Chapter

Cellular-Defined Microenvironmental Internalization of Exosomes

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Abstract

The extracellular environment exhibits a potent effect on cellular growth and development. Exosomes secreted into this milieu carry functional proteins and nucleic acids from the cell of origin to recipient cells, facilitating intercellular communication. This interaction is particularly influential in the tumor microenvironment, transporting oncogenes and oncoproteins within a tumor and to distant sites. The mechanisms by which cells internalize exosomes vary greatly and the factors dictating this process are still unknown. Most cancers show evidence of exosomal transfer of material, but differences in cell type can dictate the effectiveness and extent of the process. Improving therapeutics requires addressing specific cellular functions, illustrating the need to better understand the forces involved in exosome-cell interactions. This review summarizes what is known about the different types of cells that play a role in exosome internalization.

Keywords: exosome, endocytosis, receptors, internalization, uptake

1. Introduction

Intercellular communication is essential to homeostasis and is largely dependent on the cellular secretome [1]. An emerging awareness of the role that the extracellular environment plays is evident in the field of secreted vesicles. The vesicular contribution to the tumor microenvironment (TME) has furthered our understanding of the communication between cells and the surrounding stroma [2]. This relationship has also elucidated many potential therapeutic targets and possible transporters of chemotherapeutics [3, 4]. There are multiple extracellular vesicle types, characterized by biogenesis, size, and common protein markers [5, 6]. Of these, exosomes are the smallest, with sizes ranging from 30 to 150 nm [6]. These vesicles have the most complex synthesis, emerging from the endocytic pathway. They arise from intraluminal invaginations into a multivesicular body (MVB) and are released from the cell when the MVB fuses with the plasma membrane. Exosomes consist of intracellular material surrounded by a lipid membrane that reflects the cellular membrane of the host cell [7]. These specific vesicles have demonstrated promise in several fields of research, including rheumatoid arthritis [8, 9] and neurodegenerative disease [10], but primarily in cancer [11, 12]. Tumor-derived exosomes (TEX) contain oncoproteins and oncogenes from the cell of origin and thus are

very influential in intercellular communication. Numerous studies have used these luminal proteins and genes to better understand tumor growth and metastasis, as well as for improving diagnostic, prognostic, and therapeutic methods [13, 14].

While there has been an exponential growth in research focused on exosome biology, clarification on the mechanisms of transport between the cell of origin and the recipient cell is essential to maximizing on exosome potential in treating and diagnosing disease. The methods by which exosomes influence the cells with which they interact are still under review. Some exosomes have been shown to fuse to the recipient cell [15, 16], while others are internalized by specific receptor-ligand interactions [17, 18] or by stimulating an indirect uptake by macropinocytosis [19]. Exosome binding to cells has been seen both as a mechanism of transferring luminal contents [15, 16] and as an initial step in the endocytosis process [17, 20]. The significance of the effects of cell-exosome binding in comparison to internalization is still unknown. Most types of endocytosis have been described in the process of exosome uptake [21], but which factors determine the specific mechanism used, are still unclear. Previous reviews have clearly identified a number of ligands and receptors involved in exosome trafficking [21–23], but little is known about the dependence of uptake mechanism on cell-type. This review presents the current understanding of the endocytosis process utilized by specific cells involved in exosomal internalization.

2. Endocytosis pathways

Endocytosis is a basic cellular function that is performed by all cell types in the process of maintaining homeostasis. Many of the molecules essential for cellular function are small enough to cross the cell membrane either passively or actively, however, other structures, such as exosomes, are too large and require a more complicated process. This general process of internalization is called endocytosis and is separated into various types based on the shape [24] and the size of particles internalized [25]. There are many well-written reviews covering the specifics of the endocytic pathways [25, 26], but here we will address them only superficially. Classification under the umbrella of endocytosis varies, but the major methods include phagocytosis, macropinocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis, and clathrin/caveolin-independent or lipid raft-mediated endocytosis [25, 26]. Receptor-mediated endocytosis (RME) is an additional type that is often considered to be a subcategory under several of those previously mentioned (**Figure 1**).

2.1 Phagocytosis

Phagocytosis is the mechanism by which specialized cells (such as macrophages and monocytes) engulf large particles (>0.5 μm) by way of receptor/ligand interactions [25, 27] (**Figure 1A**). Promiscuous receptors allow for a broad range of ligand recognition and binding, facilitating a key role phagocytes play in clearing apoptotic cells [27]. Exosomes, derived from a diverse population of cells, present a vast array of available ligands that make phagocytes ideal recipient cells. This process of phagocytosis is designed to not only internalize extracellular material by enveloping it, but also to regulate the immune response by presenting degraded proteins as antigens on the phagocyte surface [25]. Tumor-derived exosomes influence immune involvement in the tumor [28, 29] which may be facilitated by this mechanism of endocytosis. Other non-phagocytic cells, such as epithelial cells, Sertoli, liver endothelial, astrocytes, and cancer cells have also been shown to perform

phagocytosis [27], potentially expanding the impact of exosomal communication. It is therefore important to define how the process of phagocytosis influences exosome function and if that influence is cell type dependent.

2.2 Macropinocytosis

While phagocytosis or "cell eating" involves ingestion of large molecules, macropinocytosis ("cell drinking") internalizes slightly smaller particles (>1 μm) [25] (**Figure 1B**). This method is a way for cells to sample the external environment without specific receptors or ligands. It is a constitutive process in specialized antigen presenting cells, but is stimulated by growth factors in most others [30]. Macropinocytosis has a unique membrane ruffling process caused by projections from the cell surface encircling extracellular fluid and fusing to the membrane [25], resulting in an increased membrane surface area and volume of engulfed material. Nakase et al., showed that stimulation of the epidermal growth factor (EGF)

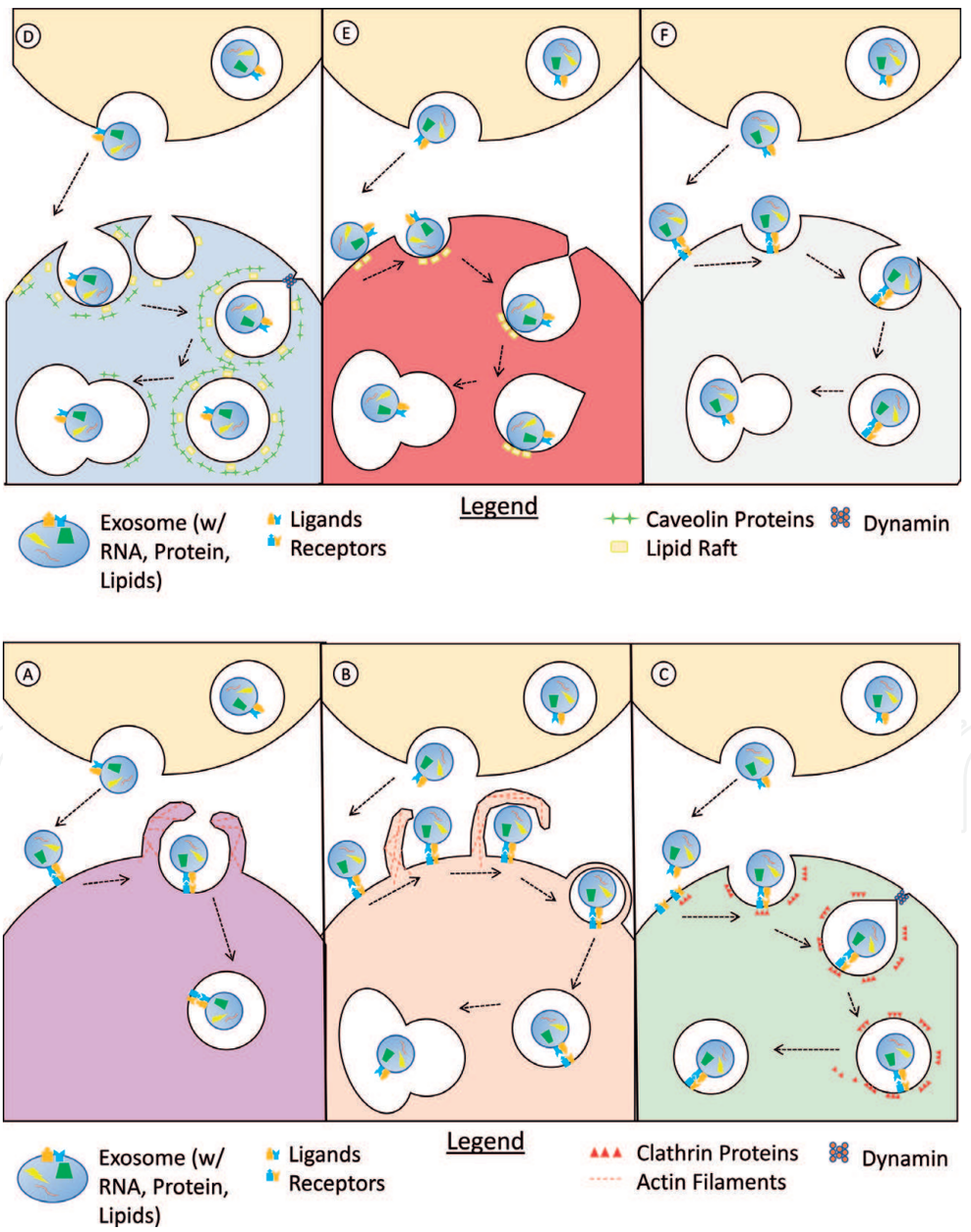

Figure 1.
Endocytosis pathways involved in exosome uptake: (A) Phagocytosis, (B) Macropinocytosis, (C) Clathrin-mediated endocytosis, (D) Caveolin-mediated endocytosis, (E) Lipid Raft-dependent or clathrin−/caveolin-independent endocytosis, (F) Receptor-mediated endocytosis.

receptor, either by soluble EGF or exosome-bound, increased exosome internalization 27-fold through the activation of macropinocytosis [19].

2.3 Clathrin-dependent endocytosis

The next three mechanisms, clathrin-dependent, caveolae-dependent, and clathrin/caveolae-independent, are facilitated by specific membrane proteins/structures: clathrin, caveolae, and lipid rafts. Clathrin is an intracellular protein that forms a coat around an invaginating vesicle facilitating formation and internalization [31] (**Figure 1C**). These vesicles internalize material around 120 nm [25], which is within the exosome size range. Stimulation can occur through receptor/ligand mediation or can be constitutive, depending on cell-type and receptor presence, but clathrin-mediated endocytosis (CME) occurs in all cell types [31]. Data continues to show that the extracellular cargo of these clathrin-coated vesicles can drive the specific mechanisms and protein interactions of internalization [32], giving way for exosome surface proteins to influence uptake. Two proteins used extensively to describe the details of CME are transferrin (Tf) and low density lipoprotein (LDL) and their respective receptors [25], which are all (except LDL) found on the surface of exosomes [33, 34]. Overexpression of transferrin receptors on cancer cells [35] may also contribute to increased exosomal uptake and clathrin-mediated endocytosis in tumors, as there have been shown to be 50–80 percent more receptors on the cancer cell compared to the non-cancer cell [36].

2.4 Caveolin-dependent endocytosis

Caveolin is similar to clathrin, as it forms a coat around membrane invaginations called caveolae and facilitates the entry of extracellular material (**Figure 1D**). These are particularly prevalent on endothelial cells but have been found on a wide distribution of cell types [25]. Caveolae are about half the size of clathrin-coated vesicles, limiting their cargo to smaller structures [25] but still covering some of the exosome size range. This type of endocytosis as well as lipid raft-dependent uptake, plays a key role in lipid transport and homeostasis [25]. One of the defining factors of the exosome membrane is its slightly altered lipid profile, which has been shown to influence internalization [37]. Two proteins commonly active in caveolae-dependent endocytosis, which have also been identified on the surface of exosomes, are the insulin receptor and albumin [34, 38, 39]. The cellular insulin receptor itself has also recently been found to influence exosome uptake [18].

2.5 Lipid raft dependent or clathrin-/caveolin-independent endocytosis

Lipid dependence is not only characteristic of caveolae-dependent endocytosis, but also clathrin/caveolae-independent processes. Lipid raft-dependent (or clathrin/caveolae-independent) endocytosis is similar to caveolae-dependent, except for the absence of the protein cav-1. Lipid rafts are 40-50 nm sections of the membrane with a high percentage of glycosphingolipids and cholesterol, and are anchoring points for many membrane proteins [40]. Lipid rafts are involved in exosome biogenesis and trafficking [41–43] and exosome uptake has been reduced by blocking lipid raft endocytosis [44] (**Figure 1E**).

2.6 Receptor mediated endocytosis

As mentioned previously, RME is an endocytosis pathway that can fit under several of the other categories (**Figure 1F**). The term and pathway were originally

Endocytosis pathway	Recipient cell type	Recipient cell line	Exosome cell of origin	References
Phagocytosis	Macrophage	RAW264.7	Leukemia cell (K562 or MT4)	[20]
	Macrophage	J774	Rat reticulocyte	[52]
	Macrophage	Primary	Trophoblast (Sw71)	[58]
	Monocytes	Primary	Activated T cell	[50]
	Macrophage	Peritoneal	Mouse melanoma cell (B16BL6)	[51]
	Macrophage	Mouse bone marrow-derived	Mouse CRC (CT-26)	[54]
	Microglia	MG6	Pheochromocytoma (PC12)	[117]
	Microglia	BV-2	Neuron (N2a)	[49]
	Dendritic cell	Mouse primary	Mouse dendritic cell	[15]
	Epithelial	Ovarian cancer (SKOV3)	Ovarian cancer cell (SKOV3)	[97]
	Epithelial	Alveolar cells (A549)	Dendritic cell	[66]
Macropinocytosis	Epithelial	Cervical cancer (HeLa)	Epidermoid carcinoma (A431)	[90]
	Epithelial	Epidermoid carcinoma (A431), Pancreatic carcinoma (MIA PaCa-2)	Cervical cancer cell (HeLa)	[19]
	Epithelial	Ovarian cancer (SKOV3)	Ovarian cancer cell (SKOV3)	[97]
	Epithelial	Breast cancer (MCF7)	Normal breast epithelial cell (MCF-10A)—exosome mimetics	[96]
	Endothelial	Cerebral vascular (hCMEC D3)	Macrophage (RAW264.7)	[89]
	Microglia	Primary mouse	Mouse oligodendrocyte (Oli-neu)	[56]
	Neuron precursor cell	Pheochromocytoma (PC12)	Pheochromocytoma (PC12)	[114]
Clathrin-mediated endocytosis	Epithelial	Ovarian cancer (SKOV3)	Ovarian cancer cell (SKOV3)	[97]
	Epithelial	Alveolar cells (A549)	Dendritic cell	[66]
	Epithelial	Gastric cancer (AGS, MKN1)	Gastric cancer cell (AGS, MKN1)	[94]
	Epithelial	Breast cancer (MCF7)	Normal breast epithelial cell (MCF-10A)—exosome mimetics	[96]
	Endothelial	Cerebral vascular endothelial (hCMEC D3)	Macrophage (RAW264.7)	[89]
	Endothelial	Brain microvascular endothelial	Embryonic kidney cell (Hek293T)	[87]

Endocytosis pathway	Recipient cell type	Recipient cell line	Exosome cell of origin	References
	Neuron	Cortical mouse neuron	Oligodendrocyte (Oli-neu)	[115]
	Neuron precursor cell	Pheochromocytoma (PC12)	Pheochromocytoma (PC12)	[114]
Caveolin-dependent endocytosis	Epithelial	Cervical cancer (HeLa)	Epidermoid carcinoma (A431)	[90]
	Epithelial	(CNE1, HONE1, NU-GC-3, A549)	EBV-infected B cells	[95]
	Epithelial	Breast cancer (MCF7)	Normal breast epithelial cell (MCF-10A)—exosome mimetics	[96]
	Endothelial	Cerebral vascular endothelial (hCMEC D3)	Macrophage (RAW264.7)	[89]
	Endothelial	Brain microvascular endothelial	Embryonic kidney cell (Hek293T)	[87]
Lipid raft-dependent endocytosis	Dendritic cell	Mouse primary	Mouse dendritic cell	[15]
	Dendritic cell (DC), T cell	Monocyte derived primary DC, T cell (Jurkat)	T cell (Jurkat)	[75]
	Epithelial, endothelial	Glioblastoma (U87), umbilical vein endothelial (HUVEC)	Glioblastoma (U87)	[43]
	Epithelial	Ovarian cancer (SKOV3)	Ovarian cancer cell (SKOV3)	[97]
	Epithelial	Breast carcinoma (BT549)	Breast carcinoma (BT549)	[44]
	Epithelial, macrophage, endothelial	Melanoma (A375), (RAW264.7), dermal microvascular endothelial (HMVEC)	Melanoma (A375)	[46]
	Endothelial	Brain microvascular endothelial	Embryonic kidney cell (Hek293T)	[87]
	B cell	Mantle cell lymphoma (Jeko1)	Mantle cell lymphoma (Jeko1)	[61]

Table 1.
Endocytosis pathways involved in exosome internalization in various cell types.

considered to be interchangeable with CME, but it is now understood that not all RME is dependent on clathrin [25]. Receptor-ligand interactions play a role in phagocytosis [25, 27], macropinocytosis [19], and lipid raft-dependent endocytosis [40]. Exosome internalization has been linked to multiple receptor-ligand interactions in each of these pathways [19, 20]. Each subtype of endocytosis has been

identified in the exosome internalization process (**Table 1**) but additional research is needed to determine the driving factors behind the specific mechanisms. One hypothesized factor is that the recipient cell type may determine the specific type of internalization.

3. Cell type-specific internalization of exosomes

3.1 Phagocytes

As introduced previously, some cells are uniquely designed to internalize extracellular material through phagocytosis. Those cells generally considered "professional" phagocytes are monocytes, macrophages, and neutrophils [25] with dendritic cells, osteoclasts, and eosinophils occasionally included [27]. Phagocytosis is dependent on receptor/ligand interactions, relying on a vast array of different receptors and ligands. Some of the established receptors include Fc receptors, integrins, pattern-recognition receptors, phosphatidylserine (PS) receptors, and scavenger receptors [45]. Macrophage uptake of exosomes has been shown to involve many of these receptors including scavenger receptors [46–48], PS/PS receptors [20, 48–51], lectins [17, 52, 53] and Fc receptors [54].

However, internalization of extracellular material by phagocytes does not always fit perfectly with the hallmarks of phagocytosis. Some phagocytic receptors, such as integrins ($\alpha_v\beta_3$), scavenger receptors (CD68 and CD36), and CD14, facilitate the tethering of apoptotic cells to the phagocyte surface, but then are unable to initiate internalization without other means, such as PS and PS receptor binding [55]. The PS/PS receptor interaction also stimulates membrane ruffling and vacuole appearance—classic hallmarks of macropinocytosis [55]. Phagocytes are primarily involved in phagocytosis, but this evidence supports the idea that multiple modes of endocytosis are operational in the same cell. This is not unique to apoptotic cell uptake, but has been seen with exosome internalization by microglia (phagocytic cells in the brain) exhibiting a dependence on PS in a macropinocytic manner [49, 56]. Cooperation between multiple receptors appears to be an important characteristic of endocytosis in phagocytic cells. Plebenak et al., showed that the scavenger receptor SR-B1 on macrophages, when blocked, reduces exosome uptake, but with further testing on melanoma cells this blocking was dependent both on the receptor as well as on cholesterol flux in the lipid rafts [46], broadening the endocytosis landscape of phagocytes to include lipid raft-dependent endocytosis.

The dependence of phagocytosis on extracellular- facing PS, which on healthy cells is expressed only on the cytosolic side of the membrane, is evidence that the material to be ingested influences the endocytic pathway of phagocytes. Further support of this interaction is found in the hypothesis that exosomes "target" specific recipient cells [48, 57]. Macrophage uptake (**Figure 2A**) of TEX is dependent on the presence of cellular scavenger receptors or exosomal PS [20, 46, 48, 51, 56], while non-tumor cell-derived exosomes require the presence of a heterogeneity of receptors. When internalized by macrophages and monocytes, hepatic stellate cell-derived exosomes require Fc receptors [54]; B cell, dendritic cell and reticulocyte-derived exosomes use lectins [52, 53]; trophoblast-derived exosomes bind to integrins [58]; and T cell-derived exosomes need scavenger receptors [50] (**Table 2**). Costa-Silva et al., showed that when comparing TEX to normal cell-derived exosomes, Kupffer cells, liver-specific macrophages, preferentially internalized TEX [57]. The significance of the exosome surface topography is therefore influential in directing a specific endocytosis pathway. Phagocytes are responsible for internalization of extracellular material and are so

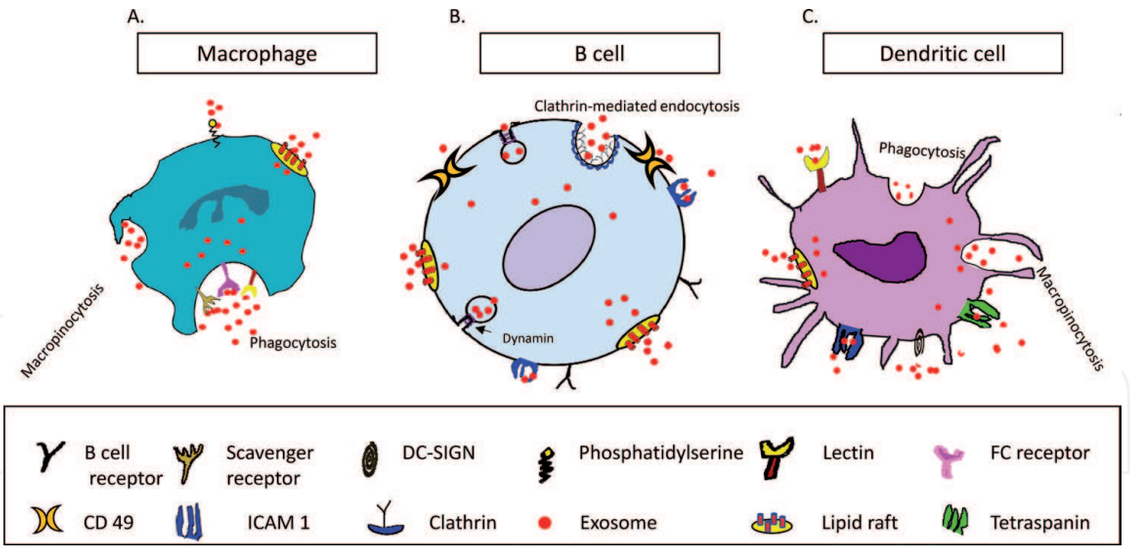

Figure 2.
Cell-specific internalization of exosomes by antigen presenting cells: (A) macrophage, (B) B cell and (C) Dendritic cells each employ multiple endocytic pathways in the uptake of exosomes. Macrophages utilize multiple endocytic pathways in the uptake of exosomes. B Cells and dendritic cells (DC) both employ multiple endocytic pathways in the uptake of exsomes. Lipid rafts, integrins and adhesion molecules are used by B cells while tetraspanins and adhesion molecules are the more common receptors found in DC-exosome interactions. Intercellular adhesion molecule 1 (ICAM-1), Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN).

named based on the primary use of phagocytosis, but as seen above, other endocytic pathways are utilized, especially in the context of exosomal internalization.

3.2 Antigen presenting cells

The antigen presenting cells (APCs) include primary phagocytes such as macrophages, but also B cells and dendritic cells [59]. The immune response is heavily dependent on the recognition of foreign structures, such as peptides, for activation. These APCs sample the extracellular environment, digest and display peptides on their surface, and then present these peptides to immune cells that can execute the response. The intercellular trafficking of immune regulating proteins, such as the major histocompatibility complexes (MHC) [28], by exosomes has the potential to either stimulate or block the immune response, dependent on the exosomal contents [17]. Uptake of exosomes plays an important role in B cell and DC cell proliferation, protein presentation, and interactions with other immune cells [17].

B cells perform multiple functions as an immune cell, including presenting antigens to T cells in order to stimulate additional immune responses. B cells traditionally operate though clathrin-mediated endocytosis, relying heavily on the B-cell receptor [60]. However, when it comes to exosome internalization, B cells have shown a greater dependence on lipid rafts and various receptors, such as adhesion molecules and tetraspanins [17] than on clathrin, indicating a preference for clathrin-independent and receptor-mediated endocytosis (**Figure 2B**). In analyzing B cell uptake of exosomes, using the mantle cell lymphoma (mutated immature B cell) cell line, Jeko-1, Hazan-Halevy et al., found dynamin, epidermal growth factor receptor (EGFR), and cholesterol to be involved in exosome internalization instead of clathrin [61]. EGFR is a well-established target in cancer therapy, particularly with lung cancer [62] and its role in exosome internalization may lend clarity and power to multiple existing and future chemotherapeutics. Additional exosomal surface proteins, with receptor functions, have been identified as participants in

Protein	Cell type	Exosome origin	References
Scavenger receptor	Macrophage	Hek293 (embryonic kidney cells)	[47]
Phosphatidylserine (PS)	Macrophage, microglia	Neuron, melanoma, oligodendrocytes	[49–51, 56]
PS receptor	Macrophage	Activated T cells	[50]
TIM4	Macrophage	K562, MT4 (leukemia cell lines)	[20]
Lectins	Lymph node cells, splenic cells, pancreatic adenocarcinoma, lung fibroblast, macrophage, dendritic cell, hCMEC/D3(brain endothelial cells), platelet, HeLa	Pancreatic adenocarcinoma, reticulocyte, B cell, macrophage, mesenchymal stem cell	[17, 48, 52, 53, 65, 89, 72, 103]
Fc receptors	Macrophage	CT26 (colon carcinoma cells)	[54]
Integrins	Macrophage, B cell	Trophoblast, pancreatic adenocarcinoma cells	[17, 58]
Tetraspanins	B cell, pancreatic adenocarcinoma, endothelial cell	Pancreatic adenocarcinoma cells	[17, 48, 106]
EGFR	A431 (epidermoid carcinoma cells)	HeLa cells	[19]
CD11c	Lymph node cells/splenic cells	Pancreatic adenocarcinoma cells	[17]
CD11b	Lymph node cells/splenic cells	Pancreatic adenocarcinoma cells	[17]
CD44	Lymph node cells/splenic cells	Pancreatic adenocarcinoma cells	[17]
CD49d/CD106	Lymph node cells/splenic cells	Pancreatic adenocarcinoma cells	[17]
Tspan8	Endothelial cell	Pancreatic adenocarcinoma cells	[48, 106]
ICAM-1/LFA-1	Dendritic cell, hCMEC/D3 (brain endothelial cells), aortic endothelium, HUVEC	Dendritic cells, pancreatic adenocarcinoma cells, T cells, macrophage	[16, 17, 37, 65, 69, 89]
DC-SIGN	Dendritic cell	Breast milk	[70]
HSPG	U87 (glioblastoma cells), CAG (myeloma), HUVEC, SW780 (bladder cancer cells)	U-87 cells, myeloma cells, SW780 cells	[63, 99, 100, 101]
Cad-11	PC3-mm2 (prostate cancer cells)	Osteoblasts	[104]
Syncytin	Choriocarcinoma cells	Trophoblasts	[105]
SNAP 25	Neuron	Mesenchymal stromal cells	[116]
CD62L	Lymph node cells, splenic cells, pancreatic adenocarcinoma, lung fibroblasts	Pancreatic adenocarcinoma	[17, 48]

Protein	Cell type	Exosome origin	References
Galectin 5	Macrophage	Reticulocyte	[52]
CD169/ α2,3-linked sialic acid	Lymph node cells, splenic cells	B cell	[53]
C-type lectin/C-type lectin receptor	Dendritic cell, brain endothelial cell (hCMEC/D3)	Macrophage	[65, 89]
P-selectin/PSGL-1	Platelet	Macrophage	[72]

Table 2.
Proteins involved in exosomal uptake.

B cell internalization of TEX, including integrins (CD49) and cell adhesion molecules (intercellular adhesion molecule 1—ICAM-1/CD54 and CD62L) [17].

These protein interactions between the cell and the exosomal membranes are essential steps in the influence the exosome has on the recipient cell. Exosomes derived from myeloma cells, cancerous plasma (mature B) cells, are dependent on the interaction between exosomal fibronectin and cellular heparan sulfate in order to form a bond between cell and exosome, resulting in modification of intracellular signaling [63]. As seen with these cells, the effects caused by the exosomes are not entirely dependent on uptake, even though the standard operation of APCs requires internalization. Some exosome-cell binding (as opposed to internalization) may be sufficient, or specifically designed, to alter intracellular processes, including signaling, as is also seen with dendritic cell-derived exosomes and T cell function [16]. While the influence of heparan sulfate on internalization in B cells is still unclear, there is evidence linking heparan sulfate proteoglycans to exosomal internalization which indicates that while it wasn't assessed in these cells, the uptake may still be present [21–23]. Whether these differing mechanisms and protein participants of uptake in the B cell population are dependent on normal versus oncologic physiology of recipient cells, or on the origin of the exosome population (tumor-derived versus non-tumor derived) is yet to be determined.

These heterogeneous protein profiles are specific to each cell type and contribute to the comparative ability of each cell to internalize exosomes. In line with the role of B cells, it was found that they readily take in exosomes, in contrast to other immune cells such as T cells and natural killer cells [61, 64]. This suggests that certain immune cells are more effective at endocytosing exosomes than others, consistent with the primary functions of these specific cell types. Additional groups have shown that while B cells internalize exosomes, the uptake is significantly less than that of macrophages and dendritic cells, but similar to T cells [17]. This was shown in non-mutated mouse cells and may also illustrate important differences between cancer cell and normal cell internalization mechanisms.

Dendritic cells (DC) can be classified as both APCs and as phagocytes since internalization of extracellular material is a crucial part of their role in the immune system. Endocytosis pathways involved in exosome uptake in these cells have been tested with various endocytic blockers, including cytochalasin D (inhibits actin polymerization), EDTA (chelates calcium), and decreased temperature (reducing active cellular processes) [15, 37, 65, 66]. As dendritic cells mature, their mode of endocytosis changes; starting first with macropinocytosis, and then in the mature cell, receptor-mediated endocytosis and phagocytosis prevails [67] (**Figure 2C**). Despite the evidence of phagocytosis in mature DCs, it was demonstrated that immature DCs are more adept at exosomal uptake [37, 68]. Developmental preference for exosome uptake may shed light on why cancer cells, which often have

similar profiles to developing cells and are subject to continuous proliferation, are so responsive to modification by exosomes. Also, immature DCs play a role in immunologic tolerance and so are less likely to activate T cells, while mature DCs activate T cell immunity [15]. This down-regulation of the adaptive immune response by immature DCs would be advantageous for tumors and so TEX may specifically target immature DCs, explaining the increase in uptake. While the mechanism is still unknown, dendritic cells are also more likely to take up TEX or DC-derived exosomes than B and T cells, as seen with fluorescent staining *in vitro* and *in vivo* in a rat model of pancreatic adenocarcinoma [17] and flow cytometry analysis of mouse bone marrow derived cells [15]. The CD11c membrane protein present on the DC and not on the other cells, was found to be involved in the internalization of TEX, as uptake decreased in the presence of an antibody to CD11c. The expression of this protein unique to DCs may contribute to the disparity in uptake among the immune cell types [17]. Recipient cell specificity in exosome uptake and DC interconnection with immune effector cells is another potential area of immune-therapeutic manipulation.

Many of the studies of exosome internalization by DCs have revealed dependence on various adhesion molecules. The ubiquity of these proteins on exosomes, leukocytes, and endothelial cells promotes the non-specific internalization characteristic of DCs. The involvement of ICAM-1 and/or its ligand, lymphocyte function-associated antigen (LFA-1), in DC-exosome interaction has been shown both *in vitro* and *in vivo* [16, 17, 37, 65, 69]. These interactions are not unique to exosome uptake as DCs regularly depend on a wide range of adhesion molecules, including a dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN) [70]. This particular adhesion molecule has been shown to be more effective at exosome uptake by DCs, when looking at breast milk-derived exosomes, than the ICAM-1/LFA-1 binding [71]. In addition to adhesion molecules, C-type lectin and its receptor have also been identified in DC-exosome binding [65]. These glycan binding proteins have also been identified as exosome uptake mediators in other cell types, including macrophages [52] and platelets [72].

In addition to binding to membrane receptors, dendritic cell endocytosis is dependent on lipid rafts and the lipid components of the cell membrane, particularly with viral or bacterial uptake [73, 74]. As viruses and exosomes are similar in size, endocytosis mechanisms are often common between these two structures [22]. Lipid-dependent endocytosis is evident in exosome uptake by DCs as illustrated with DC- and T-cell derived exosomes [15, 75]. While proteins have been the most common structure analyzed in connection with exosomal uptake, the membrane cholesterol concentration of recipient cells [15] as well as the lipid profile of the exosomal membrane [75] both play a role in uptake of exosomes by dendritic cells and need further clarification.

3.3 Circulating cells

In addition to the previously mentioned cells, two other circulating cells/structures have also been found to endocytose exosomes, platelets and T cells. Platelets are cell fragments involved in blood coagulation that are unique in their formation as they are devoid of a nucleus and some organelles. Despite a reduced intracellular load, they are involved in binding extracellular vesicles. They do so through the interaction of cellular P-selectin and vesicular P-selectin glycoprotein ligand-1 (PSGL-1) as well as PS [72]. Data suggests that binding facilitates fusion of the exosomes to the platelets, transferring of material and enhancing platelet coagulation activity [72]. This speaks to the impact of these exosomes on intracellular

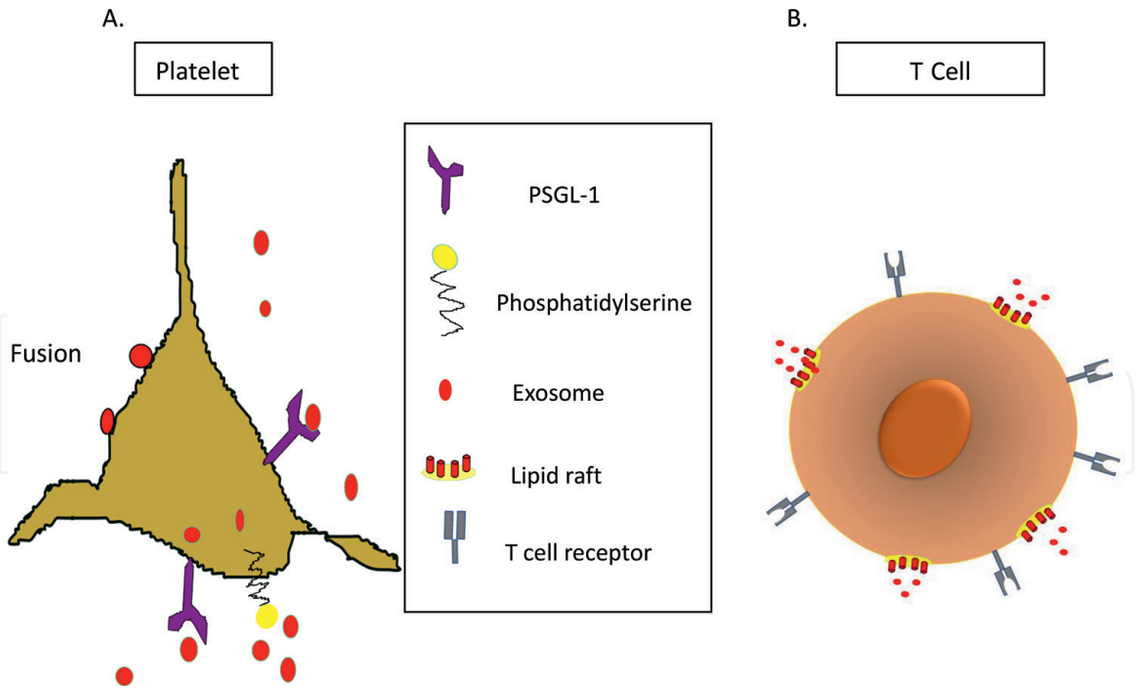

Figure 3.
Cell-specific internalization of exosomes: (A) Platelet-exosome interactions have been linked to fusion as well as the binding to PSGL-1 and phosphatidylserine, (B) T cell are influenced through their surface interactions with exosomes.

communication, both in the variability and specificity of recipient cells, since binding and fusion occurred preferentially in the activated platelets [72] (**Figure 3A**). The exosomes in this study came from monocytes, suggesting this interaction could be a key player in coagulation at a site of injury.

T cells are the effector cells of the immune system and intercellular communication is essential for activation. Endocytosis, while not a primary function of T cells, is important to T cell receptor signaling [76] as well as other functions. Dynamin-dependent endocytosis [76], phagocytosis [77], and RME [78] are some of the mechanisms involved in T cell interaction with its surrounding environment. In relation to exosomes, T cells operate through RME [17, 79, 80] and lipid raft-dependent endocytosis [75]. However, T cells do not always readily uptake exosomes as was found in a comparison with other blood cell types. In a peripheral blood mononuclear cell culture, when uptake by monocytes was blocked, internalization by T-cells increased [47], suggesting that T cell uptake may be an adaptive response to increased exosome concentration. When exosome uptake was compared to multiple splenic leukocytes [15] or peripheral blood leukocytes [64], T cells showed minimal internalization. T cell activity is often regulated by surface interactions with other cells, such as with the T cell receptor and the MHC II/antigen interaction with APCs. Exosomal influence on T cells may therefore operate similarly with surface interaction instead of exosome internalization (**Figure 3B**). When cultured with DC or DC-derived exosomes, T cells acquired functional surface molecules including MHC II from exosomes through direct exosome interaction with the T cell membrane, while still showing little evidence of internalization [81]. Mouse T cells do not express MHC II and after incubation with these exosomes, this protein was identified on the surface of the T cell, suggesting the binding of exosomes to cellular membranes is sufficient to transfer material, without internalization [81]. Further research into the transfer of material between exosomes and immune cells may elucidate the role exosomes play in immune regulation in the tumor microenvironment. Depending on the cell

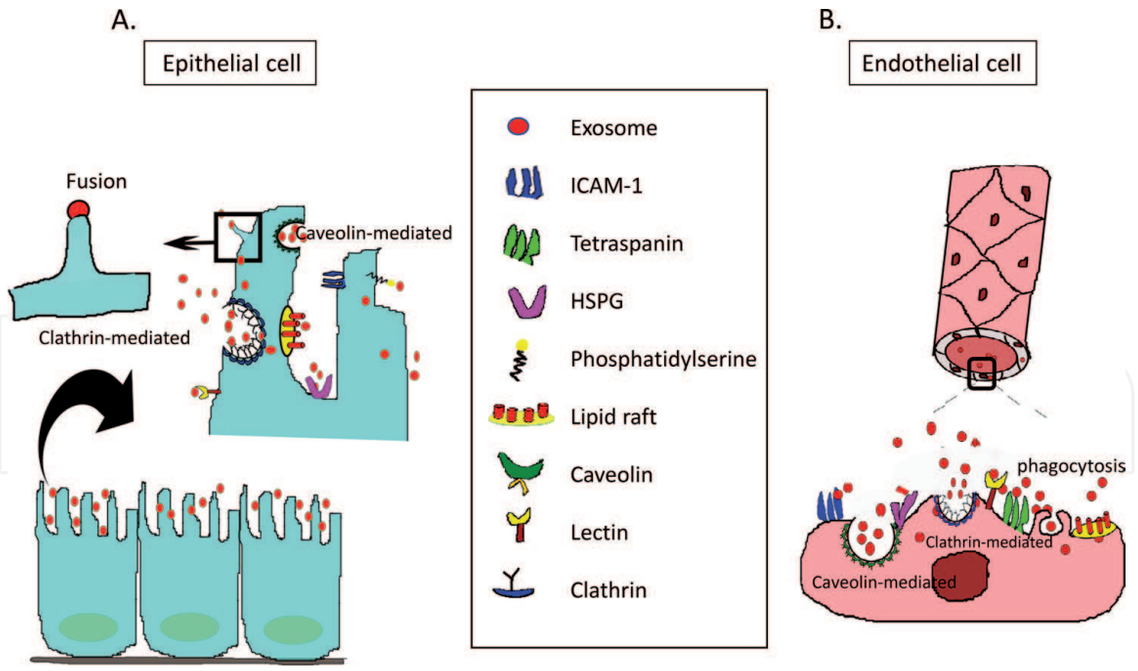

Figure 4.
Cell-specific internalization of exosomes: (A) epithelial and (B) endothelial cells. Epithelial cells and endothelial cells show the most diversity in exosome uptake of all the cell types. Multiple receptor involved in the internalization process are expressed on both cell types, including tetraspanins, adhesion molecules, and heparan sulfate peptidoglycans (HSPG). Intercellular adhesion molecule 1 (ICAM-1).

type involved, exosome-mediated communication and manipulation may not be entirely dependent on endocytosis.

3.4 Epithelial and endothelial cells

Epithelial and endothelial cells are responsible for lining most of the organs, spaces, and blood vessels in the body. They are in a prime position to be exposed to and actively endocytose a wide variety of extracellular material. Due to this broad selection, the specific mechanisms utilized are dependent on the cell subtype as well as the character of the endocytosed material [82–84]. With such variability, it is no surprise that exosome uptake by epithelial and endothelial cells is just as diverse (**Figure 4**). Cellular location of these cells is crucial in cancer biology as most of the TEX will be in close proximity to epithelial and endothelial cells either in the circulatory system or during paracrine spread in solid tumors. While there have been many studies on cell-exosome interaction in these cells, there is still much work needed to clearly understand all of the factors that dictate the endocytic mechanism of epithelial and endothelial cells from different tissues.

A unique finding in exosome studies with epithelial and endothelial cells is the dependence of uptake on intracellular signaling. Svensson et al., discovered that exosome internalization is dependent on the proper functioning of the signaling pathway, ERK1/2-HSP27 [43]. The promotion of endocytosis through intracellular signaling has been shown previously with EGFR-cSrc-ERK1/2 pathways in epithelial cells [85] and the Ras-PI3K pathway with virus uptake by fibroblasts [86]. However, little is known about how these pathways facilitate exosome internalization. The ability of exosomes to cross the blood–brain barrier and be endocytosed by the microvascular endothelial cells in the brain is also dependent on signaling. Tumor necrosis factor (TNFα) signaling, as is seen in stroke models, enhances exosome uptake [87]. Intracellular signaling may provide a regulatory mechanism

to control exosome internalization. Some studies described previously have shown that fusion of exosomes to the cell membrane, without endocytosis, can influence intracellular signaling [63], but these are the first to show how intracellular signaling specifically impacts the endocytosis mechanism of exosomes. These results illustrate the complexity of exosome-cell interactions and where additional research is needed. The interdependence of exosome-cell interactions and intracellular signaling are unexplored areas with vast therapeutic potential and are necessary to better understand how extracellular vesicles influence their environment.

Other characteristics are influential in directing endocytosis in epithelial cells including vesicle size, lipid profile, and protein profile (**Figure 4A**). In epithelial cells, particle size dictates entry mechanism with macropinocytosis as one of the pathways operative at a size range that corresponds with exosomes [88]. This pattern is supported by multiple studies where exosome internalization was decreased when key aspects of macropinocytosis were targeted. Macropinocytosis was blocked with an inhibitor of Na^+/H^+ exchange (which affects Rac1 activation and actin reorganization) in human cerebral microvascular endothelial cells (hCMEC/D3) [89] and HeLa cells, as well as with an inhibitor of phosphoinositide 3-kinase (PI3K) (influences membrane ruffling and macropinosome formation) [19, 90] with concomitant decreases in exosome internalization. Assessing the same pathway but from an activating instead of inhibiting direction, exosome internalization was stimulated by activation of epidermal growth factor receptor (which activates Rac family members) in HeLa cells [19]. Membrane extensions, or filopodia, that facilitate the formation of the macropinosome and are regulated by Rac1 activation have also been shown to influence exosome internalization in hepatocyte (Huh7) and kidney (Hek293) cells [91], furthering the support that exosomes utilize macropinocytosis in multiple epithelial cell lines.

The lipid profile of the exosomes and membrane integrity of the cell are also important contributors to vesicle uptake in several different types of epithelial and endothelial cells. While macrophages readily recognize external-facing PS, these cells can also utilize exosomal PS in the process of internalization, as was shown when pre-incubating exosomes with Annexin V inhibited uptake by HeLa cells (cervical cancer epithelial cells), A375 and A431 cells (squamous skin cancer cells) [92] and in human umbilical vein endothelial cells (HUVEC) [93]. Disruption of cellular lipid raft integrity through cholesterol depletion or sequestration reduced exosome uptake in U87 human glioblastoma epithelial cells [43], hCME/D3 human cerebral microvascular cells [89], HeLa cells [43, 90], HUVECs [43, 46], and A375 cells [46]. Lipid rafts play a key role in many of the functions of epithelial cells, including the protein binding interactions between cell and extracellular environment. Also, some of the most central components to epithelial cell function are proteins that interact closely with the environment such as integrins and adhesion molecules, and are anchored into lipid rafts.

Protein interactions are essential to epithelial and endothelial function and are closely tied to several of the most common endocytosis pathways used by these cells. Clathrin-dependent endocytosis has been shown in gastric [94], nasopharyngeal [95], breast [96], ovarian cancer epithelial cells [97] and HUVECs [98]. Caveolin-dependence was seen in breast [96] and nasopharyngeal cancer [95], however, caveolin-1 showed negative regulation in glioblastoma cell lines [43] (**Figure 4B**). General receptor-mediated uptake has been shown with several proteins including heparan sulfate peptidoglycan (HSPG) in glioblastoma cells and HUVECs [99, 100] and in the transitional epithelial cells of the bladder [101];

intercellular adhesion molecule (ICAM1) in hCMEC/D3 cells [89], rat aortic endothelial cells [48], and HUVECs [102]; lectins in cervical cancer [103], HUVECs [102], rat aortic endothelial cells [48] and hCMEC/D3 cells [89]; cad-11 in prostate cancer [104]; syncytin proteins in choriocarcinoma [105] and tetraspanins in an *in vivo* rat model of pancreatic cancer [48, 106]. The nature of cellular research has limited most of the epithelial endocytosis studies to cell lines, which consist entirely of transformed cells, and it is still unknown whether these trends are translatable to normal healthy epithelial and endothelial cells. While the mechanisms remain unknown, cultured primary normal epithelial cells take up TEX [107] highlighting a role for exosome intercellular communication in normal cell physiology.

3.5 Fibroblasts

The extracellular matrix (ECM) and stroma are important contributors to cellular homeostasis and function. This is particularly evident in tumors when evaluating the role of the tumor microenvironment (TME) on the survival and progression of the tumor cells. Fibroblasts are the major component of this extracellular environment. In normal physiology, they promote stromal stability, while in cancer, they contribute to altered ECM, increased angiogenesis, and metastasis [108]. These cells are in a pivotal position to interact with circulating exosomes and their internalization can have a compounding effect on the surrounding environment. Fibroblasts have been shown to participate primarily in clathrin-mediated endocytosis [109, 110] and occasionally receptor-mediated endocytosis [111]. Interestingly, RME [48, 106] and macropinocytosis [91] are the mechanisms by which fibroblasts have been shown to internalize exosomes (**Figure 5**). Tetraspanins are important proteins in fibroblast function and migration [112]. This protein family is well represented on the exosomal surface and is key to the uptake in many different cell types [48]. Additionally, evidence shows that the smaller the size of the vesicle, the more likely the fibroblast is to use receptors to internalize particles [111]. These three qualities lend support to the evidence of RME as a key pathway for fibroblasts to endocytose exosomes.

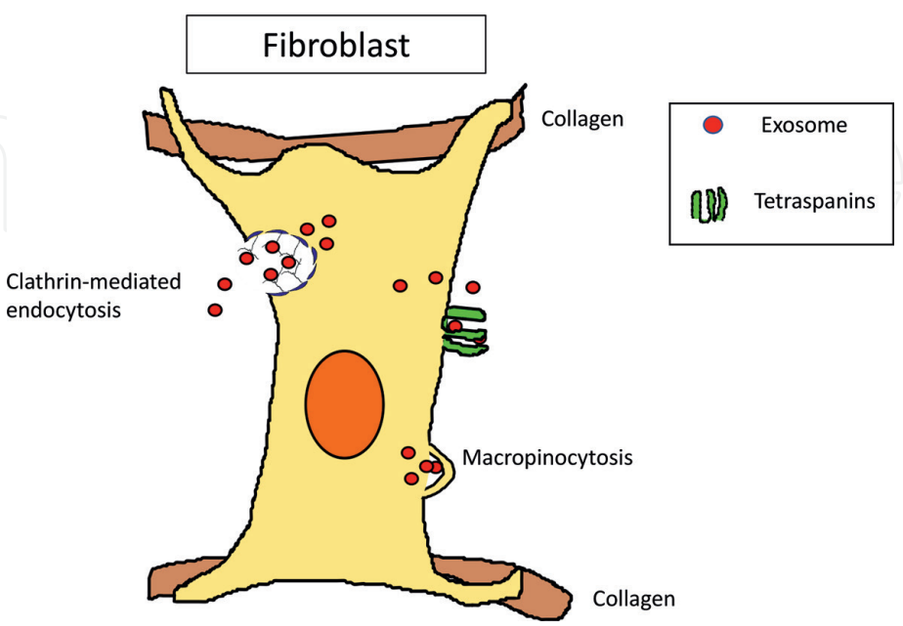

Figure 5.
Cell-specific internalization of exosomes: fibroblasts. Fibroblasts take up exosomes with tetraspanins and utilize multiple endocytic pathways.

3.6 Neurons and glial cells

The nervous system is a uniquely isolated environment with limited connection to the systemic circulation. This characteristic has long impeded therapeutic delivery for brain pathologies. The potential of exosome transport, however, is particularly poignant, as exosomes have been observed selectively targeting neurons and glial cells, successfully crossing the blood brain barrier [113]. Improving our understanding of endocytosis mechanisms involved in these particular cells is essential to therapeutic progression. Clathrin-mediated endocytosis is the most commonly observed pathway with exosomal trafficking between neurons and glial cells [114, 115]. However, some neurons also utilize macropinocytosis [114] and specific receptors, such as SNAP25 (a SNARE family protein) [116], to take up exosomes (**Figure 6**). Microglia performs phagocytosis similar to their counterparts in the extra-neuronal environment [117]. Using exosomes from two different sources, Chivet et al., illustrated the specificity of exosome targeting seen elsewhere in the body, is also evident in the nervous system. Exosomes from a neuroblastoma cell line (N2a) were preferentially internalized by astrocytes and oligodendrocytes, whereas exosomes from cortical neurons were primarily taken up by hippocampal neurons [118]. It was also shown that pre-synaptic regions were the primary site of internalization of these exosomes [118]. Endocytosis is an important process in the pre-synaptic membrane to recycle released synaptic vesicles [119], indicating that the exosomes may capitalize on this constitutive process for entrance to the neuron. Whether exosomes primarily utilize the specific clathrin-mediated endocytosis in this region [119] or are simply taken by chance with the constant bulk endocytosis [120] still remains unclear. Exosome uptake is a developing area of neuro-research, but with significant potential for therapeutics, it is growing rapidly.

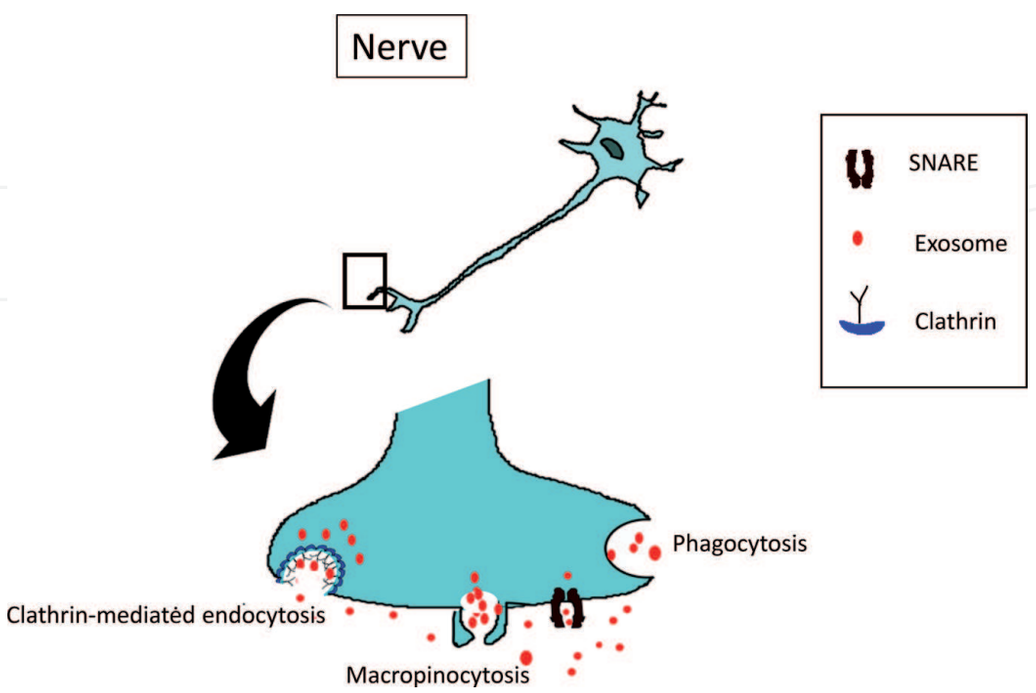

Figure 6.
Cell-specific internalization of exosomes: neurons. Neurons use similar pathways but receptor/ligand binding has less variability. Synaptosomal associated protein 25 (SNAP25).

4. Conclusion

Exosomes are internalized by a multitude of cell types and play an important role in cellular physiology. Our grasp of the mechanisms of this internalization is growing as we are better able to identify characteristics of the cell and the vesicles that facilitate uptake. Pathologic states, such as cancer, have played an integral role in our understanding of how the cellular-exosomal interaction proceeds. Clarity is still needed to better understand the mechanisms by which exosome internalization is so varied from cell to cell and within the same cell. As we have seen with fibroblasts, the vesicle size can dictate mechanism of uptake [111]. The presence or abundance of specific proteins such as scavenger receptors on macrophages [46–48] and lipid profiles in several types of cells, such as external-facing phosphatidylserine [20, 48, 49, 56] all contribute to the specificity of uptake. As has been discussed, cell type can dictate uptake mechanism, particularly with phagocytic cells and professional antigen presenting cells, but even within these specialized cells, differing mechanisms occur regularly and further evaluation is needed to parse the primary determinants.

Various types of endocytosis have been identified as possible mechanisms of intercellular transport of exosomal contents to include macropinocytosis [19, 56, 114], phagocytosis [20], clathrin-mediated [52, 114], caveolin-dependent [95], lipid raft-dependent [43, 46], and clathrin- /caveolin-independent [61] endocytosis. Though much about these processes is unique, there are some aspects where functional overlap exists between them. Macropinocytosis is a form of endocytosis that consists of membrane ruffles forming intracellular vesicles to internalize large amounts of extracellular fluid [30]. This varies from other forms of endocytosis in its formation of separate and distinct intracellular vesicles (macropinosomes) and the internalization of material that is considered non-specific exosomal has been recorded in microglia [56], human epidermoid carcinoma-derived A431 cells stimulated by endothelial growth factor receptor (EGFR) and by the pancreatic cancer MiaPaCa-2 cell line [19]. Macropinocytosis is not selective in which molecules are internalized from the extracellular environment, and so uptake may be dictated simply by proximity to the cells and not targeted by the exosome specifically [121]. However, it has been shown that some exosomes naturally induce macropinocytosis internalization [90] and others, through manipulation of exosomal content, can selectively activate this mechanism in order to increase uptake [122]. Phagocytosis is a much more common method of taking up exosomes, especially with phagocytic cells of the immune system. Feng et al., showed that two leukemia cell lines, K562 and MT4, solely utilized phagocytosis for exosome internalization [20, 121].

Four other general categories of endocytosis focus on specific cellular proteins that facilitate the uptake of particles. Clathrin and caveolin are both cytosolic proteins that form specific pits with which to internalize various substances [25]. The exact reasons why and when a cell uses clathrin, caveolin, or neither, is still incompletely understood but particle size and cell type seem to play a role [43, 115, 121]. Caveolin-dependent endocytosis is important in albumin uptake, cholesterol transport, and intracellular signaling. Due to the small size of the caveolae, its endocytosed material tends to be smaller than 60 nm [25]. Clathrin-dependent mechanisms however can internalize particles up to 120 nm. The size restrictions may indicate, with further investigation into which uptake mechanism is utilized by which cells, a possible functional difference between vesicle sizes within the current exosome size range [121]. The clathrin-dependent process is involved in many different cell types and functions ranging from vesicle

recycling in the neuronal synapse to organ development and ion homeostasis [25]. Many of the common, well-known endocytosis receptors utilize clathrin coated pits, such as low-density lipoprotein receptor (LDLR) and transferrin receptor (TfR). One of the most commonly used ways to determine which of these mechanisms is in operation is through inhibitory drugs or knocking down certain key players [121]. Dynamin, a GTPase, facilitates the fission of the intracellular clathrin coated vesicle [25, 123]. Dynasore, an inhibitor of dynamin, has been utilized to effectively block endocytosis of extracellular vesicles and establish clathrin-mediated endocytosis as a mechanism of uptake for these vesicles [21, 52, 56]. Following siRNA downregulation of caveolin-1 (the primary protein involved in caveolae-dependent endocytosis), exosome internalization was significantly reduced in B cells [95, 121]. Inhibitory drugs have also been useful in the determination of a third mechanism, lipid-raft mediated endocytosis. The lipid raft is a small portion of the plasma membrane, rich in sphingolipids and sterols, that facilitates various cellular processes [124]. Use of methyl-β-cyclodextrin (MβCD), which alters the cholesterol content of the membrane and disrupts lipid rafts, has been seen by several groups to impair exosomal internalization [43, 44, 97]. While lipid raft-dependent endocytosis is the primary clathrin- and caveolae-independent mechanism, other pathways and independent interactions have been described in the internalization of exosomes [61, 124]. Endocytosis is the primary method of exosomal delivery of its contents but research is still needed to understand what determines the specific mechanism whether it is cell type, exosome type, or condition specific [121].

Exosome stability, ubiquitous presence, and influential contents make them ideal candidates for therapeutic modalities in a wide variety of pathologies. The significance of exosomal contribution to the cellular network throughout the body still carries untapped potential for conquering some of the most pressing current health challenges including cancer and neurodegeneration. Understanding how these exosomes interact with and enter the myriad of cells in the body will empower our ability to capitalize on this natural social network.

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