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Engineered exosomes for studies in tumor immunology

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Summary

Exosomes are a type of extracellular vesicle (EV) with diameters of 30–150 nm secreted by most of the cells into the extracellular spaces and can alter the microenvironment through cell-to-cell interactions by fusion with the plasma membrane and subsequent endocytosis and release of the cargo. Because of their biocompatibility, low toxicity and immunogenicity, permeability (even through the blood–brain barrier (BBB)), stability in biological fluids, and ability to accumulate in the lesions with higher specificity, investigators have started making designer's exosomes or engineered exosomes to carry biologically active protein on the surface or inside the exosomes as well as using exosomes to carry drugs, micro RNA, and other products to the site of interest. In this review, we have discussed biogenesis, markers, and contents of various exosomes including exosomes of immune cells. We have also discussed the current methods of making engineered and designer's exosomes as well as the use of engineered exosomes targeting different immune cells in the tumors, stroke, as well as at peripheral blood. Genetic engineering and customizing exosomes create an unlimited opportunity to use in diagnosis and treatment. Very little use has been discovered, and we are far away to reach its limits.

Keywords

engineered exosomes; designer's exosomes; manipulation of biogenesis; exosomes; and immune cells; separation of exosomes; therapeutic exosomes

1 | INTRODUCTION

Exosomes are a type of extracellular vesicle (EV) with diameters of 30–150 nm secreted by most of the cells into the extracellular spaces and can alter the microenvironment through cell-to-cell interactions by fusion with the plasma membrane and subsequent endocytosis and release of their cargo.[1–6] Irrespective of the origin of parent cells, exosomes share common features such as certain tetraspanins (CD9, CD63, and CD81), heat shock proteins (HSP 60, Hsp 70, and Hsp 90), biogenesis-related proteins (Alix and TSG 101), membrane transport and fusion proteins (GTPases, annexins, and Rab proteins), nucleic

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acids (mRNA, miRNA, and long noncoding RNAs and DNAs), and lipids (cholesterol and ceramide).[2,7,8] Because of their biocompatibility, low toxicity and immunogenicity, permeability (even through the blood–brain barrier (BBB)), stability in biological fluids, and ability to accumulate in the lesions with higher specificity,[9–15] investigators have started making designer's exosomes or engineered exosomes to carry biologically active protein on the surface or inside the exosomes as well as using exosomes to carry drugs, micro RNA, and other products to the site of interest.[11,16–19]

When searched in PubMed using the term exosomes, there were only 84 publications between 1950 and 2000. However, using the same search word there were 5001 publications in 2021, and 1402 are published as review articles. When the search term is used as "exosomes in immunology" PubMed produce 581 publications in 2021 including 215 review articles. A total of 123 publications since 2010 mentioned the term "engineered exosomes" in the title or the abstract and only 21 publications dealt with engineered exosomes in immunology since 2010. Among them, only 6 review articles discuss the application of engineered exosomes in immunotherapy.[16,20–24] It is obvious that engineered exosomes in the field of immunotherapy are still in infancy and untapped.

Recently our laboratory has achieved a few milestones in exosome technology: (1) we developed a platform to make engineered exosomes using nontumorous HEK293 cells that carry and express specific cell-targeting peptides to detect specific cells in vivo when administered intravenously; (2) we used these engineered exosomes as a therapeutic probe to deplete specific cells in the body; (3) we optimized the methods to collect a uniform-sized large amount of exosomes from different cells using a combination of size exclusion and centrifugal filters in shortest possible time; (4) we showed differential biodistribution of exosomes collected from different cells in tumor-bearing animals using clinically relevant single-photon emission computed tomography (SPECT).[25,26] In this review article, we will revisit the current version of the biogenesis of exosomes using tumorous and nontumorous cells, how to manipulate the biogenesis mechanism to make engineered exosomes to express protein or RNA of interest in the exosomes and how to make designer's exosomes to carry nanoparticles, micro RNA, chemotherapeutics, and others. All possible biogenesis of engineered exosomes and their applications will be around the subject matters of immunotherapy, especially targeting tumor microenvironment (TME).

2 | CURRENT VIEW OF BIOGENESIS OF EXOSOMES

The biogenesis of exosomes starts from the process of plasma membrane invagination, the formation of early and late endosomes, the formation of the multivesicular body (MVB), the generation of exosomes as intraluminal vesicles (ILVs), and the secretion of the ILVs as exosomes extracellularly.[27] MVB is composed of ILV particles of different sizes, which range from a few nanometers (nm) to micrometers (μm). The common consensus is that size of the exosomes ranges from 30 to 150 nm.[28] The biogenesis of exosomes is a highly regulated process and involves many steps and proteins.[29] First ubiquitin-binding protein Golgi-Localized γ-Ear-Containing ARF-Binding (GGA), Vps27/Hse1, and clathrin form an endosomal clathrin coat, which acts as a cargo loading site for ESCRT machinery. The ESCRTs ESCRT-0, -I, -II, -III, and Vps4 then form the multivesicular body. Interestingly,

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the ESCRTs are also involved in the invagination of multivesicular body and formation of ILVs[7] where ESCRT III takes part in scission[1] of ILVs in the lumen. Along with ESCRTs syndecan, ceramide and tetraspanins are involved in ILVs biogenesis.[30] Several ESCRT and related proteins including HRS, STAM1, TSG101, ALIX, and VPS4 are involved in MVB docking[31] with membrane and SNAREs are responsible for fusion of MVB with membrane and release of ILVs.[32] In contrast to exosomes, microvesicles, another type of EV sizing 100–1000 nm are generated by direct outward budding of the plasma membrane with the help of several GTPases. Figure 1 shows the current view of exosome biogenesis.

2.1 | Importance of tetraspanins and their manipulation for biogenesis

EVs are secreted by all types of cells. Among the EVs, exosomes contain a specific amount and types of components based on the cell of origin. Alongside genetic materials and lipids, proteins are one of the major components in the exosomes. Exosomes show protein heterogeneity because the parent cells are secreted from having different types of protein contents. One of the large protein families present on the surface of exosomes is tetraspanins. Exosomes have their tetraspanin-enriched microdomains (TEMs) and form a cluster on the surface. By their cluster, they can interact with numerous signaling molecules.[33,34] Almost all exosomes have three major types of tetraspanins CD63, CD9, and CD81, which are also being used as exosome markers. Tetraspanins are involved in exosome biogenesis processes and sorting cargo of the exosomes. Tetraspanins are also involved in the attachment with the target cell as well as in antigen presentation.[35] They also regulate cellular motility and migration and have shown their role in the metastasis of tumors.[36] CD63 interacts by its C terminal with protein complex and attaches the exosomes with membrane to clathrin-dependent pathways.[37] CD9 marker is not specific for endosomes small vesicles (like exosomes) because the presence of this marker in large vesicles was also noted.[38] CD9 transfer from the endoplasmic reticulum to Golgi in B cell has the involvement of CD81.[39] CD9 and CD81 have been shown to interact with G proteins.[40] Additional to these, tetraspanins have different other functions.

2.2 | Manipulation biogenesis to control the contents of the exosomes in the lumen and on its surface

To meet up the protein deficiency and dysfunction, overexpression of the target protein is a way to increase the protein content of exosomes.[41] In this process, certain proteins in the donor cells are overexpressed and that overexpressed protein goes to exosomes by their normal sorting. Excessive protein-containing exosomes are released from cells and can be collected for using further studies or therapies. The downside of this process is possible cytotoxicity and the proliferation inhibition of donor cells. An alternative approach to this process is using ubiquitin. Ubiquitin is one of the most abundant proteins.[42] By using Ubiquitin, a target protein can be expressed 10-fold higher than normal by conjugating the target protein in the C terminal of ubiquitin, which has been shown in HEK 293 cell.[43] In exosomes, MHC-II β-chain cytoplasmic tail ubiquitination turns them to be sorted, therefore this ubiquitination platform could be used to package cargo protein in exosomes.[44]

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2.3 | Biogenesis of exosomes in immune cells

Similar to other cells, immune cells also release exosomes abundantly that carry membranous, cytosolic, and even nuclear molecules (DNA, RNA) characteristic of the cells of origin. Given the numerous types of immune cells, exosomes derived from immune cells play crucial and complex physiological and pathological roles within the already complex immune system. Functional molecules of exosomes, derived from various immune cells and their effects are summarized in Figure 2.

2.3.1 | Macrophages—Macrophages are innate immune cells, which exert diverse functions through their secreted exosomes and are shown to be involved in the progression of the disease by their bioactive molecules.[45,46] The biogenesis of exosomes in macrophages is the same as in other cell types. Different studies have confirmed that the exosome contents of macrophages and the surface proteins are secretory cell-specific.[47] As macrophages are of three types, M0 (nonpolarized), M1, and M2 (polarized), there are three types of macrophage-derived exosomes, and different studies investigated the role of M0, M1, and M2 macrophage-derived exosomes.[48–50] Macrophage-derived exosomes are shown to exert their effects in different pathological conditions by activating different gene signaling pathways; mostly for progression and metastasis.[51]

The main content of macrophage exosomes are miRNAs, long noncoding RNAs (lncRNAs), and proteins.[52] Some miRNAs are found in higher levels in M2 macrophage-derived exosomes than in M1 macrophage-derived exosomes.[53] Among the major miRNA in macrophage-derived exosomes for cancer progression, drug resistance and cancer inhibitions are miR-29a, miR-92a-2, miR-95, miR-125a/b, miR-142, miR-21, miR-155, miR-7, and miR-146a. The major proteins present in the macrophage-derived exosome are ApoE, IL-6, and AMAD15.[53] Other components in macrophage exosomes are mRNA, tRNA, and ribosome but there is no evidence of active DNA.[54,55]

2.3.2 | Myeloid-derived suppressor cells—MDSCs are myeloid heterogeneous cells grouped in an immature state. The major two subgroups of MDSC are monocytic MDSC (M-MDSC) and granulocytic MDSC (G-MDSC), which are differentiated based on Ly6C high (M-MDSC) or Ly6G high (G-MDSC). Annexins, tetraspanins, cytoskeletal proteins, and heat shock proteins (HSPs) are common in exosomes released by MDSC, which are similar to other cell-derived exosomes. Tetraspanins (including CD9, CD177), Hsp70, Hsp90α, Hsp90β, Alix, and the ESCRT complex are characteristic proteins of exosome biogenesis and cargo sorting are also present in MDSC exosomes.[56] Some pro-inflammatory proteins S100A8/9, CD47, and thrombospondin-1 as well as platelet factor-4 are also enriched in exosomes are also enriched in MDSC-derived exosomes. MDSC-derived exosomes contain abundant ubiquitinated proteins such as the ubiquitinated histones, and the nonhistone nuclear protein high mobility group box (HMBG).[57] Transforming growth factor-β1(TGF-β1) is 4.3 times higher in MDSC-derived exosomes than in the MDSC cell.[58] Cancer progression miRNAs are abundant in exosomes derived from MDSC, which are mainly miR-146a, miR-146b, miR-155, miR-125b, miR-100, let-7e, miR-125a, and miR-99b.[59]

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2.3.3 | T cells—Like many other cells, T cells also release exosomes but very few studies have been conducted about the T cell exosome biogenesis. T cell released exosomes mostly showed the immune modulation function.[60–62] Cytotoxic T cells release the lethal protein perforin as well as granzymes to the target cell through exosomes.[63] Even apoptosis changes the protein content of T cell released exosomes when compared between an activated T cell released exosome and apoptotic T cell released exosome.[64,65] One of the components of T cell exosomes is FasL, secreted as “lethal exosomes” following activation-induced fusion of the MVB with the plasma membrane.[65] Along with FasL, APO2 ligand (APO2L)/TNF-related apoptosis-inducing ligand (TRAIL) has also been found in T cell exosomes.[66] A study demonstrated that T cell exosomes express thrombospondin-1 receptor CD47 and it regulates endothelial cell responses to vascular endothelial growth factor (VEGF).[67]

2.3.4 | Dendritic cells—Dendritic cells (DCs) are regarded as specialized and most potent antigen-presenting cells (APC) mediating crucial functions in innate and adaptive immune responses. They can efficiently process and present antigens followed by triggering the proliferation, activation, and differentiation of naive T cells.[68] Research has shown that like DCs, exosomes released from activated DC also express MHCII complex and T cell co-stimulatory molecules and are involved in antigen presentation.[69,70] It has been found that exosomes derived from mature DCs contain CCR7, a chemokine receptor that directs mature DCs to peripheral lymphoid tissues which also analogously regulates the increased accumulation of these exosomes in the spleen and inflammatory responses upon injection in mice.[71] Although DC-derived exosomes can activate T cells through stable interactions with TCR complexes, the extent of the activation depends on DC developmental stage. Generally, T cells are more efficiently activated by mature DCs than immature DCs, and mature DCs release exosomes to facilitate immune-stimulatory responses, whereas immature DC exosomes exhibit a potent immune-suppressive response.[72,73] Immunosuppressive molecules, such as TGF-β, NKG2D, and death ligand FasL expressed by immature DCs following response to tumors, can inhibit natural killer (NK) cells, macrophages, and neutrophils.[74,75] Furthermore, DC-derived exosomes expressing HLA-B associated transcript-3 (BAT3) bound to NKp30 receptor in NK cells and stimulate the secretion of TNF-α and IFN-γ.[76] Through IL-15Rα and NKG2D, DC-derived exosomes also enhanced NK cell proliferation and activation.[20]

3 | CURRENT METHODS TO DIFFERENTIATE EXOSOMES VS EV PARTICLES

EVs are divided into three main classes[77]: Exosomes, Microvesicles (also known as microparticles or ectosomes), and apoptotic bodies. Exosomes are produced within the endosomal network as MVB, which are released upon fusion with the plasma membrane. Exosomes are identified by specific markers, for example, Alix, tetraspanin. These markers denote their specific endocytic origins and a combination of the markers is preferred. Microvesicles are formed by outward budding and fission of the plasma membrane. Apoptotic bodies are released as blebs of cells undergoing apoptosis. Characteristics and main differences between different EVs are shown in Table 1.

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3.1 | Specific markers of exosomes

Several exosomal proteins have been identified and are generally been used as exosome markers. A summary of common exosomal protein markers, their location in the exosomes, and collection and detection methods of these proteins is shown in Table 2 and Figure 3.

Alix (or PDCD6IP, also known as Programmed cell death 6-interacting protein) regulates the endolysosomal system and regulates neuronal death as demonstrated by the upregulation of Alix in degenerating hippocampal neurons after epileptic seizures.[103]

EDIL 3 (or EGF Like Repeats and Discoidin I-Like Domains Protein 3, also known as Developmental Endothelial Locus 1) is a pro-angiogenic factor and a regulator of endothelial cell adhesion and migration.[104] It is an extracellular matrix protein that contains 3 EGF-like domains. One of the domains contains an RGD (Arg–Gly–Asp) motif, which facilitates its interaction with integrins.[105]

HSP70 (heat shock proteins) are membrane-bound and extracellularly located proteins that maintain protein homeostasis as a chaperone in the cytosol. It also has cytoprotective effects. Since the synthesis of HSPs is induced by stress, heat, and other chemical and mechanical stimuli, a variety of HSPs (namely HSP70 and HSP90) have been frequently found in the plasma membrane of the tumor cells. HSPs are isolated by ultracentrifugation.[97]

Several isolation methods have been developed to detect exosomes but the combination of methods yields the best results. Exosome markers like PDCD6IP (Programmed cell death 6-interacting protein, also known as Alix), CD24, CPNE3, EDIL3, Fibronectin, FLOT1, HSP70, TEX, TfR, and TSG101 can be detected by ultracentrifugation.[82,83] Immunohistochemistry detects exosomes like CD9, CD24, CD63, CPNE3, Exo-PD-L1, and CD81.[86]

TEX (or tumor-derived exosomes) are ubiquitously present in the plasma and TME in all body fluids of cancer patients.[106] These exosomes facilitate immune-regulatory activities.[98]

Pineles et al. (2022) conducted an observational cohort study on term/near-term neonates undergoing therapeutic hypothermia (TH) for hypoxic-ischemic encephalopathy (HIE), where they purified CNS exosomes from serum using several established methods. In this study, the researchers concluded that CNS exosome cargo acts as biomarkers that correspond with the severity of brain injury, response to TH, and quantify pharmacological response to neuroactive therapeutic/adjuvant agents. Synaptopodin (SYNPO) is a protein contained within the neonatal CNS exosomes and is specific to HIE.[107]

3.2 | Specific markers for immune cell-derived exosomes

Various cellular components take part in the formation of both the innate and adaptive components of the immune system. Among the several biological functions of exosomes on immune systems, the most significant ones are immunomodulation including immune suppression and various anti-inflammatory processes; cell-to-cell communication including antigen presentation, NK cell, and T cell activation.[108]

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Among all the immune-cell-derived exosomes, DC-derived exosomes are the most vital as they exist in multiple populations, and effectively initiate the antigen-specific immune response by efficient activation and proliferation of T cells, thus promoting immunity. A combination of various cell markers is used to identify the DCs. Exosomes derived from DCs have an essential role in several diseases, including autoimmune diseases, cardiovascular diseases for example, acute MI, or transplant medicine.[109] Leone et al. demonstrated that DCs are identified by CD107a/LAMP-1 (lysosome-associated membrane protein-1) and CD107b/LAMP-2 (lysosome-associated membrane protein-2) that are present on the surface of DCs.[110] APC-derived exosomes originate from inward invagination of the internal vesicles of the MHC class II compartment (MIIC). Immunoelectron microscopy of B cells and DCs demonstrates that MVE (multivesicular endosomes) limiting membranes fuse with the plasma membranes and the internal vesicles within the MVE express MIIC-specific markers LAMP-1, MHC-II, CD63 and CD82.[111] DC-derived exosomes stimulate the proliferation of allogeneic lymphocytes. On the other hand, APC-derived exosomes express MHC-II and stimulate T cells.[112] DC-derived exosomes that express MHC-I and CD86 can effectively generate CD8+ T cell response against tumors.[69] The long-term culture method which supports the production of myeloid-like and immature myeloid DC,[113] both lack expression of MHC-II or CD40 but myeloid-like DC expresses CD11c, CD11b, CD80, CD86, and immature DC expresses FcγII/IIIR.[114]

Macrophage-derived exosomes are of monocytic lineage. These exosomes participate in immune response after cardiac injury following MI or other cardiac injuries through the recruitment of other macrophage components. Following MI (or cardiac injury), for the first few days, the M1 macrophage peaks, then macrophages shift from M1 to M2. This shift signifies the pro-inflammatory and pro-phagocytic response of M1 macrophage and the anti-inflammatory response of M2.[115] Notable microRNA contained within the exosomes taking part in this process are miR-155, miR-19, miR-21, miR-146, and miR-223. Of note, these miR-NAs inhibit fibroblast proliferation and stimulate inflammation, which in turn creates a pro-inflammatory environment in cardiac muscles. Detecting these miRNAs in macrophage-derived exosomes can provide a significant clinical understanding of myocardial diseases.[116]

B-cell-derived exosomes contain MHC-II complexes. Schroeder et al demonstrated that in HNSCC (head neck squamous cell carcinoma) involving B cells PD-1, CTLA, LAG3, and CD137 are increased in some patients.[117] PD-1 expression decreases BCR signaling, and subsets of PD-1 may also be found to be elevated in hepatocellular cancer and thyroid cancer.[91] CTLA4 expression, which is associated with inhibitory effects on immunoglobulin production, is reported to be elevated in B cell malignancies and malignant melanoma,[118] LAG3 (CD223) is a “checkpoint receptor” that regulates TCR signaling and function.[119] CD137, expressed on activated B cells in peripheral blood and on tonsillar B cells, in turn, enhances B cell proliferation, improves survival, and induces secretion of TNF-α & -β.[120] Mature B cells express CD39 (“B cell activation marker”) and CD73 on their surface. CD39 and CD73 are considered “immunological switches,” that shift from pro- to anti-inflammatory activity in the cells, create an immunocompromised environment, and contribute to the progression of cancer.[121]

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T cell-derived exosomes are determined by their surface markers, CD3, CD4, CD8, CD27, and CD28. Loss of CD27/CD28 has been associated with suppressive function and cancer cells maintain their proliferative capacity.[61] Wahlgren et al.[122] showed that exosomes from IL-2, anti-CD3, and anti-CD28 stimulated T cells to express CD9, CD63, and CD81 markers on their surface. These exosomes carry RANTES (CCL5) which promotes cytotoxic response.

4 | CURRENT METHODS OF SEPARATION/COLLECTION OF EXOSOMES

The most commonly used methods of exosome isolation are ultracentrifugation and precipitation. The gold standard for exosome isolation is ultracentrifugation. Precipitation is another most common method for exosome isolation from plasma. Coughlan et al. (2020) used ExoQuick® ULTRA EV Isolation Kit for Serum and Plasma (Systems Biosciences) for precipitation of exosomes due to the ease of extracellular vesicle preparation, significantly depleted number of both IgGs or albumin, and relative enrichment of exosomes based on Nanoparticle Tracking Analysis (NTA) assessment of size and concentration.[123] Ultracentrifugation method produces highly enriched EVs but it is low-throughput and specific infrastructure (i.e., ultracentrifuge) and expertise is required to be performed correctly.[123] Precipitation methods are significantly faster than ultracentrifugation methods and they prepare higher concentrations of exosomes. It also produces extracellular vesicles that have a significantly low number of both IgGs and albumin. A schematic summary of the processes involved in different exosome isolation techniques is shown in Figure 4.

For isolating exosomes, several techniques have been developed by exploiting a particular trait, such as the size, density, and surface markers of exosomes. However, each of these techniques comes with its own limitations which must be addressed for downstream applications. The advantages and disadvantages of commonly applied methods are shown in Table 3.

While the precipitation method provides the most effective exosome isolation (~90%), it takes a long time to achieve exosomes via this method. On the other hand, differential centrifugation takes less time (~9 hours) but the EV yield is variable, sometimes as low as 2%. AF4 (Asymmetric flow field-flow fractionation) process takes only 1 hour but its sample preparation may take up to 3 days. Newer, methods are quick, easier to detect, and can be commercially used.

Helwa et al.[143] compared different exosome extraction methods. They used 6 different volumes of human serum samples versus commercial serum samples from human donors and concluded that even with limited amounts of biological samples, commercial kits miRCURY, ExoQuick, and TEIR are suitable alternatives to ultracentrifugation. Also, exosomes isolated by these techniques and serum volumes had similar zeta potentials to previous studies. In this study, the NTA results showed that all isolation techniques produced exosomes within the expected size range (30–150 nm).

Additionally, exosome isolation methods can be categorized based on their recovery time and assay time (Tables 4 and 5).

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Newer methods have been developed that aim for better recovery and specificity. These include:

- Acoustics (or acoustic-based separation methods)[144]: This exosome separation method uses acoustic frequency (as high as ~40 MHz) through a series of cell-removal and exosome-isolation modules and can separate particles based on their physical properties such as size difference, and acoustic contrast factors.[145] Current methods are only based on biological fluids (e.g., undiluted blood samples). This method requires specialized equipment and significant time owing to its preprocessing of liquid samples.[146]
- Alternating current electrophoretic[147]: Another rapid exosome isolation technique is alternating current electrokinetic (ACE) microarray that has been shown to rapidly isolate and recover glioblastoma exosomes from undiluted human plasma samples.[147] This method requires a small plasma sample and can take up to 15 minutes to isolate exosomes. This method is used to isolate various sample types including undiluted blood, plasma, serum, high-molecular-weight DNA, viruses from high conductance buffers, and drug delivery nanoparticles. The principle of this method is based on creating an alternating current (AC) electric field by generating a dielectrophoretic (DEP) separation force generated by the ACE microarray.[148]
- Field-flow fractionation (FFF): Field-flow fractionation is a chromatography-like separation technique that is based on the principle of fractionation of macromolecules, colloids, and particles. A laminar flow of liquid between two walls is pushed by an external field force.[149] It is a rarely used method of exosome separation.[150]
- Asymmetric flow field-flow fractionation (AFFF, A4F, or AF4): The principles of AF4 isolation methods are based on the techniques of “field-flow fraction (FFF)” which was developed in 1966 by Giddings.[151] The AF4 instruments are commercially available and require minimal expertise (requires only basic knowledge of software) and can separate exomeres from other exosome subpopulations. Although the AF4 fractionation step takes only one hour, the total steps from cell culture to exosome/exomere isolation from the conditioned media by ultracentrifugation can take approximately three days. Although this method certainly has some major advantages, the significant drawbacks this method possesses are its inability to handle large samples and its inadequate separation of exosomes based on their sizes.[29]
- Deterministic lateral displacement (DLD) arrays: DLD is a passive microfluidic technique that separates particles based on their size, shape, deformability, and charge. A flat microfluidic channel is filled with a regular array of micropillar obstacles, which creates a periodic flow pattern in a “zigzag” manner, creating the potential for the separation of both cellular and nanoparticles. It is a low-cost separation method.[152]

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- (Moved up) Field-free viscoelastic flow: This method is based on the principle that particle migration is caused by size-dependent elastic forces in a viscoelastic medium. This method is more precise than other microfluidics techniques because it is possible to separate particles of submicrometer diameter from a very small volume of samples.[153]
- Fluorescence-activated sorting (especially for larger EVs including large apoptotic bodies and large oncosomes): This sorting method separates specific cell populations by phenotypes that can be detected by flow cytometry. This method is best for characterizing a single cell population without being contaminated by other cell populations.[154]
- High-throughput/high-pressure methods such as fast protein/high-performance liquid chromatography (FPLC/ HPLC) that involve some form of chromatography
- Hydrostatic filtration dialysis: Musante et al.[155] demonstrated that urine exosomal vesicles can be effectively isolated by hydration pressure pushed through a dialysis membrane and samples passing through a dialysis membrane of 1000 kDa molecular weight cut-off are separated based on their sizes.
- Ion exchange chromatography (IEX): It is a chromatographic separation method that separates molecules based on the net charge on the surface of the proteins. Depending on the ion, IEX is divided into 2 types, cation IEX and anion IEX. Since different proteins have different charges on their surface, this method of separation can easily isolate based on even the tiniest ion change on the surface of the proteins.[156]
- Microfiltration: In order to isolate urinary exosomes, microfiltration methods are developed that uses a hydrophilized, commercially available membrane. This method can isolate LMW proteins from HMW proteins irrespective of the abundance of proteins in the cell sample population.[157]
- Column-based separation protocols yield exosomes with high purity but they produce diluted exosomes and this process is time-consuming. This method of separation involves size exclusion chromatography.[158,159]

4.1 | Importance of heterogeneity of exosomes

Exosomes are a heterogeneous group of EVs and their heterogeneity is due to their varied size, constituents, function, and cellular origin, which adds complexity to their characterization. Such diversity is likely because of the limiting membrane of MVBs during ILV formation or differences in molecular routes partaken during exosome biogenesis.[160] This heterogeneity leads to differential exosome qualitative and quantitative content which in turn produces miscellaneous exosome subpopulations that are distinct in both their biophysical properties and composition. Generally, we can separate exosomes based on their sizes. Large exosomes (Exo-L) are 90–150 nm; small exosomes (Exo-S) are 60–80 nm in size, and the smallest exosomes are exomeres that are 30–35 nm in size. The exomeres are only recently discovered using asymmetric flow field-flow fractionation. Their study showed

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that exomeres can transfer functional cargo. In this study, AREG-containing exomeres and exosomes elicited prolonged EGFR effect to modulate EGFR trafficking in intestines, and significantly enhanced the growth of colonic tumor organoids. The increased activity of nanoparticle AREG elicited effects at 1:1,000th of the concentration of rAREG.[29,161] Furthermore, separation with density centrifugation exosomes can be classified as high and low-density exosomes.[78]

Lee, Sang-Soo, et al. identified a new group of EV in the P200 vesicles that were smaller than exosomes in size. Exosomes and the P200 vesicles are found in CM (conditioned medium) of human cell lines. These involve a different biogenesis pathway that is independent of the endocytic pathway. While exosome markers (e.g., Hsp70, TSG101, and CD63) are present in both P100 and P200 vesicle types, the CD81 exosome marker is not detected in the smaller EVs. The addition of the P200 vesicles to human cell cultures enhanced exosome production and cell proliferation.[162]

5 | METHODS OF ENGINEERING EXOSOMES USING DNA TECHNOLOGY

As the research enlightened exosomes' stability, low immunogenicity, and permeability in the body, the idea of using exosomes as a diagnostic and therapeutic tool has emerged. Genetic engineering became a major tool for generating modified exosomes. These engineering processes served to display a peptide/protein on the surface as a cargo or targeting sequence, load cargo into exosomes, and escape micropinocytosis in the circulation.

5.1 | Methods of designing exosomes to carry payload outside the exosomes

In one of the earliest studies of exosome engineering, Delcayre et al. reported that the lactadherin protein binds to exosome lipids with its C1C2 domain and presents on the exosome surface. They showed that engineered fusion proteins with C1C2 domains were presented in the exosomes and called this Exosome Display Technology.[163] Another group used a similar strategy, engineered lactadherin with Gaussia luciferase, and overexpressed this construct in B16-BL6 cells. Following the exosome isolation and intravenous injection into mice, they could track exosomes in mice with bioluminescence imaging.[164] Gassart et al. utilized the cytosolic domain of TM Env protein from the bovine leukemia virus and fused it with the CD8 ectodomain. Expression of this construct resulted in a CD8 enrichment in exosomes.[165]

LAMP2b is another useful exosome membrane protein expressed in murine exosomes[76] and widely engineered to present polypeptides in exosome surfaces. Inserting a polypeptide following its N terminal signal peptide results in the expression of the poly-peptide fused with Lamp2b protein and presentation on the surface of exosomes. Alverez-Erviti et al.[12] fused the neuron-specific peptide RVG to the LAMP2b DNA sequence and generated engineered exosomes with RVG peptides to target neurons. After loading exosomes with siRNA by electroporation, they observed a significant uptake of exosomes to the brain of wild-type mice, which resulted in specific knockdown of BACE1, a target in Alzheimer's disease, in mRNA and protein level. Bellavia et al.[166] utilized the Interleukin-3 fragment fused LAMP2b to target chronic myelogenous leukemia cells. By loading engineered

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exosomes with Imatinib or siRNA against BCR-ABL, they could inhibit the growth of CML cells in vitro and in vivo.

Stickney et al.[167] investigated the use of exosomal surface proteins as an anchor for fluorescent proteins and demonstrated the feasibility of CD63, CD9, and CD81 fusions with RFP. They also showed the possibility of presenting fluorescent protein either in the lumen or at the surface, depending on the location of the inserted fluorescent protein in the CD63 sequence. Besides well-known exosomal surface proteins, Ohno et al. presented GE11 peptide on exosome surface by genetic engineering of platelet-derived growth factor receptor in HEK293 cells. By inserting Let-7a miRNA into modified exosomes with liposomes, they successfully targeted EGFR-expressing cancer cells in $RAG2^{-/-}$ mice and inhibited tumor growth.[168] Curley et al.[169] also investigated the topology of CD63, exosomal membrane protein, to optimize engineering exosomes to use delivering proteins and peptides.

Dooley et al.[170] conducted a comprehensive study to identify exosomal proteins to carry proteins/peptides on the surface and inside the exosomes. Apart from previous studies, they conjugated GFP to candidate proteins to make this study a functional assay with ELISA and flow cytometry. After optimizing exosomes to work, followed by proteomics, they identified Prostaglandin F2 receptor negative regulator protein, PTGFRN, a previously unreported scaffold protein, to efficiently present GFP on the exosome's surface. Finally, they completed the study with optimization of truncated PTGFRN, which has the potential to become a standard of exosome modification. The same group used PTGFRN as a scaffold to carry IL12 on the surface of the exosome, generating engineered exosome exoIL12. Intra-tumoral injections of exoIL12 showed greater antitumor activity than recombinant IL12 in the MC38 tumor model in mice. exoIL12 also demonstrated one typical advantage that is expected from engineered exosome treatment, compared to recombinant protein counterparts, prolonged half-life/retention. The complete response to exoIL12 at a rate of 63% compared to 0% at recombinant IL12 shows exosomes have the potential to bring many protein-based therapies into the clinic.[171]

Gao et al.[172] developed a novel method to use exosomes for targeting and therapeutic purposes. They identified the CP05 peptide as a CD63 ligand using the phage display technology. By conjugating CP05 with different targeting peptides (M12 for muscle, RVG for neuron, SP94 for hepatocellular carcinoma), they achieved specific targeting of exosomes to target tissue. Furthermore, they showed dual-labeling with neuron-specific NP41 peptide and fluorescein isothiocyanate fluorescent marker, allowing feasible tracking and detecting of specific cells. Also using the amide linker, they conjugated antisense oligonucleotides for exon skipping therapy in Duchene muscular dystrophy in the mouse model and demonstrated an enhanced dystrophin expression. This approach could have great translational potential since it allows for modification of native exosomes of the organism and involves minimum disturbance. For example, it would be possible to collect exosomes from patients, label them with CP05-conjugated proteins or peptides, and give them back for diagnostic, and therapeutic purposes.

Other than genetic engineering of membrane proteins to load cargo on the surface, another method to conjugate peptides into the exosome membrane is click chemistry. Jia et

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al.[173] modified the exosome membrane by inserting (1-Ethyl-3-[3-dimethylaminoprop yl]-carbodiimide hydrochloride-N-Hydroxysuccinimide (EDC-NHS) and attaching a neuropilin-targeting RGE peptide to target glioma. With the addition of curcumin and super paramagnetic iron oxide nanoparticle (SPION) into the exosome by electroporation, they showed SPION-labeled exosomes enriched in glioma on magnetic resonance imaging (MRI). The tumor volume is decreased and survival increased in the mouse glioma model. Kim et al.[174] also used 1,2-Distearoyl-sn-glycero-3-phosphorylethanolamine (DSPE) and polyethylene glycol to incorporate AA ligand (which has a high affinity for sigma receptors in nonsmall cell lung carcinoma) into macrophage-derived exosomes. After loading paclitaxel into exosomes by sonication, they demonstrate that modified exosomes specifically target the pulmonary metastasis of the Lewis lung carcinoma mouse model and improve survival. Choi et al.[175] modified exosomes by mannose-conjugated polyethylene glycol modification of exosomal membrane to target DCs. To increase immune response, monophosphoryl lipid A (adjuvant) loaded into exosomes in the presence of DMSO, and they managed to target DCs specifically and increased inflammatory cytokines TNF-α and IL-6. Figure 5 shows currently available methods to display protein on exosome surface.

5.2 | Methods to load cargo into exosomes

Exosome lumen can be used to carry protein and nucleic acid cargoes, as the content can travel without risk of degradation or unintended interaction. In one approach, Lai et al.[176] genetically engineered cells to express nuclear localization signal (NLS) fused carrier protein (GFP) with MS2 coat protein (MS2CP) that would work as a dock inside the exosome. They also expressed a reporter mRNA with MS2 binding site (MBS), which will bind to MS2CP of docking protein. As a result, exosomes collected from these cells contained carrier protein with attached mRNA, fused with their MS2CP and MBS domains, respectively. Another docking approach utilized by Yim et al.[177] who used the interaction between photoreceptor cryptochrome 2 (CRY2) and CIBN, truncated version of CRY-interaction basic-loop-helix 1 protein in a technique they called “EXPLOR.” by genetically integrating CIBN into the luminal side of CD9 and CRY2 into cargo protein, they were able photo-activate docking of cargo protein to CD9 through CRY2 and CIBN interaction and generated exosomes filled with cargo protein in the presence of blue light. They successfully delivered Cre-carrying exosomes into the brain of lox EYFP transgenic mice and demonstrated the expression of EYFP proteins in vivo. Further, this group used the same experimental design to introduce super-repressor IκB (srIκB), an engineered protein without phosphorylation sites, which inhibits translocation of nuclear factor κB into the nucleus to prevent sepsis. After generating engineered exosomes in HEK293T cells, they have shown that local injection of engineered exosomes significantly reduced inflammatory response and mortality in the septic mouse model.[178]

Dooley et al.,[170] who identified PTGFRN protein to carry cargoes on exosome surfaces, also studied proteins to carry cargo inside the exosomes. They identified BASP1 as associated with the inner leaf-let of membranes. Further optimization with truncation of BASP1 identified eight amino acid peptides efficiently load GFP into exosomes, comparable to full-length BASP2 protein. Furthermore, an ovalbumin-loaded exosome, exoOVA, successfully induced IFNγ and OVA-reactive CD8 T cells much more efficiently than Ovalbumin alone.

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This also indicates the advantage of using engineered exosomes over recombinant protein counterparts.

Sterzenbach et al.[17] reported that the late-domain pathway could be used to load molecules into exosomes. They fused the WW tag into Cre recombinase, which is recognized by late-domain containing protein Ndfip1, ubiquitylated, and subsequently loaded into exosomes. Upon nasal administration of these engineered exosomes, exosomes were taken up by floxed reporter cells, resulting in tdTomato expression, indicating functional delivery of proteins. They also found that proteinase K treatment did not diminish WW-Cre protein in the absence of Triton X-100, showing the cargo protein is located inside the exosome. We also employed this approach in our lab and found that WW tagged Neuroglobins enriched in exosomes (unpublished).

It might be argued that protein loading techniques into exosomes with physical force and disruption may damage the exosome membrane and cause content loss. Busatto et al.[179] have used cationic amphiphilic molecules, which can penetrate membranes, to load proteins inside exosome.

There are several methods developed to load nucleic acid into the exosomes. Li et al.[180] employed Human Antigen R (HuR), an RNA binding protein, into the luminal surface of exosomal membrane protein CD9 and loaded exosomes with specific miRNA, which bound to HuR through Adenylate-uridylate-rich elements (AU-rich elements). As a result, after successfully delivering engineered exosomes to target cells, they reduced the target protein expression in vivo and in vitro. They also delivered CRISPR/dCas9 system in vivo by adding AU-rich elements to dCas9 mRNA and repressed C/ebpα expression. Kojima et al.[181] developed an RNA packaging device using archaeal ribosomal protein L7Ae that binds to the C/D_{box} RNA structure. They conjugated L7Ae into the C terminus of CD63 to place inside exosomes and inserted the C/D_{box} region in the 3′-untranslated region of reporter gene coding nanoluc bioluminescence reporter protein. Along with RVG targeting peptide attached to exosome in LAMP2b exosomal membrane protein, they demonstrated that exosomes targeted the brain and delivered their mRNA, and detected luminescence in target cells. Figure 6 shows genetic engineering and physical methods to load cargo into exosomes.

5.3 | Immunological use of engineered exosomes

Huang et al. modified HELA cells by overexpression α-Lactalbumin (α-LA), a breast-specific protein expressed in human breast cancers, and collected α-LA-enriched exosomes. After loading TLR3 agonist Hiltonol and immunogenic cell death inducer human neutrophil elastase, they treated mouse breast tumor models with this exosome. They found an increased accumulation of DCs and CD8 T cells in the tumor and reduced tumor size in MDA-MB-231 tumor-bearing mice.[182]

Antigen-presenting features of DCs are key in inducing the immune response. Dendritic cell exosomes have the potential to induce the immune system. Hong et al.[183] modified dendritic cell-derived exosome to utilize MHC-I molecule on the surface of exosome by integrating respiratory syncytial virus antigen. Despite the failure to activate CD8 T cells in vivo, it is

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in vitro success proves that it works but needs further optimization. In another effort, Kim et al.[184] genetically engineered K562 cells by overexpressing human leukocyte antigen-A2 and costimulatory molecules CD80, CD83, and 41BBL to use exosomes to stimulate antigen-specific CD8 T cells. This effort to overcome the inherent difficulty of exosome generation in DCs for the same purpose proved successful in cell culture by having a comparable CD8 stimulation. This approach could be used for adoptive cell therapies. In another attempt to employ exosomes in immune system activation, Morishita et al.[185] developed a lactadherin with streptavidin fusion protein and genetically engineered murine B16-BL6 melanoma cells. After collecting modified exosomes, they incubated exosomes with biotinylated CpG DNA (innate immune response activators) and labeled these exosomes using streptavidin on the exosome surface. They reported these engineered exosomes activated DC2.4 cells and enhanced their tumor antigen presentation.

Exosomes, through engineering surface proteins, can turn into immunological reagents. Hartman et al.[186] used the C1C2 domain of lactadherin to present carcinoembryonic antigen (CEA) and human epidermal growth factor receptor 2 (HER2) tumor-associated antigens on exosome surface to antigen-presenting cells to enhance the anti-tumor immune response. By fusing CEA and HER2 to the C1C2 domain, they expressed these antigens on the exosome surface and enhanced T and B cell responses. Shi et al.[187] turned exosomes into immunological mediators by anchoring anti-CD3 and anti-HER2 into the exosomal membrane by PDGFR. By dual targeting T cells and HER2-expressing cancer cells, the directed T cell demonstrated anti-tumor activity in the mouse breast cancer model. We used LAMP2b protein to display CD206-targeting peptide to target M2 type of macrophages and Fc fragment of mouse IgG2b to bind natural killer cells, aiming antibody-dependent cellular cytotoxicity (ADCC) to eliminate M2 type macrophages. Our engineered exosomes specifically targeted M2 macrophages and significantly reduced CD206+ cells in vivo. Further treatment with engineered exosomes reduced tumor growth and prevented early metastasis in 4T1 tumors in mice.[26] Figure 7 shows the immunological use of engineered exosomes.

Fan et al. followed a hybrid approach in engineering exosomes for immunotherapy. First, they collected ovalbumin-induced dendritic cell exosomes, which already express MHC and CD86, needed for T cell activation. Then, they enriched the exosome membrane with anti-CD3 and anti-EGFR receptors to bind T cells and B16-OVA tumor cells, respectively, and induce cytotoxicity by bringing them into the vicinity. Engineered exosome treatment resulted in an immune response augmented with a PD-L1 inhibitor, decreased tumor size, and increased survival in the B-16 OVA tumor model in mice.[188]

In the TME, one commonly studied phenomenon is the M1 and M2 macrophages and their pro-inflammatory and immunosuppressive roles, respectively. Gunassekaran et al.[189] engineered M1-derived exosomes to deliver siRNA and miRNA to M2 type of macrophages to induce M2 to M1 polarization. To achieve this, M1 exosomes were transfected with miR-5aa-3p and NK-κB siRNA. To target M2 macrophages, IL4R-binding peptide attached to exosome membrane using DOPE-PEG amine. The engineered exosomes achieved M2 to M1 polarization and reduced the tumor volume in the 4T1 mouse breast tumor model. Engineered exosomes could also modify the immune system through their displayed

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proteins. Conceição et al.[190] engineered exosomes as a decoy for interleukin 6 (IL-6), a key mediator of inflammation in skeletal muscle, to inhibit the IL-6 trans-signaling pathway and inflammation. They found engineered exosomes reduced STAT3 signaling, which indicates the inhibition of the inflammation and shows it can be used in Duchenne treatment to reduce muscle wasting as an alternative to anti-inflammatories. Duong et al.[191] have engineered exosomes by presenting the TNFα receptor on their surface to antagonize TNFα and prevent inflammation in vitro model.

5.4 | Engineered exosomes in metastasis

Tumor exosomes indicate the immune status and play a significant role in metastasis. In an indepth study, Chen et al.[192] found that PD-L1, which suppresses immune response against the tumor, on exosomes was abundant in metastatic melanoma compared to healthy donors. Exosomal PD-L1 was found to inhibit CD8 T cells and correlated with poor Pembrolizumab (antibody against PD-L1 receptor, PD-1) response. This indicates that tumors use exosomes as “decoy” to overcome immune responses.

One of the central concepts in cancer metastasis research is the “seed and soil” hypothesis. Suetsugu et al.[193] tagged CD63 exosome membrane protein with GFP in tumor cells and tracked the exosome traffic in mouse breast cancer models. They were able to track tumor-derived exosomes in organs and tumor-associated cells in the circulation and demonstrated the use of exosome tracking in investigating cancer metastasis. This approach would be particularly useful in metastasis research by tracking exosome traffic from cancer cells. Pucci et al.[194] adopted a methodical approach to investigating tumor cell communications by modifying melanoma to express luciferase. They found luciferase activity in tumor-draining lymph nodes and identified $CD169^{+}$ macrophages as a tumor suppressor that prevents tumor exosome spread. This study proves that the exosome study holds a great potential to understand metastasis. Pucci’s group further engineered exosomes to express bacterial Sortase A on PDGFR or dNGFR membrane proteins, which transfers substrate peptides (e.g., biotin-containing peptides) to N terminal glycine of surface proteins. Compared to employing GFP-labeled exosome, this method showed 10–100 fold increased sensitivity in detecting exosome-target cell interaction and a promising strategy to study specific exosome-cell interactions.[195]

5.5 | Engineered exosomes in various diseases

Organ and tissue-specific exosome delivery are achieved by also physical forces. Lee et al. loaded mesenchymal stem cell-derived exosomes with iron oxide nanoparticles by supplying them in cell culture to increase the delivery to target organs. By implanting a magnet next to the heart, they achieved an increased delivery into the infarcted myocardium.[196] In another concept study, researchers labeled exosomes through their transferrin receptor using superparamagnetic iron oxide nanoparticles conjugated to transferrin with the help of carboxylated chitosan. Exosomes are loaded with BAY55–9837 peptide for type 2 diabetes mellitus treatment and targeted to pancreatic islet cells using magnets to attract SPION labeled exosomes. They observed a significant increase in delivery of exosomes and alleviation of hyperglycemia in db/db diabetic mice.[197] Mizuta et al.[198] also used magnetic

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nanogels to increase the delivery of exosomes. After hybridizing exosomes with magnetic nanogels, magnetic force significantly improved the delivery of exosomes to cells in culture.

Liu et al. utilized the intrinsic feature of ferritin use as an MRI contrast reagent and engineered exosomes to carry ferritin in modified lactadherin transmembrane protein on the exosome surface. With this, they were able to use exosomes as MRI contrast reagents without the need for further labeling.[199] Furthermore, with further modification to target specific cells, engineered exosomes could be used to image cells or tissue in the body.

Maguire et al. found that Adeno-associated virus (AAV) generating cells also release the virus in exosomes, and called these “vexosomes.” They found vexosomes have outperformed AAV alone in transfecting the cells. Further modification of the exosome membrane with biotin attachment and magnetic bead labeling, followed by attraction with magnets in cell culture further improved the transfection efficiency.[200] Maguire’s group further explored the exosome-associated AAV gene delivery/therapy in the mouse. They found the same level of exosome-associated AAV delivery in blood, but lesser performance, still comparable in the spleen, lymph node, and liver compared to conventional AAV.[201] With the use of engineered exosome-associated AAV to target specific cells, exosomes would be a new and more effective method to be used in gene delivery and therapy.

Jhan et al.[202] fused exosomes with synthetic lipids to increase the number of vesicles and increased the vesicle amount 6–43 fold. Their siRNA cargo loading and delivery were successful. Sato et al.[203] used freeze-thaw cycles to fuse functional lipids with exosome membrane. Although these processes have the advantage of increasing quantity and modifying membranes, many cargo proteins might be lost, and surface proteins lessened through the process.

Membrane receptors are major drug targets, and molecular assays in protein’s native conformation are crucial in biotechnology and clinical research. Desplantes et al.[204] engineered exosomes to study multiple membrane proteins by directing membrane proteins to exosome membranes by conjugating patented “DCTM” peptides.

Exosomes are subject to elimination via multiple mechanisms in circulation, and there are various studies to prolong exosome half-life in the organism (Figure 8). Hung et al.[205] observed an in-sufficient peptide presentation in engineered LAMP2b of exosomes. They hypothesized that glycosylation would protect these peptides and demonstrated that glycosylation protects peptides in LAMP2b and enhances the delivery of exosomes to recipient cells. Kamerkar et al.[206] found that CD47, a ligand for signal regulatory protein (SIRPα), is crucial in protecting exosomes from micropinocytosis, the presence of CD47 on the surface protects exosomes from phagocytosis. They also showed loading exosomes with KrasG12D shRNA, which targets a common mutation in pancreatic cancer, proved to suppress pancreatic tumor growth and metastasis in mice in the presence of CD47. Another group investigated different aspect and use of CD47 interaction. Koh et al.[207] stated that CD47 is present in most tumors, making the tumors immune to phagocytosis. They overexpressed SIRPα in HEK293 cells and generated SIRPα-enriched exosomes using pDisplay. By saturating all CD47 (don’t eat me) receptors of tumors with these

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engineered exosomes, they showed a significant decrease in tumor volume of CT26.CL25 in immunocompetent BALB/c mice, but not in HT29 in BALB/c nude mice, indicates T cell immunity may be required for effective treatment in CD47 blockade. In another attempt to prevent the elimination of exosomes, Lathwal et al.[208] used cholesterol-modified DNA tethers and complementary DNA block copolymers to enhance the stability of exosomes. They found modified exosomes have fourfold higher blood circulation time. The methods to extend exosome half-life in the organism are described in Figure 8.

6 | USE OF ENGINEERED EXOSOMES FOR TARGETING SPECIFIC CELLS IN VIVO

Based on the above description and details of the engineering method of exosomes using DNA technology, it should be obvious to the readers that engineered exosomes could be the next nanotechnology that would be widely used to target specific cells in vivo not only to determine the distribution of specific cells and enhance the functional status of specific cells but also to target and deplete the specific cells. Irrespective of the origin or parent cells, exosomes share common features such as certain tetraspanins (CD9, CD63, and CD81), heat shock proteins (HSP 60, Hsp 70, and Hsp 90), biogenesis-related proteins (Alix and TSG 101), membrane transport and fusion proteins (GTPases, annexins, and Rab proteins), nucleic acids (mRNA, miRNA, and long noncoding RNAs and DNAs), and lipids (cholesterol and ceramide).[2,7,8] Investigators have started making engineered exosomes to carry biologically active protein on the surface or inside the exosomes and using exosomes to carry drugs to the site of interest.[11,16–19] Recently, our laboratory has achieved a few milestones in exosome technology.[25,26] Our laboratory is heavily engaged in the investigations of the TME and microenvironment of cerebrovascular diseases (CVD). We are working on determining the roles of myeloid cells, especially MDSC and immune suppressive M2 macrophages in the TME, and the roles of neutrophils on the exacerbation of edema in stroke or their roles in tumors following therapies. The following paragraphs will detail the methods and possible utility of immune cell-specific engineered exosomes that can be used to target and deplete cells in the TME or CVD.

6.1 | Engineered exosomes to target M2-macrophages

Depending on the stimuli, macrophages undergo a series of functional reprogramming as described by two different polarization states, known as M1 (classically activated) and M2 (alternatively activated).[209,210] Phenotypically, M1 macrophages express high levels of major histocompatibility complex class II (MHC II), the CD68 marker, and co-stimulatory molecules CD80 and CD86. On the other hand, M2 macrophages express high levels of MHC II, CD163, CD206/MRC1, Arg-1 (mouse only), and others. M2-polarized macrophages are induced by IL-4, IL-13, IL-21, and IL-33 cytokines.[211,212] M2 macrophages release high levels of IL-10, transforming growth factor-beta (TGF-β), and low levels of IL-12 and IL-23 (type 2 cytokines). M2 macrophages also produce CCL-17, CCL-22, and CCL-24 chemokines that regulate the recruitment of Tregs, Th2, eosinophils, and basophils (type-2 pathway) in tumors.[213,214] The Th2 response is associated with the anti-inflammatory and immunosuppressive microenvironment. CD206, also known as mannose receptor (MR), is a 175 kD type-I membrane protein and is

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expressed predominantly by alternatively activated M2 macrophages and resident tissue macrophages mostly in the lungs, spleen, and liver.[215] It functions in endocytosis and phagocytosis and plays an important role in immune homeostasis by scavenging unwanted mannoglycoproteins.[216] Alternately activated M2 macrophages are known to be associated with therapy-resistant, metastasis, and poor survival in different malignant tumors.[217–219] Figure 9 shows an increased number of M2 macrophages in metastatic breast cancer and there is poor overall survival and disease-free survival in tumors showing a higher number of MRC1.

In recent years, investigators have identified a peptide sequence CSPGAKVRC or its linear form CSPGAK that binds specifically to CD206+ M2 macrophages in the tumors and sentinel lymph nodes in different tumor models.[220,221] It is to note that the linear form of this peptide CSPGAK also binds to human M2 macrophages.[221] We have developed engineered exosomes that carry these peptides and precisely detected M2 macrophages both in vitro and in vivo and showed our results in recent publications.[26,222] We have used nontumorous cells (HEK-293 cells) to develop the engineered endosome carrying M2-macrophages targeting peptides as well as the Fc-portion of mouse IgG2b (Fc-mIgG2b) on the surface to target and deplete alternatively activated immunosuppressive CD206+ M2 macrophages in vivo through ADCC and apply these engineered exosomes to alter immunosuppressive TME to enhance the effect of different therapies (including immunotherapy) to decrease tumor burden and improve survival. Figure 10 shows the vector design and mechanisms of action of the engineered exosomes to initiate ADCC to kill targeted cells.

6.2 | The exosome is a better vehicle to enhance antibody-dependent cellular cytotoxicity

ADCC is a nonphagocytic mechanism by which most NK cells (effector cells) can kill antibody-coated target cells in the absence of complement and without major histocompatibility complex (MHC).[223] Targeted therapy utilizing monoclonal antibodies (mAbs) has instituted immunotherapy as a robust new tool to fight against cancer and other noncancerous disorders, such as cryoglobulinemia, Wegener's granulomatosis, and bullous pemphigoid.[224,225] As mAb therapy has revolutionized immunotherapies, ADCC has become more applicable in a clinical context. Clinical trials have demonstrated that many mAbs perform somewhat by eliciting ADCC.[226] Antibodies serve as a bridge between Fc-receptors on the effector cell and the target antigen on the cell that is to be killed. Crosslinking of receptors in both effector cells and target cells is required for triggering the cytotoxic event. ADCC occurs through various pathways, including (a) release of cytotoxic granules; (b) TNF family death receptors signaling; (c) release of pro-inflammatory cytokines, such as IFN-γ.[227] Both the uptake of perforin and granzymes by target cells and TNF family death receptor signaling induce target cell apoptosis,[228] while effector cell-released IFN-γ actuates nearby immune cells to stimulate antigen presentation and adaptive immune responses.[229] Our goal is to target the Fc gamma-receptor (FcγR)-based platform to deplete M2 macrophages (Figure 10). We have identified the sequence of the Fc-mIgG2b that triggers FcγR-mediated phagocytosis and cytotoxicity[230] and recently we have reported the utility of engineered exosomes as imaging and therapeutic probes.[26] It is to note that we have also identified the sequence Fc portion of human IgG that triggers FcγR-mediated

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phagocytosis and cytotoxicity for designing human M2 macrophages targeting engineered exosomes.

Because of the cellular origin, exosomes show enhanced permeability even through the intact BBB, which is an advantage over synthetic nanoparticles.[231–234] Exosomes are also shown to utilize enhanced permeability and retention (EPR) effects.[234,235] Due to higher stability in biological fluids and enhanced permeability, exosomes are better for targeted delivery of therapeutic payloads.[231–234,236] Investigators have used either synthetic nanoparticles or fusion protein to deliver Fc-IgG2b to initiate ADCC but because of the rigid body, synthetic nanoparticles rely most on the ERP effect and reports are showing a lack of ADCC following tagging with gold nanoparticles.[237–241] Moreover, due to a size-dependent manner, synthetic nanoparticles can be cleared by the kidneys or reticuloendothelial system, even with targeting moieties.[242–244] On the other hand, fusion protein-based ADCC did not show promise due to rapid clearance and nonspecific bindings.[245–248] Antibody-mediated ADCC also depends on the antibody design with intact Fc-portion and specific attachment to the target cells.[249] Most of the antibodies that are used to initiate ADCC are monoclonal.[250] We postulate that engineered exosomes developed in nontumorous cells, HEK293, will be a better choice to carry therapeutic payloads to enhance ADCC.

6.3 | Engineered exosomes to target Myeloid-derived suppressor cells or CSF1R+ myeloid cells

Our decade-long investigations and investigations by others proved that bone marrow-derived progenitor cells (BMDPC) influence the TME tremendously causing dynamic changes from inflammatory to the immunosuppressive milieu, neovascularization, recurrence, local invasion, and distal metastasis.[251–258] These dynamic changes are pronounced due to mobilization and accumulation of BMDPC following different therapies including radiation, chemo, and antiangiogenic causing therapy resistance.[253,254,256,259–261] Based on the status of the microenvironment such as inflammatory vs immunosuppressive, the treatment effects differ significantly and the recent addition of immunotherapy also becomes noneffective in the solid tumors.[255,256,262,263] Recently, we have pointed out the involvement of myeloid cells in the development of therapy resistance and recurrence of different tumors.[255,256,264,265] In our recent publications, we have used small molecular agents that inhibit CSF1R tyrosine kinase and showed the retardation of growth of GBM and breast cancers, which was corroborated with animal models where all CSF1R+ cells were conditionally depleted.[253,256,266,267] Therefore, using DNA engineering technology we can make exosomes to carry CSF1R targeting peptides and use payloads for depleting the myeloid cells at different stages of TME status. We have already identified a truncated version (peptide sequences from 36 aa to 147 aa) of CSF1 protein, which showed 100% sensitivity to react with CSF1R and made vectors for making engineered exosomes. We used a similar platform as shown in Figure 10 to make the vector and engineered exosomes. We are also in preparation to make targeting exosomes without inserting Fc-mIgG2b to see the distribution of CSF1R+ cells in the TME at different stages of TME following therapy. We stipulate that our engineered exosomes to target and deplete CSF1R+ cells along with established immunotherapy (anti-PD1) will show synergistic effects.

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6.4 | Engineered exosomes to target neutrophils

Each year more than 795 000 people in the United States have a stroke and it kills about 140 000 people, placing a $34 billion annual economic burden on society.[268] Though major advances in our understanding of cerebral ischemia have been made, the need for novel effective therapies remains imperative. Unfortunately, the success of different therapies is highly variable, and none can be employed early before significant vascular pathology and damage to the brain have occurred. Activated neutrophils have pivotal roles in acute ischemic brain injury, atherosclerosis, and thrombus formation.[269] Neutrophils are the most abundant polymorph nuclear (PMN) white blood cells (WBCs) in the blood and make up part of the innate immune system. Neutrophils are an essential part of the inflammatory cascade, being the first cell type to migrate from the bloodstream to the site of inflammation.[270,271] Following recruitment, neutrophils get activated and subsequently express adhesion molecules and release reactive oxygen species, cytokines/chemokines, and proteolytic enzymes causing damage to the tissues.[272,273] Infiltration of neutrophils in the ischemia-reperfusion stroke area occurs early, at the same time as brain injury. This increased accumulation of neutrophils is associated with stroke severity,[274] infarct volume,[275] and worse functional outcomes.[276] Several studies have started to evaluate broadly targeting anti-neutrophil treatments to minimize stroke injury and to improve stroke outcomes.[269,277,278]

Our laboratory studies in male B6 mice (10–12 months old) (Figure 11) show the mobilization of neutrophils (Ly6G+), natural killer (NK) cells (NKp46+CD3−), macrophages (F4/80), and M1 macrophages (CD80) in the peripheral blood and at the sites of a stroke at 3, 24, and 72 hours after stroke.

Recent studies also pointed out the involvement of tumor-associated neutrophils (TAN) and tumor-associated macrophages (TAM) in maintaining the inflammatory or immunosuppressive TME that dictates the effect of therapies.[217–219,279–286] Neutrophils are the most abundant polymorph nuclear (PMN) cells available in the peripheral blood and early accumulated tumor-associated cells following therapies that make the inflammatory milieu.[280,286–290] However, based on the tumor cell-secreted cytokines and chemokines due to therapy insults, tumor-associated TAN polarized into N1 (CD11b+Ly6G+CD206-TNF-α+) and N2 (CD11b+Ly6G+CD206+IL10+) phenotypes.

Neutrophil migration to sites of inflammation and subsequent activation and multiple functions are highly regulated and orchestrated processes that are controlled by interactions between numerous receptors and their cognate ligands. FPRs are G protein-coupled receptors that transduce chemotactic signals in phagocytes and mediate host-defense as well as inflammatory responses including cell adhesion, directed migration, granule release, and superoxide production.[291] Although there are a few ligands that are an agonist for FPRs, we cannot utilize those for targeting neutrophils because they may stimulate the neutrophils for hyper-functioning. Chemotaxis inhibitory protein of S. aureus is a native protein and part of it is FTFEPF, which shows FPR (specially FPR1) antagonistic activity.[292] A coronavirus 229E-derived 12-mer peptide (ETYIKPWWVWL) was identified as a potent antagonist of FPR1 with a Ki of 230 nM.[293] Investigators have pointed out a lower survival probability if FPR1 is highly expressed in breast cancer patients (Figure 12). We have used our platform

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(vector design, Figure 10) to make engineered exosomes to target and deplete activated neutrophils at the lesions (stroke or tumors) and in the peripheral blood. Our initial studies showed decreased number of neutrophils in the stroke areas following IV administration of the engineered exosomes.

7 | KEY TAKEAWAYS

Genetic engineering and customizing exosomes create an unlimited opportunity to use in diagnosis and treatment. Very little use has been discovered, and we are far away to reach its limits.

Exosomes, in a sense, work like hormones and transfer messages between cells. They have, along with potentially bigger extracellular vesicles, the potential to revolutionize cancer metastasis research and expand our understanding of it.

Because the human body already has exosomes, making use of their own exosomes after isolation and extracorporal modifications with treatment/diagnosis approaches that are already in use could fasten the entering of exosomes into the clinic. For example, after collecting patients' exosomes, they could be loaded with an Alzheimer's drug that has poor blood–brain barrier permeability. And at the same time, exosomes could be tagged with neuron-specific peptides to increase the permeability and targeting capabilities.

Because exosomes carry proteins, lipids, nucleic acid, and metabolites from their parental cells, it should be considered a transfusion/ transplant. Until the safety of extra-corporal sourced exosomes, its clinical use will not be possible. Once the safety of these extra-corporal exosomes is proven, we could see many different uses of it in near future. Another way to overcome this safety concern is to collect and modify exosomes with already approved treatment modalities, which could be more safe and potential translate into the clinic in a short time.

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DATA AVAILABILITY STATEMENT

Data derived from public domain resources. Data available on request from the authors.

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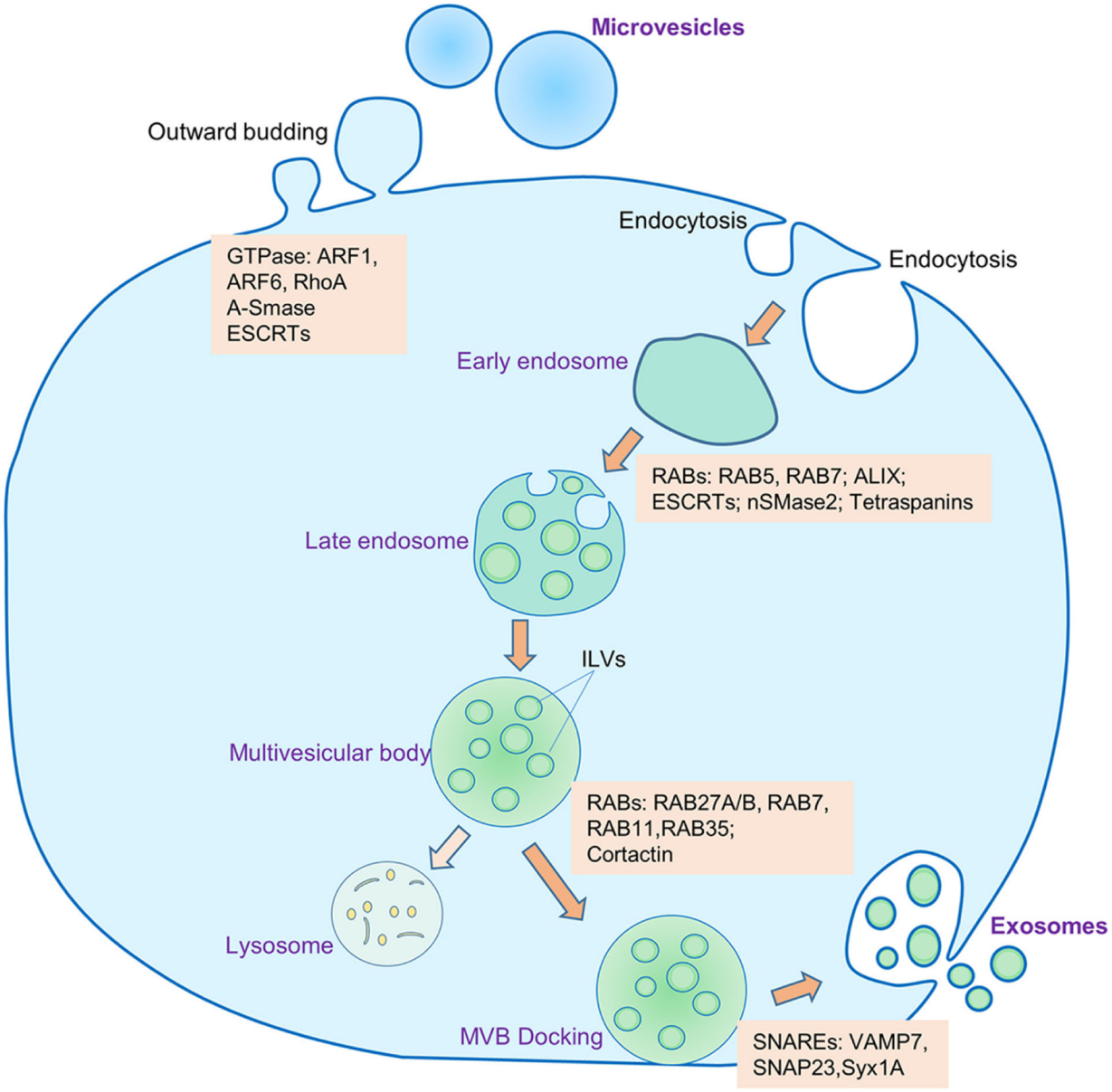

FIGURE 1.

Biogenesis and secretion of exosomes and microvesicles. Microvesicles are generated by outward budding of the plasma membrane with the help of several GTPases. The process of biogenesis and release of exosomes into the extracellular space encompasses several distinct steps: (1) invagination of plasma membrane and formation of early endosomes, (2) inward protrusion of early endosomal membrane to generate late endosomes, (3) formation of multivesicular bodies (MVBs) that contain intraluminal vesicles (ILVs), (4) docking of the MVBs to the cellular plasma membrane, (5) exocytosis of the exosomes into the extracellular milieu. Some of the MVBs may go into lysosomal degradation. Several molecules are involved in the biogenesis and release of microvesicles and exosomes. ESCRT, endosome sorting complex required for transport; RAB, RAS-related protein; ALIX, ALG-2 interacting protein X; nSMase2, neutral sphingomyelinase 2; SNARE,

soluble NSF attachment protein receptor; VAMP7, vesicle-associated membrane protein 7; SNAP23, synaptosomal-associated protein 23; Syx1A, syntaxin 1A; ARF, ADP, ribosylation factor; RohA, Ras homolog family member A; A-SMase, acid sphingomyelinase

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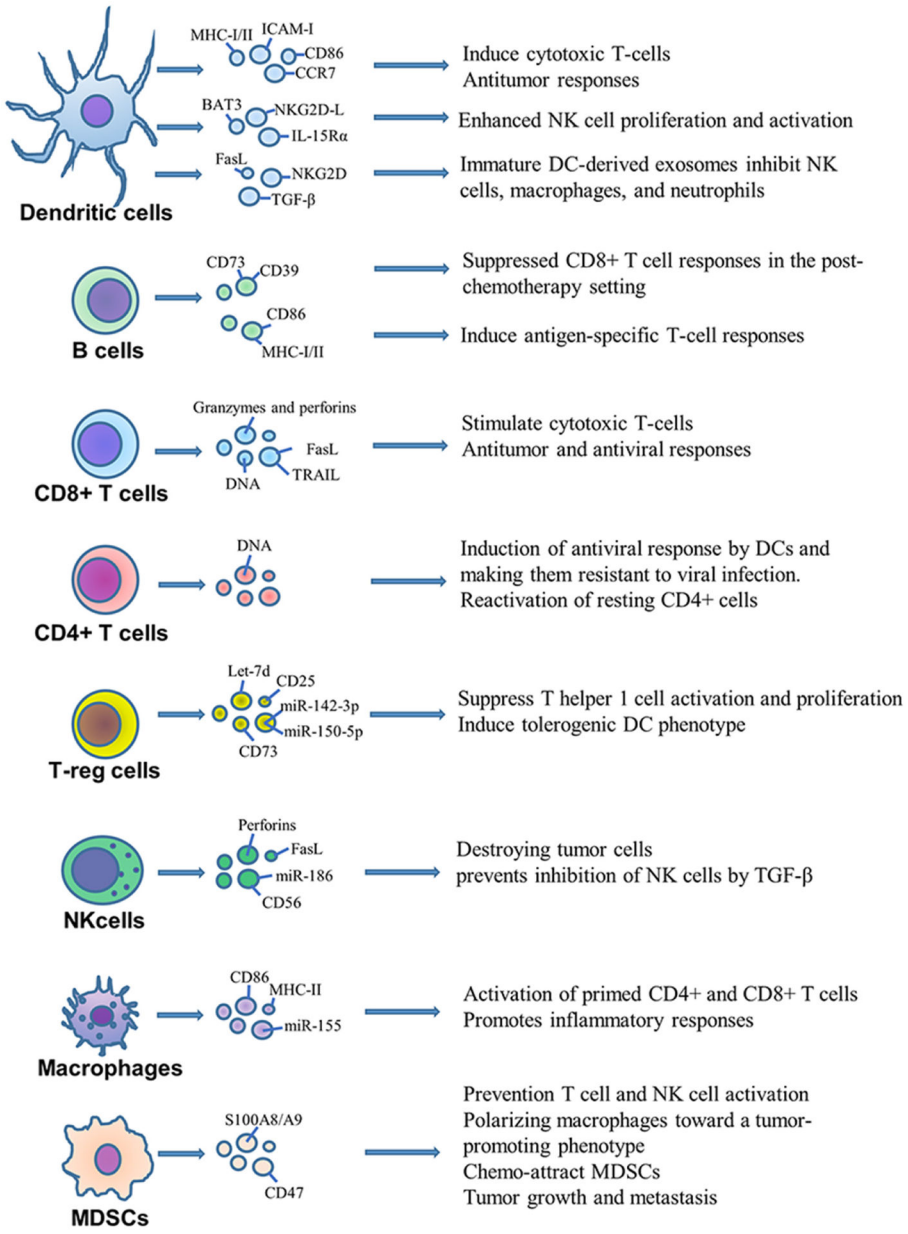

FIGURE 2.

Functional molecules in the exosomes released from different immune cells

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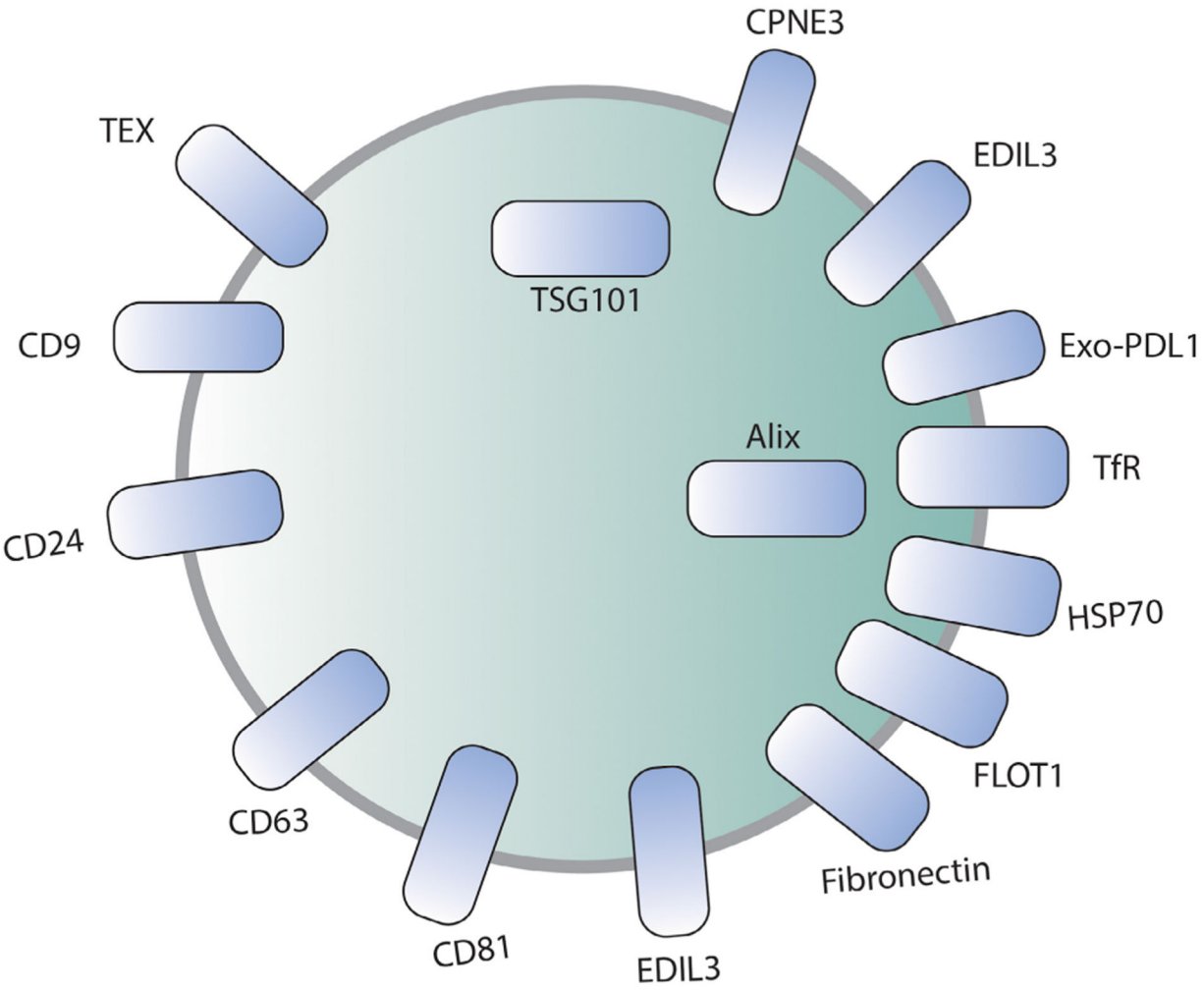

FIGURE 3.
Schematic diagram showing common exosomal markers

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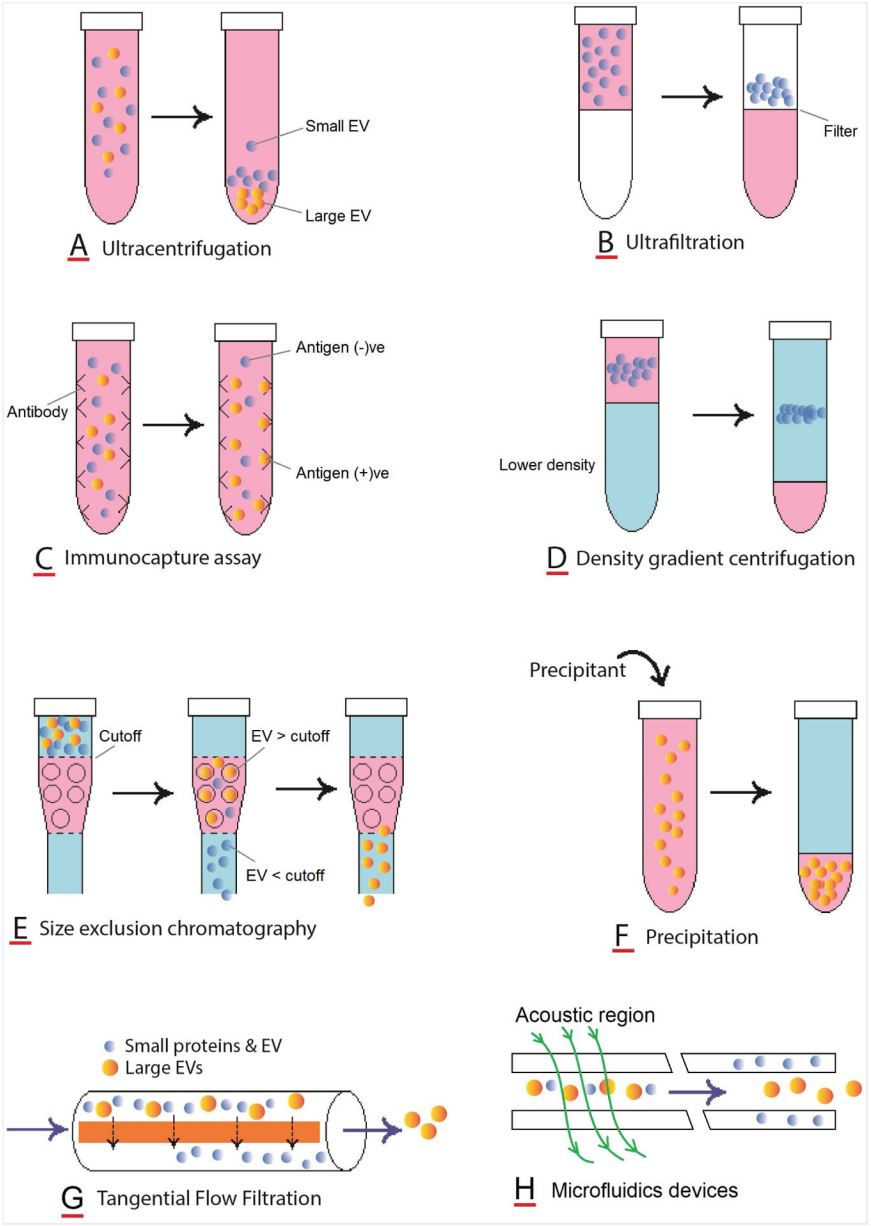

FIGURE 4.

Schematic presentation of processes involved in different techniques of exosome isolation

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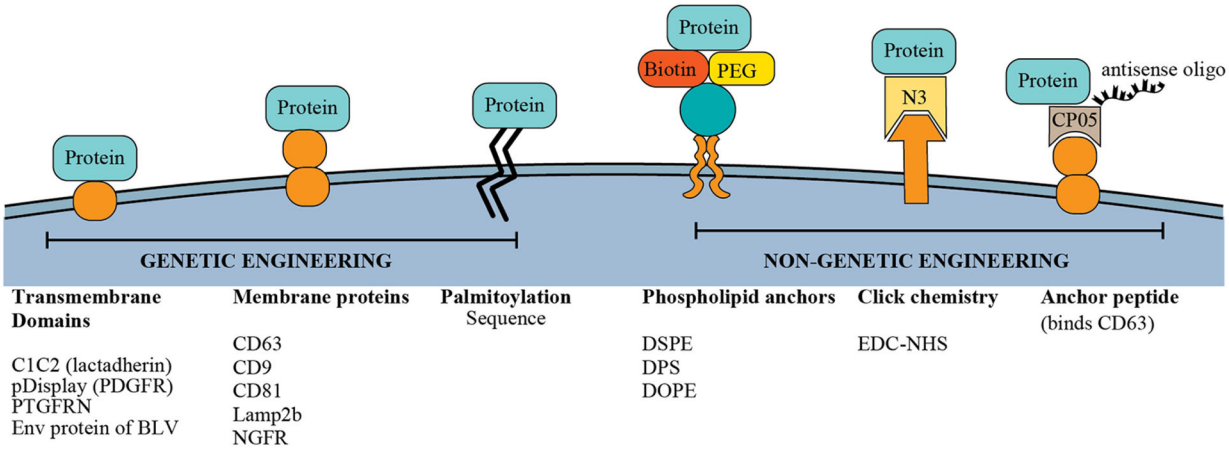

FIGURE 5.

Current methods to display a cargo on the surface of exosomes. PDGFR, Platelet-derived growth factor receptor; PTGFRN, Prostaglandin F2 Receptor Inhibitor; BLV, Bovine Leukemia Virus; LAMP2b, Lysosome-associated membrane protein 2; NGFR, Nerve Growth Factor Receptor; DSPE, 1,2-Distearoyl-snglycero-3-phosphorylethanolamine; DMPE, 1,2-Dimyristoyl-sn-glycero-3-phosphoethanolamine; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; PEG, Polyethylene glycol; EDC-NHS, (1-Ethyl-3-[3-dimethylami- nopropyl]-carbodiimide hydrochloride – N-Hydroxysuccinimide; N3, azide radical

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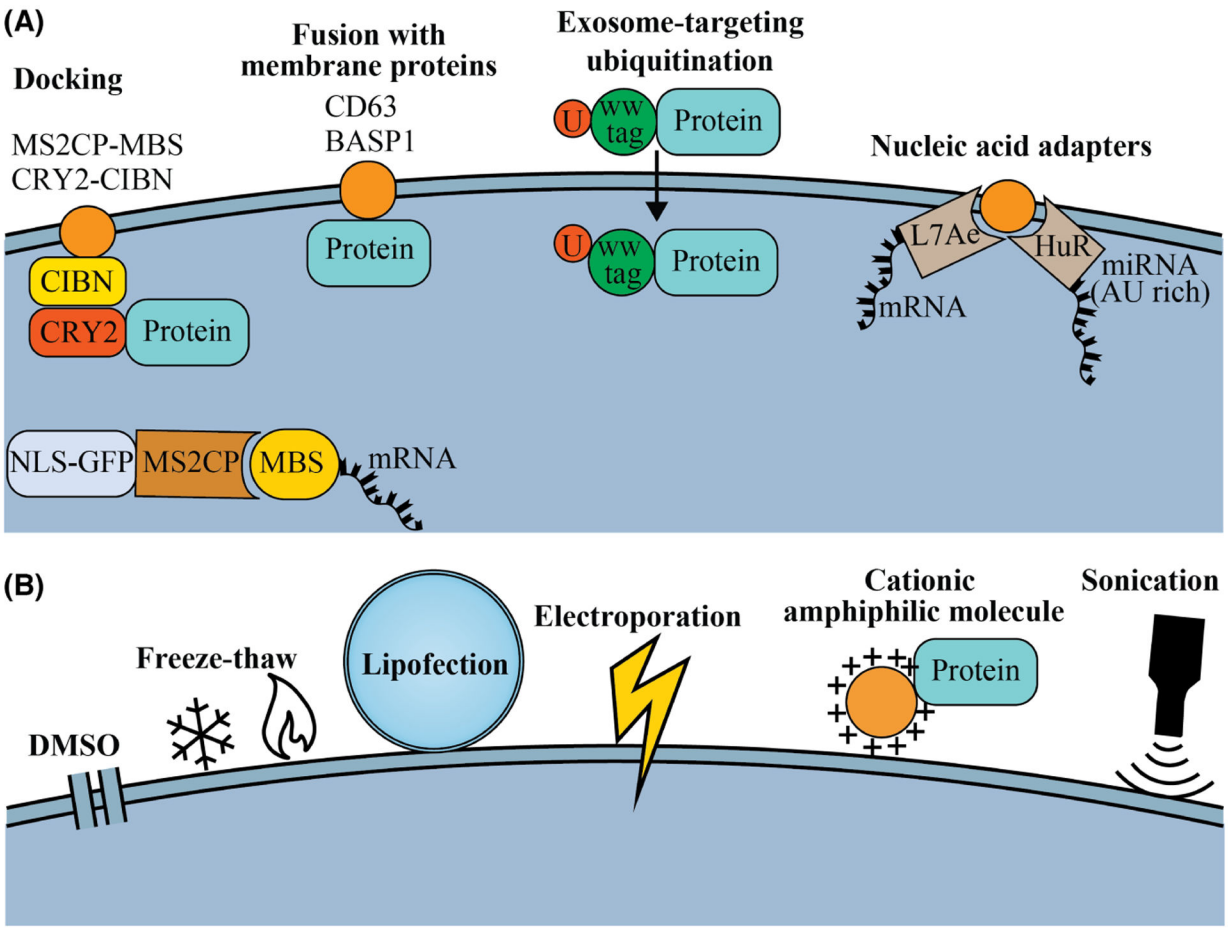

FIGURE 6.

Methods to load cargo inside exosomes. (A) Genetic engineering methods to load exosomes with protein and nucleic acid. MS2CP, MS2 coat protein; MBS, MS2 binding site; CRY2, cryptochrome 2; CIBN, truncated version of CRY-interaction basicloop- helix 1 protein; NLS, nuclear localization signal; BASP1, Brain Abundant Membrane Attached Signal Protein 1; HuR, Human Antigen R. (B) Physical methods to load proteins and nucleic acids into exosomes

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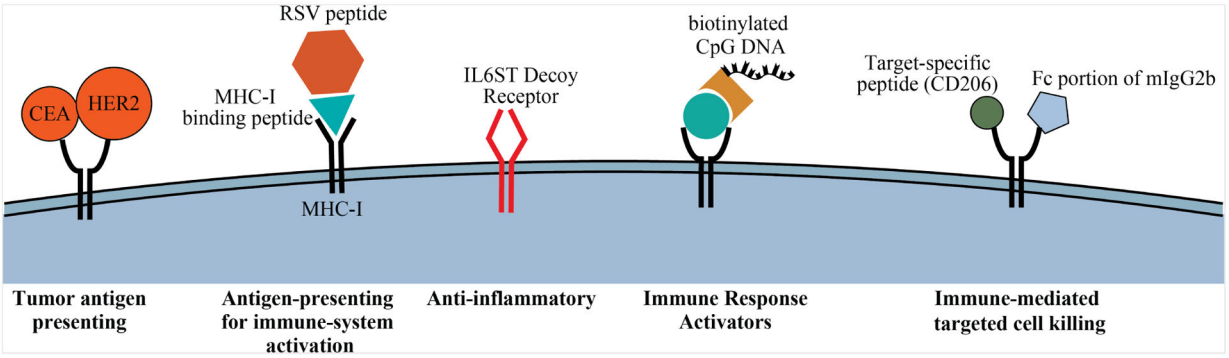

FIGURE 7.

Immunological use of engineered exosomes. CEA, carcinoembryonic antigen; HER2, human epidermal growth factor receptor 2; RSV, respiratory syncytial virus; IL6ST, Interleukin 6 Cytokine Family Signal Transducer, mIgG2b, mouse immunoglobulin G 2b

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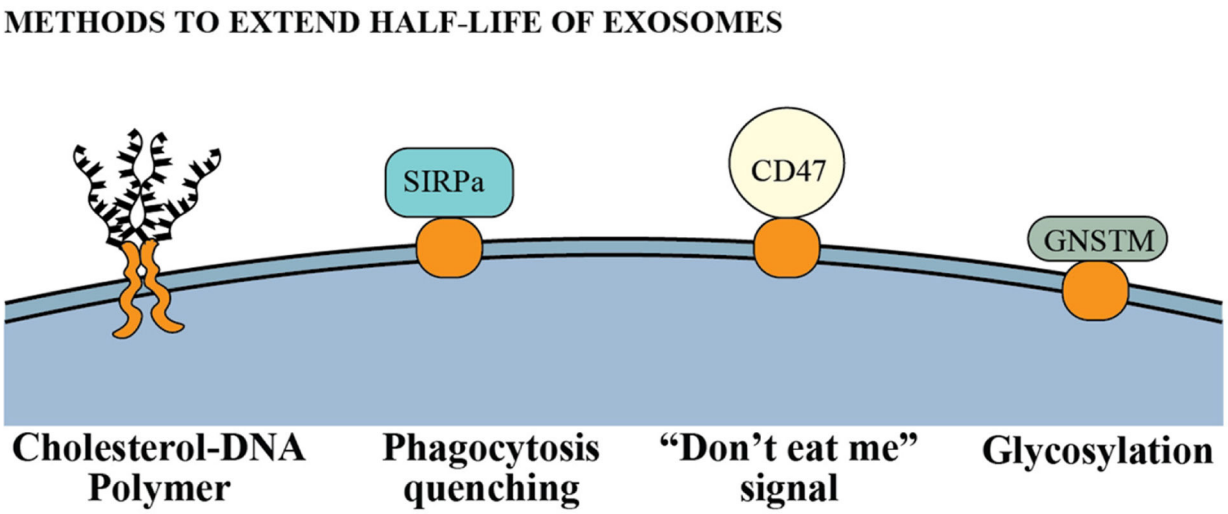

FIGURE 8.

Engineering methods to extend half-life of exosomes in circulation. SIRPα, signal regulatory protein; GNSTM, glycosylation motif

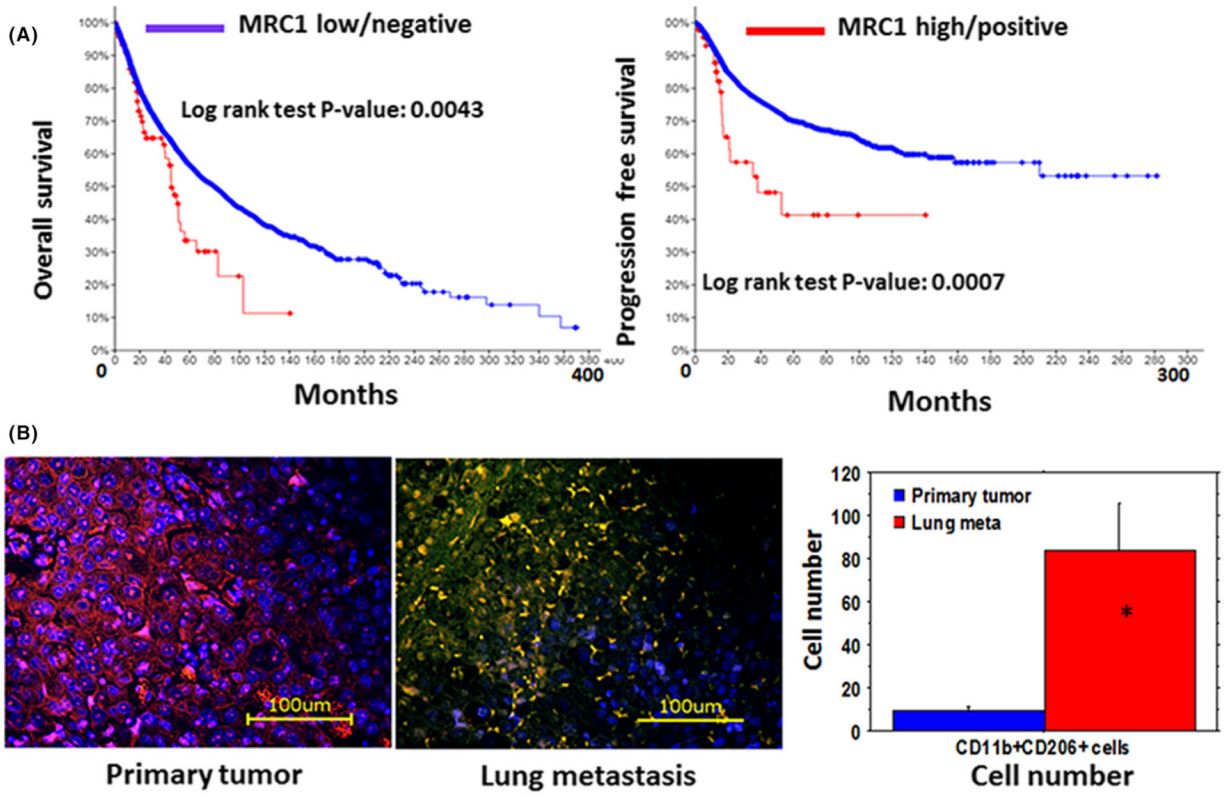

FIGURE 9.

(A) Disease-free and overall survival of patients with different cancers expressing mannose receptor (MRC1) in the tumor tissues (TCGA data). (B) Increased number of CD206+/CD11b+ cells in lung metastasis (middle panel, yellow cells) from breast cancer compared to that in the primary tumor (left panel). Quantitative analysis showed a significantly increased number of CD11b+CD206+ cells. The samples are from multiple patients and randomly selected histochemical sections ($n = 6$). * = $P < 0.01$

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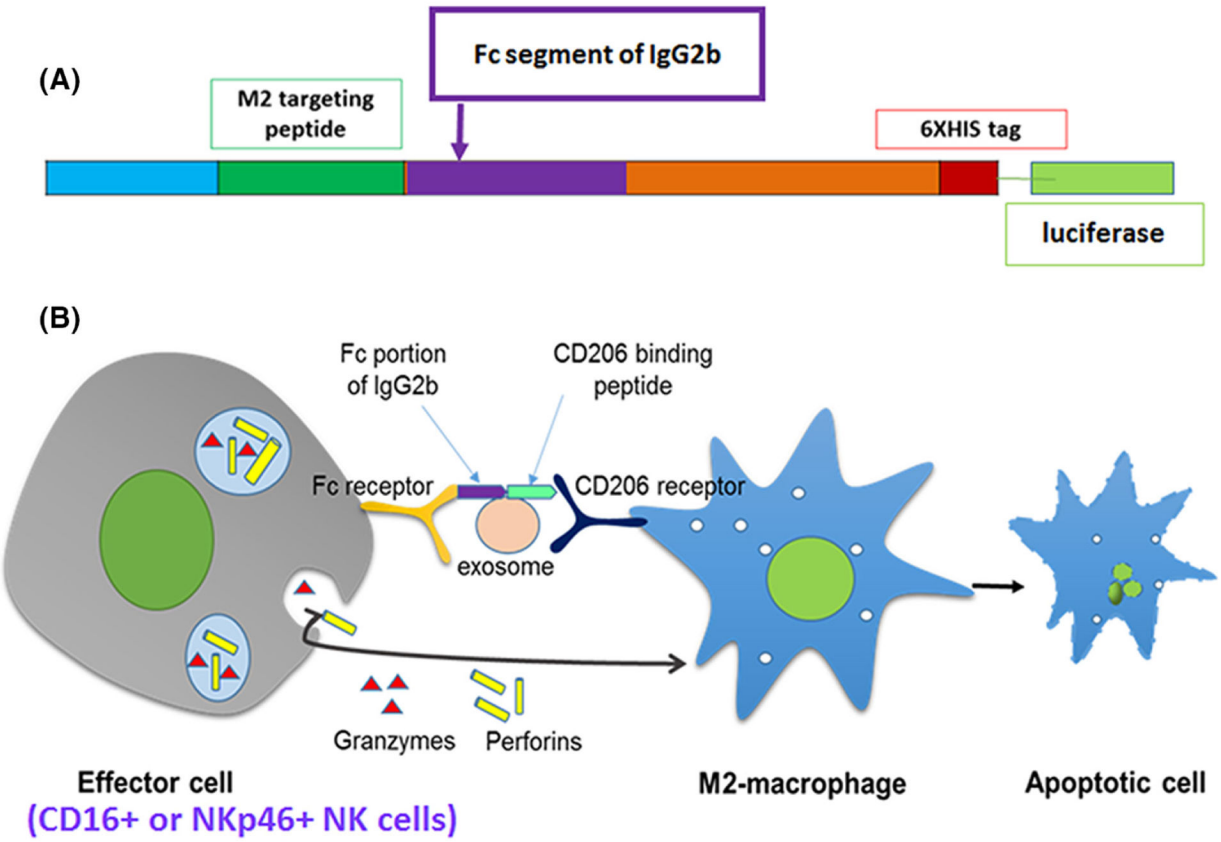

FIGURE 10.

(A) Vector design to express M2 targeting peptide and Fc-mIgG2b on exosomes. (B) Cartoon to show the mechanisms of ADCC through engineered exosomes

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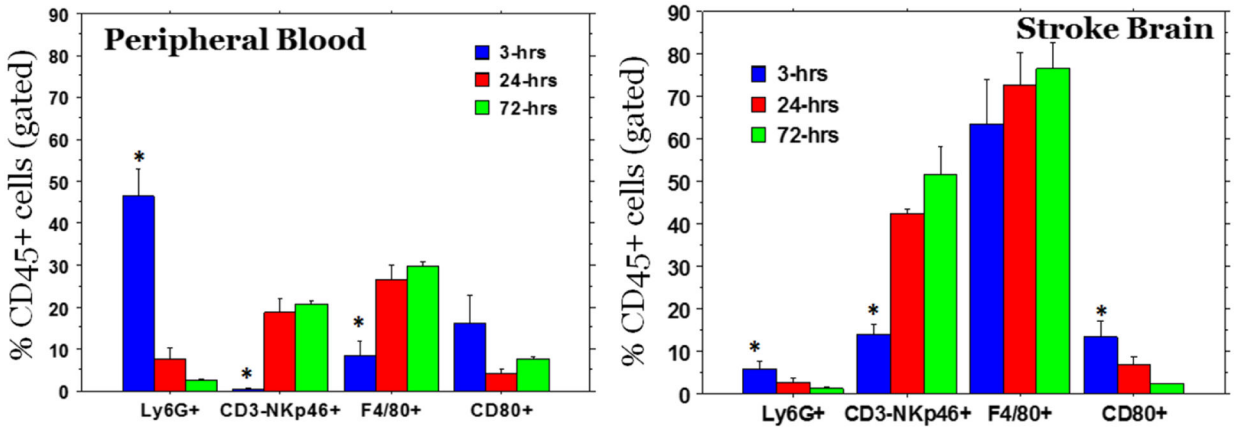

FIGURE 11.

Mobilization of neutrophils and M1 macrophages in the peripheral blood and in the stroke area was observed as early as 3 hrs. Whereas other cell types such as NK cells and macrophages (F4/80+, which also contain M2 type macrophages) gradually increased in the stroke areas. Following collection of peripheral blood from each stroke animal, animals were euthanized and perfused with ice cold PBS and the brain tissues from stroke area were collected and single cell suspensions were made for flow cytometry. * = significant differences

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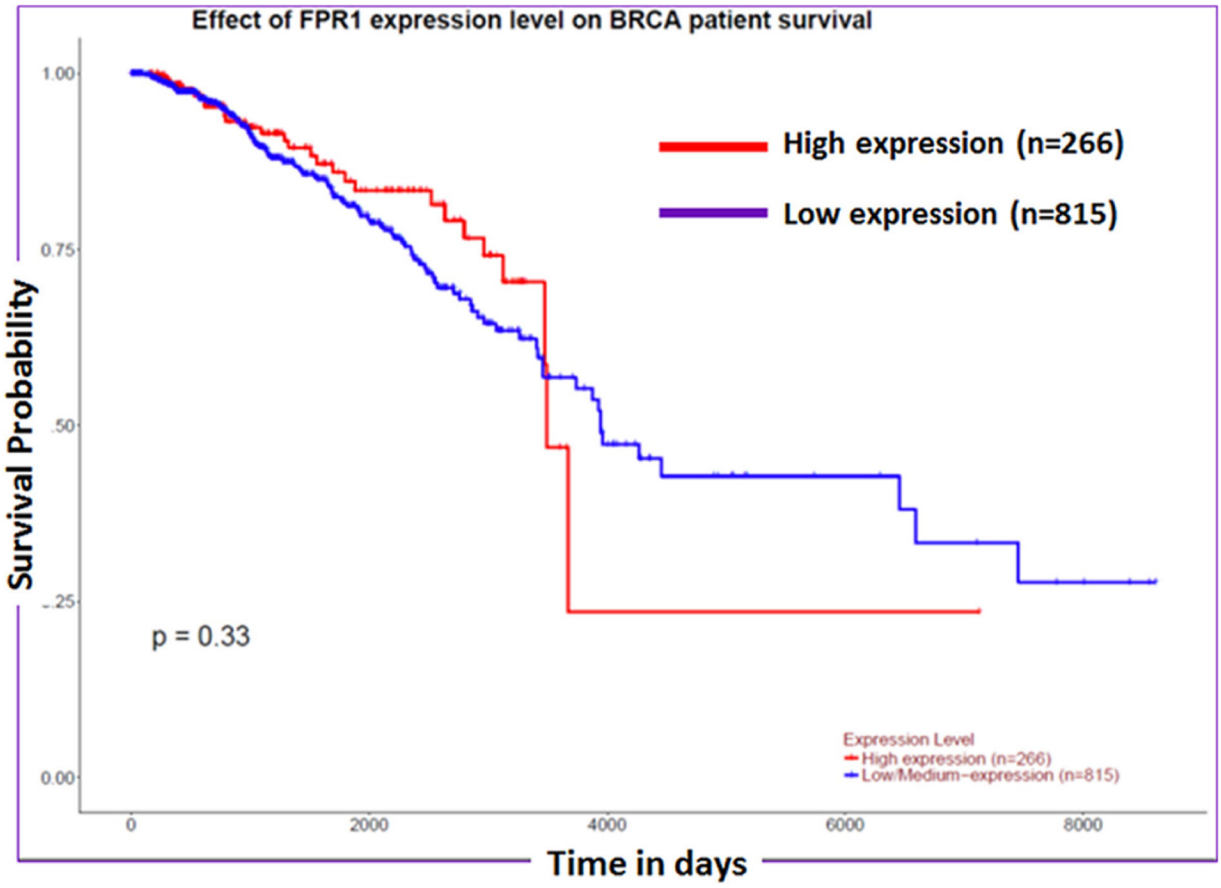

FIGURE 12.

Survival probability in BRCA+ breast cancer patients expressing FPR1. (TCGA data from UACLAN)

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TABLE 1

Characteristics and main differences between extracellular vesicles subtype[78–81]

Traits	Exosomes	Microvesicles	Apoptotic bodies
Biogenesis	Endosomal origin and exocytosis	Outward budding of the plasma membrane	Outward blebbing and fragmentation of the plasma membrane
Release time	Ten minutes or more	Few seconds	–
Pathways	ESCRT-dependent Tetraspanin-dependent Ceramide-dependent Stimuli-dependent	Ca2+-dependent Stimuli- and cell-dependent	Apoptosis-related
Size	30–150 nm	100–1000 nm	1000–5000 nm
Appearance-electron microscopy	Spheroid/cup shape	Irregular and electron-dense	Heterogeneous
Density	1.13–1.19 g/ml	1.04–1.07 g/ml	1.16–1.28 g/ml
Isolation method	ultracentrifugation, ultrafiltration, precipitation, size exclusion chromatography, immunoaffinity capture-based, microfluidics-based, polymer-based, etc.	Ultracentrifugation	No standardized protocol
Content	Proteins, nucleic acids, lipids, and metabolites	Proteins, nucleic acids, lipids, and metabolites	DNA fragments and histone, chromatin remnants, cell organelles, cytosol portions, degraded proteins
Typical constituent proteins	Tetraspanins (CD9, CD63, CD81), ESCRT proteins (Alix, TSG101), Integrins (−α, −β), heat shock proteins (HSP90, HSP70)	Anexin V, Flotillin-2, Selectins, Integrins, CD40 ligand, metalloproteinase	Anexin V, DNA, histones
Function	Cell-cell communication	Cell-cell communication	Product of programmed cell death. Removal of unwanted cells

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TABLE 2

Exosome protein markers

Exosome markers	Location	Collection and detection methods	References
Alix (PDCD6IP)	Cytoplasm	Ultracentrifugation	82,83
CD9	Plasma membrane	Immunohistochemistry	84–86
CD24	Plasma	Ultracentrifugation; Immunohistochemistry	87,88
CD63	Plasma membrane and cytosol	Immunohistochemistry	86,89,90
CD81	Plasma membrane	Immunohistochemistry	84
CPNE3	Plasma	Ultracentrifugation; Immunohistochemistry	91
EDIL3	Plasma	Ultracentrifugation	87,92
Exo-PD-L1	Serum	Immunohistochemistry	93
Fibronectin	Plasma	Ultracentrifugation	87,94
FLOT1	Plasma membrane	Ultracentrifugation, immunoblotting	95,96
HSP70	Serum, plasma membrane	Ultracentrifugation	97
TEX	Plasma	Density gradient ultracentrifugation; size exclusion chromatography (SEC); Differential centrifugation; Ultrafiltration	98
TfR	Perinuclear or plasma membrane	Ultracentrifugation; Size-exclusion chromatography (SEC)	99,100
TSG101	Cytoplasm	Ultracentrifugation, immunoblotting	101,102

Abbreviations: CPNE3, Copine III; EDIL3, EGF-Like Repeats and Discoidin I-Like Domains Protein 3; Exo-PD-L1, Exosomal programmed cell-death ligand 1; FLOT1, flotillin 1; PDCD6IP, Programmed cell death 6-interacting protein; TEX, Tumor-Derived Exosomes; TfR, Transferrin receptor; TSG101, Tumor susceptibility gene 101.

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TABLE 3

Exosome isolation methods

	Advantages	Disadvantages	References
Ultracentrifugation (Most commonly used method) [Differential centrifugation (DC)]	• Highly enriched EVs are produced. • Easiest and most widely used method. • Can analyze large volumes of samples. • Can analyze multiple samples at the same time.	• Not clinically applicable as it is laborious, time-consuming, and low throughput. • Specific equipment and expertise are required. • The absolute separation of EVs is not possible. • EVs can be damaged due to exosome clumping. • EV Recovery is only 2% to 80%. • Cannot isolate pure EV.	87,111,123–127
Precipitation	• Six times faster than ultracentrifugation. • 2.5-fold higher concentration of exosomes per ml. • Highly reproducible. • Produces extracellular vesicles that have a significantly low number of both IgGs and albumin. • Clinically applicable. • EV recovery is 90% • Fast, inexpensive, • Requires no special equipment • Can be used for both low- and high-sample volumes.	• Unable to isolate pure EVs. • Aggregation of exosomes. • Not suitable for identification of EV-associated biomarkers	123–125,128
Size exclusion chromatography (SEC)	• Clinically applicable. • Allows for size-based separation on a single column. • Can be used to obtain smaller size EVs. • EV recovery is 40%−90%. • Removes soluble components.	• Processing time is higher. • Exosomes can be degraded due to buffer selection.	124,125
Ultrafiltration (UF)	• Faster method. • Superior to other methods for using large volumes of EV-containing fluids. • Remove soluble components & separate exosomes from big particles. • Inexpensive because of cheaper equipment.	• Not clinically applicable. • Exosomes get attached to the filter pores and result in exosome loss. • Large vesicles can be damaged or deformed while passing through the membrane. • Cannot isolate pure EV.	87,124,125,129–131

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	Advantages	Disadvantages	References
Immuno-capture assays (ICA)	• Clinically applicable. • Simultaneously parallel ICA can be performed. • Isolate subpopulations of EVs. • Can efficiently detect members of the tetraspanin family, (e.g., CD81, CD9, and CD63). • Direct separation of exosomes from cell culture supernatant or bodily fluids is possible. • Highly specific. • High purity	• Time-consuming (can take up to hours). • Cannot isolate large-volume samples. • Expensive equipment. • Low exosome yield.	87,124,125,132,133
Density gradient centrifugation (DGC)	• Can separate low-density exosomes from other EVs. • Separate EVs devoid of protein contaminants. • highly purified EVs.	• Not clinically applicable as it is laborious, time-consuming, and low throughput. • Low exosome recovery rate.	87,111,134,135
Polymer-based precipitation	• Easy to use. • No need for specialized equipment. • Can be used for large samples.	• Nonexosomal contaminants may be present. • Cannot isolate pure EV.	136
Microfluidics	• Rapid analysis is possible due to the short time. • Micro devices are used.	• Low exosome yield. • Low reproducibility.	124,137,138
Tangential flow filtration	• Concentrate from large amounts of cell culture media. • Higher yield of EVs. • High reproducibility. • Produces exosome with fewer albumin contaminants. • Time-efficient.	• Costly method due to expensive equipment and the use of disposable filters.	139–142
Commercial kits miRCURY ExoQuick TEIR	• Suitable alternatives to UC. • Similar zeta potentials to UC.		143

Abbreviations: UC, Ultracentrifugation; TEIR, Total Exosome Isolation Reagent.

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TABLE 4

Categorizing isolation methods based on EV recovery

Isolation method	**% yield**	
Precipitation	90	111,125–127,129–133
Size exclusion chromatography (SEC)	40–90	
Ultrafiltration (UF)	10–80	
Differential centrifugation (DC)	2–80	
Density gradient centrifugation (DGC)	10	
Immunocapture assays (ICA)		

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TABLE 5

Categorizing isolation methods based on assay time

Isolation method	Hours	
Precipitation	0.3–12	111,125–127,129–133
Size exclusion chromatography (SEC)	0.3	
Ultrafiltration (UF)	0.5	
Differential centrifugation (DC)	3–9	
Immunocapture assays (ICA)	4–20	
Density gradient centrifugation (DGC)	16–90	