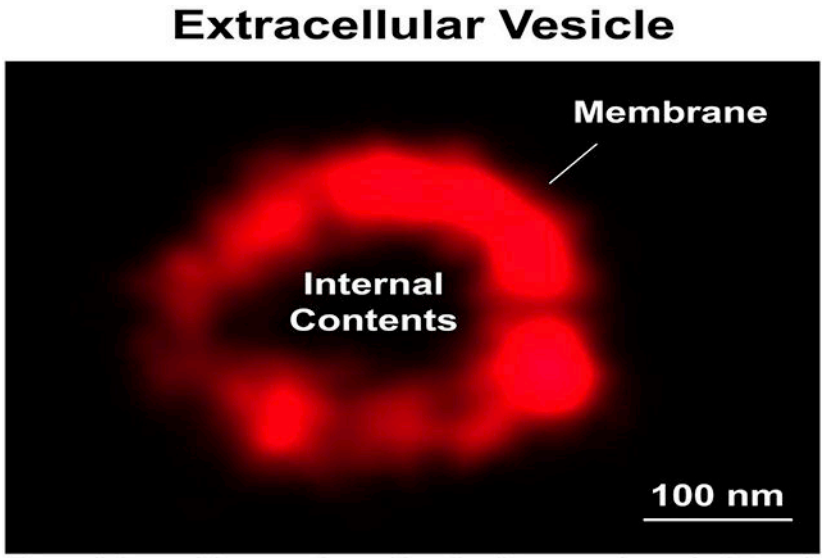

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Keywords

Viruses; exosomes; microvesicles; extracellular vesicles; HIV; KSHV; EBV; HCV; HAV; HSV; latency; pathogenesis

Introduction

As a part of the extracellular signaling network, cells release extracellular vesicles (EVs). The current model posits that EVs exist as three major subgroups: (i) apoptotic bodies, (ii) microvesicles, and (iii) exosomes. Apoptotic bodies are produced when a cell is undergoing apoptosis, and contain materials from every cellular subcompartment, and have a broad size range (100 nanometers (nm) - >1 μm in diameter). Microvesicles originate from the pinching off of the plasma membrane and are enriched for surface membrane proteins, and usually have a diameter of 80 – 500 nm). Strictly speaking, exosomes originate from the invagination of the late endosome into the multivesicular body (MVB) and are enriched for members of the endosomal sorting complex required for transport (ESCRT) machinery. Exosomes have a size range of 40 – 150 nm in diameter [1,2]. In cell supernatant or biological fluids, it is difficult to identify origin for any individual EV.

EVs function similarly to a virion in their ability to transfer active materials from one cell to another, either locally or distally. In primary fluids such as blood, EVs exist at higher concentrations (up to 10^{10} particles/mL) than even high titer viruses such as flaviviruses, filoviruses, or human immunodeficiency virus (HIV) [3,4]. Similar to a virus, contents delivered by an EV influence cell and tissue physiology such as transcription,

differentiation, migration, signaling, and metabolic state [1,5–10]. EVs from tumor cells have been linked to metastasis [10–13]. Given their function in steering local and systemic equilibrium, it is unsurprising that viruses have evolved to co-opt an EV to facilitate pathogen spread and disease progression (Figure 1).

Much of our current understanding of EVs comes from studies using viruses that deliberately manipulate EV cargo through the incorporation of viral nucleic acids, proteins, and even entire virions into EV [14–23]. Delivery of these virus-modified EVs to naïve cells alters their physiology, demonstrating that EVs can functionally deliver pathogenic materials. Given that EVs exist naturally (i.e. in the absence of virus infection) they transport these materials unbeknownst to the host's immune response. This phenomenon is believed to have occurred for millennia but has only become appreciated in recent years as a means by which viruses usurp cellular resources.

This review will focus on established models of EV hijacking by viruses, as well as recent advances in this field. We will highlight the work done on EV-virus interactions in the context of viruses that can induce chronic infections. Finally, the potential of using EV as a biomarker for chronic, persistent, and latent infection will be discussed.

Herpesviruses

A shared trait among the *Herpesviridae* family is that they persist at an equilibrium between latent and lytic replication phases. Both phases are required for viral spread and disease, but only the lytic is responsible for the production of infectious particles. Herpesviruses are some of the oldest mammalian viruses known, with estimates of their origin being hundreds of million years ago, and have host ranges extending from humans (*Homo sapiens*) to turtles (*Chelonia mydas*) [24,25].

Herpes simplex virus (HSV-1) has been shown to export viral miRNAs and the innate immune regulator stimulator of interferon genes (STING) protein into EVs post-infection [19,26]. EV transfer of STING has prompted the development of a model in which HSV-1 attenuates its shift from latency into the lytic phase through EVs. Multiple interferon stimulatory genes and pro-inflammatory regulators such as tumor necrosis factor-alpha (TNF-α) were upregulated in cells treated with EVs from HSV-1 infected cells. This led to a decrease in viral transcripts, pushing HSV-1's equilibrium towards latency [27,28]. This shift would allow the existing, chronically-infected cells to avoid detection through innate sensors triggered by cytokines like TNF-α. Other work has shown that in oligodendrocytic cells, knockdown of the MVB protein Rab27a attenuated HSV-1 infection [29,30]. This work was bolstered by the findings that HSV-1 hijacks a microvesicle-like body to transfer the virus to receptor-negative, neighboring cells. These HSV-1 particles were resistant to neutralizing antibodies and could package multiple virions into one body. Interestingly, these virus-filled vesicles contained markers of the autophagosome [31]. This phenomenon resembles poliovirus exiting through the autophagosome-mediated exit without lysing (AWOL) pathway [32].

Studies on Epstein-Barr virus (EBV) have shown an intricate subversion of EV-signaling. The viral latent membrane protein 1 (LMP1) is a driver for the transformation of B-cells [33–35]. In a B-cell lymphoma, LMP1 is expressed during the latency-II and latency-III phases in germinal center B cells. LMP1 is incorporated into exosomes and delivered to non-infected cells through LMP1's association with CD63 and lipid rafts [36–39]. LMP1 is primarily expressed in the terminal type III latency, which has a mRNAseq profile distinct from transient latency-IIb phases [40]. Exosome-mediated export of LMP1 in an EBV-transformed B-cell during latency-III to exert effects on non-infected cells or even receptor-negative cells is supported by other findings in which EVs play a role in cancer metastasis (reviewed in [10]). In addition to LMP1, EBV transfers viral miRNAs through EVs. Lymphoblastoid cells express high concentrations of EBV BHRF1 and BART cluster viral miRNAs and are trafficked into an EV. EBV miRNAs could be transferred to naïve dendritic cells and act on their known cellular target mRNA [41].

Kaposi's Sarcoma-associated herpesvirus (KSHV) is the causative agent of primary effusion lymphoma (PEL) and Kaposi's Sarcoma (KS) [42–44]. Post-infection, the KSHV genome is maintained in a mostly transcriptionally quiescent state, but a handful of latent proteins and noncoding RNAs are expressed [45–47]. During latency, KSHV packages high concentrations of viral miRNAs into EVs from infected cells (KSHV-EVs) [9,48]. Delivery of KSHV-EVs reprograms recipient, non-infected endothelial cells transcriptionally, and alters their metabolic profile into a distinct, cancer-cell defining state termed the "Warburg state" or "Warburg effect" [7,8]. The Warburg effect, named after Otto Warburg, describes a cell with a metabolic state with largely glycolytic-based energy production as opposed to oxidative phosphorylation through the electron transport chain [49]. These metabolic and transcriptome shifts occur without the transfer of the virus itself. These alterations in cellular physiology by KSHV-EV do not activate innate immune sensors such as interferon regulatory factor 3 (IRF3), STING, and nuclear factor kappa b (NF-κB). In contrast, extracellular signal-regulated kinase (ERK) is activated by KSHV-EVs. Upon chronic exposure of naïve endothelial cells to KSHV-EVs, cells transition into a hyper-proliferative state, with Ki67 positive staining similar to that of a bona fide KS tumor. These complex reprogramming events occur during latency and infectious particle production is low [7]. Therefore, the detection of KSHV miRNAs in blood has been proposed as a biomarker for virus infection [8,9].

Retroviruses

The human immunodeficiency virus (HIV) emerged into the human population in the early 20th century from spillover events of the simian immunodeficiency virus (SIV) [43,50,51]. HIV chronically infects T-lymphocytes and macrophages, depleting them over the course of years, ultimately leading to a collection of clinical symptoms referred to as AIDS.

The HIV-encoded Tat potently transactivates the viral genome and forms a positive feedback loop [52–54]. Early HIV transcripts are fully spliced, and encode for Tat, Rev, and Nef. In addition to Tat's role at the HIV promoter, a portion of Tat is enriched on membranes through its interaction with phosphatidylinositol (4–5) bisphosphate (PI(4,5)P2) [55,56]. Tat's membrane affinity is largely controlled by the first few amino acids of its RNA binding

domain (RBD). This particular domain of Tat has also doubled as a peptide transduction sequence, allowing for the cellular penetration of membrane-impermeable drugs [57,58]. The Tat-PI(4,5)P2 interaction has been proposed to allow for Tat to be exported from the cell via EV [59]. Experimentally, even particle-free Tat proteins and Tat-fusion moieties retain the ability to cross-target cell membranes and enter the cytosol directly.

In addition to Tat, the HIV-encoded 5' RNA element TAR has also been found in EVs [60–63]. TAR is transcribed at the HIV promoter by RNA Polymerase II, and Tat binds to the RNA with elongation factors with a remarkable affinity [64]. The finding of both Tat and TAR inside of EVs further highlights this unique RNA-protein binding couple. Interestingly, the delivery of Tat and/or TAR-enriched EV has been shown to activate HIV from resting CD4+T lymphocytes [65] and activate cellular migration/proliferation in an ERK-dependent manner [66].

Nef was identified as the first detectable viral protein expressed post-HIV-infection, yet the nef open reading frame is dispensable for *in vitro* growth [67,68]. Nef is nevertheless a critical driver for HIV and SIV disease progression in human and non-human primates, respectively [69–74]. Nef downregulates surface CD4 and MHC-I presumably to prevent super-infection of a cell and avoid immune detection [75–84]. A large fraction of Nef is secreted from cells through EVs *in vivo*. This is likely owed to its interactions with the endosomal recycling pathway [85,86] and its anchoring onto the inner leaflet of the plasma membrane [87–93]. The ability of this viral protein to be incorporated into EV is evolutionarily conserved between HIV and SIV. The Nef-EV can be uptaken by naïve CD4+ T cells and can induce their apoptosis [94], metabolic and lipid raft reorganization, and enhance HIV infection through inflammatory signaling [95]. Nef-EVs can also be uptaken by macrophages and endothelial cells which line the vasculature where Nef localizes to punctate intracellular structures [4,96]. Nef's ability to interact with and/or modify lipid rafts may be the mechanism by which the viral protein is trafficked into EVs [96–98]. Both HIV and SIV Nef can be visualized in complex with CD81, a known lipid raft component, by super-resolution microscopy (Figure 2) [1,4]. This has lead to a model that Nef exerts pathogenic effects via EV, contributing to co-morbidities frequently associated with long-term HIV infection and late stage AIDS. Supporting this hypothesis, Nef can be detected in EV during treatment with antiretroviral therapy (ART), which effectively abrogates virus production [4,93,99].

Hepatitis viruses

There are several taxonomically distinct hepatitis viruses that infect humans. Vaccinations exist for hepatitis A virus (HAV, a picornavirus) and hepatitis B virus (HBV, a hepadnavirus), and successful antiviral therapy regimens for hepatitis C virus (HCV, a flavivirus). Yet, these three viruses continue to infect millions of people worldwide each year. Other viruses cause viral hepatitis as well, but we will focus on HAV and HCV.

HAV belongs to the *Picornaviridae* family of viruses, which are positive-sense RNA viruses without an envelope [100,101]. However, early work showed that HAV exists on a spectrum of buoyancies, indicating interaction with lipid moieties [102]. It was later discovered that

HAV acquires a cellular envelope through the endosomal recycling pathway. This “quasi-enveloped HAV” (eHAV) is resistant to neutralizing antibodies [103–106]. Uptaken HAV and eHAV are trafficked through the endosomal pathway, but eHAV ultimately goes to the lysosome where the envelope is degraded [107]. HAV represent the prominent species in feces, and source of person-to-person transmission whereas eHAV circulates in the bloodstream and liver. Other members of the *Picornaviridae* family have also shown the ability to acquire cell membranes despite being defined as “non-enveloped” viruses [108,109]. This envelope appears to be from the late endosome due to its enrichment in phosphatidylserine (PS) [9,110]. PS binds to TIM-family of receptors on the cell surface, notably TIM1 and TIM4, allowing for phagocytosis of apoptotic cells, EV, or a virion coated with PS [111–114]. Interestingly, CRISPR-mediated knockout of TIM1 reduced eHAV infection in Vero cells but was not essential for HAV or eHAV virus infection [115]. Instead, both HAV and eHAV appear to be uptaken through a β 1 integrin-mediated clathrin and dynamin endocytic processes [107], in addition to TIM1.

HCV is a unique member of the *Flaviviridae* family in that it is transmitted from human-to-human, in contrast to other flaviviruses such as Dengue virus, West Nile virus, and Zika virus that are transmitted by arthropods [116,117]. Post-entry (see review [118]), the positive-strand viral RNA genome is sufficient to initiate an infection state in a susceptible cell. EV transfer of HCV RNA has been observed in hepatocytes, and virus constructs lacking functional structural (virion) proteins could traffic viral RNA into EVs and promote an infection state [119,120]. HCV RNA can be transferred to receptor-negative plasmacytoid dendritic cells, triggering IFN-α production [121]. This occurs through the ESCRT machinery proteins chromatin-modifying protein 4B (CHMP4B) and the tumor susceptibility gene 101 (TSG101) [122–124]. EVs from HCV-infected cells promote activation and fibrosis in hepatic stellate cells, which themselves cannot be infected with HCV. This phenomenon is believed to occur via the EV-mediated transfer of miR19a and miR192 from HCV-infected cells. These two miRNAs lead to higher levels of transforming growth factor β (TGF-β) signaling and activation of liver fibrosis [125,126].

Concluding Remarks

Extracellular signaling is paramount for organism homeostasis, and unsurprisingly viruses evolved to hijack this network. By manipulating EVs, viruses can establish a niche favorable for pathogen takeover without de novo infection or activation of innate immune cascades. Importantly, this can occur in receptor-negative cells. Additionally, the transfer of viral contents through EVs is mostly unaffected by neutralization by immunoglobulins (Figure 3). In many ways, EVs are akin to platelets, which unlike organs or cells, can be transplanted across HLA-mismatched individuals [127].

The phenomenon of reshaping the environment can occur during latency and antiviral treatment. For example, HIV and SIV Nef have been detected in EVs even during ART [4,99]. Nef-EVs may originate from leaky latent reservoirs expressing early HIV genes but not functional virions. Eradication of HIV latent reservoirs represent the single greatest hurdle towards a curative strategy for HIV, and Nef-EV may provide some utility as a biomarker for disease. Most strategies of HIV detection and quantitation rely on viral RNA

genome amplification or p24 detection, which are made in the context of full viral genome activation, and thus cells with leaky early gene expression can be missed. It has been proposed that both SIV and HIV latency is an intentional strategy by the virus to promote virus survival [128–130]. Current strategies of activating this latent reservoir to purge out SIV/HIV, particularly in resting CD4+ T lymphocytes, have seen a marked improvement in recent years [131–133]. Studies examining how EVs fit into HIV latency, curative strategies, and biomarkers for disease state seem warranted.

Given their ability to vehicle viral factors during latency, using EVs as biomarkers for infection has been explored [21]. For the oncogenic EBV and KSHV, viral genomes remain remarkably low during tissue transformation. This is in sharp contrast to viral miRNAs, which are robustly transcribed during latency. These miRNAs are trafficked into exosomes and are present at high concentrations in cultured cell supernatant and primary fluids [7–9,41,47,48,122,134,135]. This is analogous to certain tumors that have a unique exosome RNA profile [136,137]. Therefore EVs can have clinical utility in the form of liquid biopsy.

While this review focused on chronically infecting viruses, there is considerable literature showing a dynamic relationship between EVs and acutely infecting viruses. In many of these cases, EVs play an integral anti-viral role. Examples of this include infection with influenza virus [138,139], dengue virus [140], zika virus [141], and hepatitis B virus (which can elicit both acute and chronic infection) [142]. Similar to examples listed in this review, these responses can be induced through the transfer of specific factors to a naïve cell. Comparisons and contrasts in the interplay between EVs and acutely and chronically infecting viruses will be of considerable interest in the coming years as our understanding of this signaling axis continues expanding.

Other viruses that elicit chronic infections utilize EV pathogenesis, and we were unable to cover these in the depth they deserve. Examples include CMV, HPV, and HTLV-1 [15,20,143–147]. Evolutionarily distinct viruses converged to utilize EVs for the functional transfer of materials. This allows viruses to modify the local area of infection and avoid detection by the immune system and may play a previously underappreciated role in maintaining virus latency.

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Highlights

- Distinct viruses converged to usurp extracellular vesicle signaling.
- Extracellular vesicles transfer virus-encoded factors during latency.
- Extracellular vesicles can be used for biomarker detection.

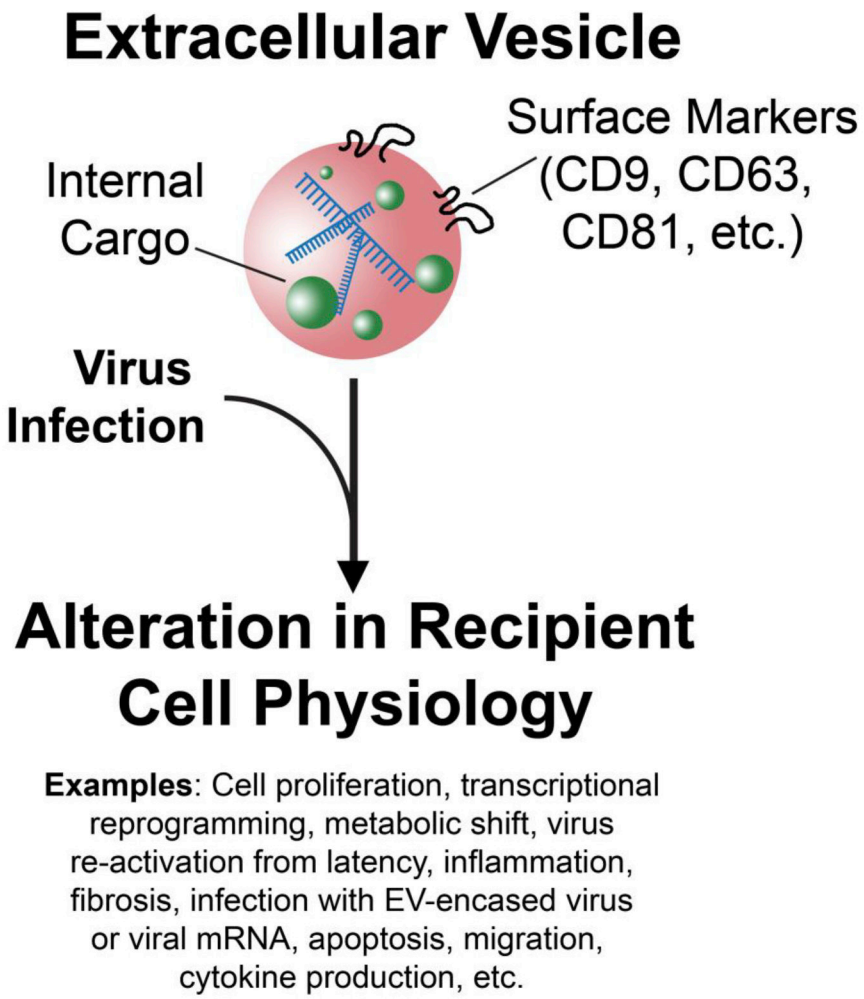

Figure 1.

Virus infection alters the cargo of an extracellular vesicle (EV) *in vivo*. The virus-modified EV is used to establish a more favorable niche for pathogen takeover through the alterations of recipient cell physiology and signaling networks such as those listed.

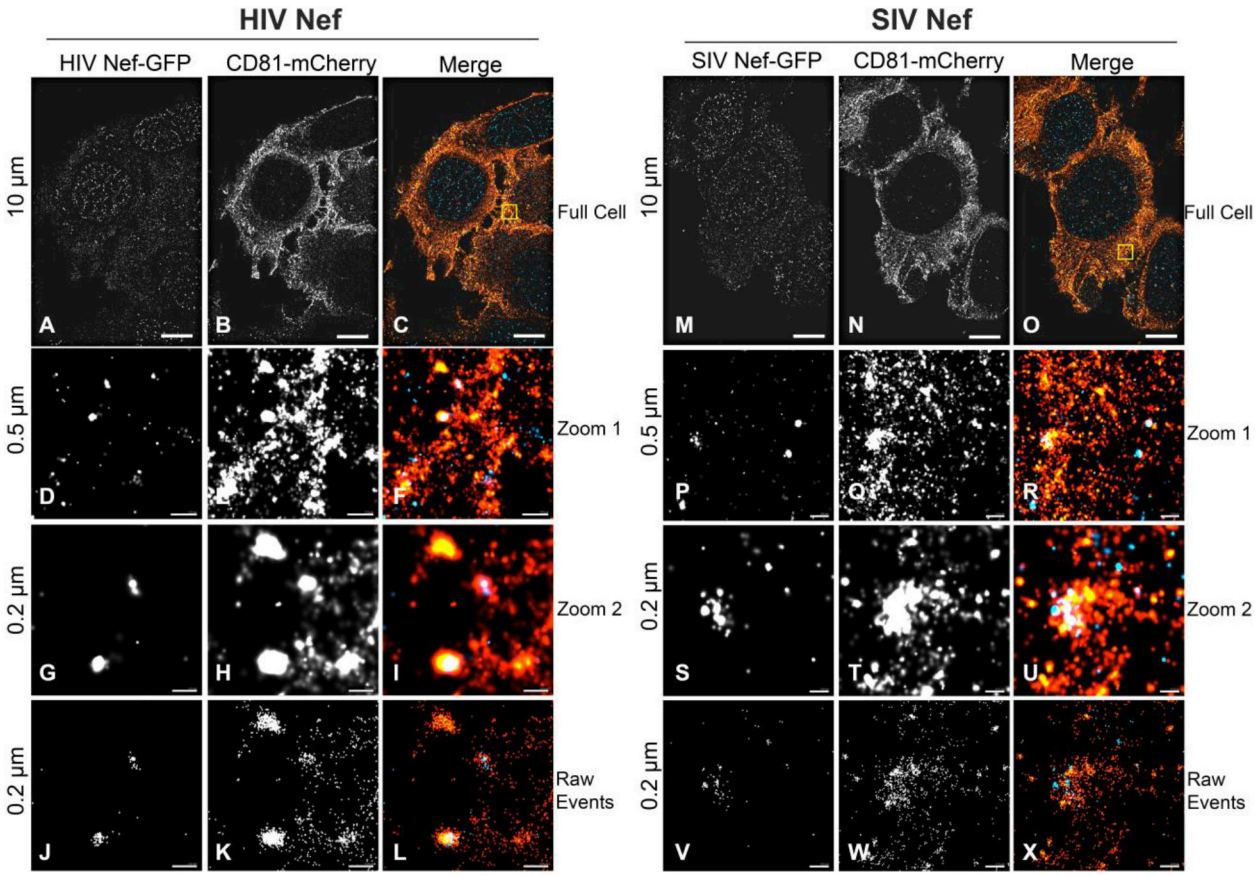

Figure 2.

Super-resolution microscopy of SIV and HIV Nef with the EV marker CD81. A-C. dSTORM of a CD81-mCherry expressing cell transfected with HIV Neg-GFP (scale = 10 µm). D-F. Zoomed in view of box in (C) (scale = 0.5 µm). G-I Further zoomed-in image of CD81-mCherry and HIV Nef-GFP co-localizing events (scale = 0.2 µm). J-L. Raw photoswitching events of -(G-I). M-X. Same as (A-L), but for SIV Nef-GFP.

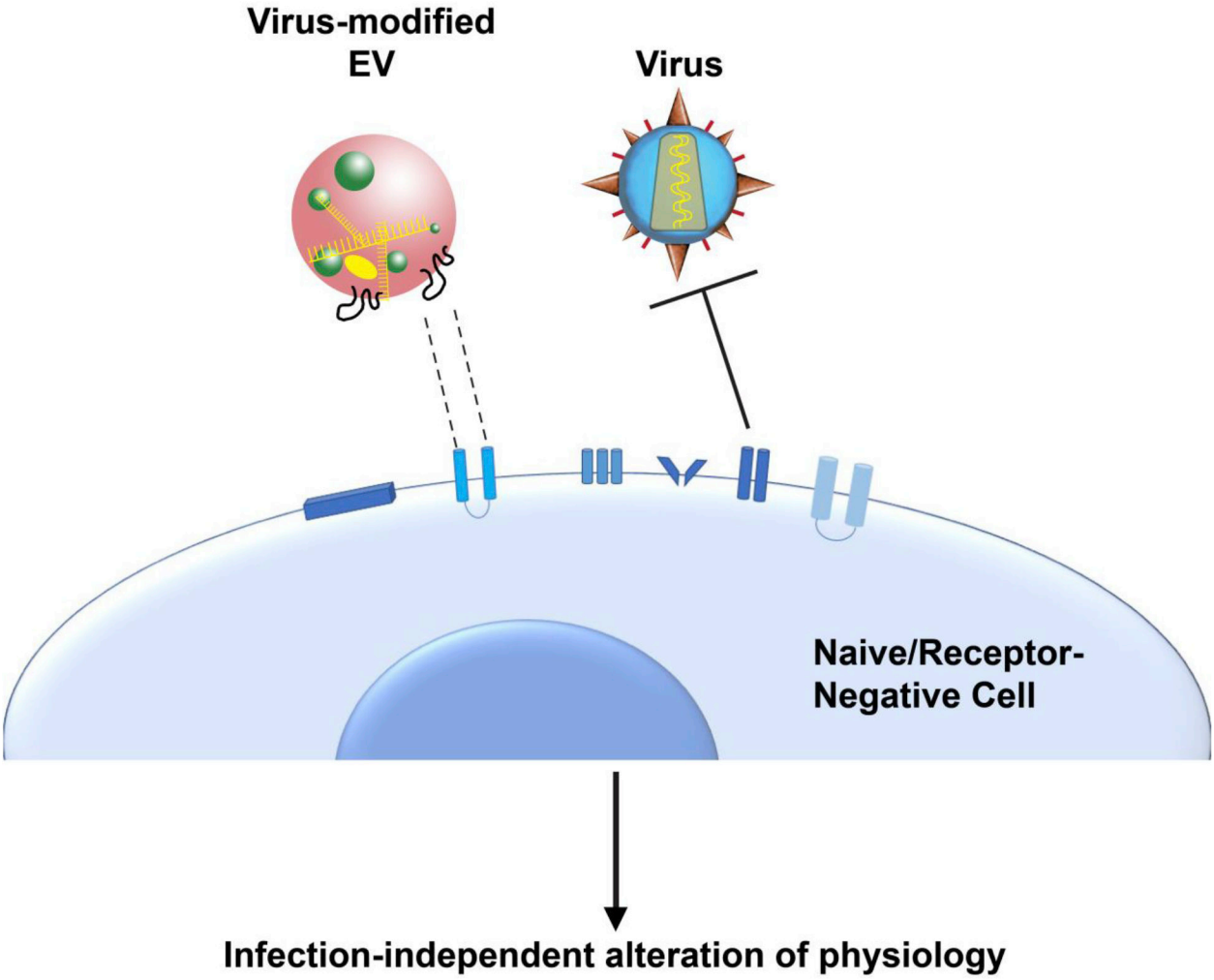

Figure 3.

Virus-modified EV alter the physiology of naïve and non-infectable (receptor-negative) cells. Cells lacking receptors for specific viruses can still be targeted by viruses through the incorporation of virus-modified cargo into EV (colored in yellow) emanating from an infected cell. This allows the virus to reprogram cellular signaling, gene expression, and metabolic state without *de novo* infection.