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Exosomal Communication during Infection, Inflammation and Virus-Associated Pathology

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Abstract

Exosomes are nanosized (30-150 nm), lipid bilayer-enclosed vesicles that serve to facilitate intercellular communication by delivering cargo from one cell to another. Exosomal cargo varies depending on the cell type, and physiological or pathological state of the originating cell. Most importantly, pathogens such as bacteria and viruses usurp exosome secretion pathways to incorporate bacterial or virion constituents. In some instances, whole virions are incorporated, in order to facilitate their dissemination and cause pathology. On the other hand, incorporated constituents can include pathogen-derived molecules or major antigens which can be processed by immune cells to induce antigen-specific immunity. This review focuses on exosomes, their biogenesis and how exosomes secreted by infected cells or Antigen presenting cells (APCs) possess the ability to induce antigen specific CD4+ and CD8+ T-cell immunity. In addition, we focused on the role played by exosomes during bacterial and viral infections and how bacteria and viruses exploit exosomal communication to facilitate infection. We conclude with a brief overview of tumor-derived exosomes and their ability to mediate immune suppression and drug resistance.

Keywords: Extracellular vesicles; Exosomes; Innate immunity; Acquired immunity; Cancer; Viruses; Bacteria

Introduction

Extracellular vesicles (EVs) are spherical, lipid bilayer limited, vesicles released from a variety of cell types into extracellular fluids. Consequently, these vesicles can be found and isolated from a range of biological fluids such as blood, serum, breast milk, saliva, urine, semen, amniotic fluid, ascites fluid, cerebrospinal fluid and bile [1-10]. Based on their size, origin, physical properties and composition, EVs are mainly classified into three types: 1. Microvesicles (a.k.a microparticles or ectosomes), which originate from the plasma membrane (PM), and are of 50-1000 nm in diameter; 2. Exosomes, which originate in endosomes or multivesicular bodies (MVBs) as intraluminal vesicles (ILVs), and are sized between 30-150 nm (density: 1.15-1.19 g/ml); and 3. Apoptotic vesicles, which arise upon blebbing of cells undergoing apoptosis and range between 50-500 nm [11].

Current protocols however, cannot distinguish between different types of EVs, due to a significant size overlap and co-purification of other EVs during commonly-employed isolation procedures such as ultracentrifugation [12]. Methods such as marker-based immune affinity isolation (i.e., anti-CD63), can enrich exosomes, although other markerless exosomes might be excluded by this procedure. As a note, the term "exosome" will be used in the rest of this description, and is the main focus of the review, although the exosome preparations described in many studies may contain minor proportions of other EVs.

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The term "exosome" was first described in 1981 to refer to microvesicles secreted by rat and mouse neoplastic cell lines that had 5' nucleotidase activity. Later in 1987, Stahl and Johnstone *et al*., independently described exosomes as vesicles of endocytic origin in maturing reticulocytes. These vesicles were shown to contain the transferrin receptor (Tfr), and were proposed to be a mechanism of surface downregulation or disposal of the Tfr upon fusion of a transferrin-Tfr-containing endosome with plasma membrane, as seen via electron microscopy [14,15]. Exosomes (or ILVs) originate in MVBs upon inward invagination of the endosomal limiting membrane. MVB-containing exosomes can either fuse with lysosomes, resulting in their degradation, or a subset of MVBs can fuse with the plasma membrane resulting in their exocytic release into the extracellular environment. Upon release, exosomes were found to serve as vehicles for intercellular communication by delivering their cargo to recipient cells upon direct fusion with their PM or via endocytosis.

Exosome Biogenesis (ESCRT-Dependent and -Independent Pathways) and Composition

Based on their endosomal origin, ILV budding is driven by Endosomal Sorting Complex Required for Transport (ESCRT) protein complexes. In fact, the process of exosome formation is analogous to the process of retroviral budding, and as such, HIV-1 proteins (*Nef, Gag, Env* and transactivation element TAR RNA) were actually found to be packaged in exosomes, transferring them to uninfected cells [16-22]. HIV-1, as well as many other RNA viruses, use cellular ESCRT components for their budding and release [16-22].

Four ESCRT complexes (ESCRT-0,-I,-II and –III), along with their associated proteins (e.g. ALIX, VPS34), mediate the cargo sorting, membrane invagination, and subsequent scission to generate ILVs [23]. ESCRT complexes were initially identified to mediate endosomal sorting of ubiquitinated proteins and their subsequent degradation upon fusion with lysosomes [24,25]. The lipid PI3P on endosomal membranes first recruits the ESCRT0 complex bound to ubiquitinated proteins, facilitating their clustering on endosomal membranes. ESCRT0 recruits the ESCRTI complex, which inturn incorporates ESCRTII subunits.

Both ESCRT-I and –II complexes drive the invagination, while ESCRTII recruits the ESCRTIII complex into the neck of nascent ILVs to mediate vesicle scission [26-30]. Upon vesicle scission, most ubiquitin chains and ESCRT subunits are removed for recycling, however, some of the ESCRT components involved in biogenesis remain retained within the exosomes, thus these components can serve as exosomal markers (e.g. ALIX, TSG101, etc.). However, MVBs are still generated in cells depleted of all four ESCRT subunits, indicative of ESCRT-independent pathways of MVB biogenesis [31,32].

Furthermore, exosomal sorting of proteins such as PMEL, MHC-II, and proteolipids in oligodendrocytes are ubiquitination- or ESCRT-independent [11,33,34]. Indeed, the tetraspanin (TSPAN) CD63 was demonstrated to sort the luminal domain of PMEL in an ESCRT-independent manner [35]. Inhibition of neutral sphingomyelinase (nSMase), an enzyme that breaks down sphingomyelin in sphingolipid-containing lipid rafts into ceramide, reduced proteolipid-bearing exosome release in oligodendrocytes [32]. Exosomes secreted from oligodendrocytes were thus enriched with high concentrations of ceramide and their derivatives.

Reduced exosomal release along with a concomitant reduction in release of their contents (CD63, CD81, TSG101 including miRNA), was observed upon treatment of various cell lines with the nSMase inhibitor manumycin or GW4869 [36-39]. These nSMase inhibitors or siRNA-mediated knock-down of nSMase is frequently used to discriminate between exosome-dependent transfer and direct effects between donor and recipient cells. Additionally, heat shock cognate protein 70 (Hsc70)-binding to the luminal domain of Tfr and KFERQ motif-containing proteins targets them to exosomes [40,41]. Exosome biogenesis and secretion can also be regulated by tumor suppressor p53 and its target gene TSPAN6, demonstrating links between cell cycle regulation and exosome biogenesis. Furthermore, p53 activity is linked to the ESCRT-III component, Chmp1A, further demonstrating a role for p53 in exosome biogenesis. Thus, proteolipid sorting is ceramide-dependent and ESCRT-independent.

Once formed, MVBs either fuse with lysosomes, leading to degradation of their contents, or traffic to the cell periphery to fuse with plasma membrane to mediate exocytic release. MVBs, fated towards either lysosomes or exocytic release, coexist in cells and differ in their biochemical and morphological characteristics. Cholesterol binding θ toxin (Perfringolysin O) from *Clostridium perfringens* is frequently use to study cholesterol localization on cellular PM and internal membranes of live or fixed sections. Cholesterol-rich MVBs, with co-localization of cholesterol with perfringolysin toxin was found on internal MVBs and on exosomes at the cell surface after secretion, whereas cholesterol-poor MVBs devoid of perfringolysin toxin appeared to be destined for lysosomal targeting [42]. On the other hand, lysobisphosphatidic acid was absent in exosomes [43], but distinctly present in epidermal growth factor–containing MVBs destined for lysosomal degradation [44].

MVBs traffic to the PM is dependent upon the coordinated action of cytoskeleton (actin filaments and microtubules), molecular motors (kinesins and dynamins), GTPase switches (Rab or RAL1), and fusion machinery (SNAREs and tethering proteins) [45]. Similar to their function in intracellular vesicle trafficking, Rab GTPases also promote MVB trafficking to the PM. Knockdown or ectopic over expression of dominant negative mutants of Rab-2b,-9a,-5a,-11, 27a,-27b,-35 in various cell lines reduced the number of exosomes secreted into the surrounding medium [46-52]. Similarly, the silencing two Rab27 effectors, Slp4 and Slac2b, inhibited exosome secretion to a similar extent as silencing of Rab27a and Rab27b, respectively [51]. In a separate study, knock-down of Rab GTPase-activating protein, TBC1D10A-C, or functional interference with its effector Rab35, led to intracellular accumulation of endosome-bearing ILVs and impaired exosome secretion [47]. Inhibition of Ral GTPase-A or -B resulted in fewer secretions of exosomes from the 4T1 mammary tumor cell line [53]. The fusion of MVBs to the PM appears to depend on SNAP (Soluble N-ethyl maleimide sensitive fusion Attachment Protein) receptors or SNAREs [11]. Both VAMP7 and NSF ATPase were found to promote MVB exocytosis in the K562 erythroleukemic cell line, whereas VAMP7-inhibition in MDCK cells was found to inhibit lysosomal exocytosis similar to other epithelial cells, suggestive of cell type-dependent requirements [54-56].

Exosome Uptake by Recipient Cells

Uptake of exosomes by recipient cells appears to be specific to some extent. For instance, exosomes released from B-cells were found selectively bound to follicular dendritic cells (DCs) in the lymphoid follicles [57]. Exosomes released by T84 intestinal epithelial cell line, and carrying HLA-DR4-loaded antigenic peptide, specifically interacted with monocyte derived DCs and were able to stimulate HLA-DR4 restricted T-cell hybridomas in the presence of monocyte derived DCs, but not alone [58].

Target cell specificity for exosome binding and uptake is likely dependent upon ~~the~~ adhesion molecules, such as integrins, incorporated into exosomes. For instance, DC MHC-II$^+$ exosomes, secreted in response to cognate DC-CD4+ T-cell interactions, are specifically-bound by activated CD4+ T-cells and this binding appears to be dependent upon high affinity state of integrin LFA-1 on the T-cells, rather than through TCR stimulation [59]. MHC-II$^+$, ICAM1$^+$ exosomes, produced by mature DCs, were specifically recruited by bystander DCs with the help of LFA1-ICAM1 interaction [60]. Differences in the tetraspanin composition of exosomes can also influence target cell binding or specificity by modulating the functions of associated proteins such as integrins [61].

Finally, EBV gp350-bearing exosomes, secreted from infected B-cells, displayed increased binding and uptake by B-cells rather than monocytes. This uptake was due to EBV gp350-CD21 receptor interaction on exosomes and B-cells respectively [62]. Upon exosome binding to target cells, they may remain stably associated with the PM, fuse with the PM, and deliver their contents into the cytosol, or get endocytosed and subsequently fuse with the endosomal limiting membrane. Stable cell surface exposure was seen by MHC-II$^+$ exosomes displayed on the surface of DCs during DC-CD4+ T cell interactions or MHC-II$^+$ exosomes associated with follicular DCs that do not inherently express MHC-II$^+$, but function in the selection and maintenance of T-cell memory [57]. Direct fusion of exosomal membranes with the PM was also observed upon incubating cells with self-quenching dye labeled exosomes, which is relieved upon fusion and dye dilution, resulting in fluorescing target cell membrane [63].

Exosome uptake via endocytosis is clathrin- or caveolae-dependent and is sensitive to actin or dynamin inhibitors [64-67]. Upon endocytosis and shuttling within the endosomes, exosomes were found to scan or localize in the viscinity of endoplasmic reticulum (ER) delivering their contents [68]. This observation is pertinent to the function of miRNA cargo carried by exosomes, which can promote post-transcriptional silencing of actively translating mRNAs on the ER. A schematic of exosome secretion and uptake pathways is depicted in figure 1.

Exosome cargo

Exosome cargo is composed of proteins (peripheral membrane-associated, transmembrane, and soluble hydrophilic), lipids, and nucleic acids (lncRNAs, miRNAs, mRNAs). Exosomal protein content varies and depends upon cellular origin, physiological and/or pathological state of the cell. Exosomes originating from antigen presenting cells (APCs) such as DCs, macrophages, and B-cells contain MHC-I, -II, co-stimulatory molecules CD80 and CD86 [69-71]. In addition, exosomes

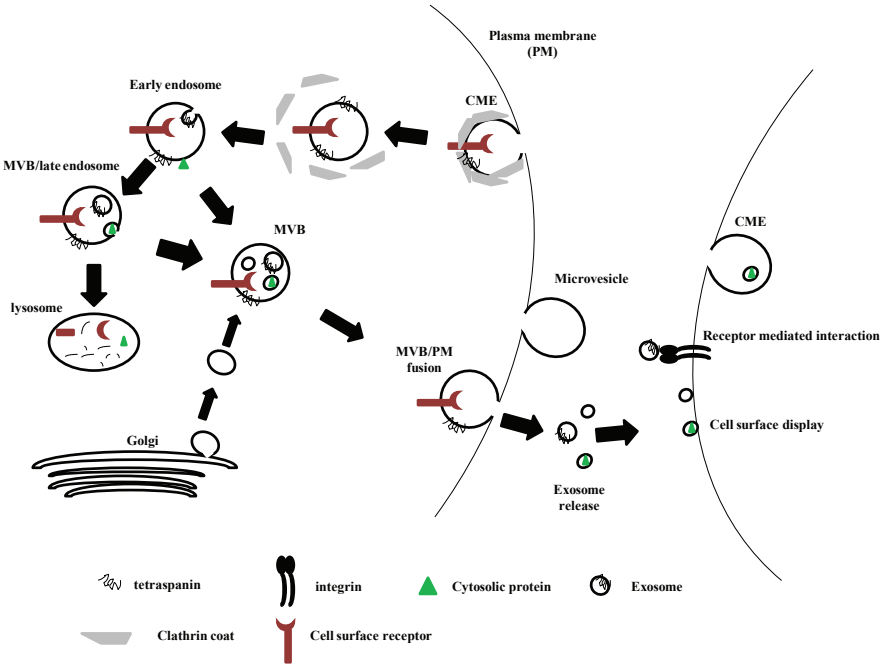

Figure 1: Mechanisms of exosome secretion and uptake: Upon Clathrin or Caveolae mediated endocytosis (CME), early endosome is formed from PM which upon maturation gives rise to late endosome. Exosomes originate in late endosome or Multivesicular body (MVB) upon inward budding of limiting membrane into the lumen. Fusion of MVB to lysosomes leads to degradation of their contents where as fusion to PM leads to the release of exosomes into the extracellular environment. Released exosomes can be displayed on the surface of cells or interact with bystander cells in a receptor dependent manner. In addition, exosomes can be internalized by the bystander cells via endocytosis

also contain some common classes of proteins such as TSPANs (CD9,-63,-81,-82), integrins (ICAM-1), chaperones (Hsc70 and Hsp90), MFGE8, membrane trafficking proteins (Rab GTPases, Annexins-I,-II, -IV, -V, -VI, -XI, syntaxin), enzymes (GAPDH, EF1α), cytoskeleton-associated proteins (actin, ezrin, moesin), MVB biogenesis proteins (Rab GTPases, TSG101, ALIX, syntenin-1), and lipid raft-associated proteins (stomatin, flotillin-1). Proteomic analyses of isolated exosomes identified proteins mainly belonging to PM, cytosol, and endosomes, whereas proteins from other cellular compartments or organelles such as the ER, Golgi, nucleus, and mitochondria were under-represented [11,12]. Exosomal proteins, lipids, coding, and non-coding RNAs identified through experimental methods have been incorporated into routinely-updated, online databases such as ExoCarta (http://www.exocarta.org) and Vesiclepedia (http://microvesicles.org/) [72].

The lipid composition of exosomes includes membrane lipids such as phosphatidylserine (PS), sphingomyelin, ceramide, GM3 ganglioside, and cholesterol [48,73-76]. The significance of the genetic composition of exosomes was first realized in 2007, when exosomes derived from human and mast cell lines (HMC-1 and MC/9, respectively), or primary bone marrow-derived mast cells from mice, were found to contain mRNAs coding for 1300 genes, along with miRNAs. The composition of these exosomes appeared specific to exosomes and contrasted the cytoplasmic m/miRNA content of the originating cells, indicating that exosome cargo selection process is specific and in some instances, exosome content may not match with that of originating cell [77]. These mRNAs were functional in *in vitro* translation assays, and protein-coding upon transfer of mouse exosomes to human cell lines. Unidirectional transfer of exosomal miRNAs from Jurkat cells to APC (Raji-SEE B-cell line) in the immunological synapse was antigen dependent, resulting in the modulation of gene expression in the recipient cells [78]. Alteration of exosomal miRNA profiles after LPS-treatment of mouse bone marrow-derived DCs (BMDCs), functionally transferred exosomal contents to other DCs by fusion or hemifusion, demonstrating that exosomes from APCs can affect the immune responses in other APCs [63]. Of particular note, is non-cell-autonomous gene silencing mediated by exosomal *let-7b,-7d* miRNAs secreted from regulatory T (T_{REG}) cells, function to suppress T_{h1} cell proliferation and IFN-γ secretion [79]. High-throughput sequencing of exosomal RNAs demonstrated that not only are miRNAs and mRNAs packaged, but also that other small ncRNAs, such as vault RNA, t-RNAs, and Y RNA and to a very limited extent, ribosomal RNAs are also packaged [80].

Immune Modulatory Role by Exosomes

Exosomes in innate immunity

Exosomes can serve as key carriers for Pathogen-associated Molecular Patterns (PAMPs), tumor antigens, and finally MHC-I/-II-antigenic peptide complexes, to facilitate antigen presentation. Exosomes isolated from *M. tuberculosis* or *M. bovis* infected macrophages and infected mice bronchoalveolar lavage fluids (BALF) were found to carry PAMPs lipoarabinomannan (LAM) and a mycobacterial-encoded 19 kDa protein [81]. PAMP-carrying exosomes stimulated TNF-α production upon *in vitro* treatment of macrophages or *in vivo* intra-nasal instillation in mice. LPS-stimulated DCs secreted exosomes carrying tumor necrosis factor (TNF) receptors, TNFR-I and TNFR-II, and as well as TNF-α, in addition to canonical MHC-II, CD40, and CD83 molecules [82]. Exosomes from mature DCs stimulated intestinal epithelial cells to produce chemokines RANTES, IL-8, MCP-1, and pro-inflammatory cytokine GM-CSF in a TNF-α-dependent manner [82]. DC-derived microvesicles were shown to activate NF-κB upon addition to microglial cells, which may be one of the potential mechanisms of CNS inflammation observed in multiple sclerosis (MS), and its model, experimental autoimmune encephalitis (EAE) [83].

Mouse DC-derived exosomes display TNF family ligands (TRAIL and FasL), and enhanced NK cell cytotoxicity by direct binding to their respective receptors [84]. Intradermal injection of mouse DC-derived exosomes increased the number of NK cells in the draining lymph nodes and facilitated their activation via IL-15Rα expressed on the exosomal surface via binding to the natural killer group 2 member D (NKG2D) receptor expressed on the surface of NK cells [85,86]. Exosomes derived from HEK293 cells, or immature DCs, displayed HLA-B-associated transcript 3 (BAT3) on their surface and facilitated NK cell activation via activating receptor NKp30, which, in turn, led to NK cell-mediated, DC maturation [87]. Exosome preparations from long-term DC cultures contaminated with *Mycoplasma* were found to stimulate B-cell proliferation in an antigen-independent manner [88]. Similarly, exosomes derived from mouse mast cell (MC) lines were mitogenic to cultured splenocytes, leading to blast formation, IL-2 and IFN- γ production, and proliferation [89]. Administration of these MC-derived exosomes to mice led to maturation of immature DCs and up-regulation of MHC-II, CD80, CD86, and CD40 on their surface [90].

Allogeneic HCMV-infected, human umbilical vein endothelial cells (HUVECs) released exosomes that stimulated the proliferation of autologous naïve and memory CD4+ T-cells in an HLA-DR-dependent manner. Exosomes from these HCMV-infected HUVECs contained the HCMV glycoprotein B (gB) and were proposed to be the source of grafted endothelium rejection [91].

Alternatively, exosomes derived from lymphocytic choriomeningitis virus (LCMV)-infected murine BMDC lacked viral antigens, and therefore failed to cross prime CD8+ CTLs [92]. These data therefore suggest that some viruses actively block or decrease the exosomal-loading of their antigens as a form of immune evasion.

In terms of their role in affecting tumor development and progression, exosomes derived from tumor cell lines, as well as pleural, and peritoneal effusions serve as vehicles for tumor antigens MART1/Melan A, Trp1, gp100, Her2/Neu. When these exosomes were used to prime DCs, they promoted the activation and expansion of autologous MHC-I –restricted, CD8+ CTLs to tumor cell lines, *in vitro,* and CTL-mediated regression of tumor size, *in vivo* [8,93].

Exosomes in acquired immunity

Due to their ability to transport antigens, exosomes can traffic antigens to APCs, such as DCs, macrophages, and B-cells, which process and present them by complexing the antigenic peptides with MHC-I and -II molecules, depending on their delivery mode (mRNA vs proteins, respectively). Antigen-bearing exosomes were able to induce antigen-specific naïve CD4+ T cell activation *in vivo*. *In vitro*, however, antigen-specific CD4+ T-cell stimulation failed to occur in the absence of intermediate DCs [94,95]. To activate naïve CD4+ T cells, APC-derived exosomes have to be initially captured by DCs. Exosomes-derived from mature DCs, however, can induce more efficient CD4+ T-cell activation than those derived from immature DCs [96-98].

This phenomenon of transferring antigens to DCs, for subsequent antigen processing and peptide loading onto endogenous MHC-I/-II for presentation to naïve CD4+ T-cells, is referred to as "indirect antigen presentation" as opposed to direct antigen presentation (see below). In addition, exosomes carrying peptide-MHC complexes can be captured by DCs, and directly presented to naïve CD4+ or CD8+ T cells, a phenomena referred to as "cross-dressing" [99].

Both indirect antigen presentation and cross-dressing require peptide or peptide/MHC complex-carrying exosomes to fuse with, or be taken up by DCs. Several surface ligands were found to facilitate this uptake via ligand-receptor interactions. For instance, ligands or adhesion molecules identified on exosomes include MFGE8, tetraspanins, ICAM1 and phosphatidylserine (PS) [60,71,94,97]. DCs and macrophages exhibit constitutive expression of integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ that serve as receptors for [100] In addition, MFGE8 also binds to cell surface PS through its PS-binding domain [100]. Furthermore, exosomal PS can also bind to cell surface PS receptors Tim-1 and Tim-4, resulting in their uptake [101].

Exosomes can serve as vehicles of direct antigen presentation by means of pre-formed peptide-MHC I complexes and co-stimulatory molecules on their surface, and thus, can directly present antigens to naïve T cells. APC-derived exosomes expressing MHC-I- peptide complexes, in addition to co-stimulatory B7 proteins and ICAM1 were found to directly activate naïve CD8+ T-cells to become effector CTLs [102].

On the other hand, exosomes presenting antigen in the absence of co-stimulatory molecules can induce anergy. Exosomes derived from DCs treated with viral immunogenic peptides (CMV, EBV and IAV), were able to directly activate autologous CD8+ T cells in a dose-dependent manner, leading to IFN-γ production [103]. Exosomes secreted by ovalbumin (OVA) peptide-pulsed mature BMDCs activated OVA-specific, MHC class-I-restricted T-cell hybridomas more efficiently than those from immature BMDCs [104]. Furthermore, cross presentation of MHC-I-OVA peptide complexes was also found in BMDC exosomes derived from $TAP^{-/-}$ (Transporter of Antigen Processing) mice [104]. Exosomes secreted by LPS-matured DCs were 50-100-fold more potent than immature DC exosomes in activating antigen specific CD8+ CTLs. Finally, mature DC exosomes added to B-cells similarly induced the ability to prime naïve CD8+ T-cells to become CTLs [97].

Exosomes during Bacterial Infection

Exosomes serve a vital role in the dissemination of bacterial components from the infected cells to bystander cells, resulting in either immune stimulation or immune suppression. As mentioned previously, exosomes secreted by macrophages infected with *M. tuberculosis* carried PAMPs such as LAM and phosphoinositolmannoside (PIM), and as a result of exosome-mediated transfer, these PAMPs were detected in bystander uninfected cells [105]. Later studies identified additional mycobacterial components such as a 19 kDa lipoprotein, glycopeptidolipids, trehalose dimycolate, and phenolic glycolipids in exosomes released from infected cells [106].

During infection of macrophages, *M. tuberculosis* resides in the phagosome and prevents fusion of the phagosome with endolysosomal compartments. However, bacterial cell wall constituents are released from phagosomes and traffic to MVBs for secretion via exocytosis in a calcium-dependent manner [81]. A later study demonstrated that *M. avium*-infected macrophage-secreted exosomes were capable of stimulating a pro-inflammatory response in bystander macrophages [81,106]. Furthermore, exosomes secreted from *M. tuberculosis* and *M. bovis* BCG-infected macrophages were also found to induce a pro-inflammatory response in macrophages [106]. *M. avium sp. Paratuberculosis* (MAP)-infection of macrophages, or exosomes isolated from infected macrophages, enhanced the expression of co-stimulatory molecules CD80 and CD86, and pro-inflammatory cytokine gene expression (TNF-α and IFN-γ) in infected macrophages as well as exosome-treated, naïve macrophages, indicating that exosomes carry key PAMPs sufficient to trigger an innate immune response [107].

Macrophages infected with *M. smegmatis* and *M. avium* displayed increased cellular expression of Hsp70 and released more exosomes with enriched Hsp70 on their surface. Treatment of macrophages with either exosomes, or exogenous Hsp70, led to NF-κB activation and subsequent TNFα production [108]. Pre-incubation of macrophages with Hsp70 led to enhanced phagocytic activity against *M. smegmatis* and *M. avium* and enhanced bacterial killing [108]. The immune stimulatory effect of

exosomes was also demonstrated *in vivo* in mice injected with exosomes derived from *M. tuberculosis* and *M. bovis* BCG-infected macrophages, which led to an induction of TNFα and IL-12p40, in addition to neutrophil and macrophage recruitment to bronchoalveolar lavage fluid (BALF) [106]. The 19 kDa protein of *M. tuberculosis* (*M. tb*), was found to be secreted via exosomes and stimulated TLR2 to induce IL-12p40, TNFα and iNOS expression. Exosomes secreted from cells infected with an *M. tuberculosis* strain deficient in 19 kDa expression failed to induce TNFα and iNOS [109].

Treatment of naïve macrophages with exosomes derived from *M. bovis*-infected macrophages induced chemokine secretion and further recruitment of macrophages, CD4+, and CD8+ T-cells, *in vitro* [110]. This observation confirms the ability of exosomes to recruit immune cells during granuloma-formation, contributing to the pathogenesis of *M. tb* [110,111]. Furthermore, exosomes from infected macrophages stimulated the proliferation of both CD4+ and CD8+ T-cells isolated from mycobacteria-sensitized mice, with maximal stimulation observed upon prior incubation of exosomes with APCs [111]. Intranasal-administration of these exosomes into mice elicited CD4+ and CD8+ memory cells, and upon *ex vivo* re-stimulation with BCG antigens, induced memory T-cell populations to secrete IFNγ. These studies demonstrate the immune-stimulatory potential of exosomes, and strategies to exploit this potential can offer therapeutic modalities against various infections [111].

On the other hand, the immune-suppressive potential of exosomes from *M. tb*-infected macrophages has also been demonstrated [112]. Exosomes from *M. tb*-infected RAW264.7 macrophages were able to inhibit anti-CD3-stimulated T-cell proliferation as well as expression of MHC-II and CD64 in mouse bone marrow-derived macrophages [112]. This suppression was partially-dependent on exosomal *M. tb* lipoproteins, but completely-dependent upon macrophage expression of TLR2 and MyD88, suggesting that the exosomes required some aspects of TLR-signaling that was affected by the mycobacterial lipoproteins downstream. IFNγ-induced expression of genes involved in macrophage activation and antigen presentation was similarly suppressed by pre-treatment with exosomes derived from *M. tb*-infected RAW264.7 macrophages [112].

Apart from mycobacteria, exosomes from *Salmonella typhimurium*-infected macrophages carried LPS and induced TNFα expression in human monocytes [81]. Exosomes secreted by mycoplasma-infected tumor cells also carried mycoplasma components and were able to activate splenic B-cells to induce pro-inflammatory IFN-γ, as well as the anti-inflammatory cytokine, IL-10 [113]. This splenic B-cell response significantly inhibited anti-CD3-stimulated T-cell proliferation and IFNγ production by T-cells, as μMT mice, deficient in B-cells, produced greater amounts of T_{H1} cytokine, IFNγ [113].

Exosomes can also serve as carriers of toxins, as seen with anthrax toxin. Anthrax toxin is a classical AB toxin, composed of the protective antigen (PA) and the lethal factor (LF). Upon receptor-mediated endocytosis, a PA-formed channel delivers LF into cytosol. In addition, LF is also incorporated into exosomes, and remains in exosomes for prolonged periods, allowing its persistence with a host [114]. LF-released in exosomes is protected from proteolytic degradation outside the cell and can be delivered to bystander cells in a manner independent of the anthrax toxin receptor-mediated trafficking pathway. Furthermore, exosome-enclosed LF is protected from antibody neutralization, underscoring the role played by exosomes during anthrax pathogenesis [114].

Finally, *Chlamydia pneumoniae*-infected cells express tissue factor (TF) that exhibits pro-coagulant properties along with functions that regulate cell proliferation, migration and apoptosis (116). *C. pneumoniae* infection resulted in an increase in microparticle release with an enhanced pro-coagulant activity within 24 hrs of infection. These TF-positive microparticles induced NF-κB activation in endothelial cells. In addition, during later stages of infection, *C. pneumoniae* elementary bodies appear to be released with microparticles with detectable amounts of *C. pneumoniae* DNA in the microparticles [115]. This finding underscores the ability of *C. pneumonia* to utilize microparticles in promoting successful dissemination in the host. Although, the publication mentioned "microparticles" as the carriers of TF, the procedure employed to purify the so called "micro particles" (ultracentrifugation at 100,000 x *g* for 60 min at 20°C) from culture supernatants is identical to exosome purification, and hence may actually be exosomes [16].

Exosomes during Viral Infection

A host of viruses such as HIV-1, EBV, HSV-1, HAV, HBV, HCV, HEV, and HTLV-I are known to exploit exosomes in order to mediate a range of effects including immune suppression, modulation, and evasion by cloaking their virions in exosomes in order to evade antibody (Ab)-mediated neutralization, as seen in HAV and HEV infections [116]. Exosomes are further employed as cargo vessels for viral proteins (LMP1, Tax, dUTPase, etc.) and viral- or specific cellular-m/miRNAs to mediate immunosuppressive effects or to contribute to viral pathogeneses [117-120]. Finally, the cargo of exosomes secreted from oncogenic virus-infected cells was found to bear genetic and protein markers involved in modifying the tumor microenvironment in an effort to promote tumor cell proliferation, immune invasion, angiogenesis, metastasis, or metabolism (i.e, eliciting the Warburg effect).

dsDNA Viruses

Herpes Simplex Virus 1

Herpes Simplex Virus 1 (HSV-1) belongs to the family *Herpesviridae*, subfamily *Alphaherpesvirinae*. Following orofacial infection, HSV-1 undergoes replication in epithelial cells before establishing latency in sensory nerves, such as trigeminal ganglia. Periodic reactivation from latency, followed by anterograde axonal transport back to the surface epithelium facilitates host transmission and disease. HSV-1-associated keratitis, encephalitis, and neurodegeneration have been linked to this reactivation. In a transiently-transfected melanoma cell line, HSV-1 glycoprotein B (gB) complexes with HLA-DR along its biosynthetic pathway and targets it to MVBs instead of cell surface, facilitating release of HLA-DR-gB complexes via CD63+ exosomes [121]. Interestingly, cellular or exosomal HLA-DR-gB complexes lack invariant chain or antigenic peptide, leading to the hypothesis that gB prevents antigenic peptide loading onto HLA-DR dimers in the endocytic pathway and diverts it for exocytic release via exosomes [121].

HSV-1 inoculum made in stimulator of interferon genes $(STING)^{hi}$ cell lines (such as Hep-2 or HEL) significantly increased the amount of STING protein in $STING^{lo}$ cell lines (Vero) upon infection [122]. Incubation of viral inoculum with antibodies against the receptor-binding glycoprotein D, in an effort to neutralize viral entry, failed to thwart this STING transfer. This was found to be due to STING transfer via exosomes that co-purified with virions upon ultracentrifugation [122].

In addition, exosomes from HSV-1-infected cells carried viral proteins ICP0 and US11, as well as HSV-1 transcripts (LAT, ICP27, VP16), and miRNAs –H3, -H5, -H6, -H28 and –H29 [122]. Of note, miRNAs –H3 and –H6 were found to be expressed late during infection upon completion of viral replication and upon reactivation from latency, but not in latently-infected ganglia. Ectopic over-expression of miRNAs –H3 and –H6 led to a reduction in viral titer in transfected cells [123]. An exciting hypothesis was developed, wherein exosomal transfer of STING or miRNAs –H3 and –H6 to uninfected cells, for instance satellite cells, or neuronal cells in the CNS, may prime them to limit replication and spread of virus upon reactivation, whereas anterograde transport of the virus allows transport

of virus to the periphery and subsequent epithelial cell infection and effective human-to-human transmission (123-125). Thus, by preventing severe pathogenesis and decreased fitness of the host, the virus has evolved to successfully disseminate among humans, while at the same time establishing persistent long-lasting infection within the individual.

Epstein-Barr Virus (EBV)

EBV (*Human herpesvirus* 4, HHV4) is a *gammaherpesvirus* that infects and is maintained in a vast majority of world's population (estimated that nearly 100% of adults are seropositive). EBV infection in an immune competent individual can range from asymptomatic, to a mild to moderate self-limiting illness (mononucleosis). In immune-compromised patients, such as those suffering from HIV-AIDS, latent EBV can contribute to lymphoid and epitheloid malignancies, such as B-cell lymphoproliferative Burkitt's lymphoma (BL), Hodgkin's and non-Hodgkin's lymphomas (HLs), post-transplant lymphoproliferative disorders (PTLD), nasopharyngeal carcinoma (NPC), and some forms of gastric carcinoma.

Predominant cellular targets of EBV include B-lymphocytes and epithelial cells, although the virus can infect other cell types such as such as T-cells, NK cells, smooth muscle cells, and follicular DCs [125]. EBV-positive lymphoblastoid cell lines (LCLs) secrete exosomes that bear glycoprotein 350 (gp350) and specifically-target complement receptor CD21 on primary B-cells. Gp-350-bearing exosomes uptake by B-cells could be blocked by either antibodies to gp350 or CD21. Furthermore, gp350-bearing exosomes significantly reduced EBV infection of cord blood mononuclear cells, suggesting a possible therapeutic potential of gp350 loaded exosomes in inhibiting EBV infection [5,6]. Exosomes bearing EBV primary oncoprotein LMP1 were found to be released into culture supernatants of EBV-positive LCLs and NPC cell lines [7-10]. LMP1-bearing exosomes significantly reduced the proliferation of PHA-stimulated primary T-cells [8]. LMP1 was found to selectively incorporate angiogenic fibroblast growth factor 2 (FGF2) into exosomes [11]. In addition, LMP1-bearing exosomes also contained immune modulatory protein galectin-9 [10].

Exosomes from both type I and type III EBV latently-infected cells induced FasL-mediated apoptosis in the recipient B-, T- and epithelial cells in a dose- and time-dependent manner [12]. Finally, latent EBV episomes in LCLs of type I, II, and III latency is sensed by nuclear IFN-γ-inducible protein 16 (IFI16) leading to activation of STING and the assembly of inflammasome complex (IFI16-ASC-pro-caspase 1) activating caspase 1[13]. Caspase 1 cleaves pro-forms of inflammatory cytokines IL-1β, IL-18, and IL-33 into their mature forms which were detected in the exosomes secreted by LCLs. Chronic inflammation triggered by the release of exosomal cytokines by EBV LCLs may contribute to tumor cell proliferation, progression, and inhibition of apoptosis [13].

EBV-encoded LMP1 was found to be associated with CD63, and CD63-mediated LMP1 trafficking significantly enhanced the quantity of LMP1+CD63+ exosome secreted by EBV+ LCLs and LMP1-transfected cell lines [9,10]. CD63-depletion by CRISPR-Cas9 led to a significant reduction in the quantity of LMP1+ exosomes. In addition, LMP1-mediated MAPK/ERK and non-canonical NF-kB signaling was greatly enhanced in the CD63-depleted cells due to impaired exosomal secretion of LMP1, whereas other LMP1-mediated pathways were relatively unaffected (canonical NF-kB and PI3K/Akt), suggesting regulation of both intracellular and intercellular signaling by exosomes [9]. Interestingly, innate sensor IFI16 was also detected in the exosome fractions from both EBV-positive and EBV-negative LCLs.

EBV-encoded dUTPase was also found secreted in exosomes from chemically-induced Raji cells at levels sufficient to induce NF-kB activation and pro-inflammatory cytokine secretion in primary DCs and PBMC, in a TLR2-dependent manner [14]. Apart from LMP1, other proteins such as LMP2A, PI3K, and EGFR, were also found to be released in exosomes [15].

In addition to viral proteins, the EBV genome encodes for non-coding RNAs, including miRNAs, in three separate clusters (BHRF1, cluster 1 and cluster 2 BARTs) [16,17]. EBV-encoded miRNAs are abundantly expressed in EBV-associated tumors and EBV-transformed LCLs. These miRNAs were detected in LCL-secreted exosomes, and were functional upon exosomal transfer to monocyte derived dendritic cells (MoDC). In addition, *in vitro* assays displayed successful exosomal transfer of EBV miRNA BHRF1-3 and post-transcriptional repression of its target, CXCL11 [17]. Exosomal transfer of EBV miR-BART15 also led to post-transcriptional repression of its target, NLRP3, in the monocytic cell line, THP1 [16]. *In vivo*, EBV miRNAs were detected in both EBV DNA positive B-cell fractions and EBV DNA negative non-B cell fractions, demonstrating exosomal transfer of EBV miRNAs to non-B cell fractions [17]. Another study demonstrated the presence of EBV miRNAs in the exosome fractions from NPC cell lines, in the plasma of the mice xenografted with NPC tumors, and the plasma of NPC-positive patients.

EBV transmission between hosts involves reactivation of latent EBV from peripheral B-cell stores (i.e. tonsils) and infection of nearby oral epithelial cells that permit lytic replication and successful dissemination. Exosomes from oral epithelial cells are enriched in mir-200 family members that were able to enhance the lytic expression cascade in neighboring, latently-infected tonsillar B-cells by relieving ZEB-1- and 2-mediated repression of the viral lytic transactivator (Zta) promoter [18].

In addition to viral miRNAs, EBV-encoded small RNAs (EBV-EBERs) were also identified in exosomes from EBV-transformed LCLs [19]. EBER1-loaded exosomes transferred 5' triphophorylated (5'pppEBER1 RNA) to the recipient plasmacytoid DCs, leading to activation of anti-viral immunity via RIG-I and MDA5. Proteomic analyses of EBV LCL-secreted LMP1+CD63+ exosomes led to the identification of several LMP1-regulated pathways. To summarize, these exosomes contained proteins affecting endocytosis, cytoskeletal signaling, integrin signaling, protein 53 (p53), JAK/STAT, NF-kB, IRF7, CD40, TNF, insulin receptor, Ras/PI3K/Akt and MAPK-signaling pathways [20]. Finally, Hodgkin Reed-Sternberg (HRS) antigen (CD30) is expressed by HRS B-lymphoma cell-derived exosomes and can serve to activate CD30L on mast and eosinophilic cells, affecting the tumor microenvironment to amplify pro-inflammatory signals and lymphoma progression [21].

Kaposi Sarcoma Herpes Virus (KSHV)

KSHV (aka, *human herpesvirus* 8, HHV8), is the etiological agent of Kaposi's sarcoma (KS), an endothelial cell proliferation and a common AIDS-associated malignancy, worldwide. KSHV is also associated with the B-cell lymphoma known as Primary Effusion Lymphoma (PEL), and with the plasmablastic variant of Multicentric Castleman's Disease (MCD) [126]. Meckes, Jr, and colleagues charactertized proteomic profiles of single positive EBV+, KSHV+, and dual (EBV+/KSHV+) PEL cell lines, in addition to uninfected LCLs, and identified a total of 871 proteins [20]. Proteomic profiles of dual (EBV+/KSHV+) PEL cell lines were more similar to KSHV+ single positive cell lines, showing very limited expression of EBV gene products in these PEL cell lines. PEL cell line-derived exosomes were significantly enriched for histones and associated variants, along with glycolytic enzymes such as pyruvate kinase, lactate dehydrogenase, and phosphoglucose isomerase that appear to significantly alter the tumor microenvironment upon exosomal transfer, contributing to viral persistence and pathogenesis [16].

Interestingly, this proteomic study identified proteins common to EBV- and KSHV-infected cells, and among these, includes innate receptors PKR and OAS3, indicative of an immune modulatory role of these exosomes.

The latent KSHV genome is sensed by IFI16, leading to STING activation, as well as inflammasome assembly and cytoplasmic redistribution. Inflammasome activation in TIVE-LTC cells and BCBL-1 cell line leads to cleavage, maturation, and exosomal secretion of cytokines IL-1β and IL-18, along with innate sensor IFI16 [22]. In fact, KSHV is known to induce aerobic glycolysis to facilitate angiogenesis in infected endothelial cells [23,24].

The KSHV genome also encodes miRNAs that are known to fine-tune cellular gene expression in order to facilitate viral pathogenesis. In studies seeking to identify biomarkers, KSHV miRNAs and cellular oncomirs, including the miR-17-92 cluster, were detected in exosomes from clinical samples. Recently, KSHV-infected cells were demonstrated to release virus like vesicles (VLVs), that are devoid of virus capsids and viral genomic DNA, yet contain significant amounts of tegument and envelope proteins [25]. In addition, cellular proteins commonly detected in virions were also found in VLVs. VLVs were similar in size to virions (~200 nm), and contained both viral and host miRNAs. VLVs induced differentiation in recipient cells. Furthermore, VLVs facilitated lytic replication by transactivating the lytic (Rta) promoter [25].

Human Herpes Virus 6

Human herpes virus 6 (HHV-6A/B) latency was described in a variety of cell types, including bone marrow progenitor cells, monocytes/macrophages, myeloid cell lines, an astrocytoma cell line, and an oligodendrocyte cell line [128]. The target cells for HHV-6A and HHV-6B differ. Individuals with chromosomally integrated HHV-6 (ciHHV6) were found with ciHHV-6 vertically transmitted from parents to children according to the Mendelian law of chromosome segregation [128,139]. This phenomenon is referred to as inherited ciHHV-6 (iciHHV-6) [128,129]. HHV-6 was found to derive its envelope from the membranes of the trans Golgi and post-trans Golgi networks, maturing to become MVBs [26]. MVBs in HHV-6A-infected HSB2 cells appear positive for virion glycoproteins gB and gM, along with cellular CD63. Furthermore, MVB fusion to the PM contributes to virion egress along with the release of exosomes. Finally, apart from exosomes, virion fractions from infected cell supernatants incorporated exosomal marker CD63 [26]. In a separate study, HHV-6B-infected cells displayed reduced cell surface and intracellular MHC-I, where a significant amount of MHC-I was incorporated into virions, and exosomes that were released upon MVB fusion to the PM [27].

Hepatitis B Virus (HBV)

HBV is an enveloped virus with partially double-stranded DNA enclosed in its nucleocapsid. It belongs to the family *Hepadnaviridae* and infects around 350 million individuals worldwide [130]. HBV transmission is mainly via vertical (perinatal) transmission, although horizontal transmission via intravenous drug use and sexual contact are also important routes of infection [130]. Acute HBV infection resolves spontaneously in 90% of infected individuals [130]. In some cases, HBV can persist, resulting in chronic infection, especially in neonates with perinatal exposure. Chronically-infected individuals are at increased risk of liver cirrhosis and hepatocellular carcinoma [130]. Recent findings have demonstrated that serum exosomes from chronic HBV-infected patients carry HBV DNA and transcripts coding for viral antigens HBx and HBs/p [131]. Serum exosomes positive for HBV DNA and RNA were able to establish productive infection in naïve hepatoma cell line (HLCZ01) [131]. NK cells serve a critical role in limiting chronic HBV infection, and HBV-infected cell-derived exosomes significantly inhibited NK cell cytolytic function and reduced their IFNγ and TNFα secretion. In addition, NK cells from healthy donors tested positive for HBV DNA upon exposure to exosomes derived from HBV-infected cells [131]. NK cell function is dependent upon the balance between activating and inhibitory signals by respective NK cell receptors. Treatment of NK cells with HBV-infected cell-derived exosomes lead to a down regulation of NK cell activating receptor NKp44, and upregulation of the killer-inhibitory receptor, NKG2A [131].

RNA viruses

Human Immune deficiency Virus 1 (HIV-1) & *Human T cell Lymphotrophic Virus type I* (HTLV-I)

HIV-1 and HTLV-I belong to the family *Retroviridae* and both have been reported to use exosomes for functional delivery of viral transcripts, proteins, and miRNAs [119,132]. Exosomes from HTLV-I-infected cells carried transcripts coding for viral proteins Tax, HBZ, and Env, to recipient cells [119].

HIV-1 is the well-studied virus in the context of exosomes. Viral accessory protein *Nef* (Negative factor), upon either HIV-1 infection or introduced via transduction, promoted enhanced secretion of exosomes by infected and also bystander cells [133,134]. Amino-terminal domains of *Nef* were found to be critical for this vesicle-mediated secretion [133]. *Nef* plays a critical role during HIV-1 pathogenesis by promoting essential functions such as CD4 and MHC-I cell surface down regulation, targeting them for lysosomal degradation, and induction of apoptosis in CD4+ T-cells [135,136]. Similarly, *Nef* also reduced the amount of exosomal CD4 and MHC-I, although *Nef* promotes an overall increase in exosome secretion.

CD4 serves as the main receptor for HIV-1, and only activated, but not resting CD4+ T-cells are permissive for productive HIV-1 infection [137]. CD4-bearing exosomes can serve as decoys to significantly inhibit HIV-1 infection by binding to HIV-1 Env [138]. *Nef* serves to promote infection by reducing CD4 expression in exosomes [139], and its expression is sufficient to activate quiescent CD4+ T cells and render them permissive for HIV-1 infection [139]. *Nef*-expressing exosomes incorporate ADAM17, a disintegrin and a metalloproteinase, (aka, TACE for TNF-α-converting enzyme) that converts pro-TNFα into its mature form, leading to activation of bystander resting CD4+ T-cells, making them permissive for HIV-1 infection [139]. *Nef*-containing microvesicles induced apoptosis in Jurkat cells, but had no effect on the U937 monocytic cell line [139].

Exosomes secreted from HIV-1-infected macrophages carry viral miRNAs vmiR88, vmiR99, and vmiR-TAR, and serum exosomes of infected patients also carried these miRNAs. Importantly, vmiR88 and vmiR99 stimulated human macrophages to release TNFα, in a TLR8-dependent manner. In addition, *Nef* expression in macrophage resulted in selective incorporation of 47 miRNAs into exosomes, suggesting active modulation of exosomal composition by *Nef* [140].

Hepatitis C virus

Hepatitis C virus (HCV) is an envelope, single-stranded, positive sense RNA virus belonging to the family Flaviviridae. HCV is distinct from other Arboviral members of Flaviviridae, in that it is not transmitted via arthropod vectors, but rather via blood-borne or sexual transmission. Approximately 170 million individuals are infected worldwide, and chronic HCV infection can lead to liver fibrosis, cirrhosis, as well as hepatocellular carcinoma. Initially, HCV RNA was detected in the plasma exosome fractions of chronically-infected patients [28]. A later study identified the ESCRT0 component, Hrs, to be critical for assembly and budding of HCV into the exosome secretion pathway [29]. Subsequently, exosomes derived from HCV-infected hepatocytes, or in HCV-positive patient sera, were found to carry the entire HCV genome, permitting receptor-independent transfer of the viral genome, capable of establishing productive infection in recipient cells [28,30-34]. Furthermore, treatment

of HCV-infected cells with intracellular cholesterol transport inhibitor, U18666a, leads to inhibition of MVB function, accumulation of viral particles in exosomes, and impairment of particle release [35]. Exosomal transfer of HCV RNA was found to activate plasmacytoid DCs to produce IFN-α [32]. The importance of exosomal transfer of HCV virions is that this form of infection is resistant to envelope protein neutralizing antibodies, in contrast to live free virus [30,33,34,36]. Consequently, exosomal transfer of HCV appears to be an important mode of spread, distinct from cell free, and cell to cell transmission.

Finally, HCV can use tetraspanin CD81 as a co-receptor for entry, which could be one of the mechanisms by which HCV accesses exosomes, which normally contain CD81 [28]. The role played by exosomes in the pathogenesis of liver fibrosis was elegantly demonstrated in experiments where exosomes from HCV-infected cells were found to carry miR-19a [37]. Upon transfer of miR-19a to non-parenchymal hepatic stellate cells, this lead to downregulation of the miR-19a target, suppressor of cytokine signaling 3 (SOCS3) [37]. Downregulation of SOCS3 lead to activation of STAT3, and induction of pro-fibrotic marker genes TGFβ1, CTGF and TIMP1 [37]. Thus, HCV appears to mediate fibrosis via an indirect mechanism, through the targeted exosomal expression of miR-19a.

Hepatitis A virus

Hepatitis A virus (HAV) is a non-enveloped, single-stranded, positive sense RNA virus belonging to the family Picornaviridae. Currently, it is the only member of the genus Hepatovirus, within the Picornaviridae family. HAV replication is slow in vitro and does not cause discernible cytopathic effect. How HAV particles are released into the extracellular medium has remained a mystery, until the recent description of two major populations of infectious HAV in the supernatants of infected cells with distinct morphologies, buoyant densities, and differential-resistance to neutralizing antibodies [141]. A dense fraction banding at 1.22-1.28 g/cm-3 contained naked viral particles under EM that were captured by capsid ELISA and displayed resistance to chloroform treatment [141], i.e., infectious HAV virions. In contrast, a lighter fraction, banding at 1.06-1.10 g/cm-3, was found cloaked in host-derived membranes as seen via EM, and remained undetected by capsid ELISA. Upon chloroform treatment, these lighter particles lost their infectivity, due to partitioning of the membrane-associated viral particles into the interface, and their disappearance from the aqueous phase. Due to their envelopment in the host membranes, these lighter particles were resistant to neutralizing antibody-mediated inhibition. Only the enveloped form of HAV was detected in the blood of infected patients with acute HAV, whereas virus shed in feces lacked the envelope [142]. These data suggest that exosomal partitioning may be an important intra-host means of HAV cell-to-cell infection, while naked infectious virions are essential for inter-host transmission and persistence in the environment.

Rift Valley Fever Virus

Rift valley fever virus (RVFV) is an enveloped tri-segmented, negative sense RNA virus, in the genus *Phlebovirus*, family Phenuiviridae, and order Bunyavirales. The genome is made up of three segments, composed of small (S), medium (M) and large (L) segments. The L segment encodes the RNA-dependent, RNA polymerase, the M segment encodes NSm, a 78 kDa protein, and the envelope glycoprotein components, Gn and Gc, and the S segment encodes the nucleoprotein N, and non-structural proteins, NSs and NSm. RVFV infection in livestock can lead to abortions in up to 100% of infected, pregnant animals [143]. In humans, RVFV infection ranges from a mild febrile illness to a hemorrhagic syndrome, encephalitis, and death. Persistent Vero clones, established from RVFV-infected Vero cells, released exosomes containing all three segments of the viral genome in varied ratios [143]. However, upon treatment of fresh Vero cultures with exosomes purified from infected Vero supernatants, no viral genome replication was observed suggesting that viral components necessary for genome replication could be missing in exosomes [143]. Furthermore, RVFV-infected Vero cell-derived exosomes, carried viral protein NSs and N [143]. Exosomal-NSs protein displayed slower migration than NSs from infected cells, suggesting a specific protein modification leading to its selective incorporation into exosomes [143]. Exosomal NSs was found to be ubiquitinated, which indicates that viral proteins usurp cellular protein modification machinery to be incorporated into exosomes [143]. Ubiquitinated-proteins are incorporated into exosomes in an ESCRT-dependent manner. Finally, exosomes or supernatants from RVFV-infected cells induced apoptosis in Jurkat T-cell and U937 monocytic cell lines [143].

Tumor Derived Exosomes (Texs)-Mediated Immune Suppression and Tumor Promotion

TEXs play a crucial role in promoting tumor angiogenesis, invasion, metastasis, immune suppression and drug resistance [144-148]. TEX numbers are significantly elevated in vivo in sera of cancer patients in general, and in vitro, in each of three breast cancer cell line supernatants exposed to hypoxic conditions [149-151]. Due to the space constraints and the vast number of publications dealing with TEX-mediated functions reported elsewhere, we will limit ourselves to outline a few examples on how TEXs promote immune suppression and mediate drug resistance towards targeted immune therapy.

TEXs from tumor cell lines and sera of leukemic-patients, when co-incubated with primary activated T-cells or NK cells (CD56+CD16+), led to: Fas/FasL-mediated CD8+ CTL apoptosis, downregulation of CD3ζ subunit and JAK3 (Janus kinase 3) expression in primary activated T-cells, induction of CD4+CD25hiFOXP3+ (TREG)-patterning, and decreased cytotoxic function of NK cells [152]. Neutralizing Abs against TGFβ1 and/or IL-10 that bound to these proteins on the exosomal surface of TEXs inhibited the ability of TEXs to expand TREG populations [152]. Furthermore, TEX-differentiated TREGS displayed significant expression of FasL, IL-10, TGF-β1, CTLA-4, granzyme B, perforin, and general repression of responder-cell (CTL, NK cell) proliferation (154). TEXs express FasL and induce Fas-dependent apoptosis of circulating CD8+ CTLs (155, 156). TEXs can carry ectonucleotidases CD39 and CD73, responsible for production of immune suppressive adenosine, to target cells in the tumor microenvironment [156]. This transferred adenosine binds adenosine A2A receptor to upregulate cAMP production and suppression of effector T-cell functions [157].

AML patient plasma-derived TEXs express MHC-I related sequences (MICA, MICB), TGF-β, latency associated peptide (LAP), and mediate down regulation of NKG2D receptors on the surface of NK cells (152, 159-161). CD14+ monocytes differentiated in the presence of TEXs, IL-4, and GM-CSF skewed their differentiation towards a suppressor phenotype (CD14+ HLA-DR−/low), with suppression of T-cell functions being mediated in a TGF-β–dependent manner [161].

Similarly, TEXs from a mesothelioma cell line decreased IL-2-responsiveness in human CD4+, CD8+ T-cell and NK cell populations [162]. Apart from impairment of IL-2-mediated CD25 upregulation in all T-cell populations except CD3+ CD8-, TEXs promoted differentiation of CD3+ CD25+ Foxp3+ TREGS. Furthermore TEXs directly hampered NK cell cytotoxic function in this study [162].

TEXs isolated from melanoma patient sera, or melanoma-derived cell lines inhibited the proliferation of activated CD8+ CTLs, including melanoma antigen-specific CD8+ CTLs (164). In addition, melanoma-expressed TEXs expanded CD4+CD25+FOXP3+ TREGS, and enhanced their suppressor activity, in vitro [163].

Exosomes from both non-cancerous and cancerous bodily fluids such

as amniotic fluid (AF), ascites from liver cirrhosis, and ovarian carcinoma patients, activated and induced differentiation of the THP-1 monocyte cell line [164]. Exosome treatment of THP-1 cell line led to activation of NF-kB, and downstream IL-6 induction in a TLR2- and TLR4-dependent manner. IL-6 auto- or paracrine actions led to STAT3 activation. Primary monocyte-derived DCs or macrophages released IL-12p40 into culture supernatants after AF exosome treatment in a Myd88-dependent manner [164], suggesting an immune developmental and patterning role for these exosomes in the developing fetus.

Finally, TEX plays a critical role in mediating drug resistance towards targeted immunotherapy. For instance, CD30 antibody drug conjugate (ADC) Brentuximab Vedotin (SGN-35, Adcetris) targets malignant Hodgkin's lymphoma cells (cHL) expressing CD30. Human Reed-Sternberg Antigen (CD30) was found on the exosomes exocytosed by malignant Hodgkin lymphoma (cHL) cell lines L540, L428, KM-H2, and L1236 and in peripheral blood of HL patients [165,166]. Exosomal CD30 facilitates communication with tumor supporting CD30L positive bystander cells. In addition, malignant cHL and their exosomes also expressed active sheddase ADAM10. ADAM10 cleaved and released soluble CD30 (sCD30). sCD30 served as a soluble competitor for ADC. Furthermore, exosomal CD30 adherence to CD30 negative cells lead to indirect binding of ADC Brentuximab Vedotin (SGN-35, Adcetris) to mediate toxicity [166].

Conclusions and Perspectives

It is now evident and clear that exosomes serve as crucial intercellular messengers by carrying cargo composed of m/miRNAs, proteins, lipids and metabolic enzymes under normal physiological and pathological conditions. During microbial infection, they serve to carry antigens necessary to promote an effective immune response. Antigen delivery function of exosomes can be harnessed in the development of novel therapeutic regimens such as exosome based vaccination against microbial infections. On the other hand, the cargo of exosomes is drastically modified during microbial infections and also in cancer. This property of exosomes can be exploited for the quest towards biomarker discovery and subsequent development of minimally invasive prognostic or diagnostic markers for diseases including cancer. Alternatively, inhibiting tumor promoting functions of exosomes such as immune suppression and establishment of pre-metastatic niche can lead to a better anti-tumor response and reduced metastasis. Finally, exosomes offer a great potential for therapy and biomarker discovery for disease diagnoses and prevention.

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