Dendritic cells and routing cargo into exosomes
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Abstract
Extracellular vesicles, released from cells, are important for intercellular communication. They are heterogeneous but fall into two broad categories based on origin and function: microvesicles formed by outward budding from the plasma membrane; and exosomes that originate as intraluminal vesicles in multivesicular endosomes that fuse with the plasma membrane to release them. Extracellular vesicles generally and exosomes in particular have powerful effects on specific immune responses, and recent advances highlight their potential therapeutic uses. Dendritic cells (DC) that have internalized antigen release exosomes that express MHC class II molecules loaded with antigenic peptides, co-stimulatory molecules and intact antigen. Depending on the setting, these stimulate CD4 T-cell proliferation either directly or only in the context of accessory antigen naïve DC. Here, we discuss the reasons for this; and review current knowledge about the loading of antigen, class II and other cargo into exosomes released by DC and other professional antigen-presenting cells in the context of advances in exosome biology more generally.

HETEROGENEITY OF EXTRACELLULAR VESICLES
Extracellular vesicles are heterogeneous in size, composition, function and origin, and vary depending on the cell type they originate from and its state of activation. Consequently, interpretation of experimental results can be challenging and is further complicated because even the best of current methods fail to adequately separate the different subtypes of EV; therefore, distinguishing between them can be difficult. Freely accessible databases have been established to help mitigate these problems; for example, ExoCarta (http://www.exocarta.org) and Vesiclepedia (http://microvesicles.org) contain curated cell- and species-specific datasets of proteins, lipids and RNA species found in different types of EV. Extracellular vesicles fall into two broad categories defined by their origin: microvesicles—also known as microparticles—are formed by outward budding from the plasma membrane; and exosomes formed as intra-luminal vesicles (ILV) within multivesicular endosomes (MVE) that are released from them after fusion with the plasma membrane.
membrane (Figure 1). Here, we briefly summarize current knowledge about EV to provide context as extensive discussion can be found in excellent recent accounts available elsewhere.4-6

MICROVESICLES

Microvesicles originate in the plasma membrane from microdomains rich in cholesterol and sphingomyelin that provide docking sites for trans-membrane proteins including tetraspanins. These, together with flipping of phosphatidylserine, induce outward budding that terminates in release of vesicles with diameters between 50 nm and 1 μm. Thus, the microvesicle limiting membrane reflects the surface of the cells they originate from, while the luminal cargo is enriched with cytoplasmic proteins and non-coding RNAs bound to molecules anchored to the lipid-rich microdomain they were generated from.4,7 Specific names have been applied to microvesicles derived from particular cells and tissues, such as oncosomes from tumors8 (described elsewhere in this issue by Manning and Danielson9) and ectosomes from neutrophils. Ectosomes provide a particularly striking example of the multiple ways in which microvesicles affect immunity. Ectosomes are rapidly released after neutrophil activation10 and are abundant at sites of neutrophil-mediated injury where they can be pathogenic, for example in anti-neutrophil cytoplasmatic antibody-associated vasculitis. Their cargo includes myeloperoxidase and proteinase-3, the autoantigens targeted by anti-neutrophil cytoplasmatic antibody, and they directly activate endothelium and injure it.11 By contrast, phosphatidylserine on the ectosome membrane has potent anti-inflammatory effects on macrophages and DC which become unresponsive to zymosan and LPS and secrete transforming growth factor-β1,12 while annexin 1 on the ectosome surface inhibits further neutrophil recruitment.13 In particular, myeloperoxidase is secreted by neutrophils either as soluble molecule or contained within ectosomes (and exosomes). Although the enzymatic activity of myeloperoxidase originating from different compartments has never been defined, uptake of myeloperoxidase inhibits activation of DC and decreases their ability to induce antigen-specific T-cell proliferation.14 Accordingly, predicting the effect of therapeutic strategies designed to manipulate microvesicles, including ectosomes and oncosomes, presents a challenge.

Figure 1. Uptake, processing and presentation of antigens by dendritic cells. Antigens internalized by professional APC enter endosomal pathways that transport them to the MHC class II loading compartment (MIIC), a specialized type of multivesicular endosome (MVE). Here, they are digested and the resulting peptides are loaded onto MHC class II molecules (pMHCII) transported to the plasma membrane. In immature DC, nascent pMHCII are rapidly ubiquinated either at the cell surface or directly from MIIC and recycled into intraluminal vesicles (ILV) within MVE that traffic to lysosomes and fuse with them for degradation. However, in mature DC, nascent pMHCII on the cell surface is not ubiquinated but persists for recognition by CD4 T cells; some nascent pMHCII is also incorporated into microvesicles (MV) that bud from the plasma membrane. An alternative pathway routes a proportion of nascent pMHCII directly into ILV of MVE that fuse with the plasma membrane to release them as exosomes.
EXOSOMES

Exosomes originate as ILV formed from inward budding of the limiting membrane of MVE. These MVE then fuse with lysosomes resulting in digestion of their contents, or alternatively with the plasma membrane to release the ILV as exosomes. Exosomes are smaller than microvesicles, with diameters between 30 and 100 nm and vary in density (1.13–1.19 g mL$^{-1}$). At least in part, this heterogeneity reflects differences in the molecular mechanisms used to generate ILV and to load them with cargo. The exosome limiting membrane is rich in cholesterol and ceramide and contains numerous transmembrane proteins whose nature and abundance depend on their cellular origin. Some, such as MHC class I (MHCI) molecules, the tetraspanins CD63, CD81 and CD9 and the lysosome-associated membrane glycoproteins (LAMP)-1 and LAMP-2 are expressed universally, although their abundance varies depending on cell type, activation state and the local microenvironment. Other proteins are more restricted: thus, MHCI class II (MHCII) and co-stimulatory molecules, such as CD86, CD80 and ICAM-1, are largely restricted to exosomes from professional APC$^{15-17}$; while CD31 and CD14 are confined to those from endothelial cells and monocytes, respectively.$^4$ In addition, the exosome lumen carries a rich cargo of soluble molecules sorted specifically into the ILV including proteins (such as antigens) and RNA species (mRNA and miRNAs). Recruitment is highly selective and the targeted molecules are delivered to specialized regions of the MVE membrane either by adapter molecules or through endocytic pathways, and again this varies greatly on the setting.

Exosomes were originally thought simply to be a cellular waste disposal mechanism,$^{18}$ and indeed they do perform this function, but more importantly, they are critical vehicles for short- and long-range intercellular communication, and sometimes utilize specific addressins.$^5,^6$ Exosomes released from some cells remain tethered to the cell surface$^{19}$ or are re-internalized by the cells they originated from. More commonly, they interact with acceptor cells in various ways: molecules expressed in the exosome membrane can directly activate receptors on the surface of acceptor cells, exosomes can be internalized non-specifically by pinocytosis or specifically utilizing endocytic receptors and either traffic directly to lysosomes for digestion or to other intra-cellular locations where they release their luminal cargo. Finally, exosomes can fuse with target cells, thus simultaneously transferring luminal cargo into the cytoplasm and incorporating molecules from its limiting membrane into the plasma membrane of the acceptor cell – in the specific case of MHC molecules, this process is known as “cross-dressing.”

The immune system provides good examples of all three processes.$^6$ Exosomes released from professional APC display pMHCII complex (signal 1) and co-stimulatory molecules (signal 2) on their surface and can carry cytokines as cargo (signal 3), and so they are potentially fully-functional antigen presenting units that can directly ligate the T-cell antigen receptor.$^{20}$ Antigen taken up by receptor-mediated pathways can be selectively routed into exosomes that can be taken up by other APC.$^1$ Finally, exosomes released from DC bind to activated T-cells adjacent to immunological synapses and induce a conformational change in LFA-1 (CD11a) which increases its affinity for ICAM-1 (CD56)$^{21}$ recruiting exosomes that transfer MHCII and CD9 to the T-cell plasma membrane by cross-dressing,$^{16}$ thus inhibiting proliferation of other lymphocytes.$^{22}$ Other examples of exosome-mediated cross-dressing include the transfer of specific pMHC from intestinal epithelial cells that lack co-stimulatory molecules to human monocyte derived DC (MoDC), which are then able to stimulate T-cells$^{23};$ the transfer of pMHC from circulating exosomes to follicular dendritic cells in lymph nodes, which are then able to prime CD4 T-cells$^{24}$ and support B-cell activation$^6;$ and cross-dressing of lymph node stromal cells with specific pMHC from DC-derived exosomes, thus facilitating antigen-specific T-cell tolerance.$^{25}$

Delivery of antigen-specific pMHCII by exosomes

Isolated exosomes were first shown to directly stimulate T-cell proliferation in studies in which EBV-transformed human B-cells were used to follow trafficking of internalized antigen along endocytic recycling pathways.$^{26}$ Purified exosomes pulsed with specific peptide antigen induced proliferation of a heat shock protein 60 (HSp60)-specific CD4 T-cell line.$^{26}$ Similarly, in independent experiments, T-cell lines specific to the major birch pollen antigen Bet v 1A proliferated and secreted Th2 cytokines in response to human peripheral blood B-cell-derived exosomes pulsed with peptide antigen, again in the absence of additional APC.$^{27}$ These studies established that in the right setting, purified exosomes express sufficient antigen-specific pMHCII to activate T-cell antigen receptor signaling and proliferation of the responding T-cell lines. This central finding has been confirmed in subsequent experiments with murine$^8$ and human DC.$^1$ However, APC-derived exosomes do not invariably provide sufficient co-stimulation for direct pMHCII induced T-cell proliferation, and in some settings they need additional support from antigen naïve DC.$^{29}$
Several studies show that murine DC-derived exosomes can stimulate transgenic CD4 T-cells that uniquely express T-cell antigen receptor specific for ovalbumin (OVA) peptide OVA323–339 (OTII cells) to proliferate in vitro, both when directly loaded with OVA323–339 or after pulsing DC with native OVA.28 The peptide loaded exosomes were much more effective in vitro, presumably because more of their pMHCII were loaded with OVA323–339; however, in neither case were additional DC required. By contrast, even high concentrations of exosomes carrying the male H-Y antigen (Dby) failed to stimulate proliferation of naïve Dby-specific T-cells unless unpulsed DC were added.29 Remarkably, MHCII deficient and sufficient DC were both equally effective, whereas DC from CD80- and CD86-deficient mice were unable to support T-cell proliferation. This indicates that the exosomes expressed sufficient specific pMHCII but provided inadequate co-stimulation. However, it is not clear from these experiments whether the exosomes and DC operated in parallel or through cross-dressing of the DC with specific pMHCII. The relative paucity of co-stimulation may also explain why exosomes from DC matured with LPS, and to a lesser extent IFN-γ, are more effective than those from immature DC.28

The way in which antigen-specific exosomes encounter responding T-cells may also influence their ability to induce proliferation. In one study,30 purified peptide-pulsed exosomes were unable to induce proliferation of hemagglutinin-specific T-cell lines in the absence of DC unless cross-linked to cell sized (3.8 μm in diameter) latex beads which decreased the concentration required for proliferation by over a hundred-fold and obviated the requirements for additional DC. The reasons for the increased effectiveness of bead-associated exosomes is not clear but, aside from purely physical differences, cross-linking would greatly increase the local concentrations of pMHC and co-stimulatory molecules at contact points with T-cells, thus providing a possible explanation. A similar effect could also explain our recent data showing that isolated keyhole limpet hemocyanin (KLH) loaded exosomes require additional DC to induce proliferation of naïve human CD4 T-cells, whereas contact with DC is dispensable as shown when DC are separated from responding T-cells into a transwell system.1

Exogenous exosomes also stimulate T-cell proliferation in vivo by delivering antigen-specific pMHCII. Exosomes loaded with OVA-specific pMHCII by incubating murine DC with whole antigen induced OT-II cell proliferation in vivo; whereas exosomes pulsed with OVA323–339 peptide did not, despite being much more effective in in vitro proliferation assays.28 Notably, the administered exosomes in the OT-II model skew immunity toward Th1 responses with high concentrations of IgG2a-2b, and the same occurs in a diphtheria toxin (DT) model31; by contrast, however, injection of native OVA and DT results in IgG1 synthesis. Similarly, exosomes isolated from mast cells pulsed with bovine transferrin induce in vivo the production of IgG1 and IgG2 also in MHC class II mismatched transfer, in contrast with injection of native antigen that did not induce any secondary response.32

### APC-derived exosomes as source of antigen

Exosomes induce adaptive immune responses by delivering specific pMHCII on their membranes and also have the potential to deliver native antigens as luminal cargo, although both processes are closely linked. Endosomes and lysosomes constitute the core antigen presentation machinery in APC. This is a highly dynamic system in constant communication with the extracellular space because exogenous antigens, internalized by pinocytosis, phagocytosis and receptor-mediated endocytosis, are processed while trafficking along distinct endocytic pathways in a journey that determines their antigenic fate.33,34 Moreover, degradation of cytoplasmic proteins by chaperon-mediated autophagy and aggregated host proteins by macroautophagy generate MHCII loaded with endogenous in addition to foreign antigens from phagosomes that constantly fuse with the endo-/lysosomal system antigen.35 Sorting and loading of peptides onto MCHII molecules occurs at the limiting membrane of MVE which then grows out to the plasma membrane of mature DC and transports nascent pMHCII to the cell surface.36 Alternatively, pMHCII can be incorporated into ILV formed by inward budding of the MVE membrane and are subsequently released as exosomes; while other MVE fuse with lysosomes and are degraded.36

Little is known about factors that regulate the balance between the different pathways nascent pMHCII can enter but the route of uptake may be important. For example, particulate antigens phagocytosed in human DC traffic rapidly to lysosomes for degradation, whereas those internalized by a distinct form of phagocytosis dependent on the autophagy protein LC3 (LC3-associated phagocytosis) are digested more slowly and routed selectively to the MHC class II loading compartment (MIIC) resulting in greater expression of pMHCII on the cell surface and enhanced T-cell proliferation.37 Similarly, internalization by certain endocytic receptors including DC-LAMP and DC-SIGN traffic soluble antigens swiftly to the MIIC which enhances T-cell responses,38,39 whereas the macrophage mannose receptor (MMR) traffics slowly to the MIIC and evokes more modest T-cell responses.40 There are no data about whether these endocytic
receptors influence the partitioning of antigen between MIIIC and exosomes or the amount of pMHC expression in the plasma membrane or the exosomes. However, our data on another LAMP family endocytic receptor, LAMP-2, show that it profoundly affects this balance.1

LAMP-2 is a lysosome membrane protein with critical roles in chaperone-mediated autophagy (CMA) and fusion of lysosomes with phagosomes.41 It is essential for transporting cytoplasmic antigens into lysosomal compartments for loading onto MHCII and for CD4 T-cell responses to them.42,43 LAMP-2 is also expressed on the surface of human (but not murine) APC1 and affects responses to exogenous antigens.42,43 Indeed, like other LAMP family members, our recent work shows that MoDC use LAMP-2 as an endocytic receptor that targets internalized antigens and nascent pMHC into exosomes for export from the cell.1 We first showed that a monoclonal antibody to LAMP-2 was rapidly and specifically internalized by MoDC and that its Fab fragments were internalized equally efficiently, thus demonstrating that ligand binding to LAMP-2 rather than antibody-induced cross-linking was responsible for internalization. The candidate antigens KLH and Bet v1 conjugated to the monoclonal antibody to LAMP-2 were internalized much more swiftly than native antigen or antigen conjugated to the isotype control. Detailed analysis using KLH showed it trafficked more swiftly to MIIIC but was retained there more transiently, and consequently that cumulative loading of MIIIC was much less for KLH internalized via LAMP-2 than for native KLH or KLH conjugated to the isotype control. This resulted in loading of significantly fewer KLH-derived peptides into MHC class II molecules that were displayed on the surface. By contrast, significantly more KLH internalized via LAMP-2 was diverted into exosomes that also expressed significantly more MHC class II. This was particularly striking because MoDC expressed significantly less MHC class II on their surface. This, together with the peptidome data, demonstrates that nascent KLH-specific pMHCII were preferentially routed away from the plasma membrane and into the exosome membrane.

The idea of selectively routing KLH-specific pMHC into the exosome membrane is supported by results from T-cell proliferation assays. These show that MoDC that had internalized KLH via LAMP-2 were unable to stimulate T-cell proliferation in standard contact dependent assays, whereas those internalizing KLH by other routes evoked strong primary responses (Figure 2). By contrast, unfractionated EVs consisting of exosomes and microvesicles from the MoDC pulsed with KLH conjugated to antibody to LAMP-2 directly stimulated T-cell proliferation, whereas EV from MoDC that had internalized KLH by other routes were unable to. Purified exosomes from these cells also stimulated T-cell proliferation but only when antigen naive DC were added. The reasons for the difference are unclear, not least because the microvesicles potentially could provide additional co-stimulation or act as a surface on which exosomes could aggregate, analogous to the latex beads.30 Regardless of the precise mechanism, the experiments establish the principle that LAMP-2-mediated endocytosis re-routes nascent antigen-specific pMHCII away from the plasma membrane for expression in the membranes of exosomes that also carry native antigen as cargo.1 It will now be important to ascertain whether other endocytic receptors for antigen selectively influence routing of pMHCII and antigens into exosomes.

Delivery of antigen by exosomes in vivo

Exosomes have repeatedly been shown to deliver antigen in vivo. For example, endosomes bearing an MHC class II allopeptide (IE\textsubscript{E52-68}) bind to \(\alpha\varepsilon\) (CD51)/\(\beta\varepsilon\) (CD61) integrin on murine DC and are internalized together with CD11a (LFA-1), CD9 and CD81. The IE\textsubscript{E52-68} traffics to the early endosome and then to the MHCII where it is loaded onto MHCII and efficiently presented by DC both in vivo and in vitro to restricted CD4 T-cells independently from the MCHII present onto the exosomes.44 Similarly, rejection of murine heart transplants is dependent on release of exosomes from donor DC which are taken up by host DC in local lymph nodes\textsuperscript{45} while activated human lymphatic endothelial cells secrete exosomes that express C-X-C chemokine receptor type 1 (CXCR1) ligand increasing migration of DC.46 Finally, iDC internalize exosomes released from mast cells pulsed with BSA or OVA via CD91 dependent pathway and this promotes their maturation and enables them to induce efficient CD4 T-cell proliferation\textsuperscript{45}; by contrast, exosomes from macrophages and B-cells were ineffective.

Biogenesis of exosomes

Multivesicular endosomes are factories where exosomes are generated and loaded with cargo. The quantity and composition of exosomes released from cells are governed by two intimately linked processes that occur at the MVE membrane: loading of specific molecules into the membranes and lumens of developing ILV; and furnishing of the MVE membrane with the cues that dictate whether it traffics to lysosomes for destruction or to the plasma membrane to release exosomes. These processes are increasingly known in outline, but many of the critical steps have yet to be defined, not least the loading of molecules directly affecting immunity.
Morphologically identical MVE can differ greatly in lipid content, and this correlates with their fate: MVE rich in the phospholipid metabolite lysobiphaditic acid (LBPA) but low in cholesterol are biased to migrate toward lysosomes and to fuse with them, whereas those rich in cholesterol but low in LBPA selectively traffic to the plasma membrane and fuse with it to release their ILV as exosomes. Notably, ubiquitinated pMHCII retrieved from the plasma membrane of immature DC are routed selectively into the ILV in LBPA-rich MVE that traffic to lysosomes for degradation; by contrast, pMHCII from mature DC is not ubiquitinated and is routed directly from MIIC to the cholesterol-rich MVE and subsequently discharged as exosomes.47

More than half of the cholesterol present in the endocytic pathways is associated with secreted ILV (exosomes), suggesting that high cholesterol content might be necessary for routing MVE to release exosomes.49 In line with this, saturation of oligodendroglial cells with a soluble cholesterol analog increases release of exosomes50 and treatment with an inhibitor of lysosomal cholesterol export (U18666A) inhibits exosome-dependent release of viral particles.51 By contrast, exosome release was increased by acute cholesterol depletion induced by methyl-β-cyclodextrin (MBCD).52 These apparently contradictory results demonstrate the complexity of the influence of the cholesterol pathway and are also reflected in our system in which U18666A-treated MoDC incorporated more KLH into exosomes by endocytosis using LAMP-2 as a receptor but less LAMP-2. Instead, exosomes released from them induce strong proliferation in the presence of antigen naïve accessory MoDC, either because they ligate T-cell antigen receptor directly or because they “cross-dress” the plasma membranes of the accessory DC after fusing with them.

Figure 2. Antigen endocytosed by LAMP-2 is diverted into exosomes that express antigen specific pMHCII. Upper panel: After internalization by human monocyte derived dendritic cells (MoDC), native keyhole limpet hemocyanin (KLH) and KLH conjugated to mouse IgG (isotype*KLH) traffic to the MHC class II loading compartment (MIIC) where KLH-derived peptides are loaded onto class II molecules (pMHCII). Nascent KLH-specific pMHCII traffics to the plasma membrane where it ligates the T-cell receptor and stimulates CD4 T-cell proliferation; exosomes released from these MoDC do not induce T-cell proliferation. Lower panel: KLH conjugated to an antibody to LAMP-2 (anti-LAMP-2*KLH) and internalized using LAMP-2 as an endocytic receptor also traffics to MIIC. However, nascent KLH-specific pMHCII – and antigen – traffics selectively to multivesicular endosomes (MVE) rather than to the plasma membrane and is exported in exosomes; these MoDC express little KLH specific pMHCII on their surface and do not stimulate CD4 cell proliferation. Instead, exosomes released from them induce strong proliferation in the presence of antigen naïve accessory MoDC, either because they ligate T-cell antigen receptor directly or because they “cross-dress” the plasma membranes of the accessory DC after fusing with them.
rich microdomains on the MVE membrane and are critical to both major pathways used to load molecules into ILV: the endosomal sorting complex required for transport (ESCRT) dependent ALIX/heparanase pathway (Figure 3a) and the ESCRT independent pathway based on flotillin/ceramide/sphingosyne 1 phosphate (S1P) (Figure 3b).

The ESCRT pathway internalizes multiply ubiquitinated proteins destined mainly for degradation in lysosomes and comprises over 20 proteins assembled into four complexes: ESCRT-0 that bind ubiquitinated proteins at the endosomal membrane; ESCRT-I and ESCRT-II that induce inward membrane deformation and bud formation; and ESCRT-III which is responsible for the cutting leading to the release of the vesicles into the endosome lumen. Thus, the ESCRT complex both selects and incorporates target molecules into ILV. For example: ubiquitinated pMHC retrieved from the plasma membrane is recognized by ESCRT-0 and sorted into MVE destined to fuse with lysosomes.53 ESCRT-0 also binds substrate tagged with other ubiquitin-like molecules, such as SUMO (small ubiquin-like modifier) and ISG15 (interferon-stimulated gene 15): for example, SUMOylation of hnRNPA2B1 (heterogeneous nuclear ribonucleoprotein A2B1) enables it to bind to specific miRNAs and to sort them into ILV destined to be released as exosomes.54 By contrast, ISGylation of the ESCRT I component TSG101 (tumor susceptibility gene 101) decreases exosome release from multiple cell lines including monocyte-derived macrophages.55 Conjugation of ISG15 to TSG101 in the MVE membrane induces their aggregation and consequently the MVE movement to lysosomes and autophagosomes for degradation independently of the remaining ESCRT machinery. Notably, type 1 interferon (IFN)-a/b) activate the ISGylation pathway to promote viral destruction within lysosomes while minimizing release of viral particles into the extracellular environment.56

The functions of the 23 proteins that compose the ESCRT pathway have been analyzed individually using RNAi-mediated knock down in a HeLa cell line stably expressing HLA-DR (HeLa-CIITA).57 Knock down of ESCRT-0 components HRS and STAM1 or the ESCRT-1 component TSG101 caused a global reduction of exosome release, as well as the abundance of MHCII and CD63 within them. By contrast, knock down of the ESCRT-associated protein VPS4B enhanced exosome release, presumptively by facilitating the degradative pathway. The study also highlighted the effect of individual ESCRT components on exosome heterogeneity. The HeLa-CIITA exosomes vary in size and CD63 and MHCII expression. Knock down of HRS reduced the total number of exosomes released without affecting those smaller than 50 nm; whereas knock down of

![Figure 3. (a) Syndecan, ALIX pathway for exosome biogenesis. Syndecans bind exogenous ligands through heparin sulfate; the whole complex is internalized into endosomes where heparans cleave the long heparin sulfate releasing C-terminal fragment (CTF), allowing the clustering of syndecan on the surface of endosomes that recruit syntenin and ALIX, inducing invagination and ILV formation involving the small GTPase ARF675 and the recruitment of ESCRT-III induces scission releasing the ILV containing the cargo, CTF, syntenin and ALIX. (b) ESCRT-independent pathway for exosome biogenesis. Aggregation of flotillin within lipid-rich domains in the plasma membrane induces endocytosis of cargo, in this case myelin proteolipid protein (PLP) from the surface of oligodendroglial cells. Sphingomyelinase digests sphingomyelin to ceramide in the endosomal membrane, facilitating flotillin dependent inward budding with formation of intraluminal vesicles (ILV) and the development of multivesicular endosomes (MVE). In parallel, ceramide is digested to sphingosine which is phosphorylated by sphingosine kinase to sphingosine-1-phosphate (S1P) and activates the S1P receptor to promote ILV maturation.](image-url)
TSG101 or STAM1 reduced their number. In addition, STAM1 knock down increased the number of double negative exosomes, suggesting its critical role in sorting CD63 and MHCII into exosomes. Knock down of apoptosis-linked gene-2 interacting protein X (ALIX), an ESCRT-associated protein, increased the intracellular abundance of MHCII in human DC, presumably by interrupting its normal loading onto ILV destined for lysosomal degradation, but without changing its abundance in released exosomes. However, depletion of ALIX in HeLa-CIITA cells results in accumulation of MHCII within exosomes, again highlighting cell type-/line-specific differences. Results of studies of exosome release by DC from mice genetically deficient for Hrs (the mouse equivalent of HRS) are somewhat confusing, especially with regard to MHCII (I-A$^\beta$) loading. ALIX is also involved in a recently defined ESCRT-dependent pathway, in which cargo selection is determined by the enzyme heparanase. Syndecan is the main heparin sulfate bearing proteoglycan expressed in the plasma membrane and has a short cytoplasmic tail that binds syntenin which links it to the cytoskeleton. Syndecan internalization occurs only after its polymeric heparin sulfate has been degraded to oligosaccharides by heparanase, which facilitates its aggregation. Syntenin bound to the cytoplasmic tail of syndecan recruits ALIX and links it to an ESCRT III subunit (CHMP4): this induces scission of budding vesicles both at the plasma membrane and on MVE. Overexpression of syntenin increases incorporation of syndecan and ALIX into exosomes, together with CD63. Silencing syntenin reduces the abundance of ALIX and CD63 without affecting a separate subpopulation of exosome characterized by the expression of flotillin. Interestingly, proteoglycan internalization is independent of clathrin and caveolin, but absolutely dependent on flotillin which promotes inward budding of membranes.

Flotillin is a scaffold protein concentrated in lipid-rich microdomains in the plasma membrane and is associated with clathrin- and caveolin-independent endocytosis. It also accumulates on the surface of MVE and is essential also for the flotillin/ceramide/SIP pathway that loads molecules into ILV to be released as exosomes. The pathway is functionally distinct and independent of the ESCRT ubiquitin-binding complexes, as exemplified by studies of oligodendroglial cells. In these cells, ubiquitinated epidermal growth factor receptor is retrieved from the plasma membrane and routed via the canonical ESCRT pathway into MVE that are then degraded by lysosomes. By contrast, myelin proteolipid protein internalization is uniquely dependent on flotillin and it is sorted into flotillin and ceramide-rich ILV that are released as exosomes. Inhibition of ceramide generated from sphingomyelin with a neutral sphingomyelinase inhibitor attenuates proteolipid protein incorporation into ILV, as well as that of CD63, and flotillin. Ceramide is also a precursor of S1P which recruits the tetraspanin CD63 to exosomes without affecting their size or quantity, demonstrating its importance in selecting cargo for this pathway.

Recent studies suggest that there may be yet other exosome loading pathways in which tetraspanins are critical. For example, it is essential for the luminal domain of pre-melanosome protein to interact CD63 for it to be loaded into melanocyte exosomes via an ESCRT and ceramide-independent pathway because in its absence pre-melanosome protein containing ILV are routed to lysosomes and digested. This identifies new possibilities for manipulating exosome biogenesis to influence adaptive immune responses. It also emphasizes the need for more knowledge.

**FINAL REMARKS**

Advances over the past decade have highlighted the ability of exosomes from human and murine professional APC both to augment specific immune responses and to facilitate tolerance, making them an obvious potential therapeutic target. Antigen-specific pMHCII on the surface of exosomes activates T-cell antigen receptor on CD4 T cells and, depending on the setting, stimulates them to proliferate directly or in the context of accessory DC. The requirement for additional DC is usually dictated by the abundance of co-stimulatory molecules expressed in the exosome membrane rather than the pMHCII. This emphasizes the heterogeneity of exosomes which is becoming increasingly apparent.

Much has been learnt about the biogenesis of exosomes and the nature of their heterogeneity. However, the pathways used to load specific molecules onto the limiting membrane remain incompletely defined, as do those responsible for the selective loading of luminal cargo. Open questions remain about the extent to which endocytic receptors other than LAMP-2 can be utilized for specifically loading antigen and antigen-specific pMHCII onto exosomes, and how expression of co-stimulatory molecules can best be manipulated. It will be equally important to optimize ways to prevent the non-specific removal and destruction of administered exosomes by phagocytes, for example by expressing “don’t eat me” molecules such as CD47; and for targeting them to cells of interest using specific addressins.

Finally, in addition to their direct effect on adaptive immune responses, exosomes (and microvesicles more generally) have potential as biomarkers for disease activity and as vectors for targeting gene therapy to specific cells.
This emphasizes the need for deeper knowledge about the biogenesis, secretion and uptake of exosomes as a foundation for novel specific therapeutic strategies.

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CONFLICT OF INTEREST

The authors declare that they have no competing financial interests.

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