

Extracellular vesicles : lipids as key components of their biogenesis and functions

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Intercellular communication has been known for decades to involve either direct contact between cells or to operate via circulating molecules such as cytokines, growth factors, or lipid mediators. During the last decade we have begun to appreciate the increasing importance of intercellular communication mediated by extracellular vesicles released by viable cells either from plasma membrane shedding (microvesicles, also named microparticles), or from an intracellular compartment (exosomes). Exosomes and microvesicles circulate in all biological fluids and can trigger biological responses at a distance. Their effects include a large variety of biological processes such as immune surveillance, modification of tumor microenvironment, or regulation of inflammation. Extracellular vesicles can carry a large array of active molecules, including lipid mediators such as eicosanoids, proteins and nucleic acids, able to modify the phenotype of receiving cells. This review will highlight the role of the various lipidic pathways involved in the biogenesis and functions of microvesicles and exosomes.

1. Exosome and microvesicle biogenesis: is it an interconnected process ? (Figure 1)

Exosome biogenesis begins by a process of microautophagy (1) occurring at the level of late endosomes (Figure 1). This process ends by an inward budding of the endosome membrane generating the accumulation of nano-vesicles (30-150 nm diameter) inside the late endosome subset of **MutiVesicular Bodies** (MVB). Moving along the microtubules by a dynamic process regulated by cholesterol (2) MVBs reach the plasma membrane, fuse with it and release their content of intraluminal vesicles (ILVs) which become named exosomes (3). A comprehensive review updating the cell biology of extracellular vesicles was recently issued (4). However the difference between intraluminal vesicles and exosomes might be not just a matter of name since intraluminal vesicles are generated in an acidic pH environment (pH5.5) inside the MVB, and they are released into a neutral pH environment to become “exosomes”. Such a pH variation might affect their membrane organization because it has been shown that the membrane rigidity of exosomes increases between pH 5 to 7 (5).

ILV biogenesis involves interactions between the protein sorting machinery of the MVB membrane, *i.e* the Endosomal Sorting Complex Required for Transport (ESCRT), and various lipidic molecules. Associated with the ESCRT complexes I-III are the **Vacuole protein sorting Vps4** and **Alix (ALg2-Interacting protein X)**. Vps4 has been shown in yeast to interact with the oxysterol binding proteins Osh6 and Osh7 (6). Oxysterols have specific pharmacological and physiological properties (7). Alix is recruited on the endosome membrane *via* a specific domain binding the endosomal lipid **Bis(Monacylglycero)Phosphate (BMP)(8)**, also inappropriately named LBPA for **Lyso Bis Phosphatidic Acid**. An ESCRT-independent pathway involving ceramides and the neutral sphingomyelinase 2 has also been characterized (8). Remarkably another sphingomyelinase, the acid sphingomyelinase, is involved in microvesicle formation following translocation of the enzyme to the plasma membrane outer leaflet where it generates ceramides triggering microvesicle budding (10).

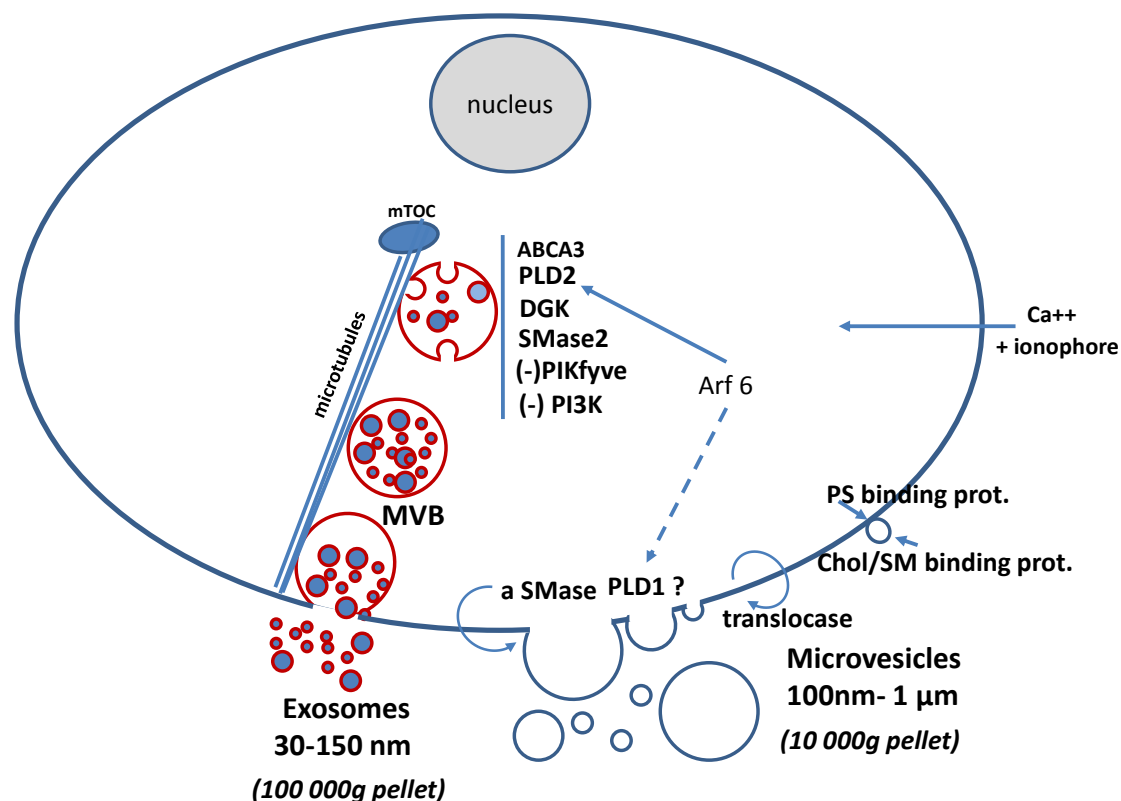


Figure 1. Lipid-related partners of exosome and microvesicle biogenesis.

Exosomes and microvesicles (also called ectosomes or microparticles) are recovered by ultracentrifugation from viable cells. Microvesicles sediment in the 10 000g pellet and their size ranges between 100 to 1000 nm. The 10 000g supernatant is then used to recover exosomes by a 100 000 g centrifugation (11); exosome size ranges from 30 to 150 nm, with an average around 100 nm. Some markers to discriminate between these two types of extracellular vesicles have been reported (12-14). MVB: **M**ulti**V**esicular **B**odies; mTOC: **m**icro**T**ubule **O**rganizing **C**enter.

Enhanced exosome production involves lipid transporters such as ABCA3 (15) and requires the activities of phospholipase D (PLD2)(16), diglyceride kinase (DGK)(17), neutral sphingomyelinase (9) but the inhibition of phosphoinositide kinases such as the PI3kinase (18, 19) and PIKfyve (20). Translocation on the outer leaflet of the plasma membrane of the acid sphingomyelinase (aSMase) promotes the budding of microvesicles (10). This budding process also involves the small G proteins such as Arf6, RhoA (21), which are activators of PLD1 and PLD2, supporting the proposition that in some situations the production of both exosomes and microvesicles could be coordinated via PLD activity. Microvesicles can also be produced by modification of plasma membrane asymmetry by the aminophospholipid translocases (22), or by modification of the lateral pressure of phospholipids *via* phosphatidylserine binding protein on the inner leaflet (23) or sphingomyelin/cholesterol binding protein (24) on the outer leaflet. Calcium loading into cells by means of a calcium ionophore can trigger the production of microvesicles (25) or exosomes (26). MVB and exosomes are circled in red in the figure to represent the Bis(Monoacylglycerol)Phosphate (BMP) content of their membrane, which definitely discriminates between exosomes and microvesicles because BMP intracellular localization is strictly restricted to late endosomes and lysosomes (27) (28).

More recently another pathway for exosome biogenesis involving the syndecan/syntenin complex has been characterized. This pathway requires the activity of the phospholipase D2 (PLD2) (16). Indeed the inactivation of PLD2 prevents the formation of intraluminal vesicles inside the MVBs (16). Phospholipase D (PLD) activities could be a coordinating process between exosomes and microvesicle formation. PLD2 is activated by the small G protein Arf6, and interestingly, Arf6 is also involved in microvesicle formation from plasma membrane shedding (22). Arf6 activity leads to the localization of the myosin-light chain kinase at the neck of the newly forming vesicles, promoting their release by fission from the plasma membrane. The plasma membrane contains mainly the PLD1 isoform. Although

Arf1 is the most common PLD1 activator, stimulation by Arf6 has also been reported (29). Thus, a coordinated secretion of exosomes and microvesicles involving Arf6/Arf1/PLD2/PLD1 pathways deserves further study. These pathways could occur in tumor cells, which constitutively release exosomes and microvesicles. It is known that PLDs are overexpressed in cancer cells (30). However, the pioneering study showing that the same cell can produce both exosomes and microvesicles was performed using activated platelets (31).

Disruption of the plasma membrane lipid organization appears critical to allow microvesicle (microparticle) formation. Indeed modification of the outer membrane leaflet by a cholesterol/sphingomyelin binding protein promotes microvesicle formation (24), which can occur following outer membrane sphingomyelin hydrolysis (10). On the inner leaflet, proteinase 3, an autoantigen that binds to phosphatidylserine (PS) promotes the formation of microvesicles (23). Translocation of PS to the outer leaflet upon cellular activation is a prerequisite for microvesicle biogenesis (25, 32). This occurs during platelet activation by natural agonists or calcium ionophore (32); notably, a calcium ionophore also promotes exosome production (26).

In summary, although the biogenesis of microvesicles and exosomes may be coordinated, the molecular pathways involved in their respective formation appear different. Indeed, discrimination between microvesicles and exosomes can be demonstrated through use of a lipid marker (13) the Bis(Monoacylglyero)Phosphate (BMP) which is present on the peripheral membrane of the MultiVesicular Bodies (MVB) (28) and on the intraluminal vesicles (33). BMP has been shown to trigger the inward budding of a reconstituted MVB membrane *in-vitro*, leading to the formation of intra-liposomal vesicles similar to the ILVs observed in cells (34). In this respect, we previously reported the presence of BMP in exosomes with a similar amount as in the parental cells, *i.e.* about 1% of total phospholipids (5). However, a recent report indicated an enrichment of BMP on exosomes released upon inactivation of the class III PI3 kinase Vps34 in parental cells (19). On the other hand, no BMP labeling has been observed in the cell plasma membrane by immunoelectron microscopy (28) or by immunostaining using the highly specific anti-BMP antibody 6C4 (27,35,36), thus ruling out the possibility that this lipid is present on microvesicles. Moreover, the negatively charged BMP could account for the increase in exosome electronegativity as compared with the plasma membrane. Indeed exosomes (77-93 nm diameter) (26) derived from rat mast cells exhibited a zeta-potential comprised between -12.9 to -15.7 mV whereas that of parent cell was of -8,5 mV (26). In summary, both the BMP content and electronegativity may allow discrimination between vesicles having endosomal versus plasma membrane origins.

2. Exosomes and microvesicles concentrate signaling molecules in distinct populations from multiple biogenesis pathways (Figure 2).

Exosomes were originally described about 30 years ago as a mechanism for clearing unneeded molecules from reticulocytes during their maturation into erythrocytes (37). This led to the concept that the function of exosomes was to eliminate biological material. The idea expanded when exosomes were shown to participate in immune surveillance (38) and to transport molecules such as miRNA and mRNA (39) able to initiate functional responses in target cells. In the meantime it was shown that lipid molecules and related enzymes can participate in the signaling function of exosomes because they carry eicosanoids together with functional phospholipases and enzymes of the prostaglandin (3,26) and leukotriene pathways (41). The same conceptual evolution occurred for microvesicles, moving from the idea of "artefact no more" (40) to the demonstration of their involvement in various biological events (22).

Comparative proteomic and lipidomic analysis of exosomes and microvesicles has identified about 3500 proteins and 2000 lipid species (42). A recent review of lipids in exosomes has been released (43). Also it was reported that exosomes contained 1300 mRNAs (i.e about 10% of mRNAs from the parent cells) and 120 miRNAs (39) together with various other types of RNAs and of double-stranded DNA fragments (44). The presence of so many molecules in a single type of vesicle seemed unlikely. Instead, it has been shown that the bulk of material isolated as an “exosome pellet” or a “microvesicle pellet” is representative of several populations of microvesicles or exosomes (45) (46) (47) (14) .

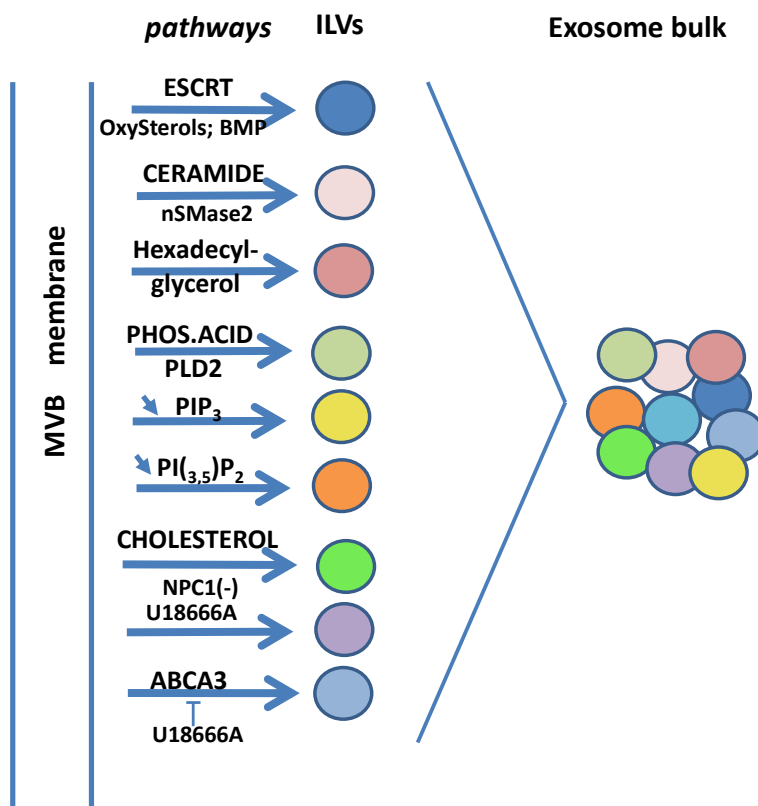


Figure 2. Lipid pathways involved in exosome biogenesis processes

(MVB=MultivesicularBody; ILVs=intraluminal vesicles).

Connections with lipid pathways are present within the Endosomal Sorting Complex Required for Transport (ESCRT), the first pathway described for ILV biogenesis (46). Indeed Vps4 a member of the AAA family of proteins (ATPases Associated with a variety of Activities) interacts with oxysterol binding proteins (Osh6 and Osh7) (6), and Alix (ALG2-Interacting protein X) interacts with BMP (Bis[Monoacylglycerol]Phosphate) *via* a specific sequence domain (8). Independent of the ESCRT machinery are lipid pathways involving ceramides (nSMase 2) (9) and to some extent phosphatidic acid (phos.acid /PLD2) (16). Another neutral lipid, precursor of ether-linked phospholipids, hexadecyl-glycerol, stimulates exosome production (48). The phosphoinositides PIP₃ and PI_{3,5}P₂ act in a negative way since inhibition of PI3K/Akt (18, 19) and PIKfyve (20) favor exosome production. Because PI3K is involved in macroautophagy (49), this observation indicates that there is a requirement to block macroautophagy to promote the microautophagy process involved in exosome biogenesis (50). Indeed, rapamycin, the inhibitor of mTORC1 (mammalian Target Of Rapamycin) induces macroautophagy (50, 51) and decreases exosome production (15). However an alternative pathway for exosome production via a secretory autophagy process, distinct from the degradative autophagy, has been reported following PIKfyve inhibition (20). Because PIKfyve activity promotes mTORC1 translocation to the plasma membrane (52), the reverse PIKfyve inhibition might retain mTORC1 on the late endosome membrane. mTORC1 is located on the endosome membrane (53) and could have a direct function at this location to generate ILVs. mTORC1 is activated by the endosomal cholesterol (53) and an enhanced exosome production was reported when cholesterol was supplied to glial cells either directly or by means of U18666A (54). mTORC1 activation by cholesterol is reversed by NPC1 which

effluxes cholesterol from endosomes (53). Consistently NPC1 inactivation is required to enhance exosome production (54). U18666A displays an opposite effect on lymphoblastoma cells where it inhibits exosome production, which is mediated in these cells by the lipid transporter ABCA3 (15). It is worth noting that NPC1 and ABCA3-containing endosomes are distinct populations (55). In addition, the effect of U18666A cannot be restricted to cholesterol since U18666A also promotes the accumulation of oxysterols in cells (cholesterol 5,6 epoxides) and cholesterol precursors (zymosterol, desmosterol) (56).

In the figure each pathway leading to a specific vesicle content is a graphical simplification. Combination of the various pathways reported could depend upon cell type and activation conditions or mobilization of distinct late endosome populations, and several pathways can probably be triggered at the same time.

The existence of these various exosome populations suggests that there are distinct biogenesis pathways for different populations (Figure 2). It has been shown that exosomes containing miRNA were preferentially produced *via* the SMase2/ceramide pathway, independently of the canonical ESCRT pathway (57). Mobilization of the various pathways reported in the Figure 2 might operate differently according to the cell type and cell activation conditions, making a combination of pathways leading to a combination of exosomes populations constituting the bulk of exosomes. In that respect the ESCRT-associated member Alix binds the endosomal lipid BMP (8) but also syntenin, which interacts directly with phosphatidic acid produced by the PLD2 (16). Whereas the syndecan/syntenin pathway enhanced angiopoietin-containing exosome secretion from endothelial cells, the PI3K/Akt/eNOS (endothelial Nitric Oxide Synthase) pathway had an opposite effect in the same cells (18) reducing angiopoietin secretion. Thus, the two opposite pathways promote the release of distinct exosome populations with different contents including angiopoietin, which plays multiple roles in blood and lymphatic vessel growth. Differential labeling of exosome lipids and proteins had already indicated the existence of various exosome populations (45). This observation was confirmed by inactivation of different elements of the ESCRT machinery (46, 47) and by means of fine-tuned gradient fractionations to accurately characterize exosomes and microvesicles populations (14). With respect to microvesicles, it is also conceivable that distinct parts of the plasma membrane generate several different microvesicle populations.

Microvesicles have lose the plasma membrane phospholipid asymmetry of the parent cell (25). Consequently microvesicles expose their phosphatidylserine (PS) on the outer membrane leaflet. Similarly, exosomes lack transmembrane phospholipid asymmetry as demonstrated by the presence of 50 to 70% of aminophospholipids (PE and PS) on the outer leaflet (5). This is consistent with the presence of a phospholipid scramblase in the exosome membrane (26, 58) and with the rapid flip-flop reported between the two membrane leaflets (5). Consistently, exosomes were shown to be stained by annexin V (59). Only some transmembrane asymmetry of aminophospholipids was reported in exosomes derived from guinea pig reticulocytes (60), although how this asymmetry was maintained was not determined. Indeed, exosomes lack an organized cytoskeleton that participates in maintaining the phospholipid asymmetry of the cellular plasma membrane.

Exosomes are enriched in disaturated molecular species of phospholipids (5, 9, 61). This accounts in part for their increased membrane rigidity relative to parent cell membranes. The high ratio of protein/lipid in exosomes (62, 63) also enhances their membrane rigidity. The increased membrane rigidity of exosomes could be important in ensuring that exosomes are not easily degraded and are readily able to circulate in biological fluids (64). Consistently, exosomes have been found to be more resistant than microvesicles toward detergent treatments, representative of a higher lipid order (63). The only elimination process reported so far for circulating exosomes occurs *via* their surface lipid lysophosphatidylcholines which bind IgM-type immunoglobulins. This facilitates interaction with apoptotic circulating cells whose elimination can lead to exosome clearance (65). Pharmacokinetics of injected exosomes in mice revealed an average half-life of 4 min with 10% of the initial amount remaining at 4 hours (66). In contrast, direct degradation of microvesicles can occur in the circulation

because their membrane lipids can be hydrolyzed by secreted phospholipases (25, 67) suggesting a shorter half-life than exosomes in biological fluids.

In summary, many lipid-related pathways appear to be involved in the biogenesis of exosomes (Figure 2). Mobilization of these pathways may depend upon the type of parent cell, the nature of the initial stimulus, and the micro-environment, making possible combinations of biogenesis pathways with leading to vesicles with diverse contents. Material packaging into exosomes and microvesicles appears as a highly dynamic process.

3. Exosomes and microvesicles are intercellular transporters of lipid molecules and related enzymes involved in various pathophysiologies (Figure 3).

The interaction of exosomes and microvesicles with surrounding cells is a stochastic process (*i.e* it is both random and organized). The vesicles randomly move in the intercellular space, or in biological fluids until they find a way to interact with cells. If they fail to interact, they are eliminated by degradation or clearance. Interaction with cells is the “organized process” which can involve either receptor mediated endocytosis, macropinocytosis, or membrane fusion (Figure 3).

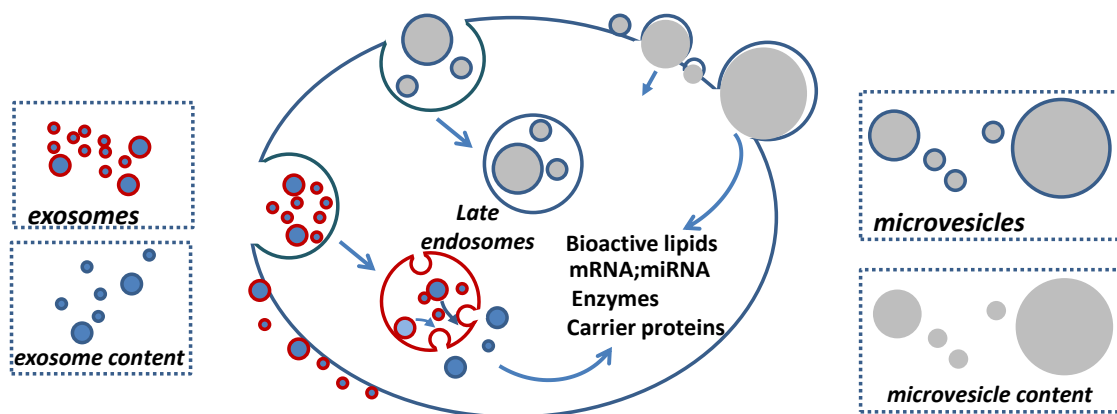


Figure 3. Exosome and microvesicle-mediated transfer of biologically active material into recipient cells.

Importantly, both exosomes (39) and microvesicles (22) carry functional molecules able to modify the phenotype of recipient cells. Exosomes are preferentially endocytosed and may release their contents by fusion with the recipient endosomal membrane by a process called back-fusion (8). This may be mediated by the BMP (highlighted in red) present both on the exosome and endosome membrane, because BMP is fusogenic in an acidic pH environment (33). Even though some microvesicles are internalized by receptor-mediated endocytosis (68), the microvesicle membrane is devoid of BMP and how microvesicles transfer their contents inside target cells is unclear. Instead the fusion of large microvesicles with the peripheral cell membrane has been observed (69) and fusion between microvesicles and the cellular plasma membrane might represent the preferential transfer mechanism of material from microvesicles to recipient cells.

Because exosomes and microvesicles expose peripheral PS, they can interact with cells exposing the PS receptors Tim1 or Tim4 on their surfaces (70). Exosomes released from activated T cells and internalized via the PS receptor on monocytes have been shown to induce cholesterol accumulation, suggesting that exosomes could be an atherogenic factor (71). Other endocytic receptors are involved in exosome internalization by recipient cells, such as the scavenger receptor CD36 (72) or the Low density lipoprotein receptor-Related Protein 1 (CD91/LRP1) (73), which binds the heat-shock proteins Hsp90 or

Hsp70 that are enriched in exosome membranes (3). Interestingly LRP1 regulates phosphorylation of cPLA2 as well as cholesterol efflux (74).

Microvesicles recovered in the 10 000g pellet from ATP-activated microglia cells have been shown to stimulate synaptic activity in neurons by activation of sphingolipid metabolism (75). Microglia cell activation provokes the acid sphingomyelinase to move to the plasma membrane outer leaflet where it generates ceramides that trigger microvesicle budding (10). Functional effects of microglia-derived microvesicles involve lipid components of their membrane (75) because liposomes prepared from the microvesicle lipid extract reproduce the effects. Microvesicle treatment with annexin V prevented their biological effect suggesting that the PS receptor is required for their interaction with and activity on the target cells (75). Remarkably, microvesicles released from ATP-stimulated microglia cells carry the endocannabinoid molecule N-arachidonyl ethanolamine (anandamide), and such microvesicles are able to activate the type 1 cannabinoid receptor CB1 (76). Microglia cells released both microvesicles and exosomes, but the anandamide was three times more enriched in microvesicles than in exosomes (76).

In platelet-derived microvesicles (microparticles) the lipid component triggering the functional effect was identified as being 12-HETE (12[S]-hydroxyeicosatetraenoic acid) (68). BLT2 is the HETE receptor that mediates the internalization of microparticles into neutrophils (68). In this work microparticles are designated as microvesicles with an average size of 350 nm. HETE biosynthesis occurs in platelet microparticles themselves. The successive steps of this process involve the action of a phospholipase A2 (secreted sPLA2-IIA) present in the extracellular milieu that acts on phospholipid microparticles to release free arachidonic acid that is then converted to HETE by the 12-lipoxygenase present in microparticles (68). Interestingly, this study suggests a possible cooperation between exosomes and microparticles. Activated platelets release both exosomes and microvesicles (31), and it has been shown that exosomes carry the sPLA2-IIA (26). Exosomal sPLA2-IIA could act on microparticle phospholipids to release arachidonic acid required for HETE formation, leading to amplification of inflammation in arthritis (77). Platelet microparticles are a critical component of inflammation and immunity-induced thrombotic disorders (78). In addition to its importance in arachidonate mobilization, PLA2 activity generates lysophosphatidylcholine (LPC) that acts as a substrate for autotaxin activity. Autotaxin generates lysophosphatidic acid (LPA) that acts as an agonist for cell surface G-protein-coupled receptors. Autotaxin has been demonstrated to be bound to a population of exosomes (79). Thus, the PLA2 associated with microparticles and/or exosomes promotes activation of two (or more) signaling cascades.

Exosomes carry functional enzymes related to eicosanoid biosynthesis as well as their products. Antigen presenting cell (APC)-derived exosomes from human macrophages and dendritic cells (DC) incubated with the leukotriene precursor LTA4 synthesized the end-products LTB4 and LTC4 in amounts exceeding those generated by the parent cells (40). Exosomes contain enzymes of the leukotriene pathway (i.e. 5-lipoxygenase (weakly active), LTA4 hydrolase and LTC4 synthase). Activated APC-derived exosomes promoted granulocyte migration (40). Exosomes recovered from fluids of patients with allergic asthma contributed to leukotriene production (80).

Exosomes also transport prostaglandins. Exosomes produced from rat basophil leukemia cells contain three types of phospholipases A2 (cPLA2, iPLA2, sPLA2) and cyclooxygenases-1 and -2 (COX-1 and COX-2). These exosomes are also enriched in free fatty acids (including arachidonic acid) and in the immunosuppressive prostaglandin PGE2 (26). Tumor-derived exosomes enriched in PGE2 participate largely to turn the tumor micro-environment into one that permits tumor growth. Indeed PGE2 inhibits

immune responses towards the tumor at different steps of its development (81). For instance PGE2-enriched exosomes released from the intestinal mucosa can be transferred to the liver where they induce energy of Natural Killer T cells (82).

Other enzymes related to fatty acid metabolism play a role in exosome function. Exosomes derived from adipocytes supply to melanoma cells two enzymes of the β -oxidation pathway (83), namely enoyl CoA hydratase (ECHA) and hydroxyacyl-CoA dehydrogenase (HCDH). Adipocyte exosome internalization into melanoma cells increased fatty acid oxidation and promoted melanoma cell migration. Cell migration was suppressed upon inhibition of fatty acid oxidation (83). Thus exosomes may be central in linking obesity and cancer (84). Beside enzymes related to fatty acid metabolism, exosomes also carry lipid kinases such as diacylglycerol-kinase (85) or sphingosine kinase that promote hepatocyte proliferation and liver regeneration (86). Exosomes also contain phospholipid hydrolases such as phospholipases (26, 65), and the phosphoinositide (PIP3)-phosphatase (PTEN) (87). Secretion of PTEN by exosomes requires its ubiquitination, and PTEN retains its PIP3 phosphatase activity when exosomes are transferred into recipient cells (87). Consequently exosomes can participate in reducing tumor growth through their delivered PTEN content. Whereas some exosomes contain PTEN, others derived from astrocytes in the central nervous system carry miRNAs triggering PTEN degradation in brain infiltrating tumor cells, allowing tumors to develop in the brain (88). Thus, the delivery of different exosome contents to recipient cells can produce opposing physiological outcomes.

Conclusion:

In summary, both exosomes and microvesicles contribute their lipid molecules or their lipid-related enzymes to several pathophysiologicals, including inflammation, tumor development, and atherogenesis. However, extracellular vesicles also act positively within the immune system in lymph nodes (89) or in the physiology of brain through neuron activation (10) and endocannabinoid transfer (76). Whereas research on exosomes and microvesicles has previously been conducted somewhat independently by distinct scientific communities, more recent publications deal with parallel studies on both types of vesicles and have demonstrated that both be produced by the same cells, suggesting a possible coordinated biogenesis. Exosome and microvesicle functions appear either complementary or in opposition. Complementary, for instance, in the case of transfer of oncogenic material to recipient cells (90) but in opposition in the immune system. Indeed exosomes act positively in the immune response by extending the duration of antigen presentation in lymph nodes (89) and appear as a tool for immunotherapy against cancer (91), whereas microvesicles (ectosomes) turn off the immune response by inducing differentiation of CD4 T cells into T regulatory lymphocytes (Treg) (92). Dendritic cell (DC)-derived exosomes are more efficient than DC-derived microvesicles in eliciting an antigen-specific immune response *in-vivo* (93). Similar to agonist/antagonist molecules interacting with receptors, further studies on extracellular vesicles may reveal the presence of “ago-vesicles” and “antago-vesicles” in the regulation of several physiological processes.

Another aspect of the growing interest in extracellular vesicles is the detection of biomarkers borne by circulating exosomes or microvesicles in the blood. Indeed isolating circulating exosomes might allow enrichment of a specific marker by 10^5 times.¹ In this respect an exosomal marker of pancreatic cancer has been recently identified (94). One can conceive that isolating PGE2-carrying exosomes or 12-HETE-carrying microvesicles will permit monitoring of the efficiency of a related therapy. Thus, “nano-devices” able to monitor extracellular vesicles have begun to appear (95), and a continuous-flow chip with multiplexed exosomal marker detection for blood-based ovarian cancer detection has recently been devised (96). In conclusion, intercellular communication by exosomes and microvesicles has opened a new era in studying pathophysiological processes.

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