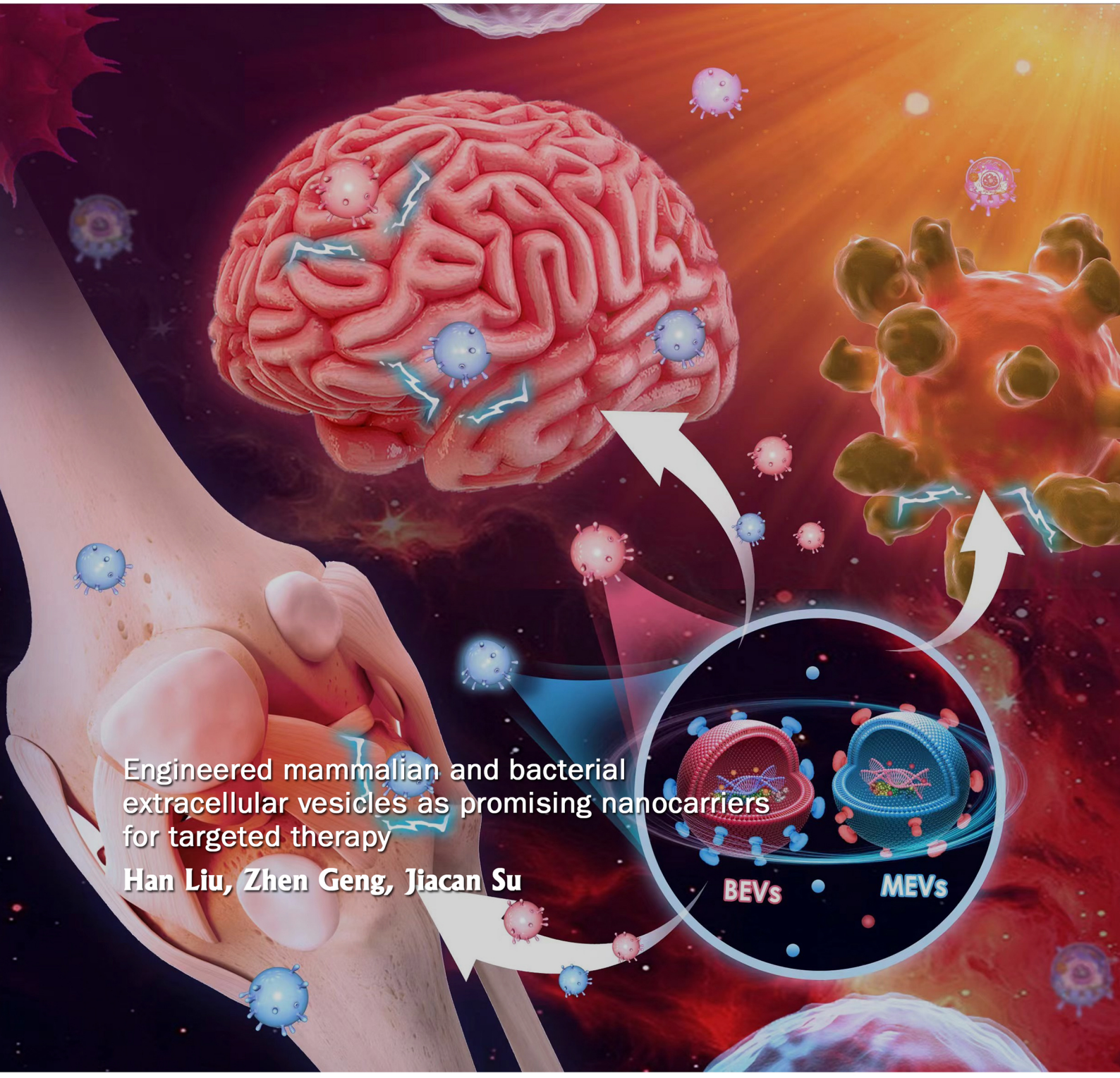
ISSN 2767-6641 (Online)
Volume 3 | Issue 2 | June 2022

Extracellular Vesicles and Circulating Nucleic Acids



Engineered mammalian and bacterial extracellular vesicles as promising nanocarriers for targeted therapy

Han Liu, Zhen Geng, Jiacan Su

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DOI: 10.20517/evcna.2022.04

Extracellular Vesicles and Circulating Nucleic Acids

Review

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Engineered mammalian and bacterial extracellular vesicles as promising nanocarriers for targeted therapy

Han Liu[#], Zhen Geng[#], Jiacan Su

Institute of Translational Medicine, Shanghai University, Shanghai 200444, China.
[#]Authors contributed equally.

Correspondence to: Prof. Jiacan Su, Institute of Translational Medicine, Shanghai University, Shanghai 200444, China. E-mail: drsujiacan@163.com

How to cite this article: Liu H, Geng Z, Su J. Engineered mammalian and bacterial extracellular vesicles as promising nanocarriers for targeted therapy. *Extracell Vesicles Circ Nucleic Acids* 2022;3:63-86. https://dx.doi.org/10.20517/evcna.2022.04

Received: 17 Jan 2022 **First Decision:** 17 Mar 2022 **Revised:** 21 Mar 2022 **Accepted:** 7 Apr 2022 **Published:** 13 Apr 2022

Academic Editor: Yoke Peng Loh, Cheng Jiang **Copy Editor:** Jia-Xin Zhang **Production Editor:** Jia-Xin Zhang

Abstract

Extracellular vesicles (EVs), which are nanocarriers with phospholipid bilayer structures released by most cells, play a key role in regulating physiological and pathological processes. EVs have been investigated due to their loading capacity, low toxicity, immunogenicity, and biofunctions. Although EVs have shown good potential as therapeutic vehicles, natural EVs have a poor targeting ability, which substantially reduces the therapeutic effect. Through the addition of a targeting unit into the membrane surface of EVs or inside EVs by engineering technology, the therapeutic agent can accumulate in specific cells and tissues. Here, we focus on mammalian EVs (MEVs) and bacterial EVs (BEVs), which are the two most common types of EVs in the biomedical field. In this review, we describe engineered MEVs and BEVs as promising nanocarriers for targeted therapy and summarize the biogenesis, isolation, and characterization of MEVs and BEVs. We then describe engineering techniques for enhancement of the targeting ability of EVs. Moreover, we focus on the applications of engineered MEVs and BEVs in targeted therapy, including the treatment of cancer and brain and bone disease. We believe that this review will help improve the understanding of engineered MEVs and BEVs, thereby promoting their application and clinical translation.

Keywords: Biological engineering, chemical modification, mammalian extracellular vesicles, bacterial extracellular vesicles, targeted therapy

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INTRODUCTION

According to the International Society for Extracellular Vesicles, extracellular vesicles (EVs) are defined as "particles naturally released from the cell that is delimited by a lipid bilayer and cannot replicate"[1]. Notably, the term "exosomes" is often used as a general description of EVs[2,3]. EVs can transmit a variety of biologically active components, such as proteins, nucleic acids, lipids, and metabolites, to affect the performance of recipient cells[4-7]. Many studies have found that EVs play an important role in regulating the physiological and pathological processes of the body by participating in cell-to-cell communication, cell proliferation, cell migration, angiogenesis, and immune regulation[6,8]. Due to their unique nanosized structures, loading capacity, biochemical properties, and good biocompatibility, EVs have been widely used in various applications in the biomedical field, such as vaccines, cancer agents, and drug delivery vehicles[9-11]. Although EVs have shown good potential as therapeutic vehicles, natural EVs were shown to have a poor targeting ability in animal experiments, which substantially reduced the therapeutic effect[12,13]. Therefore, many engineered methods have been applied to improve the targeting ability of EVs[14-16].

In our previous study, we focused on engineering EVs derived from mammalian cells such as endothelial cells[17] and NIH-3T3 cells[18]. Although these engineered mammalian EVs (MEVs) were shown to have an excellent targeting ability and therapeutic effect, the low extraction yield (requiring many mammalian cells) is still a limiting factor. The current complex and low yield protocols for purification and extraction of EVs, such as ultracentrifugation, gradient ultracentrifugation, co-precipitation, size-exclusion chromatography, and field flow fractionation, pose a tremendous challenge in the mass production of EVs. Therefore, we recently paid more attention to bacterial EVs (BEVs), which can be easily obtained through fed-batch fermentation and purification procedures[4,13]. Moreover, according to the latest minimal information for studies of extracellular vesicles (MISEV) in 2021, the topic of "nonmammalian EVs, especially BEVs" ranked fourth[1]. Although engineered BEVs have also been used in the field of biomedicine[19,20], BEV research is less developed than that of MEVs. The number of MEV and BEV studies has increased rapidly in recent years (PubMed.gov). In general, MEVs and BEVs are the two most common types of EVs in the biomedical field.

Due to the importance of MEVs and BEVs in the field of biomedicine, we focus on these two types of EVs and their engineering and applications in this review. Here, to elucidate engineered MEVs and BEVs as promising nanocarriers for targeted therapy, we first summarize the biological basis of MEVs and BEVs, including different mechanisms of biogenesis, isolation, and characterization. We then present approaches for modifying BEVs and MEVs, which are physical engineering (membrane fusion and membrane coating), biological engineering [membrane fusion, lysosome-associated membrane glycoprotein 2B (LAMP-2B), and CD63], and chemical engineering (covalent reaction and noncovalent reaction), to enhance the targeting ability. Finally, we conclude with the application of engineered MEVs and BEVs in targeted therapy of tumors (chemotherapy, gene therapy, photothermal therapy, and immunotherapy), brain disease [Alzheimer's disease (AD), Parkinson's disease (PD), and ischemic stroke], and bone disease [osteoarthritis (OA) and osteoporosis (OP)]. This review will help improve our understanding of the importance of MEVs and BEVs and thus promote targeted therapy for various diseases.

THE BIOGENESIS OF MEVS AND BEVS

EVs are a general term for nanovesicles with phospholipid bilayer structures secreted by most cells[2,3,21]. EVs can be secreted by almost all cells and are widely present in cell supernatants and various body fluids[22]. As early as the 1960s, BEVs were first reported in the Gram-negative bacteria *Escherichia coli*[23-26]. In the 1980s, Pan and Harding *et al.*[27,28]successively observed the release of MEVs in reticulocytes. At this stage, both BEVs and MEVs were regarded as "garbage bags" for cells to discharge metabolic waste[29]. In 1996, Raposo

et al.[30] found that EVs derived from B lymphocytes can present antigens and activate T lymphocytes to participate in the regulation of immune cells. Soon after, EVs that could transfer nucleic acids such as mRNA and miRNA were also found in archaea[31,32]. Gradually, researchers discovered that the role of EVs is much more than clearance of cell waste; EVs also transmit signals to distant parts of the body, where they can affect multiple dimensions of cell life[10]. A detailed description of the mechanisms of MEVs and BEVs would provide an important theoretical basis for the treatment of disease.

The biogenesis of MEVs

According to the size, biological characteristics, and formation process, MEVs can be classified into three major subtypes: exosomes, microvesicles, and apoptotic bodies [Figure 1A][33]. Exosomes are EVs with a diameter of 40-160 nm formed by the fusion of multivesicular bodies (MVBs) and cell membranes. Moreover, microvesicles are EVs with a size range of 200-1000 nm in diameter that are directly formed by cell membrane budding. Apoptotic bodies are vesicular bodies with larger diameters (500-2000 nm in diameter) formed by cell fragmentation during the process of cell apoptosis. Among these subtypes, exosomes have received widespread attention due to their sizes, biological composition, and cell-to-cell communication ability[10]. Therefore, we use exosomes to represent MEVs in this review.

MEVs are formed by the endosomal system in a process involving three stages [Figure 1A][34]. First, the plasma membrane invaginates to form endocytic vesicles, which fuse with each other to form early endosomes. Second, early endosomes invaginate again to encapsulate intracellular cargos, forming multiple intraluminal vesicles (ILVs), which are further transformed into late endosomes, MVBs. Finally, the MVBs fuse with the plasma membrane and excrete their contents into the extracellular space. The formation, sorting of cargos, and release of exosomes are a series of finely regulated processes that require the participation of many proteins. The formation of exosomes involves proteins such as endosomal sorting complex required for transport (ESCRT), transmembrane proteins (CD9, CD63, and CD81), apoptosis-linked gene 2-interacting protein X (Alix), and tumor susceptibility gene 101 protein (TSG101). Moreover, the intracellular transport of exosomes involves the participation of many molecular switches such as the RAB GTPase protein and cytoskeletal proteins such as actin and tubulin[35]. In addition, the secretion of exosomes requires the participation of SNARE protein complexes and the synaptic binding protein family[36]. The protein composition of exosomes can reflect the characteristics of their endosomal origin. In different types of cells and body fluids, exosomes all contain the same marker protein molecules, such as Alix, TSG101, SNARE, and RAB GTPase, and the transmembrane proteins, CD9, CD63, and CD81[37]. In addition to specific protein composition, exosomes also have a special lipid composition. Exosome membranes are enriched with cholesterol, ceramide, and sphingolipids[38]. These lipids are also involved in the formation and secretion of exosomes. For example, ceramide is involved in the budding of ILVs and MVBs[38].

The biogenesis of BEVs

Bacteria are divided into Gram-negative (G^-) bacteria and Gram-positive (G^+) bacteria based on their structure, morphology, and staining properties. Both G^+ and G^- bacteria can release EVs without energy consumption[39,40]. BEVs are EVs with sizes of 20-400 nm in diameter and can be divided into four types: outer-membrane vesicles (OMVs), explosive outer-membrane vesicles (EOMVs), outer-inner membrane vesicles (OIMVs), and cytoplasmic membrane vesicles (CMVs). The first three EVs are formed by G^- bacteria, and the latter are formed by G^+ bacteria. G^+ bacteria produce CMVs by endolysin-triggered cell lysis (bubbling cell death) [Figure 1B][26]. OMVs are formed by blebbing of the outer membrane of G^- bacteria, while OIMVs and EOMVs are released by explosive cell lysis of G^- bacteria [Figure 1B][25].

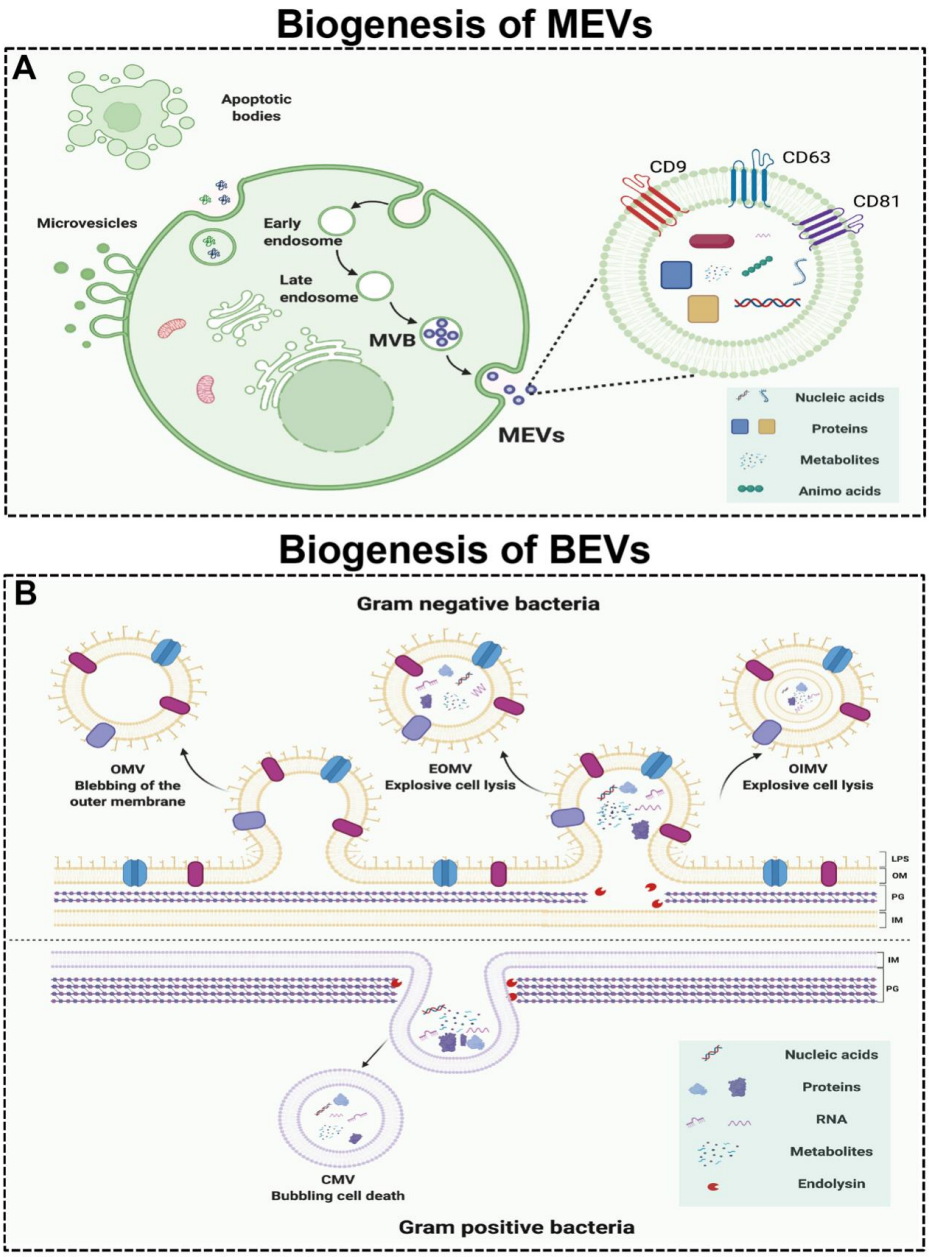

Figure 1. The biogenesis of mammalian extracellular vesicles (MEVs) and bacterial extracellular vesicles (BEVs). (A) The biogenesis of MEVs. (B) The biogenesis of BEVs. Figures were created with Biorender.com.

Similar to MEVs, BEVs are lipid bilayer-enclosed structures containing various biomolecules released by cells and are increasingly regarded as the main form of cell-to-cell communication[41-43]. Due to the diversity of their contents, BEVs have a key role in bacteria-bacteria and bacteria-host communications. Generally, BEVs contain high levels of proteins, nucleic acids, metabolites, small molecules, *etc.* G^- BEVs are enriched in periplasmic proteins such as the multidrug efflux pump subunit AcrA and outer membrane proteins such as outer membrane protein F (OmpF). Notably, Vanaja *et al.*[44] used OmpF as a specific (surface) marker for *E. coli*-derived EVs. However, the lack of specific markers is still a challenge in the field of BEVs[39]. Moreover, lipids are an important structural component of bacterial cell membranes. The most significant difference between G^+ and G^- BEV contents is lipopolysaccharide (LPS, or endotoxin), which can cause an

innate immune response[45]. Knockout of *msbB* in G^- bacteria *E. coli* for less endotoxic EVs is a common approach[15,19,20,46]. Interestingly, the nonpathogenic G^- bacteria *E. coli* Nissle 1917 lacks definite virulence factors such as LPS, so it can be used as a probiotic for the treatment of various gastrointestinal diseases[47-49]. Due to its easy genetic manipulation and probiotic characteristics, *E. coli* Nissle 1917 and its BEVs are promising candidates for medical engineering. In addition, BEVs have been reported to transfer nucleic acids, such as DNA and RNA, into other bacterial cells[50] and mammalian cells, which trigger different host immune responses and cellular processes[51,52]. RNA, especially miRNA and siRNA, can be protected from degradation through BEVs, which promotes delivery to mammalian cells[53]. BEVs selectively package different metabolites depending on the strains. Gujrati *et al.*[15] reported that the BEVs secreted by strains overexpressing melanin (Mel) also contain Mel. These findings indicate the applications of EVs in biomedicine.

THE ISOLATION OF MEVS AND BEVS

The isolation and characterization of MEVs and BEVs is an indispensable step for their further application in biomedicine. In fact, the isolation of such nanoparticles is generally difficult. MEVs can be derived from a variety of biological fluids, such as blood serum[54], breast milk[55], urine[56], tears[57], saliva[58], and sperm[59]. However, BEVs are found in many kinds of media, such as LB, MRS broth[60], and BHI broth (which always requires porcine mucin for *Akkermansia muciniphila*)[61]. Therefore, there is a major difference in viscosity, which causes difficulties in isolation and purification. Moreover, the amount of sample available for isolation is another factor that affects efficiency. Recently, various isolation methods, such as differential centrifugation, precipitation, size exclusion chromatography, and magnetic capture, have been established based on the differences in size, density, charge, and surface ligands[62-65]. Here, we summarize the most commonly used and effective MEV and BEV isolation techniques [Figure 2].

Isolation of MEVs

Ultracentrifugation-based MEV isolation is the gold standard, thus one of the most commonly used and reported techniques[66-69]. According to the first large-scale detailed survey of current global MEV isolation practices, 81% of researchers used ultracentrifugation (including differential centrifugation) for MEVs isolation[68]. Li *et al.*[67] summarized the use of ultracentrifugation and differential centrifugation to remove other impurities in the sample through a combination of different speeds and times to finally achieve the isolation of MEVs. Low-speed centrifugation (300-2000 *g* or 2000-10,000 *g*) is generally used to remove cells, dead cells, cell debris, *etc.* Ultracentrifugation (100,000-200,000 *g*) is generally applied to collect MEVs [Figure 2B]. In addition, for better purification of MEVs, density gradient centrifugation such as iodixanol can be used [Figure 2A]. After the isolation of MEVs, the most commonly used characterization methods are transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA), and Western blotting (WB)[17,18,70,71]. TEM and NTA are used to show the sizes, shapes, and concentrations of EVs. The transmembrane proteins CD9, CD63, and CD81, as well as TSG101, are often used as specific markers in WB[17,18,70,71].

Isolation of BEVs

The standard protocol for BEVs purification is to physically separate EVs from cell culture through a series of steps [Figure 2B][72-74]. Simply, low-speed centrifugation (2000-10,000 *g*) is used to remove bacteria and their debris in the fermentation broth. Then, a 0.22 μm sterile filter is applied to remove residual bacteria. Subsequently, a 100 kDa ultrafiltration membrane is required to remove non-BEV-associated proteins. Furthermore, ultracentrifugation and density gradient centrifugation are used together for the separation and purification of BEVs. Using the above method, we successfully obtained multiple BEVs, such as EVs derived from *Lactobacillus rhamnosus* GG[4]. Similar to the characterization of MEVs, TEM and NTA are common methods used to assess BEVs. The outer membrane proteins OmpA[20] and OmpF[44] are used as

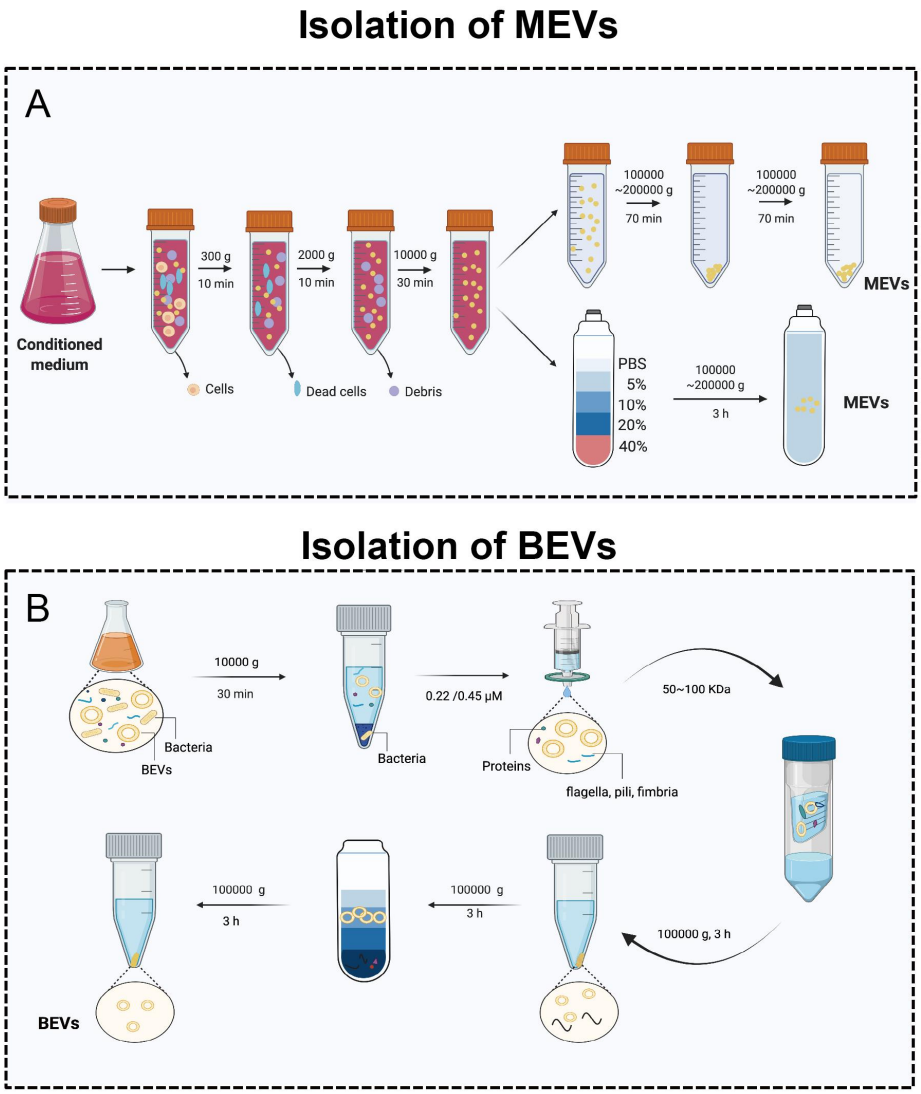

Figure 2. The isolation of MEVs and BEVs. (A) The isolation of MEVs. (B) The isolation of BEVs. Figures were created with Biorender.com.

specific markers for *E. coli*-derived EVs. However, many studies involving BEVs do not use WB[15,19,75]. The selection of specific markers is still a major challenge in the BEV field.

ENGINEERING TECHNIQUES TO IMPROVE THE TARGETING ABILITY OF MEVS AND BEVS

Nanosized EVs have been investigated as therapeutic vehicles due to their loading capacity, low toxicity, immunogenicity, and biofunctions[10,76]. However, the poor targeting ability of natural EVs is not conducive to therapy. With the introduction of the concept of precision medicine in 2015[77], researchers are increasingly investigating the targeting ability of EVs. Targeted delivery could increase the local concentration of the therapeutic agent and minimize side effects. Through the addition of a targeting unit into the membrane surface of EVs or inside EVs by engineering technology, the therapeutic agent could accumulate in specific cells and tissues. Many engineering technologies such as biological engineering and chemical modification have been used to modify EVs to enhance their targeting ability. The membrane

surface of MEVs is rich in lipoproteins (such as phosphatidylserine, cholesterol, sphingomyelin, and ceramide) and membrane proteins (adhesion molecules, integrins, membrane transport proteins, MHC class I/II, tetraspanins, and transferrin receptor)[78]. At present, the targeting engineering of MEVs is intensively studied[13,16,66], but research on the targeting engineering of BEVs has just started[15,20]. Their similar phospholipid bilayer structure makes most engineering methods universal. Here, we summarize the techniques to improve the targeting ability of MEVs and BEVs [Figure 3 and Table 1].

Physical engineering

Physical methods mainly include membrane fusion [Figure 3A] and membrane coating [Figure 3B]. The liposome mediated MEVs and BEVs membrane fusion strategy is an important engineering approach that endows EVs with specific functional ligands. Liposomes with targeting molecules on the surface can be delivered into EVs through membrane fusion. A mixture of MEVs and liposomes incubated at 37 °C for 12 h could form hybrid nanocarriers. Lin *et al.*[79] developed MEV-liposome hybrid nanoparticles to accurately deliver large plasmids, such as CRISPR-Cas9, into mesenchymal stem cells (MSCs). Similarly, our team constructed MEV-liposome hybrid nanoparticles with the ability to target bone through spontaneous membrane fusion[18]. Yang *et al.*[91] reported that virus-mimetic fusogenic MEVs could deliver membrane proteins to the target cell membrane by membrane fusion. Gao *et al.*[92] also developed a virus-mimicking fusogenic vesicle with fusogenic proteins that could target sialic-acid-containing receptors on MEVs and promote membrane fusion. In addition, the fusion of MEVs derived from different cells and functionalized liposomes could be triggered by polyethylene glycol (PEG)[93].

On the other hand, membrane coating is a promising nanotechnology for disease-relevant targeting. The biological characteristics of cell membranes endow nanoparticles with broader applications, including targeting ability[94]. Various cell types, including mammalian cells (such as red blood cells[95], platelet[96], and cancer cells[97]) and bacterial cells[40,98], have been used for membrane sources. The bacterial membrane could be used for vaccination because of the immunogenic caused by peptidoglycan and outer membrane proteins[99]. Recently, BEV-coated multi-antigenic nano-vaccines have been developed. BEV coating and indocyanine green (ICG)-loaded magnetic mesoporous silica nanoparticles (MSN) were developed by Chen *et al.*[80] to regulate antigen presentation pathways in dendritic cells. The *in vivo* data show that the BEV-ICG-MSNs vaccine could target lymph nodes from the injection site[80].

Biological engineering

We can use biological engineering to fuse the gene sequence of the protein with the gene sequence of the selected membrane protein[75]. The most commonly used methods of biological engineering are LAMP-2B [Figure 3C][71,83,85,100,101] and CD63 [Figure 3D][86,87,102]. LAMP-2B, a member of the lysosome-associated membrane protein family, is the most widely used MEV membrane protein for displaying targeting motifs. The N-terminus of LAMP-2B is present on the outer surface of MEVs, and any targeting sequences can be added [Figure 3B]. Alvarez-Erviti *et al.*[83] used EVs derived from dendritic cells containing Lamp2B-RVG (neuron-specific rabies viral glycoprotein) to achieve neuronal cell (Neuro-2a) targeting. To obtain colorectal tumor (HCT-116) targeting abilities, Liang *et al.*[84] fused a human epidermal growth factor receptor 2 (HER2) affibody to the N-terminus of LAMP-2B. Xu *et al.*[71] fused peptide E7 and LAMP-2B to produce MEVs with the ability to target synovial fluid-derived MSCs. By fusing a chondrocyte-affinity peptide with LAMP-2B, Liang *et al.*[85] generated chondrocyte-targeting MEVs. The transmembrane protein CD63 can also be used to display targeting sequences [Figure 3C]. Engineered MEVs with hepatocellular carcinoma (HepG2)-targeting ability were developed by expression in 293T cell hosts and gene fusion between the CD63 and ApoA-1 sequences[86]. For targeting $CD8^+$ T cells, Kanuma *et al.*[87] constructed engineered MEVs by fusing ovalbumin (OVA) antigen to CD63.

Table 1. Summary of the techniques to improve the targeting ability of MEVs and BEVs

Methods	Target cells/Tissue	Ref.
Physical engineering		
Membrane fusion	Mesenchymal stem cells Bone mesenchymal stem cells	[79] [18]
Membrane coating	Lymph nodes	[80]
Biological engineering		
LAMP-2B	Neuronal cell Colorectal cancer (HCT-116) Synovial mesenchymal stem cells Chondrocyte	[83] [84] [71] [85]
CD63	Hepatocellular carcinoma (HepG2) $CD8^+$ T-cells	[86] [87]
Chemical engineering		
Covalent reactions	Glioma Cerebral vascular endothelial cell	[88] [12]
Noncovalent reactions	Hepatoma 22 subcutaneous cancer cells Cancer cells (SKOV3, HCC-1954)	[89,90] [20]

MEVs: Mammalian extracellular vesicles; BEVs: bacterial extracellular vesicles.

Chemical engineering

Chemical engineering is another common method that allows various ligands to be displayed in the membrane by covalent reactions [Figure 3E][103-105] or noncovalent reactions [Figure 3F][106-108]. The most applied covalent reactions include click chemistry bioconjugation and aldehyde amine condensation. Click chemistry has been the most used method for attaching targeting peptides to the surface of MEVs in recent years[12,88,109]. Sulfhydryl groups, widely present in membrane proteins, can react with maleimide by Michael addition reaction, which is usually used to selectively modify protein sites. Therefore, various functional molecules are added to the surface of MEVs by conjugating sulfhydryl groups. Jia *et al.*[88] and Tian *et al.*[12] also applied click chemistry to develop targeting MEVs, which have glioma-targeting and cerebral vascular endothelial cell-targeting abilities, respectively. In addition, the binding of the anchor peptides CP05 and CD63 via covalent bonding is an example of bioconjugation, indicating that engineered MEVs have emerging prospects in targeted therapy[103]. Tran *et al.*[16] reported that the combination of aptamers and molecularly targeted MEVs is an intelligent engineering nanovesicle for precision medicine. Moreover, MEVs could be conjugated to aptamers by N-ethyl-N'-[3-(dimethylamino) propyl] carbodiimide/N-hydroxysuccinimide amidation and aldehyde amine condensation reactions[82].

The most commonly applied noncovalent reactions include hydrophobic insertion, multivalent electrostatic interactions, and receptor-ligand binding [Figure 3E]. Lipids or amphiphilic molecules can be inserted into the lipid bilayer of MEVs, and the hydrophilic part is displayed on the exterior. The hydrophobic insertion can be easily accomplished with cells and MEVs at different temperatures. The commercial amphiphilic molecule DSPE-PEG can couple with ligands such as aminoethylanisamide (AA)[110], RGD[107,111], folate[112], *etc.*, to enhance the targeting ability of MEVs. Multivalent electrostatic interactions and receptor-ligand binding are less frequently applied to MEVs. Nakase *et al.*[113] utilized the negatively charged characteristics of MEVs to bind cationic lipids, which promoted the formation of pH-sensitive fusion peptides and MEVs. Qi *et al.*[89,90] constructed targeted and magnetic MEVs by receptor-ligand binding. These researchers coincubated reticulocyte-derived EVs (containing transferrin receptors on the membrane surface) and transferrin-conjugated multiple superparamagnetic iron oxide nanoparticles. Importantly, Gujrati *et al.*[20] used this method to construct engineered BEVs with an anti-HER2 affibody on the outer membrane surface. The engineered BEVs could target and kill cancer cells without nonspecific side effects. Although

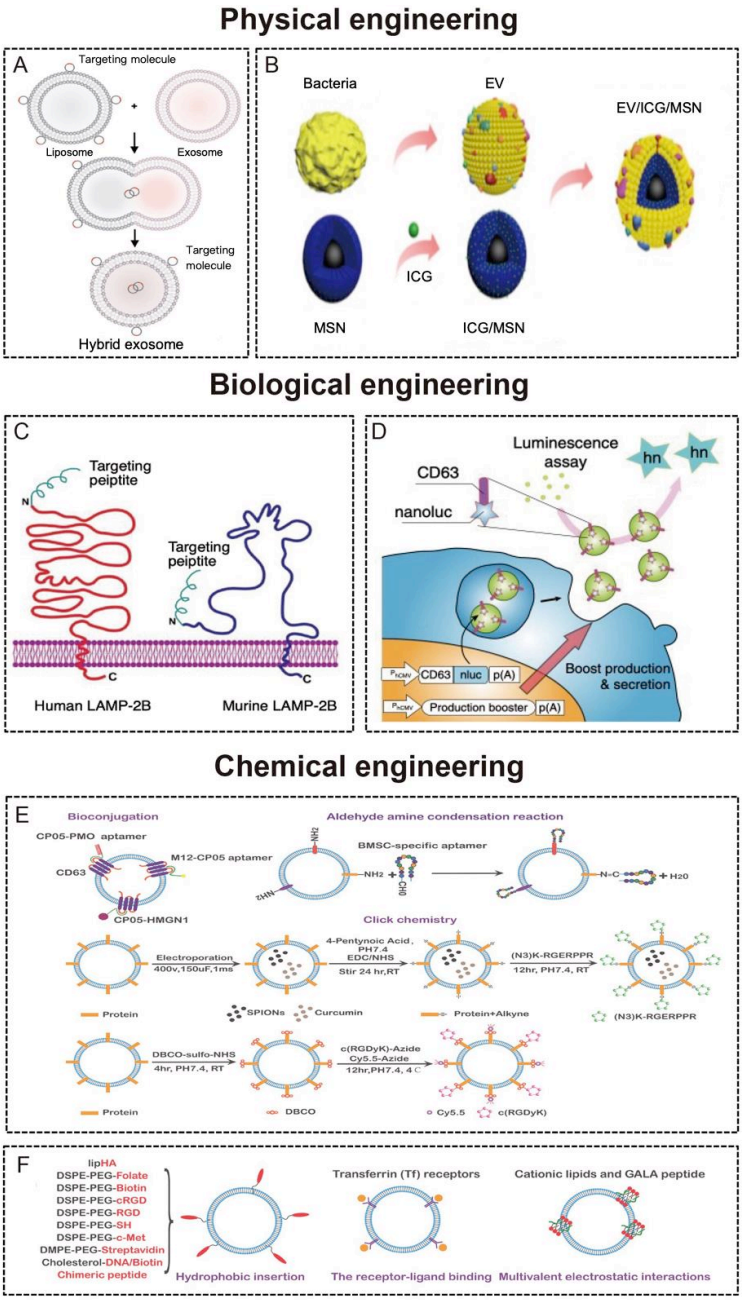

Figure 3. (A) The procedure to produce hybrid nanoparticles by membrane fusion. Liposomes with targeting molecules on the surface can be delivered into EVs through membrane fusion[79]. Copyright 2018 WILEY-VCH. (B) The procedure to produce hybrid nanoparticles by membrane coating[80]. Copyright 2020 Ivyspring International Publisher. (C) The fusion of the targeting peptide with LAMP-2B[66]. Copyright 2021 Ivyspring International Publisher. (D) The fusion of the delivery molecule with CD63[81]. Copyright 2018 Springer Nature. (E) Targeted modification of EVs based on chemical covalent reactions[82]. Copyright 2021 Elsevier. (F) Targeted modification of EVs based on chemical non-covalent reactions[82]. Copyright 2021 Elsevier. LAMP-2B: Lysosome-associated membrane glycoprotein 2B.

research on BEVs is not as developed as that on MEVs, their membrane structures are similar. Therefore, the engineering techniques of MEVs provide a good foundation for the in-depth study of BEVs in the future.

THE APPLICATION OF ENGINEERED MEVS AND BEVS IN TARGETED THERAPY

We summarize above the biogenesis, isolation, and characterization methods of MEVs and BEVs, as well as the various engineering methods, and engineered MEVs and BEVs can be used for targeted therapy in a variety of tissues. Next, we summarize the applications of engineered MEVs and BEVs with an enhanced targeting ability in tumors, brain, and bone diseases [Figure 4 and Table 2].

Cancer

Various natural nanoscale MEVs and BEVs have been applied as drug delivery nanocarriers in cancer therapy[125-129]. The engineered targeting MEVs and BEVs show enhanced therapeutic effects for future cancer therapy, including chemotherapy, gene therapy, photothermal therapy, and immunotherapy.

Chemotherapy

The treatment of tumor diseases routinely involves chemotherapeutic drugs, but chemotherapy drugs do not show specific targeting and have significant cytotoxic side effects, resulting in poor therapeutic effects. Targeted drug delivery based on engineered MEVs and BEVs could increase the local concentration and minimize cytotoxic side effects, consequently improving efficacy. Tian *et al.*[100] constructed iRGD-MEVs for the delivery of doxorubicin to the breast cancer cell line MDA-MB-231. The chemotherapeutic drug doxorubicin was encapsulated in targeting iRGD-MEVs by electroporation technology. Intravenous injection of iRGD-MEVs specifically delivered doxorubicin to tumor tissues and resulted in inhibition of tumor growth. Qi *et al.*[89] developed dual-functional (magnetic and targeting ability) MEVs loaded with doxorubicin to target hepatoma 22 subcutaneous cancer cells. Dual-functional MEVs enhanced the cancer-targeting ability under a magnetic field and suppressed tumor growth [Figure 5A]. Similarly, A33 antibody functionalized MEVs with doxorubicin were used to target the colorectal cancer cell line LIM1215[114].

Gene therapy

Gene therapy is a strategy to correct or compensate for abnormal gene expression in tumor cells by delivering nucleic acids such as siRNAs, miRNAs, *etc.*, to achieve the purpose of treatment, which has proven to be a promising cancer treatment approach[130]. Specifically, EVs can protect RNA from degradation, which ensures the stability and bioactivity of RNA after targeting cells[131]. Bai *et al.*[101] reported engineered targeting tLyp-1 MEVs for efficient delivery of *SOX2* siRNA to HEK293T cells. The engineered tLyp-1 MEVs had high transfection efficiency in non-small-cell lung cancer (NSCLC) and a high *SOX2* gene silencing ability in NSCLC stem cells. Zhao *et al.*[115] exploited biomimetic CBSA-MEV nanoparticles loaded with S100A4 siRNA, which effectively targeted the lung and showed excellent gene-silencing effects. Moreover, bioengineered BEVs have been used for targeted therapy of tumors. Gujrati *et al.*[20] constructed BEVs with low immunogenicity that can target cancer cells by delivering KSP siRNA [Figure 5B].

Photothermal therapy

The problems of recurrence, drug toxicity, and multidrug resistance are still difficult to overcome with traditional surgical intervention, chemotherapy, and gene therapy. Photothermal therapy is a nontoxic and noninvasive tumor-targeted treatment method[111,116,132,133]. The combination of engineered bioactive material loaded EVs and photothermal therapy is a promising method for cancer therapy. Bose *et al.*[116] developed MEVs loaded with anti-miRNA-21-coated gold-iron oxide nanoparticles (GIONs). MEV-GIONs showed strong T2 contrast in magnetic resonance imaging and photothermal effects in breast cancer 4T1 cells. Cao *et al.*[111] constructed Arg-Gly-Asp (RGD) peptide-MEVs coated with vanadium carbide quantum dots. The resulting MEVs could target cancer cells and access the nucleus to induce low-temperature photothermal therapy, which showed effective tumor destruction. In addition, Mel is highly suitable for photothermal therapy due to its good photothermal conversion efficiency[134,135]. Gujrati *et al.*[15,117] introduced engineered

Table 2. Summary of the application of engineered MEVs and BEVs in cancer, brain, and bone disease

Disease/Therapy	EV source	Ref.
Cancer		
Chemotherapy	Mouse immature DCs Serum MDA231/B16F10 cells	[100] [89] [114]
Gene therapy	HEK293T Mouse 4 T1 cells *E. coli* K-12 W3110	[101] [115] [20]
Photothermal therapy	4T1/SKBR3/HepG2 cells MCF-7 cells *E. coli* K-12 W3110	[116] [111] [15,117]
Immunotherapy	CAR-T cells HEK-293T cells	[118] [119]
Brain		
Alzheimer's disease	Dendritic cells Mesenchymal stem cells	[83] [120]
Parkinson's disease	Dendritic cells HEK293T cells HEK293T cells	[121] [81] [122]
Ischemic stroke	HEK293T Mesenchymal stem cells	[123] [124]
Bone		
Osteoarthritis	Mesenchymal stem cells Chondrocyte	[71] [85]
Osteoporosis	Endothelial cells NIH-3T3 cells.	[17] [18]

MEVs: Mammalian extracellular vesicles; BEVs: bacterial extracellular vesicles; EV: extracellular vesicles.

BEVs derived from *E. coli* W3110△*msbB* to carry Mel [Figure 6C]. The engineered BEV-Mel is an excellent anticancer therapy due to its targeting ability, biocompatibility, and scalability. Importantly, BEV-Mel did not induce chronic systemic toxicity or side effects [Figure 5C].

Immunotherapy

Immunotherapy is another promising method in the field of tumor therapy[119,136]. Precision targeted therapy with chimeric antigen receptor T (CAR-T) cells is a new type of tumor treatment that has achieved good results in clinical tumor treatment. Fu *et al.*[118] introduced engineered MEVs derived from CAR-T cells. CAR-containing MEVs could express many cytotoxic molecules and target and kill cancer cells. Shi *et al.*[119] developed synthetic multivalent antibodies retargeted MEVs (SMART-MEVs), which could specifically target CD3-positive T cells and HER-2 breast cancer cells. The SMART-MEVs exhibited valid and specific antitumor effects [Figure 5D].

Brain

In addition to their strong loading capacity, low toxicity, and low immunogenicity, EVs can also cross the blood-brain barrier. Therefore, EVs can be widely used as a therapeutic vehicle for brain and neurodegenerative diseases[13,137,138]. Here, we summarize the therapeutic effects of engineered targeting EVs in brain diseases, such as AD, PD, and ischemic stroke.

Alzheimer's disease

AD is a progressive neurodegenerative disease involving the superfluous accumulation of β-amyloid, which is produced by the BACE1 protein[139]. Therefore, controlling the expression of the BACE1 protein is an effective way to control AD. Alvarez-Erviti *et al.*[83] described engineered RVG (central nervous system-specific peptide)-MEVs (derived from DCs) to specifically deliver GAPDH siRNA to neurons,

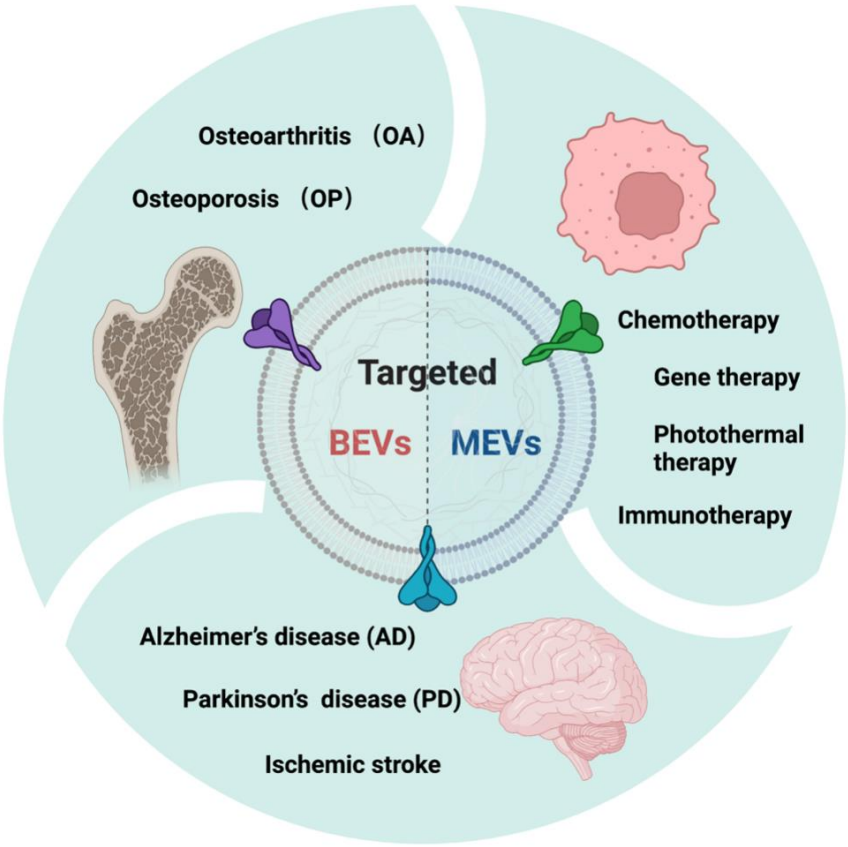

Figure 4. The application of engineered MEVs and BEVs in targeted therapy. The figure was created with Biorender.com. MEVs: Mammalian extracellular vesicles; BEVs: bacterial extracellular vesicles.

oligodendrocytes, and microglia in the brain. Mice were injected intravenously with RVG-MEVs, and the engineered MEVs resulted in a significant decrease in the expression of BACE1 mRNA and protein [Figure 6A]. Cui *et al.*[120] also demonstrated that intravenously infused RVG-MEVs (derived from MSCs) show strong targeting to the cortex and hippocampus, effectively improving learning and memory abilities.

Parkinson's disease

PD is another progressive neurodegenerative disease that involves the formation of Lewy bodies, which is affected by excessive accumulation of α-synuclein (α-Syn)[140-142]. Similarly, decreasing α-synuclein in brain cells could delay PD. Cooper *et al.*[121] delivered α-Syn siRNA by RVG-MEVs (derived from murine dendritic cells) to reduce α-Syn accumulation in the brain. Kojima *et al.*[81] developed MEVs with targeting, cytoplasmic delivery capabilities, and specific RNA encapsulation by EV production booster devices. The delivery of therapeutic catalase mRNA significantly alleviated neurotoxicity and neuroinflammation in mice [Figure 6B]. Liu *et al.*[122] also modified the membrane surface with the RVG peptide for the targeting ability of MEVs, which delivered MOR siRNA to Neuro2A cells in the brain, leading to decreased morphine addiction.

Ischemic stroke

Ischemic stroke is a disease caused by cerebral arterial stenosis that releases high-mobility group box 1 (HMGB1) to the extracellular spaces and results in inflammatory reactions[143]. The knockdown of HMGB1

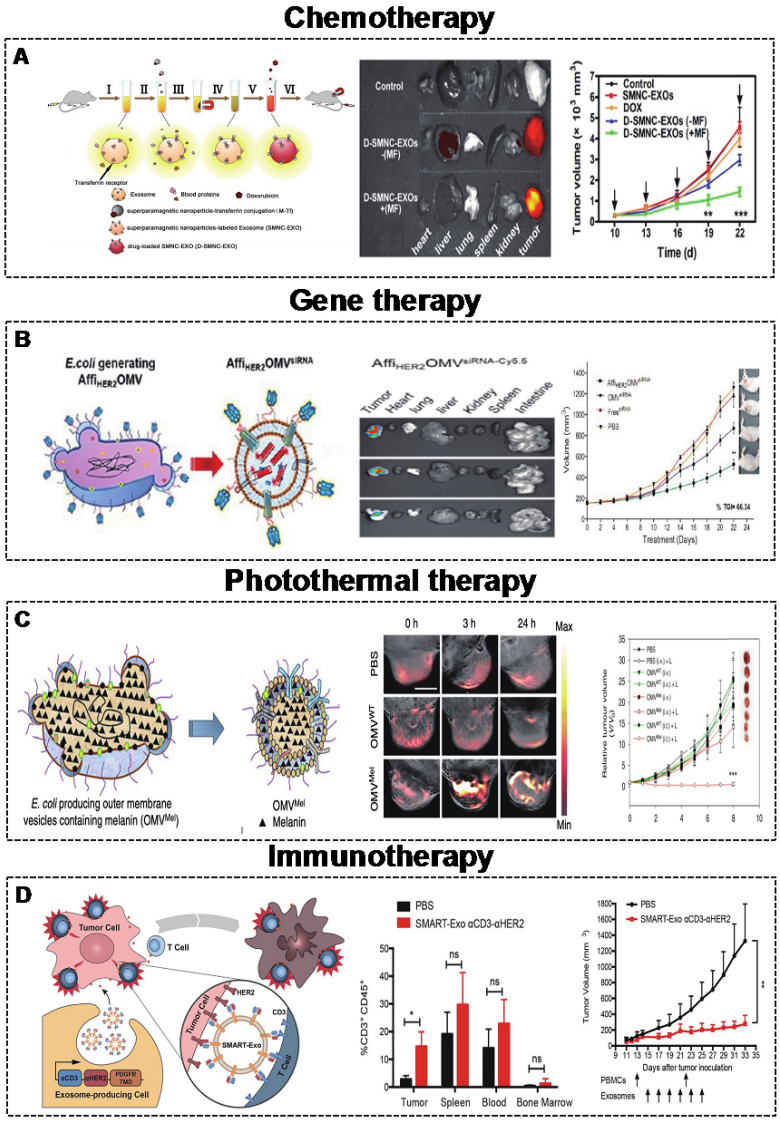

Figure 5. (A) Engineered MEVs for tumor chemotherapy. Schematic illustration of the construction and delivery of doxorubicin loaded in MEVs, which show tumor targeting and antitumor effects[89]. Copyright 2016 American Chemical Society. (B) Engineered BEVs for tumor gene therapy. Schematic illustration of the construction and delivery of siRNA loaded in BEVs, which show tumor targeting and antitumor effects[20]. Copyright 2014 American Chemical Society. (C) Engineered MEVs for tumor photothermal therapy[15]. Schematic illustration of the construction of BEV-Mel, which shows tumor targeting and antitumor effects. Copyright 2019 Springer Nature. (D) Engineered MEVs for tumor immunotherapy[119]. Schematic illustration of the construction of SMART-MEVs, which show tumor targeting and antitumor effects. Copyright 2020 Elsevier. MEVs: Mammalian extracellular vesicles; BEVs: bacterial extracellular vesicles; SMART-MEVs: synthetic multivalent antibodies retargeted MEVs. Significance of finding was defined as follows: not significant, $^{ns}P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

in the brain may be an effective anti-inflammatory strategy to improve ischemic stroke. Kim[123] applied brain-targeting RVG-MEVs (derived from HEK293T cells) to precisely deliver HMGB1 siRNA. HMGB1 siRNA was loaded by electroporation technology. Engineered RVG-MEVs with HMGB1 siRNA successfully reduced the expression of HMGB1 protein and apoptosis levels in the brain. In addition, the delivery of miRNAs such as miR-124 is involved in the neuro-remodeling process[144,145]. Using this strategy, Yang *et al.*[124] constructed RVG-MEVs to deliver miR-124 to the infarct site and protect against ischemic injury [Figure 6C].

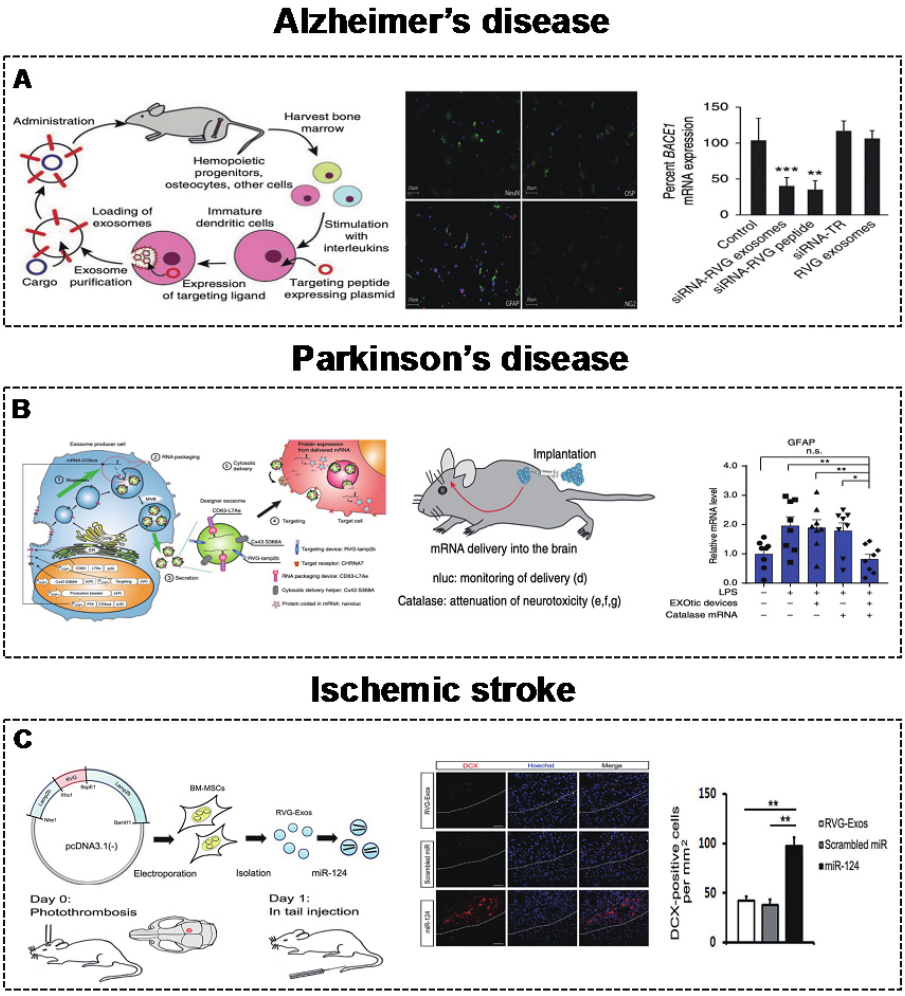

Figure 6. (A) Engineered MEVs for AD. Schematic illustration of the construction of MEVs to deliver BACE1 mRNA with targeting and anti-AD effects[83]. Copyright 2011 Springer Nature. (B) Engineered MEVs for PD. Schematic illustration of the construction of MEVs to deliver catalase mRNA with targeting and anti-PD effects[81]. Copyright 2018 Springer Nature. (C) Engineered MEVs for ischemic stroke. Schematic illustration of the construction of MEVs to deliver catalase miR-124 with targeting and anti-ischemic stroke effects[124]. Copyright 2017 Elsevier. MEVs: Mammalian extracellular vesicles. AD: Alzheimer's disease. PD: Parkinson's disease. Significance of finding was defined as follows: not significant, $^{ns}P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Bone

Bone is an internal support system that provides the structural foundation for the human body and muscle[146-149]. The most common bone diseases, such as OA, OP, bone fractures, and bone defects, have been linked to MEVs and BEVs[150-153]. The application of engineering techniques to enhance the bone targeting ability of EVs has substantially increased their therapeutic efficacy in these bone-related diseases. Conventional fractures or bone defects often require biomaterials, such as hydrogels[154-156] and scaffolds[157-160], for therapeutic effects. Here, we review the application of targeted EVs in OA and OP.

Osteoarthritis

OA is a common joint disease with no recognized mechanism[161]. Cartilage degeneration, subchondral bone sclerosis, and synovial inflammation are prominent features of OA[162,163]. There are no effective OA treatments approved by official agencies, except for joint replacement. Although the precise mechanism of OA is still unclear, EVs, especially targeted EVs, play a vital role during the progression of OA, indicating

their exciting therapeutic prospects[164]. The small molecule drug kartogenin (KGN) was shown to induce synovial fluid-derived MSCs (SF-MSCs) to differentiate into chondrocytes[165-167]. Xu *et al.*[71] reported the targeted delivery of KGN to SF-MSCs by engineered MEVs to accelerate chondrogenesis [Figure 7A]. The targeting ability of engineered MEVs is due to E7-Lamp2B. KGN was loaded inside MEVs by electroporation. Moreover, miR-140 is regarded as a promising agent for the treatment of OA due to its dual roles in both homeostasis and cartilage[168,169]. Liang *et al.*[85] reported a similar targeted strategy to deliver miR-140 to chondrocytes by engineered MEVs to alleviate the progression of OA. The targeting ability of engineered MEVs is due to CAP-Lamp2B. miR-140 was also introduced into MEVs by electroporation.

Osteoporosis

OP is a systemic metabolic disease of the skeletal system characterized by fragility fracture[170-172]. The main cause of OP is an imbalance in the metabolism of osteoblasts and osteoclasts. Postmenopausal women suffer from osteoporosis-related fractures throughout their lifetime[173]. Bone-targeted EVs are optimal interventions to improve postmenopausal OP (PMO). Our group demonstrated that MEVs derived from endothelial cells have a better targeting ability than those from osteoblasts[17]. In addition, our team developed targeting MEVs by displaying C-X-C motif chemokine receptor 4 (CXCR4) on their surface[18]. miR-188 was shown to inhibit osteogenesis of bone marrow mesenchymal stem cells (BMSCs), and the knockdown of miR-188 also improved bone loss[174]. Therefore, we combined $CXCR4^+$ with liposomes containing antagomir-188 to generate hybrid nanoparticles for OP treatment [Figure 7B].

CONCLUSION AND PERSPECTIVE

Over time, the pivotal role of EVs in cell-to-cell communications, in contrast to their initial roles as "garbage bags", has been established. Nanosized EVs have many advantages, such as rich functional contents, a stable membrane structure, good biocompatibility, low immunogenicity, *etc.* Such cell membrane-derived vesicles have been explored in prokaryotic and eukaryotic cells[95,175,176]. As the two most common types of EVs in the biomedical field, MEVs and BEVs have been studied, resulting in major progress in the biogenesis, isolation, and characterization of these vesicles. For biogenesis, MEVs are formed by endosomal systems including sequential plasma membrane invagination and membrane fusion of eukaryotic cells. BEVs are generated by endolysin-triggered cell lysis and membrane blebbing of bacteria. The isolation and characterization of MEVs and BEVs determine their further application in biomedicine. Here, an ultracentrifugation-based isolation protocol is described for MEVs. An effective isolation method based on ultracentrifugation and density gradient centrifugation is also described for BEVs. For characterization, TEM and NTA are commonly used to characterize the sizes, shapes, and concentrations of these vesicles. However, different types of MEVs contain the same protein molecules, such as TSG101, CD63, and CD81, which are always used as specific markers by WB. In contrast, although several membrane proteins, such as OmpF and OmpA, have been used for the characterization of *E. coli* EVs, specific markers of BEVs are still a major challenge.

The use of MEVs and BEVs also has many challenges, such as poor targeting specificity. Targeted drug delivery of EVs was proposed in 2011 and has since received increasing attention due to their excellent characteristics[177]. Targeted modification methods have been applied in MEVs and BEVs to increase the targeting ability and healing efficacy. Targeted engineering aims to increase the local concentration of EVs at diseased sites, thereby reducing toxicity and side effects and maximizing healing efficacy. Both MEVs and BEVs are lipid bilayer-enclosed structures containing various biomolecules. Therefore, most engineering methods are universal. Here, physical, biological, and chemical engineering methods based on membranes to modify MEVs are described, which could also guide the modification of BEVs. Furthermore, the applications of engineered MEVs and BEVs in targeted therapy, such as therapy for tumors and brain and

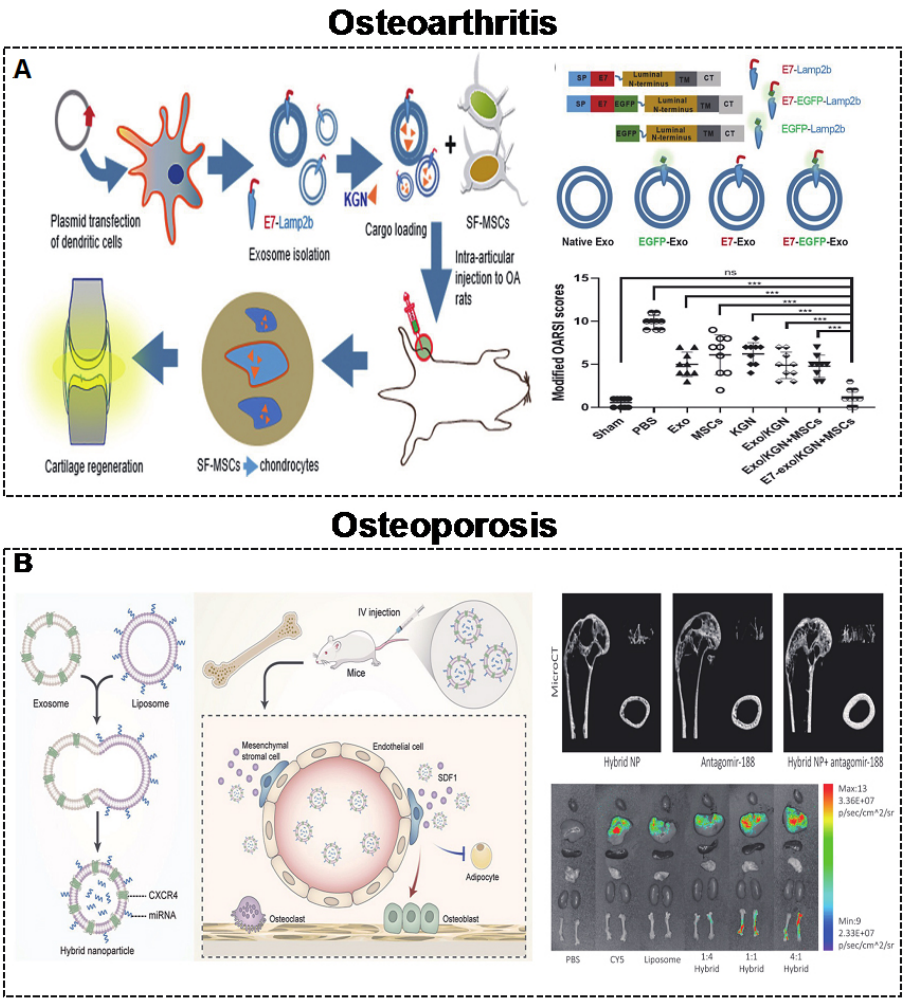

Figure 7. (A) Engineered MEVs for OA. Schematic illustration of the construction of MEVs to deliver KGN with targeting and anti-OA effects[71]. Copyright 2021 Springer Nature. (B) Engineered MEVs for OP. Schematic illustration of the construction of hybrid nanoparticles to deliver antagomir-188 with targeting and anti-OP effects[18]. Copyright 2021 Elsevier. MEVs: Mammalian extracellular vesicles; OA: osteoarthritis; KGN: kartogenin; OP: osteoporosis. Significance of finding was defined as follows: not significant, $^{ns}P > 0.05$; $^{***}P < 0.0001$.

bone diseases, are summarized. MEVs and BEVs with targeting capabilities are usually administered systemically intravenously, and they will accumulate at the target site for better therapeutic efficiency. In addition to the direct injection, the incorporation of MEVs and BEVs with biomaterials is also a promising approach to enhance the healing efficacy[160]. Different types of biomaterials, such as hydrogels[154,178-181] and scaffolds[157,159,182,183], have been developed to achieve high retention rates of EVs and healing efficacy of tissue. In situ injection is another targeted technique, allowing MEVs and BEVs to act directly at the injured tissues. However, some hard-to-reach tissues still require targeted EVs. The many big deals recently by large pharmaceutical companies indicate that the industry expects MEVs and BEVs to deliver drugs to hard-to-reach tissues[184].

Although MEVs have been more extensively studied than BEVs, one of the challenges of MEVs is the limited yield. BEVs are easily available due to the rapid proliferative abilities, mature culture methods, and gene editing techniques of bacteria[185,186]. In addition, the scalability, low cost, and environmental

friendliness of bacterial fermentation culture indicate that the industrialization of BEVs is possible[187,188]. Synthetic biology can also be used to confer additional functions on bacteria and their associated BEVs[189,190]. Moreover, several biotherapeutic bacteria, especially human commensal bacteria, such as *E. coli* Nissle 1917[48], *A. muciniphila*[191], and *L. rhamnosus* GG[192], are being investigated in clinical trials[193,194]. Therefore, BEVs derived from probiotics are promising pharmaceutical agents in the biomedical field. Importantly, BEVs are safe because they are cell-free. Both oral and intravenous BEVs were well tolerated and resulted in low immunogenic responses[15,19,20]. Therefore, the topic of "nonmammalian EVs, especially BEVs" is receiving more attention in the latest MISEV. The mature application system of MEVs can also lay a solid foundation for BEVs in biomedical fields. Studies on MEVs and BEVs can inspire each other and draw important elements from each type to enhance functional and therapeutic effcacy. Overall, the rise of targeted therapeutics of engineered MEVs and BEVs shows promise for future clinical translation of EVs.

DECLARATIONS

Authors' contributions

Liu H and Geng Z contributed equally to this work
Drafted the manuscript: Liu H, Geng Z
Reviewed the manuscript: Liu H, Geng Z, Su J
Approved the submitted manuscript: Su J
All authors have read and agreed to the published version of the manuscript.

Availability of data and materials

Not applicable.

Financial support and sponsorship

This work was supported by the National Key R&D Program of China (2018YFC2001500); National Natural Science Foundation of China (NSFC) Key Research Program in Aging (91749204); National Natural Science Foundation of China (82172098, 81771491, 81972254).

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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REFERENCES

1. Witwer KW, Goberdhan DC, O'Driscoll L, et al. Updating MISEV: evolving the minimal requirements for studies of extracellular vesicles. *J Extracell Vesicles* 2021;10:e12182. DOI PubMed PMC
2. Witwer KW, Théry C. Extracellular vesicles or exosomes? *J Extracell Vesicles* 2019;8:1648167. DOI PubMed PMC
3. Théry C, Witwer KW, Aikawa E, et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement Of The International Society For Extracellular Vesicles and update of the MISEV2014 guidelines. *J Extracell Vesicles* 2018;7:1535750. DOI PubMed PMC
4. Liu H, Zhang Q, Wang S, et al. Bacterial extracellular vesicles as bioactive nanocarriers for drug delivery: advances and perspectives. *Bioact Mater* 2022;14:169-81. DOI PubMed PMC
5. O'Brien K, Breyne K, Ughetto S, Laurent LC, Breakefield XO. RNA delivery by extracellular vesicles in mammalian cells and its

applications. *Nat Rev Mol Cell Biol* 2020;21:585-606. DOI PubMed PMC
6. Herrmann IK, Wood MJA, Fuhrmann G. Extracellular vesicles as a next-generation drug delivery platform. *Nat Nanotechnol* 2021;16:748-59. DOI PubMed
7. Liu C, He D, Cen H, et al. Nucleic acid functionalized extracellular vesicles as promising therapeutic systems for nanomedicine. *EVCNA* 2022. DOI
8. Duan L, Xu L, Xu X, et al. Exosome-mediated delivery of gene vectors for gene therapy. *Nanoscale* 2021;13:1387-97. DOI PubMed
9. Li M, Zhou H, Yang C, et al. Bacterial outer membrane vesicles as a platform for biomedical applications: an update. *J Control Release* 2020;323:253-68. DOI PubMed
10. Sharma S, Masud MK, Kaneti YV, et al. Extracellular vesicle nanoarchitectonics for novel drug delivery applications. *Small* 2021;17:e2102220. DOI PubMed
11. Jiang C, Fu Y, Liu G, et al. Multiplexed profiling of extracellular vesicles for biomarker development. *Nanomicro Lett* 2021;14:3. DOI PubMed PMC
12. Tian T, Zhang HX, He CP, et al. Surface functionalized exosomes as targeted drug delivery vehicles for cerebral ischemia therapy. *Biomaterials* 2018;150:137-49. DOI PubMed
13. Xu M, Feng T, Liu B, et al. Engineered exosomes: desirable target-tracking characteristics for cerebrovascular and neurodegenerative disease therapies. *Theranostics* 2021;11:8926-44. DOI PubMed PMC
14. Liao Z, Liu H, Ma L, et al. Engineering extracellular vesicles restore the impaired cellular uptake and attenuate intervertebral disc degeneration. *ACS Nano* 2021;15:14709-24. DOI PubMed
15. Gujrati V, Prakash J, Malekzadeh-Najafabadi J, et al. Bioengineered bacterial vesicles as biological nano-heaters for optoacoustic imaging. *Nat Commun* 2019;10:1114. DOI PubMed PMC
16. Tran PHL, Xiang D, Tran TTD, et al. Exosomes and nanoengineering: a match made for precision therapeutics. *Adv Mater* 2020;32:e1904040. DOI PubMed
17. Song H, Li X, Zhao Z, et al. Reversal of osteoporotic activity by endothelial cell-secreted bone targeting and biocompatible exosomes. *Nano Lett* 2019;19:3040-8. DOI PubMed
18. Hu Y, Li X, Zhang Q, et al. Exosome-guided bone targeted delivery of Antagomir-188 as an anabolic therapy for bone loss. *Bioact Mater* 2021;6:2905-13. DOI PubMed PMC
19. Kim OY, Park HT, Dinh NTH, et al. Bacterial outer membrane vesicles suppress tumor by interferon-γ-mediated antitumor response. *Nat Commun* 2017;8:626. DOI PubMed PMC
20. Gujrati V, Kim S, Kim SH, et al. Bioengineered bacterial outer membrane vesicles as cell-specific drug-delivery vehicles for cancer therapy. *ACS Nano* 2014;8:1525-37. DOI PubMed
21. Kalluri R, LeBleu VS. function**,** and biomedical applications of exosomes. *Science* 2020;367:eaau6977. DOI PubMed PMC
22. Kim J, Song Y, Park CH, Choi C. Platform technologies and human cell lines for the production of therapeutic exosomes. *EVCNA* 2021. DOI
23. Work E, Knox KW, Vesk M. The chemistry and electron microscopy of an extracellular lipopolysaccharide from escherichia coli. *Ann N Y Acad Sci* 1966;133:438-49. DOI PubMed
24. Knox KW, Vesk M, Work E. Relation between excreted lipopolysaccharide complexes and surface structures of a lysine-limited culture of Escherichia coli. *J Bacteriol* 1966;92:1206-17. DOI PubMed PMC
25. Schwechheimer C, Kuehn MJ. Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions. *Nat Rev Microbiol* 2015;13:605-19. DOI PubMed PMC
26. Brown L, Wolf JM, Prados-Rosales R, Casadevall A. Through the wall: extracellular vesicles in Gram-positive bacteria, mycobacteria and fungi. *Nat Rev Microbiol* 2015;13:620-30. DOI PubMed PMC
27. Harding C, Heuser J, Stahl P. Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. *J Cell Biol* 1983;97:329-39. DOI PubMed PMC
28. Pan B, Johnstone RM. Fate of the transferrin receptor during maturation of sheep reticulocytes. *in vitro* ;33:967-78. DOI PubMed
29. Vidal M. Exosomes: Revisiting their role as "garbage bags". *Traffic* 2019;20:815-28. DOI PubMed
30. Raposo G, Nijman HW, Stoorvogel W, et al. B lymphocytes secrete antigen-presenting vesicles. *J Exp Med* 1996;183:1161-72. DOI PubMed PMC
31. Prangishvili D, Holz I, Stieger E, et al. Sulfolobicins, specific proteinaceous toxins produced by strains of the extremely thermophilic archaeal genus Sulfolobus. *J Bacteriol* 2000;182:2985-8. DOI PubMed PMC
32. Valadi H, Ekström K, Bossios A, et al. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol* 2007;9:654-9. DOI PubMed
33. Fonseka P, Marzan AL, Mathivanan S. Introduction to the Community of Extracellular Vesicles. *Subcell Biochem* 2021;97:3-18. DOI PubMed
34. Mathieu M, Martin-Jaular L, Lavieu G, Théry C. Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication. *Nat Cell Biol* 2019;21:9-17. DOI PubMed
35. Kowal J, Arras G, Colombo M, et al. Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes. *Proc Natl Acad Sci U S A* 2016;113:E968-77. DOI PubMed PMC
36. Kowal J, Tkach M, Théry C. Biogenesis and secretion of exosomes. *Curr Opin Cell Biol* 2014;29:116-25. DOI PubMed
37. Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. *J Cell Biol* 2013;200:373-83. DOI PubMed

PMC
38. Trajkovic K, Hsu C, Chiantia S, et al. Ceramide triggers budding of exosome vesicles into multivesicular endosomes. *Science* 2008;319:1244-7. DOI PubMed
39. Palomino RA, Vanpouille C, Costantini PE, Margolis L. Microbiota-host communications: Bacterial extracellular vesicles as a common language. *PLoS Pathog* 2021;17:e1009508. DOI PubMed PMC
40. Wang S, Gao J, Wang Z. Outer membrane vesicles for vaccination and targeted drug delivery. *Wiley Interdiscip Rev Nanomed Nanobiotechnol* 2019;11:e1523. DOI PubMed PMC
41. Garcia-Martin R, Wang G, Brandão BB, et al. MicroRNA sequence codes for small extracellular vesicle release and cellular retention. *Nature* 2022;601:446-51. DOI PubMed
42. Díaz-Garrido N, Badia J, Baldomà L. Microbiota-derived extracellular vesicles in interkingdom communication in the gut. *J Extracell Vesicles* 2021;10:e12161. DOI PubMed PMC
43. Bittel M, Reichert P, Sarfati I, et al. Visualizing transfer of microbial biomolecules by outer membrane vesicles in microbe-host-communication. *in vivo* ;10:e12159. DOI PubMed PMC
44. Vanaja SK, Russo AJ, Behl B, et al. Bacterial outer membrane vesicles mediate cytosolic localization of LPS and caspase-11 activation. *Cell* 2016;165:1106-19. DOI PubMed PMC
45. Deo P, Chow SH, Han ML, et al. Mitochondrial dysfunction caused by outer membrane vesicles from gram-negative bacteria activates intrinsic apoptosis and inflammation. *Nat Microbiol* 2020;5:1418-27. DOI PubMed
46. Somerville JE Jr, Cassiano L, Darveau RP. Escherichia coli msbB gene as a virulence factor and a therapeutic target. *Infect Immun* 1999;67:6583-90. DOI PubMed PMC
47. Grozdanov L, Raasch C, Schulze J, et al. Analysis of the genome structure of the nonpathogenic probiotic escherichia coli strain nissle 1917. *J Bacteriol* 2004;186:5432-41. DOI PubMed PMC
48. Yan X, Liu XY, Zhang D, et al. Construction of a sustainable 3-hydroxybutyrate-producing probiotic escherichia coli for treatment of colitis. *Cell Mol Immunol* 2021;18:2344-57. DOI PubMed PMC
49. Praveschotinunt P, Duraj-Thatte AM, Gelfat I, et al. Engineered E. coli Nissle 1917 for the delivery of matrix-tethered therapeutic domains to the gut. *Nat Commun* 2019;10:5580. DOI PubMed PMC
50. Domingues S, Nielsen KM. Membrane vesicles and horizontal gene transfer in prokaryotes. *Curr Opin Microbiol* 2017;38:16-21. DOI PubMed
51. Gilmore WJ, Johnston EL, Zavan L, Bitto NJ, Kaparakis-Liaskos M. Immunomodulatory roles and novel applications of bacterial membrane vesicles. *Mol Immunol* 2021;134:72-85. DOI PubMed
52. Lee HJ. Microbe-host communication by small RNAs in extracellular vesicles: vehicles for transkingdom RNA transportation. *Int J Mol Sci* 2019;20:1487. DOI PubMed PMC
53. Dauros-Singorenko P, Blenkiron C, Phillips A, Swift S. The functional RNA cargo of bacterial membrane vesicles. *FEMS Microbiol Lett* 2018:365. DOI PubMed
54. Kumar SR, Kimchi ET, Manjunath Y, et al. RNA cargos in extracellular vesicles derived from blood serum in pancreas associated conditions. *Sci Rep* 2020;10:2800. DOI PubMed PMC
55. la Torre Gomez C, Goreham RV, Bech Serra JJ, Nann T, Kussmann M. "Exosomics"-a review of biophysics, biology and biochemistry of exosomes with a focus on human breast milk. *Front Genet* 2018;9:92. DOI PubMed PMC
56. Zhu Q, Cheng L, Deng C, et al. The genetic source tracking of human urinary exosomes. *Proc Natl Acad Sci U S A* 2021;118:e2108876118. DOI PubMed PMC
57. Liu H, Yuan W, Pang Q, Xue C, Yan X. Single-particle analysis of tear fluid reveals abundant presence of tissue factor-exposing extracellular vesicles with strong coagulation activity. *Talanta* 2022;239:123089. DOI PubMed
58. Lin Y, Dong H, Deng W, et al. Evaluation of salivary exosomal chimeric. *GOLM1-NAA35* ;25:3035-45. DOI PubMed
59. Höög JL, Lötvall J. Diversity of extracellular vesicles in human ejaculates revealed by cryo-electron microscopy. *J Extracell Vesicles* 2015;4:28680. DOI PubMed PMC
60. Han X, Lee A, Huang S, Gao J, Spence JR, Owyang C. Lactobacillus rhamnosus GG prevents epithelial barrier dysfunction induced by interferon-gamma and fecal supernatants from irritable bowel syndrome patients in human intestinal enteroids and colonoids. *Gut Microbes* 2019;10:59-76. DOI PubMed PMC
61. Liu JH, Chen CY, Liu ZZ, et al. Extracellular vesicles from child gut microbiota enter into bone to preserve bone mass and strength. *Adv Sci (Weinh)* 2021;8:2004831. DOI PubMed PMC
62. Maeki M, Kimura N, Sato Y, Harashima H, Tokeshi M. Advances in microfluidics for lipid nanoparticles and extracellular vesicles and applications in drug delivery systems. *Adv Drug Deliv Rev* 2018;128:84-100. DOI PubMed
63. Zhu L, Xu N, Zhang ZL, Zhang TC. Cell derived extracellular vesicles: from isolation to functionalization and biomedical applications. *Biomater Sci* 2019;7:3552-65. DOI PubMed
64. Hartjes TA, Mytnyk S, Jenster GW, van Steijn V, van Royen ME. Extracellular vesicle quantification and characterization: common methods and emerging approaches. *Bioengineering (Basel)* 2019;6:7. DOI PubMed PMC
65. Li X, Corbett AL, Taatizadeh E, et al. Challenges and opportunities in exosome research-perspectives from biology, engineering, and cancer therapy. *APL Bioeng* 2019;3:011503. DOI PubMed PMC
66. Liang Y, Duan L, Lu J, Xia J. Engineering exosomes for targeted drug delivery. *Theranostics* 2021;11:3183-95. DOI PubMed PMC
67. Li P, Kaslan M, Lee SH, Yao J, Gao Z. Progress in exosome isolation techniques. *Theranostics* 2017;7:789-804. DOI PubMed

PMC
68. Gardiner C, Di Vizio D, Sahoo S, et al. Techniques used for the isolation and characterization of extracellular vesicles: results of a worldwide survey. *J Extracell Vesicles* 2016;5:32945. DOI PubMed PMC
69. Xiong Y, Chen L, Yan C, et al. Circulating exosomal miR-20b-5p inhibition restores Wnt9b signaling and reverses diabetes-associated impaired wound healing. *Small* 2020;16:e1904044. DOI PubMed
70. Jiang S, Tian G, Yang Z, et al. Enhancement of acellular cartilage matrix scaffold by Wharton's jelly mesenchymal stem cell-derived exosomes to promote osteochondral regeneration. *Bioact Mater* 2021;6:2711-28. DOI PubMed PMC
71. Xu X, Liang Y, Li X, et al. Exosome-mediated delivery of kartogenin for chondrogenesis of synovial fluid-derived mesenchymal stem cells and cartilage regeneration. *Biomaterials* 2021;269:120539. DOI PubMed
72. Prados-Rosales R, Brown L, Casadevall A, Montalvo-Quirós S, Luque-Garcia JL. Isolation and identification of membrane vesicle-associated proteins in Gram-positive bacteria and mycobacteria. *MethodsX* 2014;1:124-9. DOI PubMed PMC
73. Chutkan H, Macdonald I, Manning A, Kuehn MJ. Quantitative and qualitative preparations of bacterial outer membrane vesicles. *Methods Mol Biol* 2013; 966:259-72. DOI PubMed PMC
74. Klimentová J, Stulík J. Methods of isolation and purification of outer membrane vesicles from gram-negative bacteria. *Microbiol Res* 2015;170:1-9. DOI PubMed
75. Kim W, Lee EJ, Bae IH, et al. DOI PubMed PMC
76. Wang X, Zhang H, Yang H, et al. Cell-derived exosomes as promising carriers for drug delivery and targeted therapy. *Curr Cancer Drug Targets* 2018;18:347-54. DOI PubMed
77. Collins FS, Varmus H. A new initiative on precision medicine. *N Engl J Med* 2015;372:793-5. DOI PubMed PMC
78. Mathivanan S, Fahner CJ, Reid GE, Simpson RJ. ExoCarta 2012: database of exosomal proteins, RNA and lipids. *Nucleic Acids Res* 2012;40:D1241-4. DOI PubMed PMC
79. Lin Y, Wu J, Gu W, et al. Exosome-liposome hybrid nanoparticles deliver CRISPR/Cas9 system in MSCs. *Adv Sci (Weinh)* 2018;5:1700611. DOI PubMed PMC
80. Chen G, Bai Y, Li Z, et al. Bacterial extracellular vesicle-coated multi-antigenic nanovaccines protect against drug-resistant. *Staphylococcus aureus* ;10:7131-49. DOI PubMed PMC
81. Kojima R, Bojar D, Rizzi G, et al. Designer exosomes produced by implanted cells intracerebrally deliver therapeutic cargo for Parkinson's disease treatment. *Nat Commun* 2018;9:1305. DOI PubMed PMC
82. Wu P, Zhang B, Ocansey DKW, Xu W, Qian H. Extracellular vesicles: a bright star of nanomedicine. *Biomaterials* 2021;269:120467. DOI PubMed
83. Alvarez-Erviti L, Seow Y, Yin H, et al. Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. *Nat Biotechnol* 2011;29:341-5. DOI PubMed
84. Liang G, Zhu Y, Ali DJ, et al. Engineered exosomes for targeted co-delivery of miR-21 inhibitor and chemotherapeutics to reverse drug resistance in colon cancer. *J Nanobiotechnology* 2020;18:10. DOI PubMed PMC
85. Liang Y, Xu X, Li X, et al. Chondrocyte-targeted microRNA delivery by engineered exosomes toward a cell-free osteoarthritis therapy. *ACS Appl Mater Interfaces* 2020;12:36938-47. DOI PubMed
86. Liang G, Kan S, Zhu Y, et al. Engineered exosome-mediated delivery of functionally active miR-26a and its enhanced suppression effect in HepG2 cells. *Int J Nanomedicine* 2018;13:585-99. DOI PubMed PMC
87. Kanuma T, Yamamoto T, Kobiyama K, et al. CD63-mediated antigen delivery into extracellular vesicles via DNA vaccination results in robust $CD8^+$ T cell responses. *J Immunol* 2017;198:4707-15. DOI PubMed
88. Jia G, Han Y, An Y, et al. NRP-1 targeted and cargo-loaded exosomes facilitate simultaneous imaging and therapy of glioma. *in vitro* ;178:302-16. DOI PubMed
89. Qi H, Liu C, Long L, et al. Blood exosomes endowed with magnetic and targeting properties for cancer therapy. *ACS Nano* 2016;10:3323-33. DOI PubMed
90. Yang L, Han D, Zhan Q, et al. Blood TfR+ exosomes separated by a pH-responsive method deliver chemotherapeutics for tumor therapy. *Theranostics* 2019;9:7680-96. DOI PubMed PMC
91. Yang Y, Hong Y, Nam GH, et al. Virus-mimetic fusogenic exosomes for direct delivery of integral membrane proteins to target cell membranes. *Adv Mater* 2017;29:1605604. DOI PubMed
92. Gao X, Li S, Ding F, et al. Rapid detection of exosomal microrna using virus-mimicking fusogenic vesicles. *Angew Chem Int Ed Engl* 2019;58:8719-23. DOI PubMed
93. Piffoux M, Silva AKA, Wilhelm C, Gazeau F, Tareste D. Modification of extracellular vesicles by fusion with liposomes for the design of personalized biogenic drug delivery systems. *ACS Nano* 2018;12:6830-42. DOI PubMed
94. Fang RH, Kroll AV, Gao W, Zhang L. Cell membrane coating nanotechnology. *Adv Mater* 2018;30:e1706759. DOI PubMed PMC
95. Hu CM, Zhang L, Aryal S, et al. Erythrocyte membrane-camouflaged polymeric nanoparticles as a biomimetic delivery platform. *Proc Natl Acad Sci U S A* 2011;108:10980-5. DOI PubMed PMC
96. Su T, Huang K, Ma H, et al. Platelet-inspired nanocells for targeted heart repair after ischemia/reperfusion injury. *Adv Funct Mater* 2019;29:1803567. DOI PubMed PMC
97. Rao L, Bu LL, Cai B, et al. Cancer cell membrane-coated upconversion nanoprobes for highly specific tumor imaging. *Adv Mater* 2016;28:3460-6. DOI PubMed
98. Wang S, Gao J, Li M, Wang L, Wang Z. A facile approach for development of a vaccine made of bacterial double-layered membrane

vesicles (DMVs). *Biomaterials* 2018;187:28-38. DOI PubMed PMC
99. Pore D, Chakrabarti MK. Outer membrane protein A (OmpA) from shigella flexneri 2a: a promising subunit vaccine candidate. *Vaccine* 2013;31:3644-50. DOI PubMed
100. Tian Y, Li S, Song J, et al. A doxorubicin delivery platform using engineered natural membrane vesicle exosomes for targeted tumor therapy. *Biomaterials* 2014;35:2383-90. DOI PubMed
101. Bai J, Duan J, Liu R, et al. Engineered targeting tLyp-1 exosomes as gene therapy vectors for efficient delivery of siRNA into lung cancer cells. *Asian J Pharm Sci* 2020;15:461-71. DOI PubMed PMC
102. Rountree RB, Mandl SJ, Nachtwey JM, et al. Exosome targeting of tumor antigens expressed by cancer vaccines can improve antigen immunogenicity and therapeutic efficacy. *Cancer Res* 2011;71:5235-44. DOI PubMed
103. Gao X, Ran N, Dong X, et al. Anchor peptide captures, targets, and loads exosomes of diverse origins for diagnostics and therapy. *Sci Transl Med* 2018;10:eaat0195. DOI PubMed
104. Zuo B, Qi H, Lu Z, et al. Alarmin-painted exosomes elicit persistent antitumor immunity in large established tumors in mice. *Nat Commun* 2020;11:1790. DOI PubMed PMC
105. Fan Z, Xiao K, Lin J, Liao Y, Huang X. Functionalized DNA enables programming exosomes/vesicles for tumor imaging and therapy. *Small* 2019;15:e1903761. DOI PubMed
106. Di H, Zeng E, Zhang P, et al. General approach to engineering extracellular vesicles for biomedical analysis. *Anal Chem* 2019;91:12752-9. DOI PubMed
107. Wang J, Li W, Lu Z, et al. The use of RGD-engineered exosomes for enhanced targeting ability and synergistic therapy toward angiogenesis. *Nanoscale* 2017;9:15598-605. DOI PubMed
108. Wang J, Li W, Zhang L, et al. Chemically edited exosomes with dual ligand Purified by microfluidic device for active targeted drug delivery to tumor cells. *ACS Appl Mater Interfaces* 2017;9:27441-52. DOI PubMed
109. Khongkow M, Yata T, Boonrungsiman S, et al. Surface modification of gold nanoparticles with neuron-targeted exosome for enhanced blood-brain barrier penetration. *Sci Rep* 2019;9:8278. DOI PubMed PMC
110. Kim MS, Haney MJ, Zhao Y, et al. Engineering macrophage-derived exosomes for targeted paclitaxel delivery to pulmonary metastases:. *in vitro* ;14:195-204. DOI PubMed
111. Cao Y, Wu T, Zhang K, et al. Engineered exosome-mediated near-infrared-II region V_2C quantum dot delivery for nucleus-target low-temperature photothermal therapy. *ACS Nano* 2019;13:1499-510. DOI PubMed
112. Pi F, Binzel DW, Lee TJ, et al. Nanoparticle orientation to control RNA loading and ligand display on extracellular vesicles for cancer regression. *Nat Nanotechnol* 2018;13:82-9. DOI PubMed PMC
113. Nakase I, Futaki S. Combined treatment with a pH-sensitive fusogenic peptide and cationic lipids achieves enhanced cytosolic delivery of exosomes. *Sci Rep* 2015;5:10112. DOI PubMed PMC
114. Li Y, Gao Y, Gong C, et al. A33 antibody-functionalized exosomes for targeted delivery of doxorubicin against colorectal cancer. *Nanomedicine* 2018;14:1973-85. DOI PubMed
115. Zhao L, Gu C, Gan Y, et al. Exosome-mediated siRNA delivery to suppress postoperative breast cancer metastasis. *J Control Release* 2020;318:1-15. DOI PubMed
116. Bose RJC, Uday Kumar S, Zeng Y, et al. Tumor cell-derived extracellular vesicle-coated nanocarriers: an efficient theranostic platform for the cancer-specific delivery of Anti-miR-21 and imaging agents. *ACS Nano* 2018;12:10817-32. DOI PubMed PMC
117. Gujrati V, Ntziachristos V. Bioengineered bacterial vesicles for optoacoustics-guided phototherapy. *Methods Enzymol* 2021;657:349-64. DOI PubMed
118. Fu W, Lei C, Liu S, et al. CAR exosomes derived from effector CAR-T cells have potent antitumour effects and low toxicity. *Nat Commun* 2019;10:4355. DOI PubMed PMC
119. Shi X, Cheng Q, Hou T, et al. Genetically engineered cell-derived nanoparticles for targeted breast cancer immunotherapy. *Mol Ther* 2020;28:536-47. DOI PubMed PMC
120. Cui GH, Guo HD, Li H, et al. RVG-modified exosomes derived from mesenchymal stem cells rescue memory deficits by regulating inflammatory responses in a mouse model of Alzheimer's disease. *Immun Ageing* 2019;16:10. DOI PubMed PMC
121. Cooper JM, Wiklander PB, Nordin JZ, et al. Systemic exosomal siRNA delivery reduced alpha-synuclein aggregates in brains of transgenic mice. *Mov Disord* 2014;29:1476-85. DOI PubMed PMC
122. Liu Y, Li D, Liu Z, et al. Targeted exosome-mediated delivery of opioid receptor Mu siRNA for the treatment of morphine relapse. *Sci Rep* 2015;5:17543. DOI PubMed PMC
123. Kim M, Kim G, Hwang DW, Lee M. Delivery of high mobility group box-1 sirna using brain-targeting exosomes for ischemic stroke therapy. *J Biomed Nanotechnol* 2019;15:2401-12. DOI PubMed
124. Yang J, Zhang X, Chen X, Wang L, Yang G. Exosome mediated delivery of mir-124 promotes neurogenesis after ischemia. *Mol Ther Nucleic Acids* 2017;7:278-87. DOI PubMed PMC
125. Abhange K, Makler A, Wen Y, et al. Small extracellular vesicles in cancer. *Bioact Mater* 2021;6:3705-43. DOI PubMed PMC
126. Zhuang Q, Xu J, Deng D, et al. Bacteria-derived membrane vesicles to advance targeted photothermal tumor ablation. *Biomaterials* 2021;268:120550. DOI PubMed
127. Chronopoulos A, Kalluri R. Emerging role of bacterial extracellular vesicles in cancer. *Oncogene* 2020;39:6951-60. DOI PubMed PMC
128. Huang S, Jiang C, Mughal MJ, Wang G, Xing F. An. *in vivo* ;10:839078. DOI PubMed PMC

129. Han M, Pang B, Zhou C, et al. Liquid biopsy of extracellular vesicle biomarkers for prostate cancer personalized treatment decision. *EVCNA* 2022. DOI
130. Mulligan RC. The basic science of gene therapy. *Science* 1993;260:926-32. DOI PubMed
131. Vader P, Mol EA, Pasterkamp G, Schiffelers RM. Extracellular vesicles for drug delivery. *Adv Drug Deliv Rev* 2016;106:148-56. DOI PubMed
132. Zheng L, Hu X, Wu H, et al. DOI PubMed
133. Webb JA, Ou YC, Faley S, et al. Theranostic gold nanoantennas for simultaneous multiplexed Raman imaging of immunomarkers and photothermal therapy. *ACS Omega* 2017;2:3583-94. DOI PubMed PMC
134. Stritzker J, Kirscher L, Scadeng M, et al. Vaccinia virus-mediated melanin production allows MR and optoacoustic deep tissue imaging and laser-induced thermotherapy of cancer. *Proc Natl Acad Sci U S A* 2013;110:3316-20. DOI PubMed PMC
135. Fan Q, Cheng K, Hu X, et al. Transferring biomarker into molecular probe: melanin nanoparticle as a naturally active platform for multimodality imaging. *J Am Chem Soc* 2014;136:15185-94. DOI PubMed PMC
136. Cheng Q, Shi X, Han M, et al. Reprogramming exosomes as nanoscale controllers of cellular immunity. *J Am Chem Soc* 2018;140:16413-7. DOI PubMed PMC
137. Xiao L, Hareendran S, Loh YP. Function of exosomes in neurological disorders and brain tumors. *Extracell Vesicles Circ Nucl Acids* 2021;2:55-79. DOI PubMed PMC
138. Cuesta CM, Guerri C, Ureña J, Pascual M. Role of microbiota-derived extracellular vesicles in gut-brain communication. *Int J Mol Sci* 2021;22:4235. DOI PubMed PMC
139. Hampel H, Vassar R, De Strooper B, et al. The β-Secretase BACE1 in Alzheimer's disease. *Biol Psychiatry* 2021;89:745-56. DOI PubMed PMC
140. Jiang C, Hopfner F, Katsikoudi A, et al. Serum neuronal exosomes predict and differentiate Parkinson's disease from atypical parkinsonism. *J Neurol Neurosurg Psychiatry* 2020;91:720-9. DOI PubMed PMC
141. Jiang C, Hopfner F, Berg D, et al. Validation of α-Synuclein in L1CAM-immunocaptured exosomes as a biomarker for the stratification of parkinsonian syndromes. *Mov Disord* 2021;36:2663-9. DOI PubMed PMC
142. Fu Y, Jiang C, Tofaris GK, Davis JJ. Facile Impedimetric analysis of neuronal exosome markers in Parkinson's disease diagnostics. *Anal Chem* 2020;92:13647-51. DOI PubMed PMC
143. Powers WJ. Acute ischemic stroke. *N Engl J Med* 2020;383:252-60. DOI PubMed
144. Dharap A, Bowen K, Place R, Li LC, Vemuganti R. Transient focal ischemia induces extensive temporal changes in rat cerebral microRNAome. *J Cereb Blood Flow Metab* 2009;29:675-87. DOI PubMed PMC
145. Jeyaseelan K, Lim KY, Armugam A. MicroRNA expression in the blood and brain of rats subjected to transient focal ischemia by middle cerebral artery occlusion. *Stroke* 2008;39:959-66. DOI PubMed
146. Ren X, Liu H, Wu X, et al. Reactive oxygen species (ROS)-responsive biomaterials for the treatment of bone-related diseases. *Front Bioeng Biotechnol* 2021;9:820468. DOI PubMed PMC
147. Chen S, Chen X, Geng Z, Su J. The horizon of bone organoid: a perspective on construction and application. *Bioact Mater* 2022;18:15-25. DOI PubMed PMC
148. Hu Y, Chen X, Wang S, Jing Y, Su J. Subchondral bone microenvironment in osteoarthritis and pain. *Bone Res* 2021;9:20. DOI PubMed PMC
149. Gu J, Zhang Q, Geng M, et al. Construction of nanofibrous scaffolds with interconnected perfusable microchannel networks for engineering of vascularized bone tissue. *Bioact Mater* 2021;6:3254-68. DOI PubMed PMC
150. Puhm F, Boilard E, Machlus KR. Platelet extracellular vesicles: beyond the blood. *Arterioscler Thromb Vasc Biol* 2021;41:87-96. DOI PubMed PMC
151. Wu X, Wang Y, Xiao Y, et al. Extracellular vesicles: potential role in osteoarthritis regenerative medicine. *J Orthop Translat* 2020;21:73-80. DOI PubMed PMC
152. Liu Y, Ma Y, Zhang J, Yuan Y, Wang J. Exosomes: a novel therapeutic agent for cartilage and bone tissue regeneration. *Dose Response* 2019;17:1559325819892702. DOI PubMed PMC
153. Tao SC, Guo SC. Extracellular vesicles in bone: "dogrobbers" in the "eternal battle field". *Cell Commun Signal* 2019;17:6. DOI PubMed PMC
154. Zhang Y, Xie Y, Hao Z, et al. Umbilical mesenchymal stem cell-derived exosome-encapsulated hydrogels accelerate bone repair by enhancing angiogenesis. *ACS Appl Mater Interfaces* 2021;13:18472-87. DOI PubMed
155. Re F, Gabusi E, Manferdini C, Russo D, Lisignoli G. Bone regeneration improves with mesenchymal stem cell derived extracellular vesicles (EVs) combined with scaffolds: a systematic review. *Biology (Basel)* 2021;10:579. DOI PubMed PMC
156. Pishavar E, Luo H, Naserifar M, et al. Advanced hydrogels as exosome delivery systems for osteogenic differentiation of MSCs: application in bone regeneration. *Int J Mol Sci* 2021;22:6203. DOI PubMed PMC
157. Fan J, Lee CS, Kim S, et al. Generation of small rna-modulated exosome mimetics for bone regeneration. *ACS Nano* 2020;14:11973-84. DOI PubMed PMC
158. Zha Y, Li Y, Lin T, et al. Progenitor cell-derived exosomes endowed with VEGF plasmids enhance osteogenic induction and vascular remodeling in large segmental bone defects. *Theranostics* 2021;11:397-409. DOI PubMed PMC
159. Zhai M, Zhu Y, Yang M, Mao C. Human mesenchymal stem cell derived exosomes enhance cell-free bone regeneration by altering their miRNAs profiles. *Adv Sci (Weinh)* 2020;7:2001334. DOI PubMed PMC

160. Lazar S, Mor S, Chen J, Hao D, Wang A. Bioengineered extracellular vesicle-loaded bioscaffolds for therapeutic applications in regenerative medicine. *Extracell Vesicles Circ Nucl Acids* 2021;2:175-8. DOI PubMed PMC
161. Xue S, Zhou X, Sang W, et al. Cartilage-targeting peptide-modified dual-drug delivery nanoplatform with NIR laser response for osteoarthritis therapy. *Bioact Mater* 2021;6:2372-89. DOI PubMed PMC
162. Glyn-jones S, Palmer AJR, Agricola R, et al. Osteoarthritis. *The Lancet* 2015;386:376-87. DOI PubMed
163. Martel-Pelletier J, Barr AJ, Cicuttini FM, et al. Osteoarthritis. *Nat Rev Dis Primers* 2016;2:16072. DOI PubMed
164. Ragni E, Orfei CP, Papait A, de Girolamo L. Comparison of miRNA cargo in human adipose-tissue vs. amniotic-membrane derived mesenchymal stromal cells extracellular vesicles for osteoarthritis treatment. *EVCNA* 2021. DOI
165. Johnson K, Zhu S, Tremblay MS, et al. A stem cell-based approach to cartilage repair. *Science* 2012;336:717-21. DOI PubMed
166. Kang ML, Ko JY, Kim JE, Im GI. Intra-articular delivery of kartogenin-conjugated chitosan nano/microparticles for cartilage regeneration. *Biomaterials* 2014;35:9984-94. DOI PubMed
167. Xu X, Shi D, Shen Y, et al. Full-thickness cartilage defects are repaired via a microfracture technique and intraarticular injection of the small-molecule compound kartogenin. *Arthritis Res Ther* 2015;17:20. DOI PubMed PMC
168. Zhang R, Ma J, Yao J. Molecular mechanisms of the cartilage-specific microRNA-140 in osteoarthritis. *Inflamm Res* 2013;62:871-7. DOI PubMed
169. Miyaki S, Sato T, Inoue A, et al. MicroRNA-140 plays dual roles in both cartilage development and homeostasis. *Genes Dev* 2010;24:1173-85. DOI PubMed PMC
170. Black DM, Rosen CJ. Clinical Practice. Postmenopausal osteoporosis. *N Engl J Med* 2016;374:254-62. DOI PubMed
171. Li X, Wang L, Huang B, et al. Targeting actin-bundling protein L-plastin as an anabolic therapy for bone loss. *Sci Adv* 2020;6:eabb7135. DOI PubMed PMC
172. Zhao H, Li X, Zhang D, et al. Integrative bone metabolomics-lipidomics strategy for pathological mechanism of postmenopausal osteoporosis mouse model. *Sci Rep* 2018;8:16456. DOI PubMed PMC
173. Brown C. Osteoporosis: staying strong. *Nature* 2017;550:S15-7. DOI PubMed
174. Li CJ, Cheng P, Liang MK, et al. MicroRNA-188 regulates age-related switch between osteoblast and adipocyte differentiation. *J Clin Invest* 2015;125:1509-22. DOI PubMed PMC
175. Gujrati V, Lee M, Ko YJ, et al. Bioengineered yeast-derived vacuoles with enhanced tissue-penetrating ability for targeted cancer therapy. *Proc Natl Acad Sci U S A* 2016;113:710-5. DOI PubMed PMC
176. MacDiarmid JA, Mugridge NB, Weiss JC, et al. Bacterially derived 400 nm particles for encapsulation and cancer cell targeting of chemotherapeutics. *Cancer Cell* 2007;11:431-45. DOI PubMed
177. Gudbergsson JM, Jønsson K, Simonsen JB, Johnsen KB. Systematic review of targeted extracellular vesicles for drug delivery - considerations on methodological and biological heterogeneity. *J Control Release* 2019;306:108-20. DOI PubMed
178. Xue X, Hu Y, Deng Y, Su J. Recent advances in design of functional biocompatible hydrogels for bone tissue engineering. *Adv Funct Mater* 2021;31:2009432. DOI
179. Chen W, Zhou Z, Chen D, et al. Bone regeneration using MMP-cleavable peptides-based hydrogels. *Gels* 2021;7:199. DOI PubMed PMC
180. Xiong Y, Chen L, Liu P, et al. All-in-one: multifunctional hydrogel accelerates oxidative diabetic wound healing through timed-release of exosome and fibroblast growth factor. *Small* 2022;18:e2104229. DOI PubMed
181. Li L, Yu F, Zheng L, et al. Natural hydrogels for cartilage regeneration: Modification, preparation and application. *J Orthop Translat* 2019;17:26-41. DOI PubMed PMC
182. Zou Y, Huang B, Cao L, Deng Y, Su J. Tailored mesoporous inorganic biomaterials: assembly, functionalization, and drug delivery engineering. *Adv Mater* 2021;33:e2005215. DOI PubMed
183. Geng Z, Sang S, Wang S, et al. Optimizing the strontium content to achieve an ideal osseointegration through balancing apatite-forming ability and osteogenic activity. *Mater Sci Eng C Mater Biol Appl* 2022:112647. DOI PubMed
184. Zipkin M. Big pharma buys into exosomes for drug delivery. *Nat Biotechnol* 2020;38:1226-8. DOI PubMed
185. Liu H, Wang Y, Hou Y, Li Z. Fitness of chassis cells and metabolic pathways for l-cysteine overproduction in escherichia coli. *J Agric Food Chem* 2020;68:14928-37. DOI PubMed
186. Liu H, Hou Y, Wang Y, Li Z. Enhancement of sulfur conversion rate in the production of l-cysteine by engineered escherichia coli. *J Agric Food Chem* 2020;68:250-7. DOI PubMed
187. Yang D, Park SY, Park YS, Eun H, Lee SY. Metabolic engineering of escherichia coli for natural product biosynthesis. *Trends Biotechnol* 2020;38:745-65. DOI PubMed
188. Choi KR, Jang WD, Yang D, et al. Systems metabolic engineering strategies: integrating systems and synthetic biology with metabolic engineering. *Trends Biotechnol* 2019;37:817-37. DOI PubMed
189. Service RF. Synthetic biology. Synthetic biologists design 'living materials' that build themselves. *Science* 2014;343:1421. DOI PubMed
190. Tang T, An B, Huang Y, et al. Materials design by synthetic biology. *Nat Rev Mater* 2021;6:332-50. DOI PubMed
191. Depommier C, Everard A, Druart C, et al. Supplementation with akkermansia muciniphila in overweight and obese human volunteers: a proof-of-concept exploratory study. *Nat Med* 2019;25:1096-103. DOI PubMed PMC
192. Riehl TE, Alvarado D, Ee X, et al. Lactobacillus rhamnosus GG protects the intestinal epithelium from radiation injury through release of lipoteichoic acid, macrophage activation and the migration of mesenchymal stem cells. *Gut* 2019;68:1003-13. DOI

PubMed PMC

193. Ozdemir T, Fedorec AJH, Danino T, Barnes CP. Synthetic biology and engineered live biotherapeutics: toward increasing system complexity. *Cell Syst* 2018;7:5-16. DOI PubMed

194. Charbonneau MR, Isabella VM, Li N, Kurtz CB. Developing a new class of engineered live bacterial therapeutics to treat human diseases. *Nat Commun* 2020;11:1738. DOI PubMed PMC

Zhang *et al. Extracell Vesicles Circ Nucleic Acids* 2022;3:87-92
DOI: 10.20517/evcna.2022.16

Extracellular Vesicles and Circulating Nucleic Acids

Commentary

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Summary of Prof. Yin's CSEMV-EVCNA award lecture 2021

Ying Zhang[1], Hang Yin[1,2,3]

[1]School of Pharmaceutical Sciences, Key Laboratory of Bioorganic Phosphorous chemistry and Chemical Biology(Ministry of Education), Tsinghua University, Beijing 100084, China.
[2]Tsinghua-Peking Center for Life Sciences, Tsinghua University, Beijing 100084, China.
[3]Beijing Advanced Innovation Center for Structural Biology, Beijing Frontier Research Center for Biological Structure, Tsinghua University, Beijing 100084, China.

Correspondence to: Prof. Hang Yin, School of Pharmaceutical Sciences, Tsinghua-Peking Center for Life Sciences, Tsinghua University, Beijing 100084, China. E-mail: yin_hang@tsinghua.edu

How to cite this article: Zhang Y, Yin H. Summary of Prof. Yin's CSEMV-EVCNA award lecture 2021. *Extracell Vesicles Circ Nucleic Acids* 2022;3:87-92. https://dx.doi.org/10.20517/evcna.2022.16

Received: 24 Mar 2022 **Accepted:** 8 Apr 2022 **Published:** 13 Apr 2022

Academic Editor: Yoke Peng Loh **Copy Editor:** Jia-Xin Zhang **Production Editor:** Jia-Xin Zhang

Abstract

Extracellular vesicles (EVs) have been regarded as influential intracellular delivering parcels that possess tremendous potential because of their strict and complex secretion regulation processes. However, traditional detection methods cannot monitor the secretion of EVs due to their small particle diameters. Inspired by their peculiar diverse appearances and lipid membranes ingredients, we developed an innovative strategy to detect EVs in any kind of fluids by using rationally designed peptide probes that particularly recognize the highly curved surface of EVs. These peptide probes also serve as novel tools to selectively target cancerous cells with specific lipid compositions and distributions. With this strategy, we discovered a series of EV-secreting regulation mechanisms and identified their roles within physiological processes. Recently, we found that the transportation of oligodeoxynucleotides and cell division control protein 42 homolog from TLR9-activated macrophages to naïve cells via EVs exerts synergetic effects in the propagation of the intracellular immune response, which suggests a general mechanism for EV-mediated uptake of pathogen-associated molecular patterns.

Keywords: Extracellular vesicles (EVs), peptide probe, detection methods

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INTRODUCTION

Microvesicles and exosomes are two subsets of extracellular vesicles (EVs) with diameters of 30-1000 nm[1,2]. EVs are secreted by all living cells and then maintained in fluids or taken up by cells after being packed with certain proteins, nucleotides, metabolites, *etc.*[3,4]. The secretion and uptake of EVs are precisely manipulated by regulation systems under strict and highly conserved mechanisms for intercellular communication and resource exchange in normal physiology[5,6]. Although normal cells use EVs for intercellular communications, they also transport molecules that promote disease progression and immune system modulation, for instance, in inflammatory autoimmune diseases, cardiovascular diseases, neurodegenerative diseases, and cancer[7,8]. Therefore, EVs are currently considered to be promising but underexploited potential biomarkers. Large amounts of bioactive molecules such as proteins, DNA, mRNA, microRNA, lncRNA, and lipids are found in EVs, rendering them potential biomarkers for the diagnosis of carcinomas, cardiovascular disorders, autoimmune disorders, *etc.*[7,9]. Furthermore, understanding the functions of EVs in the physiological environment and disease progression would shed light on EVs' mechanism as biomarkers in these diseases.

Beginning two decades ago, we focused on developing the technology for EV peptides sensing through chemical and biological tools. In conducting this work, we have sought to elucidate previously obscure regulatory mechanisms of EVs in diverse diseases. Our curvature-sensing biotechnology works by targeting protein-lipid interactions. EV-enabled biofluid diagnostics such as our curvature-sensing biotechnology are potentially applicable to tumor metastasis and other diseases. Our laboratory has also successfully developed several promising peptides and peptidomimetic agent candidates that can sense and induce membrane curvature, providing a potential measure of the concentration of exosomes in solution and blood plasma. With these tools, we have broadened the horizon for EVs' diagnostic and treatment functions and characterized the relevant machinery that EVs use to influence the onset and progression of diseases.

Curvature and lipid-sensing peptide probes for EV detection

It has been demonstrated that EVs exist in various kinds of body fluids, such as plasma, saliva, urine, breast milk, and cerebrospinal fluid, and that biomarkers remain stable inside the EVs because of the protection afforded by their lipid bilayers. Conventional diagnostics for cancer, such as biopsy or antinuclear antibody tests, have drawbacks, including the invasive nature of biopsies and their limitation in specificity and sensitivity. The challenges posed by conventional methods call for a breakthrough to address existing technical bottlenecks. Examination of EVs in human bodily fluid may allow for a new method of non-invasive liquid biopsy and could therefore serve as a pivotal diagnostic tool for various diseases. The development of specific, efficient, and minimally invasive probes for the detection of disease biomarkers offers a remarkable opportunity for advancing both basic, applied, and clinical research.

Nonetheless, despite the crucial role EVs could play in liquid biopsy developments, there are still problems with their efficient isolation. Current techniques for EV isolation, such as size exclusion, ultracentrifugation, immunoaffinity, or polymeric precipitation, are tedious, expensive, or have limitations for acquiring adequate EV quantities or purity. To deal with these problems, we have designed peptide probes to capture EVs by sensing their highly curved membranes and specific lipid compositions, independent of protein and oligonucleotide cargoes, thus enabling a paradigm shift from the conventional immunoaffinity approach to the universal recognition of ubiquitous phospholipids in EV membranes based on *peptide-lipid interactions*.

Diagnostic EVs are believed to be superior to some traditional methods in terms of sensitivity and specificity. More importantly, they can be collected from and detected in bodily liquids such as blood serum and urine, so their extraction is, at the most, minimally invasive[10]. The most recognized standard for EV

isolation is ultracentrifugation, but the capital outlay cost of the instrument and the long EV isolation process (up to 12 h of continuous centrifugation) prohibit its utility in routine clinical use. New EV-based diagnostic kits have been approved by the US Food and Drug Administration for clinical use. However, there are still several limitations in exosome diagnostics. The quick, efficient, and effective isolation of EV from a bodily fluid sample is a prerequisite for their translation into clinical use[10], as contaminants may affect the accuracy of the work. Newly developed techniques such as size exclusion, immunoaffinity, or polymeric precipitation are tedious, limited to specific protein recognition, or yield samples contaminated with materials that adversely affect downstream analyses. Therefore, developing more efficient and standard separation methods for clinical diagnostics is a necessity.

Several proteins or peptides that interact with the membrane bilayer, including but not limited to Bin-Amphiphysin-Rvs domains[11], Arf-GAP lipid packing sensors[12], synaptotagmin I[13], myristoylated alanine-rich protein kinase C substrate effector domain (MARCKS-ED)[14,15], and bradykinin (BK)[16], have been reported to possess lipid-binding domains. We demonstrated that the C2B loop 3 of synaptotagmin could be truncated and cyclized using solid-phase "click chemistry" to recuse its lipid and EV curvature sensing properties[13]. MARCKS-ED was investigated via empirical and theoretical processes to elucidate its interactions with lipid located in membrane bilayers [14,15], providing insights into the correlation of its structure and functions. We further found that the curvature sensing behavior of BK may be due to its negatively charged phosphatidylserine (PS) lipid component, which aids in its binding affinity and its lipid packing effects in smaller vesicles, and which may even allow for hydrophobic Phe interactions with the membrane bilayers. We found that the peptides' synthetic lipid vesicle binding ability was also translated to the detection of EVs, which offers an exciting new direction in the study of cell-derived lipid vesicles that carry membrane-protected information for intercellular communications.

Previously, we showed that the monomeric and trimeric forms of BK bind on synthetic nanovesicles and EVs, with multimerization yielding an increased binding affinity of up to 7 uM[16]. BK (RPPGFSPFR) is a cationic peptide ligand for B_1 and B_2 G-protein coupled receptors. It is believed that the conformation adopted by peptide ligands such as BK is facilitated by interactions with membrane phospholipids prior to receptor binding and activation[17,18]. Previous studies reported that this molecule shows differential interactions with lipid vesicles[17] and micelles[19] and has a stronger preference for mixtures with higher anionic phospholipid composition[17], which makes it an excellent candidate for capturing EVs.

The proline-induced beta-turn orients the arginine residues to a claw-like conformation that is critical for lipid recognition and attachment. Since the outer leaflets of EVs are enriched with anionic lipids as a consequence of their biogenesis[20,21], it is possible that BK analogs could sense PS-enriched lipid vesicles such as EVs and work as capture and isolation agents. Moreover, the rational design of peptides can significantly alter the degree of peptide-lipid interactions. Overall, the EV-sensing peptide technique is innovative and significant because its successful implementation will offer a new approach for rapid isolation of EVs, independent of their biomolecule cargoes, and fulfill an unmet need for a simple, efficient, and high throughput EV isolation method for disease diagnosis.

The role of EVs in intercellular innate immune responses

Another research focus in our laboratory has been to decipher the function of EVs in response to immune stimuli. Using the above-mentioned chemical biology tools and others to detect and track EVs, we recently deciphered the mechanism of innate immunity and pro-inflammatory signaling. Sensing pathogen-associated molecular patterns and danger-associated molecular patterns is a vital step in innate immune responses. Because intracellular innate immunity has already attracted attention, we chose to investigate the

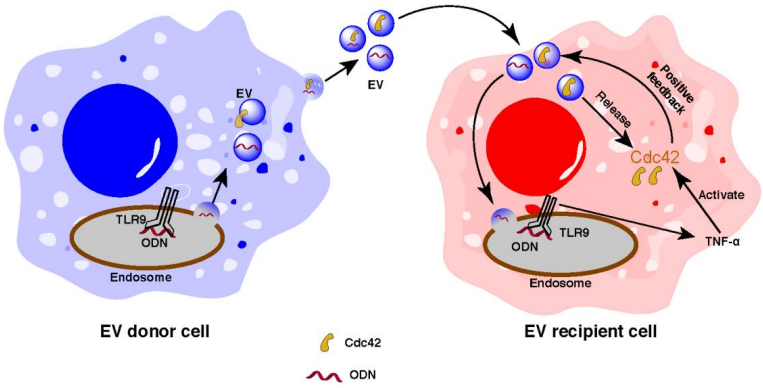

Figure 1. Roles of extracellular vesicles (EVs) in the communication between macrophages and propagation of the intracellular immune response (this figure is reprinted from *Science Advances* journal)[22].

role of EVs in intercellular communication and the activation of innate immunity.

Endosomal Toll-like receptors (TLR) are an important family of proteins in innate immune responses capable of recognizing foreign nucleic acid sequences and then mediating innate immune responses. In our study, aided by real-time tracking of EVs, we showed that EVs transported a DNA ligand, oligodeoxynucleotides (ODN), for TLR9 activation, revealing the role of EVs in innate immune surveillance[22]. In addition, we found that cellular uptake of EVs is increased upon TLR9 activation, which was demonstrated by a biological tool showing real-time EV transfer between cells called the Cre-LoxP reporter system[22]. Further, we found that cell division control protein 42 homolog (Cdc42) is responsible for the increased cellular uptake of ODN-induced EVs from macrophages. In particular, we found that EV-carried ODN induced the release of tumor necrosis factor-α (TNF-α) from macrophages, transforming Cdc42 into its activated form, which is bound to guanosine triphosphate inside cells and accounts for the further uptake of EVs. In this study, we elucidate a new mechanism that facilitates the understanding of the positive feedback loop influenced by the uptake of EV protein cargo[22]. Our findings not only shed light on a new mechanism via which EVs may shuttle protein to increase their cellular uptake but also point out that Cdc42 could be a novel target for developing more efficient therapeutic approaches to regulate EV uptake [Figure 1].

Discussion and perspectives

Here, we briefly introduce our previous work using chemical and biological tools to enable EV sensing and facilitate the understanding of the functions and mechanisms in EVs' role in physiological or pathological conditions. Moreover, EVs have been characterized and investigated in various diseases including cancer, and this work was summarized and discussed in our recent publication[23]. In addition, EVs show great potential in drug delivery by acting as a Trojan horse, even penetrating biological barriers such as the blood-brain barrier. Notably, in the current pandemic situation, EVs have been approved in clinical trials as therapeutic entities and are demonstrating great potential in disease treatment, in addition to serving as biomarkers. Very soon, EVs may lead to a new era in both the pharmaceutical and biomedical fields, affecting mechanisms for the surveillance of disease, drug delivery systems, or even drug development and treatment for a variety of diseases.

DECLARATIONS

Authors' contributions

Made substantial contributions to conception: Zhang Y, Yin H
Drafted the manuscript: Zhang Y, Yin H
Approved the final version: Zhang Y, Yin H

Availability of data and materials

Not applicable.

Financial support and sponsorship

This work was supported by funds from the National Key R&D Program of China (Grant No. 2021YFE0109300, 2017YFA0505200), the National Natural Science Foundation of China (Grant No. 21825702, 22137004, 21977061), the Tsinghua University Spring Breeze Fund (Grant No. 2020Z99CFY036), the International Research Project of Independent Research of Ministry of Education Grant (UTokyo-Tsinghua Collaborative Research Fund) (Grant No. 2019Z02UTY), Beijing Advanced Innovation Center for Structural Biology Funding (Grant No. 20151551402), and the Beijing Outstanding Young Scientist program (Grant No. BJJWZYJH01201910003013).

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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REFERENCES

1. Colombo M, Raposo G, Théry C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annu Rev Cell Dev Biol* 2014;30:255-89. DOI PubMed
2. Kowal J, Tkach M, Théry C. Biogenesis and secretion of exosomes. *Curr Opin Cell Biol* 2014;29:116-25. DOI PubMed
3. Kalluri R, LeBleu VS. The biology, function and biomedical applications of exosomes. *Science* 2020;367:eaau6977. DOI PubMed PMC
4. Andaloussi S, Mäger I, Breakefield XO, Wood MJ. Extracellular vesicles: biology and emerging therapeutic opportunities. *Nat Rev Drug Discov* 2013;12:347-57. DOI PubMed
5. Niel G, D'Angelo G, Raposo G. Shedding light on the cell biology of extracellular vesicles. *Nat Rev Mol Cell Biol* 2018;19:213-28. DOI PubMed
6. Maas SLN, Breakefield XO, Weaver AM. Extracellular vesicles: unique intercellular delivery vehicles. *Trends Cell Biol* 2017;27:172-88. DOI PubMed PMC
7. Shah R, Patel T, Freedman JE. Circulating extracellular vesicles in human disease. *N Engl J Med* 2018;379:2179-81. DOI PubMed
8. Marar C, Starich B, Wirtz D. Extracellular vesicles in immunomodulation and tumor progression. *Nat Immunol* 2021;22:560-70. DOI PubMed
9. Théry C. Cancer: Diagnosis by extracellular vesicles. *Nature* 2015;523:161-2. DOI PubMed
10. Xiao Y, Zhong J, Zhong B, et al. Exosomes as potential sources of biomarkers in colorectal cancer. *Cancer Lett* 2020;476:13-22. DOI PubMed
11. Zimmerberg J, McLaughlin S. Membrane curvature: how BAR domains bend bilayers. *Curr Biol* 2004;14:R250-2. DOI PubMed
12. Drin G, Casella JF, Gautier R, et al. A general amphipathic alpha-helical motif for sensing membrane curvature. *Nat Struct Mol Biol* 2007;14:138-46. DOI PubMed
13. Saludes JP, Morton LA, Ghosh N, et al. Detection of highly curved membrane surfaces using a cyclic peptide derived from

synaptotagmin-I. *ACS Chem Biol* 2012;7:1629-35. DOI PubMed PMC

14. Morton LA, Yang H, Saludes JP, et al. MARCKS-ED peptide as a curvature and lipid sensor. *ACS Chem Biol* 2013;8:218-25. DOI PubMed PMC
15. Yan L, de Jesus AJ, Tamura R, et al. Curvature sensing MARCKS-ED peptides bind to membranes in a stereo-independent manner. *J Pept Sci* 2015;21:577-85. DOI PubMed PMC
16. Saludes JP, Morton LA, Coulup SK, et al. Multivalency amplifies the selection and affinity of bradykinin-derived peptides for lipid nanovesicles. *Mol Biosyst* 2013;9:2005-9. DOI PubMed PMC
17. Bonechi C, Ristori S, Martini G, Martini S, Rossi C. Study of bradykinin conformation in the presence of model membrane by Nuclear Magnetic Resonance and molecular modelling. *Biochim Biophys Acta* 2009;1788:708-16. DOI PubMed
18. Langelaan DN, Rainey JK. Membrane catalysis of peptide-receptor binding. *Biochem Cell Biol* 2010;88:203-10. DOI PubMed PMC
19. Chatterjee C, Mukhopadhyay C. Conformational alteration of bradykinin in presence of GM1 micelle. *Biochem Biophys Res Commun* 2004;315:866-71. DOI PubMed
20. Février B, Raposo G. Exosomes: endosomal-derived vesicles shipping extracellular messages. *Curr Opin Cell Biol* 2004;16:415-21. DOI PubMed
21. Subra C, Laulagnier K, Perret B, Record M. Exosome lipidomics unravels lipid sorting at the level of multivesicular bodies. *Biochimie* 2007;89:205-12. DOI PubMed
22. Zhang Y, Jin X, Liang J, et al. Extracellular vesicles derived from ODN-stimulated macrophages transfer and activate Cdc42 in recipient cells and thereby increase cellular permissiveness to EV uptake. *Sci Adv* 2019;5:eaav1564. DOI PubMed PMC
23. Zhang Y, Xiao Y, Sun G, et al. Harnessing the therapeutic potential of extracellular vesicles for cancer treatment. *Semin Cancer Biol* 2021;74:92-104. DOI PubMed

Gurrieri *et al. Extracell Vesicles Circ Nucleic Acids* 2022;3:93-101
DOI: 10.20517/evcna.2022.07

Extracellular Vesicles and Circulating Nucleic Acids

Review

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Strategies to functionalize extracellular vesicles against HER2 for anticancer activity

Elena Gurrieri, Vito Giuseppe D'Agostino

Department of Cellular, Computational and Integrative Biology, University of Trento, Trento 38123, Italy.

Correspondence to: Prof. Vito Giuseppe D'Agostino, Department of Cellular, Computational and Integrative Biology, University of Trento, Via Sommarive 9, Trento 38123, Italy. E-mail: vito.dagostino@unitn.it

How to cite this article: Gurrieri E, D'Agostino VG. Strategies to functionalize extracellular vesicles against HER2 for anticancer activity. *Extracell Vesicles Circ Nucleic Acids* 2022;3:93-101. https://dx.doi.org/10.20517/evcna.2022.07

Received: 14 Feb 2022 **First Decision:** 7 Mar 2022 **Revised:** 22 Mar 2022 **Accepted:** 12 Apr 2022 **Published:** 18 Apr 2022

Academic Editors: Yoke Peng Loh, Michael Graner **Copy Editor:** Tiantian Shi **Production Editor:** Tiantian Shi

Abstract

Cell-secreted extracellular vesicles (EVs) are membranous particles highly heterogeneous in size and molecular cargo. Comprehensively, released EV sub-populations can show a wide range and selection of different protein, RNA, and lipid species, complementing cell communication signals. Recently, EVs represent a new source for developing targeted delivery systems. EVs are stable in biofluids, intrinsically biocompatible with low immunogenicity, and capable of transferring cargo molecules into "recipient" cells. The immune-mediated recognition represents a popular approach to functionalize and direct EVs towards receptor-positive cell populations. The human epidermal growth factor receptor 2 (HER2, also known as *neu* or ERBB2) is a tyrosine kinase of clinical relevance, targeted by several available antibodies, and a model receptor used to test the biodistribution and anticancer activity of bioformulations, including EVs. Here, we focus on recent strategies adopted for EV functionalization with fusion ligands able to recognize HER2, covering the enhanced expression of membrane-fusion proteins in "EV-donor" cells as well as post-isolation EV-surface modifications.

Keywords: Extracellular vesicles, human epidermal growth factor receptor 2 (HER2/ERBB2), EV engineering, fusion proteins

INTRODUCTION

The establishment of programmable and versatile delivery systems that could control the dosage of the

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therapeutic agent in the tumor site and bypass biological barriers represents a current challenge in biomedical research. Extracellular vesicles (EVs) can serve as a new biological source for targeted drug delivery and the development of nanoparticle-based technologies[1,2]. EVs are cell-secreted membranous particles highly heterogeneous in size and molecular cargo. Ranging from nanometer to micrometer scale, EVs are classified as exosomes or microvesicles according to the cellular pathways responsible for their release from the endosomal system or the plasma membrane, respectively[3]. As cell-derived material, secreted EV sub-populations can show a wide range and selection of different species of proteins, RNA, and lipids, complementing cell communication signals[4,5,6]. Substantiated by the observed stability in biofluids, intrinsic biocompatibility, low immunogenicity, and capacity to convey the cargo molecules into "recipient" cells, EVs have been proposed as advantageous delivery vehicles compared to liposomes or other synthetic biomaterials[7,8]. For example, EVs were successfully employed *in vitro* and *in vivo* for miRNA delivery, siRNA-based gene silencing, or shuttling mRNAs encoding for reporter proteins[9,10,11].

The immune-mediated interaction is commonly exploited to direct EVs towards specific membrane receptors on the desired cell populations[12]. The family of epidermal growth factor receptors (EGFRs) includes well-known functional ligands and clinically relevant biomarkers in solid tumors, such as breast, ovarian, and stomach cancers. The proto-oncogene human epidermal growth factor receptor 2 (HER2, also known as *neu* or ERBB2) is a member of tyrosine kinase receptors involved in cell differentiation, proliferation, and migration[13]. Overexpression of this glycoprotein can be due to *HER2* gene amplification or aberrant protein expression in tumor tissues[14,15,16]. Since the secreted EV sub-populations display proteins that mirror the plasma-membrane identity of the donor cells, the HER2 receptor has been detected in EVs recovered by different methods, from media of cultured cells or the blood of patients with HER2-positive tumors. Indeed, Nanou *et al.*[17] (2020) encouraged the screening of vesicular HER2 in clinical settings as they found blood circulating EVs extremely informative on the presence of HER2-positive primary tumors and proposed them as valuable prognostic factors complementing circulating tumor cells (CTCs)[17]. Kim *et al.*[18] (2020) described a positive correlation between cellular HER2 and receptor enrichment in the smallest EV fractions, confirming the presence of HER2 in EVs circulating in breast cancer patients with different tumor stages[18]. Vesicular HER2 was also detected in human serum using a primary antibody with the same variable region of trastuzumab[19,20], an antibody under clinical use since 1998 for treating HER2-positive breast cancer patients[13]. Interestingly, Quinn *et al.*[21] (2021) reported that EVs released from cells overexpressing HER2 could horizontally transfer the protein to receptor-negative cells, sensitizing them to paclitaxel[21].

Different monoclonal antibodies or fusion moieties have been developed to target the extracellular domain of HER2[22], leading to cytostatic effects or cell death when in conjugation with cytotoxic compounds[23,24,25]. These moieties were explored for their targeted delivery potential within different bioformulations, including unilamellar liposomes loaded with chemotherapy agents[26], gold nanoparticles[27], or silk nanospheres[28].

In this review, we summarize the different strategies of EV-functionalization with ligands able to recognize the HER2 receptor, including seminal examples designed on EGFR, ranging from the enhanced expression of membrane-fusion proteins in EV-donor cells to post-isolation approaches.

STRATEGIES TO FUNCTIONALIZE EVS BY MANIPULATING DONOR CELLS

The manipulation of specific cells conceived as EV-producers is one of the most common strategies adopted to enrich vesicle sub-populations with specific surface proteins. This approach finds a rationale, for example, with chimeric antigen receptor (CAR)-T cells which can be directed against a tumor-expressing

antigen. Indeed, there is evidence that heterogeneous EVs deriving from CAR-T cells, over-expressing an anti-HER2 ligand, can penetrate and induce apoptosis in target cells, albeit with a delayed timing compared to donor cells[29].

As already demonstrated for the anti-EGFR strategy, donor cells can be used to transiently or stably overexpress multi-domain proteins subsequently detected on secreted EVs. These constructs generally encode for a receptor transmembrane (TM) domain, a linker, and an antibody-derived targeting moiety. This last portion can also be fused with a reporter, such as the green fluorescent protein (GFP)[30]. Leading examples of HER2-targeting strategy include the lysosome-associated membrane protein 2b (LAMP2b), the platelet-derived growth factor receptor domain (PDGFR TM), and the membrane-associated C1-C2 domains of lactadherin.

LAMP2b

Limoni *et al.*[31] (2019) isolated exosomes from HEK293T cells expressing a fusion protein constituted by LAMP2b followed by designed ankyrin repeat protein (DARPin) G3, a 14 kDa-engineered peptide targeting HER2. The authors observed that the concentration of particles used to treat recipient cells was crucial for targeting HER2-positive SKBR3 cells compared to HER2-negative MDA-MB-231 cells. In addition, the isolated exosomes were able to deliver siRNAs specifically, ultimately impacting the levels of *TPD52* in recipient cells[31]. The same group used the construct to transduce bone marrow mesenchymal stem cells (MSCs) and isolate exosomes that were electroporated in the presence of doxorubicin (exo-DOX). In this case, LAMP2b-DARPin-exo-DOX confirmed a specific targeting profile up to 0.1 μg/μL, while not at 0.2 μg/μL, and induced death of TUBO cells to a greater degree than free doxorubicin[32].

In the study by Molavipordanjani *et al.*[33] (2020), radiolabeled 99mTc-DARPins were used to decorate exosomes with an affinity for HER2. They tested the uptake ratio of their preparations using SKOV-3, MCF-7, U87-MG, HT-29, and A549 cell lines characterized by different levels of HER2 expression. They profiled an accumulation of particles proportional to the higher expression of the receptor (SKOV-3 cells). The authors also evaluated the biodistribution of their particles injected in normal and SKOV-3 xenografted nude mice and reported a high liver uptake at one hour, which gradually decreased at four hours. In addition, the intestines and kidneys showed a consistent level of radioactivity, while it decreased in a time-dependent manner in other tissues, including the spleen, lungs, and blood. The same pattern of biological distribution, including accumulation in the tumor site, was observed in the xenografted mice subjected to tumor tissue visualization by planar imaging[33].

A LAMP2b-directed fusion protein approach was also adopted by Liang *et al.*[34] (2020), who fused a HER2-binding affibody (or antibody mimetic) at the C-terminus with the addition of GFP. They reported the complete sequence of the fusion protein, including a flexible peptide linker $(GGGGS)_3$, and exploited exosomes to co-deliver oligonucleotides and drugs to HER2-positive colorectal cancer cells. Besides the cell-targeting properties of the preparations reaching HER2-mcherry-SGC-7901 cells or the tumor *in vivo*, encapsulated 5-fluorouracil (5-FU) together with miR-21 inhibitor nucleotide (miR-21i) were found to be more effective than exosomes loaded with either miR-21i or 5-FU alone[34].

PDGFR TM

Another interesting strategy exploited EVs co-expressing CD3 and HER2 ligands to enhance the proximity of T cells to tumor cells and elicit an anticancer effect in the presence of non-activated human peripheral blood mononuclear cells (PBMCs) *in vivo*[35]. In this study, the authors exploited the human PDGFR TM domain fused to an antibody single-chain variable fragments (scFv) connected by a $(GGGGS)_3$ linker in the

CD3/HER2 bi-specific chimeric protein. By confirming cellular uptake *in vitro*, the authors demonstrated cytotoxic activity selectively enhanced in HER2-expressing breast cancer cells mediated by T cell activation compared with native vesicles still isolated by ultracentrifugation. The same qualitative data were obtained using mouse xenografts with no significant effects reported in total body weight or specific organ damage, especially liver or kidney[35].

Lactadherin C1-C2 domains

Longatti *et al.*[36] (2018) transduced HEK293 cells to express anti-HER2 scFvs fused to C1-C2 domains of lactadherin, which associate with phosphatidylserine (PS) in exosome fractions. The authors used three different scFvs covering a range of high (Kd~15 pM), intermediate (Kd~1 nM), and low (Kd~317 nM) affinity for HER2. They compared the different preparations by also varying the HER2 expression level on recipient cells, finding that both high-affinity scFv and high receptor expression were parameters positively influencing the selective uptake[36]. Another targeting moiety to direct exosomes against the receptor consisted of two copies of HER2 ligand still fused to the C-terminal C1-C2 domains[37]. The authors reported ELISA experiments showing a four-fold enhanced binding of recovered exosomes compared to untargeted ones. *In vivo* experiments demonstrated a tumor volume reduction of implanted SKOV-3 cells upon injection of targeting exosomes carrying a HER2-downregulating miRNA[37]. The lactadherin C1-C2 domains fused to anti-HER2 scFv were used by Forterre *et al.*[38] (2020) to deliver prodrugs that exert cytotoxic activity in recipient cells. The authors showed a near-complete growth arrest of human HER2-positive breast cancer xenografts upon systemic administration in athymic mice, with no reported injury to other tissues and absence of "off-target mRNA delivery"[38].

STRATEGIES TO FUNCTIONALIZE EVS POST-ISOLATION

Since biochemical strategies focused on HER2 emulate approaches already presented for targeting EGFR, we include the most recent post-isolation designs to direct vesicles against EGFR. Seminal approaches include the use of lactadherin C1-C2 domains, protein ligation, and antibody-receptor binding.

In the study by Kooijmans *et al.*[39] (2016), the nanobody EGa1 was conjugated to PEG-phospholipid micelles, subsequently incorporated into EVs, and then purified by size-exclusion chromatography. The authors reported that PEGylation increased circulation time in the blood of tumor-bearing mice and presented this method as a versatile tool to increase the stability of targeting EVs[39]. A different study followed in 2018 and reported EVs decorated with C1-C2 fusion proteins[40]. The authors expressed in HEK293 a fusion protein connecting the EGa1 sequence to the PS-binding domains of lactadherin (C1-C2) via a GGGS2 linker. The EGa1-C1-C2 protein was generated together with R2-C1-C2 protein as a negative control, and the native recombinant proteins were purified from cell culture media, still retaining binding activity. The final preparation was obtained by incubating fusion proteins with PS-bearing EVs following the nanobody:EVs ratio of ng:μg. Exosomes isolated from red blood cells (RBCs) and mouse neuroblastoma cells were reported to maintain their size and integrity after decoration with the fusion proteins. The authors showed enhanced specific binding (incubation at 4 °C for 1 h) and uptake (incubation at 37 °C for 4 h) by receptor-positive cells[40].

Wang *et al.*[41] (2018) provided an example of enzyme/prodrug therapy mediated by targeted EVs. They designed a chimeric protein containing a high-affinity anti-HER2 scFv antibody fused to lactadherin C1-C2 domains. To engineer EVs, they showed that post-isolation incubation of EVs from HEK293 with the protein alone was more efficient than HEK293 transfection with the plasmid encoding for the protein. They showed that the PKH26-labeled targeted EVs could bind to HER2-positive cells with a selective cytotoxic profile *in vitro* when combined with the mRNA of the enzyme responsible for the prodrug activation. In

addition, their preparations inhibited HER2-positive tumor xenografts growth in mice[41].

Other authors exploited the protein ligase OaAEP1 to conjugate a biotinylated EGFR-targeting peptide (Biotin-YHWYGYTPQNVI-GGGGS-NGL)[42] to EVs recovered from human red blood cells (RBCEVs)[43]. They proved an increased internalization of functionalized EVs in EGFR-positive cells after 2 h of incubation at 37 °C. The authors performed a similar enzymatic ligation to conjugate RBCEVs with an α-EGFR camelid biparatopic nanobody (VHH) and reported the importance of a linker peptide with the ligase binding site at the C-terminus (GGG-Myc-GLPETGG), necessary as a bridge to reduce the steric hindrance between the VHH and the RBCEV surface. *In vivo* experiments with immunodeficient NOD scid gamma (NSG) mice bearing luciferase-mCherry-H358 lung tumors demonstrated that tail vein-injected EVs preferentially accumulated in the lung, as expected, as compared to the liver, and enhanced the effect of paclitaxel, pre-loaded through sonication[43].

Focusing directly on the HER2 receptor, Sato *et al.*[44] (2016) produced hybrid particles through membrane fusion between exosomes derived from a mouse macrophage cell line and liposomes after up to 10 freeze-thaw cycles. They used zwitterionic, cationic, or anionic lipids to explore different lipidic compositions and found that exosomes from mouse fibroblast sarcoma cells, overexpressing HER2, maintained the receptor expression after fusion with PEGylated anionic lipids. These preparations showed increased uptake in HeLa cells[44].

A different approach was proposed by Barok *et al.*[45] (2018), who decorated exosomes from HER2-positive cancer cells with the antibody–drug conjugate trastuzumab emtansine (T-DM1). They isolated EVs from the secretome of several HER2-positive cancer cell lines and incubated them with T-DM1 (25 ug/mL at 4 °C for 30 min). Confocal microscopy imaging showed that EV preparations, exposed overnight, preferentially entered HER2-positive cells, inhibiting proliferation and inducing caspase activation[45].

CONCLUSION

EVs represent a valuable source in liquid biopsy studies as cargo of clinically relevant biomarkers, including HER2 and EGFR. Recent reports also indicate that EVs decorated with anti-HER2 ligands hold promise for targeted therapy, expanding the repertoire of nanoparticle-based technologies[46].

In Figure 1, we summarize the pre- and post-isolation strategies thus far presented to direct EVs against HER2/EGFR receptors. The lactadherin C1-C2 domains emerged as the most versatile system used either in pre- or post-isolation strategies. However, we could not determine comparative relationships based on the yield of specific EV sub-populations, relative *in vitro* and *in vivo* stability, HER2-binding efficiency, or tissue-specific EV internalization rates. Table 1 highlights the liquid biopsy studies reporting the detection of blood circulating HER2-positive EVs in breast cancer patients and the outcomes described in preclinical models with the corresponding targeting strategy. In xenograft studies, the LAMP2b- and lactadherin C1-C2-based strategies effectively inhibited tumor growth with a certain degree of tissue specificity.

Considering the qualitative proof-of-principles reported with both *in vitro* and *in vivo* models using cell-secreted EVs, the preparation of the vesicle suspension represents a relevant aspect. A repeatable and homogenous suspension of the bioformulation is a crucial requirement to develop EV-based nanotechnologies. On the one side, we cannot exclude that the high heterogeneity of cell-secreted EVs could be directly responsible for their stability in circulation or the biological effects exerted in recipient cells due to a sort of induced "parallel, cumulative signaling/internalization". On the other side, technical challenges exist in establishing single-step, multi-parametric methodologies that efficiently separate vesicle sub-

Gurrieri *et al. Extracell Vesicles Circ Nucleic Acids* 2022;3:93-101 | https://dx.doi.org/10.20517/evcna.2022.07

Table 1. Summary of the liquid biopsy studies reporting the detection of blood circulating HER2-positive extracellular vesicles (EVs) in breast cancer patients and the outcomes described in preclinical models with the corresponding targeting strategy

Source	Liquid biopsy studies	Targeting strategy	Preclinical model	Outcome description	Ref.
Metastatic breast cancer patients with HER2-positive tumors	Blood			Detection of HER2-positive EVs and prognostic significance	Nanou *et al.*[17], 2020
Breast cancer patients at different tumor stage	Blood			Detection of HER2-positive EVs	Kim *et al.*[18], 2020
Breast cancer patients	Blood			Detection of HER2-positive EVs	Jiang *et al.*[19], 2019; Ciravolo *et al.*[20], 2012
		PDGFR TM + GE11	Breast cancer xenograft in RAG2$^{-/-}$ mice	Migration of exosomes to tumor tissues and inhibition of tumor development	Ohno *et al.*[12], 2013
		Targeting peptide ligated to EV-membrane proteins	Mice bearing luciferase-mCherry-H358 EGFR-positive lung tumors	Tail vein-injected EVs preferentially accumulated in the lung more than in the liver; enhanced the efficacy of paclitaxel	Pham *et al.*[43], 2021
		GPI conjugated to EGa1 nanobody	A431-xenografted mice	Increased EV circulation time	Kooijmans *et al.*[39], 2016
		LAMP2b + DARPin	TUBO-xenografted mice	Selective tissue distribution and reduction of the tumor growth rate	Gomari *et al.*[32], 2019
		LAMP2b + DARPin G3	SKOV-3-xenografted mice	Tumor site accumulation together with other organs	Molavipordanjani *et al.*[33], 2020
		LAMP2 + affibody	Colon cancer xenografts in BALB/c mice	Significant reduction of tumor growth	Liang *et al.*[34], 2020
		PDGFR TM + CD3/HER2 ligands	Mice bearing HCC 1954 tumors, engrafted with human PBMCs	Pharmacokinetic profile; significant inhibition of tumor growth; no systemic cytotoxicity; significant T cell infiltrations in the tumor site	Shi *et al.*[35], 2020
		Lactadherin C1-C2 + targeting peptide	Mice with HER2-negative or -positive tumor xenografts	Reduction of HER2-positive tumor volume	Wang *et al.*[37], 2020
		Lactadherin C1-C2 + scFv	BALB/C athymic mice with human HER2-positive BT474 xenografts	Growth arrest of xenografts; no reported injury to other tissues	Forterre *et al.*[38], 2020
		Lactadherin C1-C2 + scFv ML39	Mice with HER2-positive BT474 xenografts	Inhibition of HER2-positive tumor growth; EV-mediated delivery of exogenous mRNA	Wang *et al.*[41], 2018

populations characterized by homogeneous biophysical (size/concentration distribution) and biological (lipid/protein/nucleic acid content) profiles.

After manipulating EV-donor cells, one of the relevant challenges is the efficient recovery of the desired EV sub-populations. Conversely, the post-isolation methods often suffer from an induced increment of size and/or particle aggregation. A deeper characterization and separation of EV sub-populations using isogenic cell models could improve our knowledge of the internalization performance or the degradation/recycling pathways upon endocytosis. A direct, systematic comparison among the designed tools will contribute knowledge on improving the preparations' stability, how the receptor's expression level

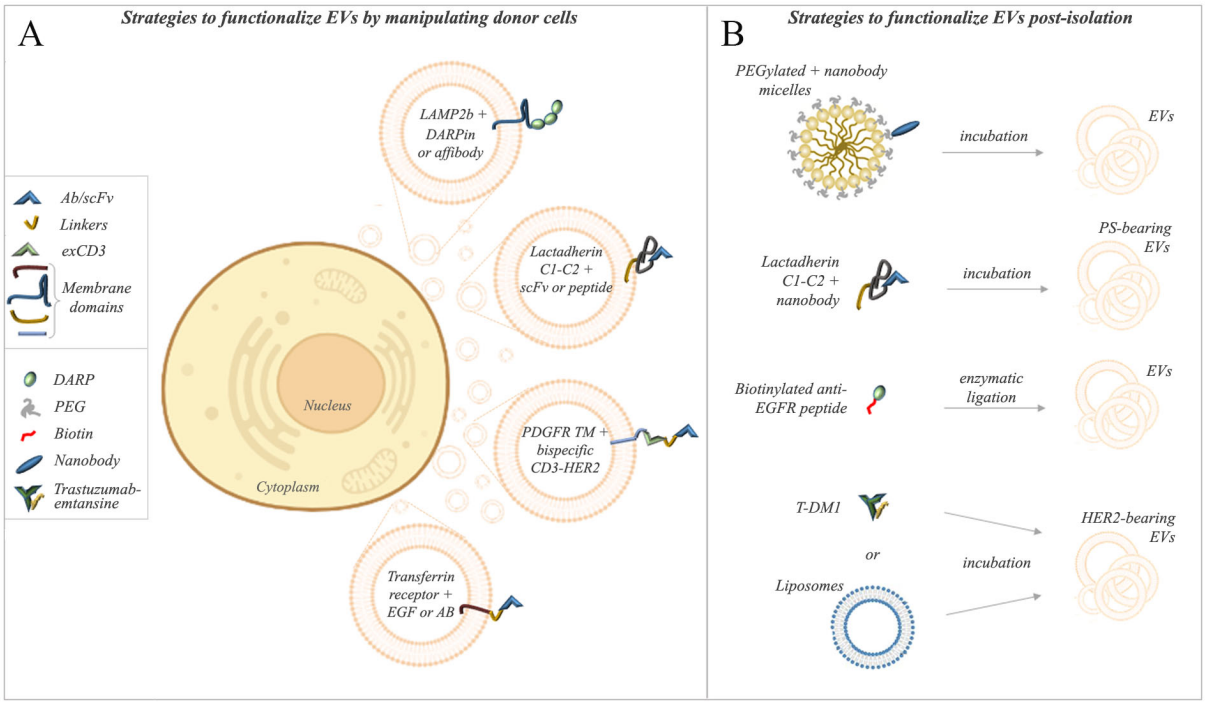

Figure 1. Strategies for EV targeting against HER2/EGFR. Pre-isolation strategies (A): manipulation of EV-producing cells to enrich targeting proteins in EVs. TM proteins (LAMP2b), TM domains (PDGR, transferrin receptor), or membrane-associated domains (C1-C2 of lactadherin) are exploited to anchor the targeting moieties to the EV membrane. Post-isolation strategies (B): EVs are directly functionalized by incubation with nanobodies or targeting peptides alone or fused to membrane-associated domains (C1-C2 of lactadherin). Post-isolation modifications also include the fusion of EVs with micelles or liposomes, forming hybrid particles. Both pre- and post-isolation approaches include scFvs, affibodies, nanobodies, DARPins, or peptides as targeting moieties. The figure was partially created on www.biorender.com.

influences the EV up/intake, or the advantages conferred by specific linkers. In this regard, the presence of linkers of different lengths that confer distance and flexibility to the targeting moiety on the EV surface has been evaluated in synthetic immunoliposomes[26] as well as engineered EVs, favoring the particle uptake[43,34,40]. Nevertheless, the storage, freezing cycles, and potential cryopreserving agents could be relevant to correlating morphological changes and quality of proteins/RNAs with bioactivity[47,48].

We believe these are fundamental aspects contributing to the development of promising routes for establishing EV-based, innovative delivery systems.

DECLARATIONS

Authors' contributions

Both the authors (Gurrieri E and D'Agostino VG) made substantial contributions to conception and design of the study, performed literature screening, data interpretation, and reporting, manuscript writing and revision.

Availability of data and materials

Not applicable.

Financial support and sponsorship

This work was supported by Fondazione Caritro, Cassa Risparmio Trento e Rovereto.

Conflicts of interest

Both authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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REFERENCES

1. Herrmann IK, Wood MJA, Fuhrmann G. Extracellular vesicles as a next-generation drug delivery platform. *Nat Nanotechnol* 2021;16:748-59. DOI PubMed
2. Duncan R, Richardson SC. Endocytosis and intracellular trafficking as gateways for nanomedicine delivery: opportunities and challenges. *Mol Pharm* 2012;9:2380-402. DOI PubMed
3. Niel G, D'Angelo G, Raposo G. Shedding light on the cell biology of extracellular vesicles. *Nat Rev Mol Cell Biol* 2018;19:213-28. DOI PubMed
4. Doyle LM, Wang MZ. Overview of extracellular vesicles, their origin, composition, purpose, and methods for exosome isolation and analysis. *Cells* 2019;8:727. DOI PubMed PMC
5. Fabbiano F, Corsi J, Gurrieri E, Trevisan C, Notarangelo M, D'Agostino VG. RNA packaging into extracellular vesicles: an orchestra of RNA-binding proteins? *J Extracell Vesicles* 2020;10:e12043. DOI PubMed PMC
6. Sork H, Corso G, Krjutskov K, et al. Heterogeneity and interplay of the extracellular vesicle small RNA transcriptome and proteome. *Sci Rep* 2018;8:10813. DOI PubMed PMC
7. Andaloussi S, Mäger I, Breakefield XO, Wood MJ. Extracellular vesicles: biology and emerging therapeutic opportunities. *Nat Rev Drug Discov* 2013;12:347-57. DOI PubMed
8. Kim OY, Lee J, Gho YS. Extracellular vesicle mimetics: novel alternatives to extracellular vesicle-based theranostics, drug delivery, and vaccines. *Semin Cell Dev Biol* 2017;67:74-82. DOI PubMed
9. Wang Y, Chen X, Tian B, et al. Nucleolin-targeted extracellular vesicles as a versatile platform for biologics delivery to breast cancer. *Theranostics* 2017;7:1360-72. DOI PubMed PMC
10. Munir J, Yoon JK, Ryu S. Therapeutic miRNA-Enriched extracellular vesicles: current approaches and future prospects. *Cells* 2020;9:2271. DOI PubMed PMC
11. O'Brien K, Breyne K, Ughetto S, Laurent LC, Breakefield XO. RNA delivery by extracellular vesicles in mammalian cells and its applications. *Nat Rev Mol Cell Biol* 2020;21:585-606. DOI PubMed PMC
12. Ohno S, Takanashi M, Sudo K, et al. Systemically injected exosomes targeted to EGFR deliver antitumor microRNA to breast cancer cells. *Mol Ther* 2013;21:185-91. DOI PubMed PMC
13. Nuti M, Bellati F, Visconti V, et al. Immune effects of trastuzumab. *J Cancer* 2011;2:317-23. DOI PubMed PMC
14. Ahn S, Woo JW, Lee K, Park SY. HER2 status in breast cancer: changes in guidelines and complicating factors for interpretation. *J Pathol Transl Med* 2020;54:34-44. DOI PubMed PMC
15. Iqbal N, Iqbal N. Human epidermal growth factor receptor 2 (her2) in cancers: overexpression and therapeutic implications. *Mol Biol Int* 2014;2014:852748. DOI PubMed PMC
16. Ooi A, Takehana T, Li X, et al. Protein overexpression and gene amplification of HER-2 and EGFR in colorectal cancers: an immunohistochemical and fluorescent in situ hybridization study. *Mod Pathol* 2004;17:895-904. DOI PubMed
17. Nanou A, Zeune LL, Bidard FC, Pierga JY, Terstappen LWMM. HER2 expression on tumor-derived extracellular vesicles and circulating tumor cells in metastatic breast cancer. *Breast Cancer Res* 2020;22:86. DOI PubMed PMC
18. Kim D, Woo HK, Lee C, et al. EV-Ident: Identifying tumor-specific extracellular vesicles by size fractionation and single-vesicle analysis. *Anal Chem* 2020;92:6010-8. DOI PubMed
19. Jiang Q, Liu Y, Wang L, Adkins GB, Zhong W. Rapid enrichment and detection of extracellular vesicles enabled by CuS-enclosed microgels. *Anal Chem* 2019;91:15951-8. DOI PubMed PMC
20. Ciravolo V, Huber V, Ghedini GC, et al. Potential role of HER2-overexpressing exosomes in countering trastuzumab-based therapy. *J Cell Physiol* 2012;227:658-67. DOI PubMed
21. Quinn Z, Mao W, Xia Y, John R, Wan Y. Conferring receptors on recipient cells with extracellular vesicles for targeted drug delivery. *Bioact Mater* 2021;6:749-56. DOI PubMed PMC
22. Salimi F, Forouzandeh Moghadam M, Rajabibazl M. Development of a novel anti-HER2 scFv by ribosome display and in silico evaluation of its 3D structure and interaction with HER2, alone and after fusion to LAMP2B. *Mol Biol Rep* 2018;45:2247-56. DOI

PubMed
23. Krop I, Winer EP. Trastuzumab emtansine: a novel antibody-drug conjugate for HER2-positive breast cancer. *Clin Cancer Res* 2014;20:15-20. DOI PubMed
24. Xu Z, Guo D, Jiang Z, et al. Novel HER2-targeting antibody-drug conjugates of trastuzumab beyond T-DM1 in breast cancer: trastuzumab deruxtecan(DS-8201a) and (Vic-)trastuzumab duocarmazine (SYD985). *Eur J Med Chem* 2019;183:111682. DOI PubMed
25. Gajria D, Chandarlapaty S. HER2-amplified breast cancer: mechanisms of trastuzumab resistance and novel targeted therapies. *Expert Rev Anticancer Ther* 2011;11:263-75. DOI PubMed PMC
26. Kirpotin D, Park JW, Hong K, et al. Sterically stabilized anti-HER2 immunoliposomes: design and targeting to human breast cancer cells in vitro. *Biochemistry* 1997;36:66-75. DOI PubMed
27. Van de Broek B, Devoogdt N, D'Hollander A, et al. Specific cell targeting with nanobody conjugated branched gold nanoparticles for photothermal therapy. *ACS Nano* 2011;5:4319-28. DOI PubMed
28. Florczak A, Deptuch T, Kucharczyk K, Dams-Kozlowska H. Systemic and local silk-based drug delivery systems for cancer therapy. *Cancers (Basel)* 2021;13:5389. DOI PubMed PMC
29. Aharon A, Horn G, Bar-Lev TH, et al. Extracellular vesicles derived from chimeric antigen receptor-T cells: a potential therapy for cancer. *Hum Gene Ther* 2021;32:1224-41. DOI PubMed
30. Zhao C, Busch DJ, Vershel CP, Stachowiak JC. Multifunctional transmembrane protein ligands for cell-specific targeting of plasma membrane-derived vesicles. *Small* 2016;12:3837-48. DOI PubMed PMC
31. Limoni SK, Moghadam MF, Moazzeni SM, Gomari H, Salimi F. Engineered exosomes for targeted transfer of siRNA to HER2 positive breast cancer cells. *Appl Biochem Biotechnol* 2019;187:352-64. DOI PubMed
32. Gomari H, Forouzandeh Moghadam M, Soleimani M, Ghavami M, Khodashenas S. Targeted delivery of doxorubicin to HER2 positive tumor models. *IJN* 2019;14:5679-90. DOI PubMed PMC
33. Molavipordanjani S, Khodashenas S, Abedi SM, Moghadam MF, Mardanshahi A, Hosseinimehr SJ. ^{99m}Tc-radiolabeled HER2 targeted exosome for tumor imaging. *Eur J Pharm Sci* 2020;148:105312. DOI PubMed
34. Liang G, Zhu Y, Ali DJ, et al. Engineered exosomes for targeted co-delivery of miR-21 inhibitor and chemotherapeutics to reverse drug resistance in colon cancer. *J Nanobiotechnology* 2020;18:10. DOI PubMed PMC
35. Shi X, Cheng Q, Hou T, et al. Genetically engineered cell-derived nanoparticles for targeted breast cancer immunotherapy. *Mol Ther* 2020;28:536-47. DOI PubMed PMC
36. Longatti A, Schindler C, Collinson A, et al. High affinity single-chain variable fragments are specific and versatile targeting motifs for extracellular vesicles. *Nanoscale* 2018;10:14230-44. DOI PubMed
37. Wang L, Zhou X, Zou W, et al. Exosomes containing miRNAs targeting HER2 synthesis and engineered to adhere to HER2 on tumor cells surface exhibit enhanced antitumor activity. *J Nanobiotechnology* 2020;18:153. DOI PubMed PMC
38. Forterre AV, Wang JH, Delcayre A, et al. Extracellular vesicle-mediated. *in vitro* ;19:858-67. DOI PubMed PMC
39. Kooijmans SAA, Fliervoet LAL, van der Meel R, et al. PEGylated and targeted extracellular vesicles display enhanced cell specificity and circulation time. *J Control Release* 2016;224:77-85. DOI PubMed
40. Kooijmans SAA, Gitz-Francois JJJM, Schiffelers RM, Vader P. Recombinant phosphatidylserine-binding nanobodies for targeting of extracellular vesicles to tumor cells: a plug-and-play approach. *Nanoscale* 2018;10:2413-26. DOI PubMed PMC
41. Wang JH, Forterre AV, Zhao J, et al. Anti-HER2 scFv-Directed extracellular vesicle-mediated mRNA-based gene delivery inhibits growth of HER2-positive human breast tumor xenografts by prodrug activation. *Mol Cancer Ther* 2018;17:1133-42. DOI PubMed PMC
42. Li C, Tang Z, Hu Z, et al. Natural single-domain antibody-nanobody: a novel concept in the antibody field. *J Biomed Nanotechnol* 2018;14:1-19. DOI PubMed
43. Pham TC, Jayasinghe MK, Pham TT, et al. Covalent conjugation of extracellular vesicles with peptides and nanobodies for targeted therapeutic delivery. *J Extracell Vesicles* 2021;10:e12057. DOI PubMed PMC
44. Sato YT, Umezaki K, Sawada S, et al. Engineering hybrid exosomes by membrane fusion with liposomes. *Sci Rep* 2016;6:21933. DOI PubMed PMC
45. Barok M, Puhka M, Vereb G, Szollosi J, Isola J, Joensuu H. Cancer-derived exosomes from HER2-positive cancer cells carry trastuzumab-emtansine into cancer cells leading to growth inhibition and caspase activation. *BMC Cancer* 2018;18:504. DOI PubMed PMC
46. Nahta R, Yu D, Hung MC, Hortobagyi GN, Esteva FJ. Mechanisms of disease: understanding resistance to HER2-targeted therapy in human breast cancer. *Nat Clin Pract Oncol* 2006;3:269-80. DOI PubMed
47. Wu Y, Deng W, Klinke DJ 2nd. Exosomes: improved methods to characterize their morphology, RNA content, and surface protein biomarkers. *Analyst* 2015;140:6631-42. DOI PubMed PMC
48. Staufer O, Dietrich F, Rimal R, et al. Bottom-up assembly of biomedical relevant fully synthetic extracellular vesicles. *Sci Adv* 2021;7:eabg6666. DOI PubMed PMC

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DOI: 10.20517/evcna.2022.08

Extracellular Vesicles and Circulating Nucleic Acids

Review

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Mesenchymal stem cell-derived extracellular vesicles for cell-free therapy of ocular diseases

Xiaoling Liu[1], Liang Hu[1], Fei Liu[1,2]

[1]Eye Hospital, School of Ophthalmology and Optometry, School of Biomedical Engineering, Wenzhou Medical University, Wenzhou 325000, Zhejiang, China.
[2]Wenzhou Institute, University of Chinese Academy of Science, Wenzhou 325000, Zhejiang, China.

Correspondence to: Dr. Liang Hu and Prof. Fei Liu, Eye Hospital, School of Ophthalmology and Optometry, School of Biomedical Engineering, Wenzhou Medical University, 270 West Xueyuan Road, Wenzhou 325000, Zhejiang, China. E-mail: lianghu@wmu.edu.cn; E-mail: feiliu@wmu.edu.cn

How to cite this article: Liu X, Hu L, Liu F. Mesenchymal stem cell-derived extracellular vesicles for cell-free therapy of ocular diseases. *Extracell Vesicles Circ Nucleic Acids* 2022;3:102-17. https://dx.doi.org/10.20517/evcna.2022.08

Received: 16 Feb 2022 **First Decision:** 14 Mar 2022 **Revised:** 31 Mar 2022 **Accepted:** 18 Apr 2022 **Published:** 24 Apr 2022

Academic Editors: Yoke Peng Loh, Cheng Jiang **Copy Editor:** Peng-Juan Wen **Production Editor:** Peng-Juan Wen

Abstract

Mesenchymal stem cells-derived extracellular vesicles (MSC-EVs) have noticeably attracted clinicians' attention in treating ocular diseases. As the paracrine factor of MSCs and an alternative for cell-free therapies, MSC-EVs can be conveniently dropped over the ocular surface or diffused through the retina upon intravitreal injection, without increasing the risks of cellular rejection and tumor formation. For clinical translation, a standardized and scalable production, as well as reprogramming the MSC-EVs, are highly encouraged. This review aims to assess the potential approaches for EV production and functional modification, in addition to summarizing the worldwide clinical trials initiated for various physiological systems and the specific biochemical effects of MSC-EVs on the therapy of eye diseases. Recent advances in the therapy of ocular diseases based on MSC-EVs are reviewed, and the associated challenges and prospects are discussed as well.

Keywords: Mesenchymal stem cells, extracellular vesicles, ophthalmic diseases, therapy

INTRODUCTION

Mesenchymal stem cells (MSCs) are a heterogeneous population of stromal stem cells that can be isolated from various tissues, including bone marrow, adipose tissue, umbilical cords, and even urine[1]. Recently,

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MSCs have been extensively recognized as an experimental and therapeutic tool spanning from physiological regulation to organ remodeling, due to their superiority in low antigenicity and tumorigenicity[2-3]. When the term "Mesenchymal Stem Cell" was searched on ClinicalTrials.gov, more than 1000 clinical trials could be retrieved, which were directly associated with various diseases, suggesting the great potential of the mentioned term in regenerative medicine.

The roles of MSCs may include straight differentiation into target cells to replace injured tissues and generate various bioactive substances including extracellular vesicles (EVs), especially nano-sized exosomes[4,5]. EVs are generally classified as exosomes (30-150 nm) formed inwardly during the maturation of multiple vesicle endosomes[6-7], microvesicles (50-1000 nm) directly shed from plasma membrane, and apoptotic bodies (1000-5000 nm) released by dying cells[8,9] [Figure 1A]. However, due to the overlapping size range and the lack of specific markers, current "exosome" preparations are a mixture of EVs with undefined biogenesis origin and undetermined purity. According to the MISEV2018 position paper from ISEV[10], in this review, we use the term "MSC-EVs" to describe the MSC-derived exosomal preparations. EVs are released from living cells and can be found in almost all body fluids, including blood, urine, breast milk, tears, saliva, vitreous fluid, and aqueous humor[11-14]. The top two most studied body fluids are still blood and urine observed in 143 clinical trials [www.clinicaltrials.gov (Accessed: August 2021)] [Figure 1B]. These nanoparticles carry plenty of bioactive molecules, such as proteins, lipids, RNAs [messenger RNAs (mRNAs), circular RNAs (circRNAs), small RNAs (sRNAs), long non-coding RNAs (lncRNAs)], and DNAs [genomic DNA (gDNA), complementary DNA (cDNA), and mitochondrial DNA (mDNA)][15-19] that are delivered to recipient cells mediating intercellular response. Compared with MSCs, EV-based therapeutics have shown unique advantages, including cell-free therapy, large-scale EV production, low immunogenicity, and high bioavailability, making these vesicles possible drugs in treating various diseases (e.g., eye diseases).

The human eye has a localized array of surface molecules and cytokines, and it is a sensory organ that reacts with visible light and enables us to use visual information for various purposes[20-22]. The intercellular signaling pathway is critical to maintaining the homeostasis of the intraocular microenvironment. EVs from both non-immune and immune cells play important roles in immune regulation[23]. At present, for the visual system, researchers have mainly concentrated on the application of EV-based therapeutics in a variety of ocular diseases, such as physical injuries, immune-related diseases, and other eye diseases. These nanoparticles migrate to injured or inflammatory sites, releasing genetic materials or proteins to repair damage by participating in signaling pathways[24-29]. As a new model of cell-free therapy, EVs have been evaluated in preliminary clinical trials and have shown great efficacy[30]. In addition, EV-associated products have also been applied in the treatment of ocular diseases[31,32]. With the continuous exploration of the physical and chemical properties and functions of the EVs, these naturally occurring nanoparticles have been feasibly applied to clinical medicine.

The present review aims to assess the biological characteristics of MSC-EVs and consider novel methods for EV isolation. More importantly, the translational application of MSC-EVs in eye diseases and the current challenges are discussed.

MSC-EVs

Secretion of the cells in the form of EVs was traditionally considered as unimportant waste material, cellular "garbage bags", or dust particles, while it was later found that these nano-vesicles are vital messengers and participate in diverse physiological and pathological processes, such as bone tissue regeneration[33], tumor defense[34], nerve signal transmission[35], endothelial cell migration[36], and immune tolerance[37], as

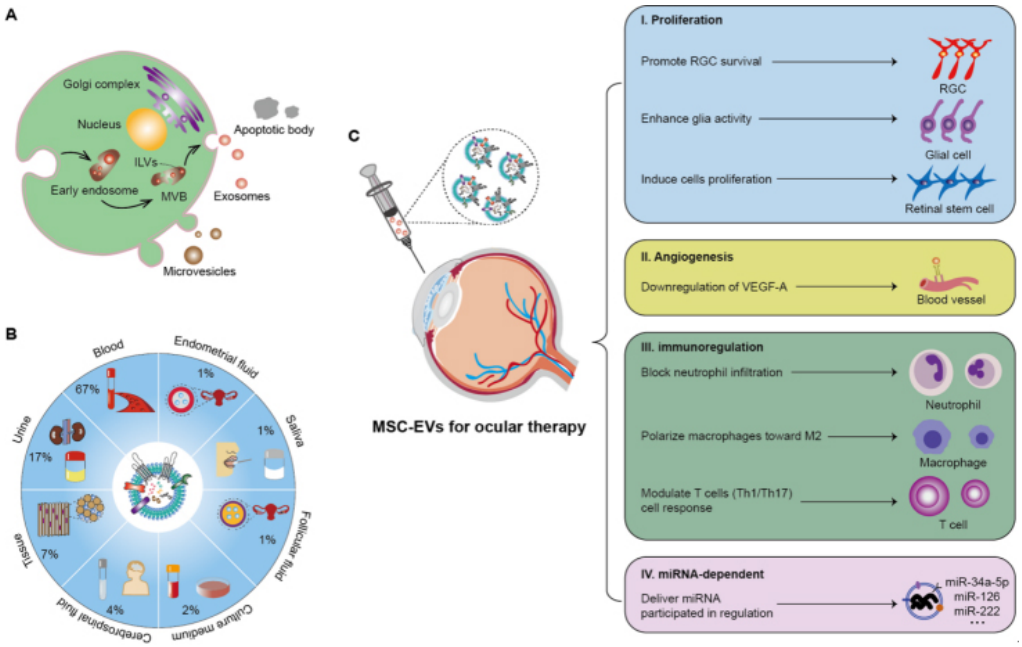

Figure 1. Illustration of biogenesis of EVs and MSC-EVs for ocular therapy. (A) EVs mainly include microvesicles, apoptotic bodies, and exosomes based on their biogenesis. (B) EV sources and the proportion of EV sources derived from 143 clinical trials [www.clinicaltrials.gov (Accessed: August 2021)]. (C) MSC-EVs, which are used for ocular therapy, are associated with the mechanisms of cell proliferation, angiogenesis, immunoregulation, and miRNA-dependent regulation. EVs: Extracellular vesicles; MSC-EVs: mesenchymal stem cells-derived EVs; ILVs: intraluminal vesicles; MVB: multivesicular body; RGC: retinal ganglion cells; VEGF-A: vascular endothelial growth factor A; M2: macrophage of type 2; T cells: thymus-dependent lymphocyte; Th cells: helper T cells; miRNA: microRNA.

summarized in Table 1. The paracrine effect of MSCs was first described by Haynesworth *et al.*[51], who reported the synthesis and secretion of various growth factors, chemokines, and cytokines by MSCs. In 2009, Bruno *et al.*[52] demonstrated that microvesicles derived from MSCs may activate a proliferative program in surviving tubular cells after injury via a horizontal transfer of mRNAs. MSC-EVs were first fractionated with a particle size of 55-65 nm by high-performance liquid chromatography. In total, 857 related proteins and 151 microRNAs (miRNAs) of MSC origin have been detected by mass spectrometry, antibody array technology, and microarray analysis[53,54]. Besides, among the functional elements of these EVs, the roles of miRNAs in EV-based therapeutics have been widely investigated[55-57]. We mainly summarize several mechanisms achieved by recent studies, including proliferation, angiogenesis, immunoregulation, and miRNA-dependent activity [Figure 1C].

Preparation and modification of EVs

The important role of EVs in the diagnosis, prognosis, and regeneration of diseases promotes the development of EV isolation techniques. As EVs are nanoparticles and originate from a complex fluid environment, obtaining homogeneous and high purity therapeutic EVs remains a great challenge. The current methods for isolating EVs are mainly based on physical (size and density) and chemical (affinity) properties, as well as immunoaffinity chromatography (combining the use of liquid chromatography with the specific binding of antibodies)[58-60]. However, the most ubiquitous adopted method for EV preparation is still dominated by ultracentrifugation (UC). Methods based on micromachining technology, due to label-free processing, cost-effectiveness, and amenability to automation, have emerged as a promising method for label-free EV separation. Inglis *et al.*[61] designed and implemented theoretical models for the critical particle size of fractionation in deterministic lateral displacement (DLD) separation arrays, aiming to provide a

Table 1. Summary of the mechanisms and applications of the MSC-derived EV therapies

Application	Disease model	EV source	Dose	Effector molecule
Tissue regeneration	Nephrectomy	UMSCs	10 μg	Proteins: ANG-1, etc[38]
	Renal ischemia reperfusion	hP-MSCs	100 μg	miRNA let-7a-5p[39]
	Calvarial bone defect	BMSCs	5×10^8 particles	Protein: BMP2[40]
Tumor defense	Metastatic lung nodules	AD-MSCs	2.32×10^9 particles	miR-101[41]
	Gastric cancer	UMSCs	64 μg	Protein: L-PGDS[42]
	LC	BMSCs	50 μg	Protein: caspase 3[43]
Nerve injury	Sciatic nerve transection	UMSCs	100 μg	Protein: IL-10, etc[44]
	Brain injury	BMSCs	200 μL (unknown)	miR-140-5p[45]
	AD	BMSCs	30 μg	miR-29c-3p[46]
Endothelial cell migration	Angiogenesis	OM-MSCs	50 μg	miR-612[47]
	Myocardial ischemia	DPSC	3.5×10^{10} particles	miR-4732-3p[48]
immunoregulation	Knee osteoarthritis	SMSC	5 μL (unknown)	miR-31[49]
	Chronic asthma	UMSCs	40 μg	miR-146a-5p[50]

EV: Extracellular vesicle; UMSCs: Umbilical cord-derived mesenchymal stem cells; ANG-1: angiopoietin-1; hP-MSCs: human placenta-derived MSCs; BMP2: bone morphogenetic protein 2; AD-MSCs: adipose tissue-derived mesenchymal stromal cells; L-PGDS: lipocalin-type prostaglandin D2 synthase; LC: lung carcinoma; AD: Alzheimer's disease; OM-MSCs: olfactory mucosa MSCs; DPSC: dental pulp-derived MSC; SMSC: synovial MSC.

theory and experimental measurements for critical conditions. Wunsch *et al.*[62] applied this technique to the true nanoscales, where they could function in EV separation, such as exosomes. This study revealed a potential for the on-chip sorting of these nanoparticles. For fast EV enrichment, a technology that integrates 1024 nanoscale DLD (nano-DLD) arrays on a single chip allows parallel processing to reach 900 μL/h[63]. Moreover, compared with other methods, including UC, UC plus density gradient, size-exclusion chromatography, and co-precipitation, the chip showed a superior efficiency. Recently, our team reported a novel exosome detection method via the ultrafast-isolation system (EXODUS) that allowed automated label-free purification of exosomes from various biofluids[64]. We also reported a size-based EV isolation tool, namely ExoTIC, to efficiently isolate EVs from small sample volumes, providing an analytical tool for preclinical studies[65]. These techniques are advantageous for the standardized preparation of MSC-EVs and can accelerate the clinical translation of MSC-EVs.

The massive production of MSC-EVs is another research hotspot for the cell-free treatment model. To expand the clinical translation of MSC-EVs, the methods used for large-scale production of EVs with a good manufacturing practice (GMP) level are necessary. To date, the efficiency of EVs has been improved by changing the culture method of donor cells, such as three-dimensional (3D) environmental culture[66-68]. Natural extracellular matrix and 3D biological scaffolds were used for cell attachment, cell growth, and production of functional EVs[69]. Using the 3D spheroid culture method based on photolithography and micro-pattern technologies, gene expression profiles of MSCs were confirmed with a high differentiation efficiency[70]. Cone *et al.*[71] assessed the potential therapeutic effects of EVs from a 3D culture of bone marrow-derived MSCs (BMSCs) in an Alzheimer's disease (AD) model, and it was revealed that intranasally administration of MSC-EVs ameliorated pathology and cognitive deficits of AD. Mend *et al.*[72] reported a bioreactor-based and clinical-grade production of engineered exosomes with the ability to target oncogenic KRAS. The clinical-grade product was tested in multiple *in vitro* and *in vivo* experiments to confirm the feasibility of various therapies for human diseases.

In addition to natural EV agents, the development of different modifications of MSC-EVs may provide new approaches for gene therapy and drug delivery. Exogenous nucleic acids, such as miRNA, siRNA, DNA carrier, and DNA probe, are loaded into EVs by electroporation, accompanied with favorable biocompatibility and biostability. For instance, the engineered MSC-EVs can serve as a promising anti-osteoporosis therapy via loading Shn3 gene-targeted siRNA[73]. Angiopep-2 (Ang) is a ligand that binds specifically to the lipoprotein receptor-related protein 1 receptor and improves the high efficiency of transport across the blood-brain barrier (BBB)[74-76]. Several scholars designed a multifunctional exosome-mimetics decorated with Ang and load docetaxel for anti-glioblastoma therapy[77]. This personalized approach also achieved the purpose of targeted therapy.

Overall, with the advances of nanomedicine in molecular cell biology, pharmaceutical science, and nano-engineering[78], higher requirements for engineering transformation of MSC-EVs are demanded, especially standardized production and storage of MSC-EVs.

EV-based therapeutics

EVs have been extensively studied in clinical trials. A statistical analysis of 143 EV-dependent clinical trials was performed, and significant conclusions were obtained, as shown in Figure 2 [www.clinicaltrials.gov (Accessed: August 2021)]. The majority of clinical studies are conducted in the United States and China, and respiratory, tumor, and gland-related diseases were research hotspots [Figure 2A and B]. Based on research purposes, we divide all studies into four groups, which are followed by diagnosis, monitoring, treatment, and mechanisms [Figure 2C]. Then, we calculate the percentages of EVs involved in 108 studies that are mainly related to exosomal RNAs and proteins [Figure 2D]. Treatment-related research accounted for 15% of all items, which are mostly derived from MSCs [Figure 2E]. As shown in Figure 2F, most clinical trials are still in the infancy stage. At present, therapeutic vesicles are widely used in cardiovascular and cerebrovascular diseases, respiratory diseases, neurological diseases, cancer, and bone regeneration by affecting cell cycle arrest or apoptosis[79-84]. As a good example of the application of EVs in bone regeneration, osteoarthritis (OA) is a joint degenerative disease characterized by synovial inflammation and articular cartilage damage. The treatment of OA mainly depends on surgery and drugs. Several studies have shown that EVs maintain homeostasis and improve the severity of osteoarthritis pathologically through local and distal intercellular and intracellular signaling pathways[84-86]. In a rat model of glucocorticoid-induced femoral head necrosis, human umbilical cord-derived MSC-EVs (UMSC-EVs) could reduce the apoptosis of bone cells through the miR-21-PTEN-AKT signaling pathway[87].

MSC-EV-BASED THERAPY FOR OPHTHALMIC DISEASES

Corneal disease

Corneal disease is the major cause of vision loss, which may be caused by several clinical conditions, including physical trauma, chemical burns, infections, limbal stem cell defects, age-related degeneration, and corneal malnutrition[26]. Although corneal transplantation has made significant progress in the past decade, there are still problems, such as few donors, immune rejection, and long-term use of immunosuppressant agents[88,89]. The role of MSCs in corneal regeneration therapy can be directly attributed to cell replacement[90] and delivering targeted biomolecules[91-94]. Several scholars attempted to incorporate hydrogel with exogenous recombinant human stromal cell-derived factor-1 alpha for corneal epithelial regeneration[95]. EVs have a promising prospect of therapeutic applications, as they inherit parental cell-derived molecules. Thus, MSC-EVs have also been applied in the therapy of corneal disease.

Many studies have confirmed the therapeutic efficiency of MSC-EVs for eye diseases including corneal and retinal models *in vitro* and *in vivo*, as presented in Table 2. Overall, tissue sources of MSCs for ocular

Table 2. Studies on the therapy of ocular diseases using MSC-EVs

Position	EV source	Administration route/dose	Results	Effector molecule
Cornea	BMSCs	Viscoelastic gel carrier/unclear	Enhance HCECs proliferation and wound healing; reduce scar formation, neovascularization, and hemorrhage	Unclear[96]
	BMSCs	Co-culture/unclear	Induce proliferation and migration of damaged HCECs; inhibit cell apoptosis	Unclear[97]
	ADSCs	Topical administration/unclear	Promote proliferation and migration of HCECs, reduce inflammatory cytokine levels, polarize infiltrating macrophages toward M2	Unclear[98]
	CSSCs	EVs drop/5.0 × 10^6 particles	Accelerate wound healing	Unclear[99]
	CSSCs	Topical fifibrin gel/1 × 10^7 particles	Decreased expression of fibrotic genes Col3a1 and Acta2, blocked neutrophil infiltration	miRNA[100]
	ADSCs	Co-culture/1.61 × 10^{10} particles	Toxicological testing	Unclear[101]
	BMSCs	Co-culture/unclear	facilitate wound healing	Unclear[102]
Retina	UMSCs	IV/2.5 μg	Inhibition of MCP-1	MCP-1[28]
	BMSCs	IV/3 × 10^9 particles	Through miRNA dependent mechanisms	miRNA[56]
	UMSCs	Tail vein/55 μg	MiR-126 expression and downregulating the HMGB1 signaling pathway	miR-126[103]
	ADSCs	IS/unclear	Delivering microRNA-222 acts as mediators in retinal tissue repair	miRNA-222[104]
	BMSCs	IV/4 × 10^9 particles	Reduce neuroinflammation and neuronal apoptosis	Unclear[105]
	BMSCs	Tail vein/30 μg	Inhibit activation of antigen-presenting cells and suppress the development of Th1 and Th17 cells	Unclear[106]
	UMSCs	IV/0.05 μg	Ameliorate retinal injury via downregulation of VEGF-A	Unclear[107]
	UMSCs	IV/1 × 10^9 particles	Promoting the RGCs survival and glia cells activation	Unclear[108]
	BMSCs	IV/1 × 10^9 particles	Preserving RGC numbers and protecting against axonal degeneration	Unclear[109]
	ES-MSCs	IO/15 μg	Improved Brn3a+ RGCs survival and improved cognitive visual behavior	Unclear[110]

MSC-EVs: Mesenchymal stem cells-derived extracellular vesicles; BMSCs: bone marrow-derived MSCs; HCECs: human corneal epithelial cells; ADSCs: adipose tissue-derived mesenchymal stem cells; CSSCs: corneal stromal stem cells; IV: intravitreal injection; UMSCs: umbilical cord-derived MSCs; IS: intravenous subconjunctival; RGCs: retinal ganglion cells; ES-MSCs: embryonic stem cell-derived MSCs; MCP-1: monocyte chemoattractant protein-1; HMGB1: high mobility group 1; IO: intravenous.

diseases mainly originate from human corneal stroma, bone marrow, umbilical cord, and adipose tissues. In-depth analysis and generalization of the mechanism of MSC-EVs for corneal disease can be summarized in the following aspects: (1) MSC-EVs enhance the proliferation of human corneal epithelial cells (HCECs) and promote the migration of HCECs after corneal disease[96-98]; (2) MSC-EVs reduce scar formation, neovascularization, and hemorrhage after corneal disease[96,97,99]; (3) MSC-derived products decrease the levels of inflammatory cytokines, such as interleukin (IL)-1β, IL-6, and IL-10[98]; (4) MSC-EV-based treatment can inhibit neutrophil infiltration and polarize M2 macrophage infiltration[98,100]; and (5) MSC-EVs depress the expression levels of fibrotic genes (Col3a1 and Acta2) and serve as a delivery vehicle for miRNA in blocking corneal scarring blocking scarring and initiating regeneration after wound healing[100].

EV-encapsulated natural lipid bilayers were considered as a good carrier to protect miRNAs from degradation. The differences in certain miRNA or miRNA expressions of EVs showed the diversity of receptor phenotypic regulation by non-coding RNA[111-113]. To elucidate the molecular mechanisms of EVs in the treatment of corneal wounds, researchers have performed numerous cutting-edge experiments. Using small-interfering RNAs (siRNAs) to knock down the mRNA of ESCRT protein Alix resulted in a reduction of 85% of EV miRNA; thus, EVs lacking miRNA lost their regeneration function[100]. The finding indicates that miRNA is a key adjustable molecule for EVs to exert restorative effects. Some studies have concentrated on the exosomal miRNA functions (i.e., regulating angiogenesis and anti-fibrotic immunosuppressive agents)[114,115], suggesting that miRNAs play an important role in maintaining homeostasis. Moreover, the

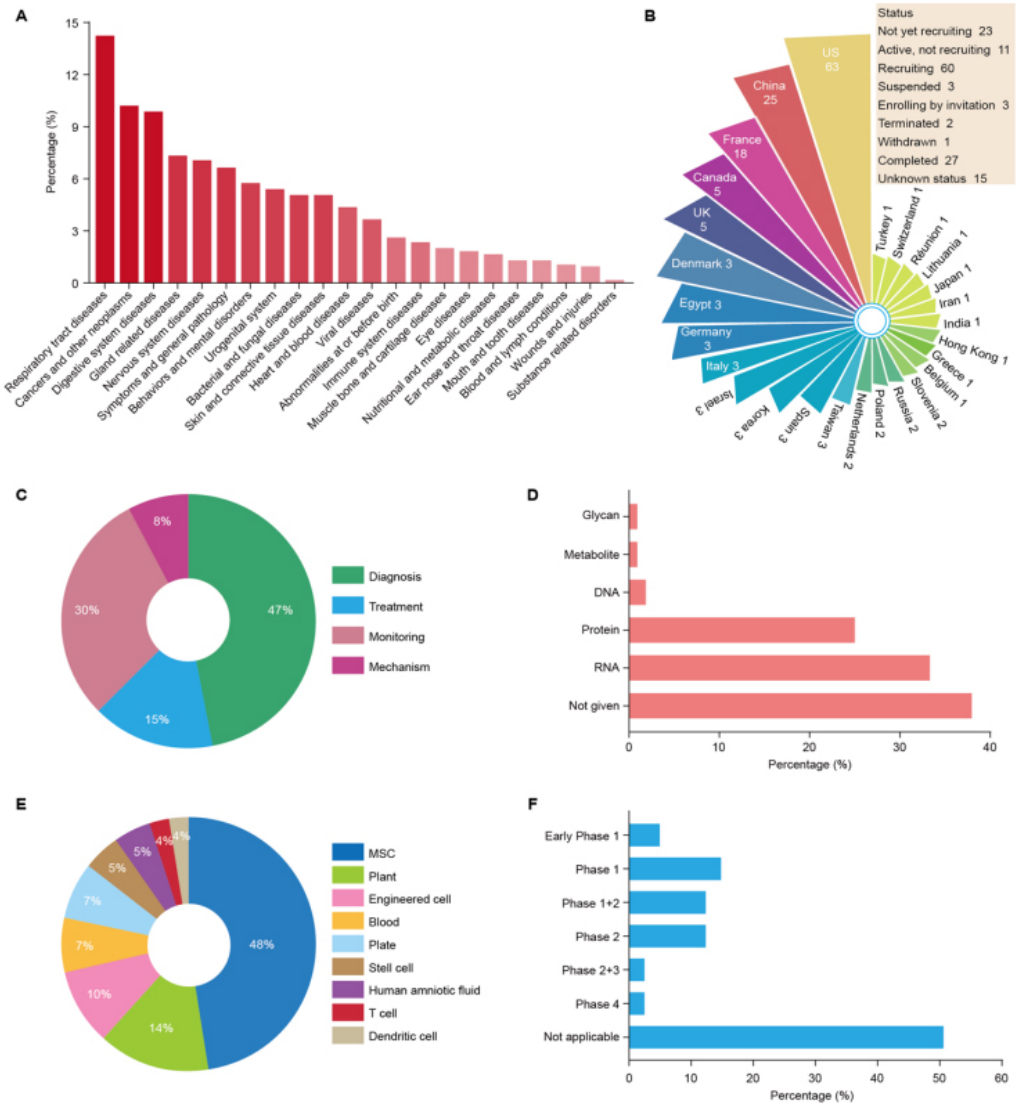

Figure 2. The progress of 143 clinical trials involving EVs worldwide [www.clinicaltrials.gov (Accessed: August 2021)]. (A) The number of studies on EVs by physiological structure. There are 22 categories according to the body structure in 143 studies. (B) The number of studies on EVs is classified according to country and project progress status. According to country classification, 27 countries participated in the 143 studies on exosomes, and there are nine research statuses in 143 studies. (C) The percentage is presented based on the research purpose. There are four different directions of EV-based research, including diagnosis, treatment, monitoring, and mechanism. (D) The contents of EVs were evaluated in 108 studies. Others were used to illustrate an unclear description. (E) The proportion of cell sources used for the treatment with EVs in 42 studies. There are nine therapeutic sources of EVs, mainly originating from MSCs. (F) According to the definition presented by the US Food and Drug Administration (FDA), the number of studies in each clinical trial stage is shown. "Not Applicable" is used to describe trials without FDA-defined phases. EVs: Extracellular vesicles; MSCs: mesenchymal stem cells.

miRNA expression profiles are variable in different cell types, which were mainly reflected in the number and category[116].

The therapeutic potential of MSCs can be related to cultivation conditions and cellular microenvironment. The influences of two-dimensional (2D) and 3D culture conditions on the therapeutic efficacy of MSC secretomes on corneal wound healing were studied with *in vitro* cell and organ culture experiments[84]. Notably, the secretomes from the MSC 3D environment facilitate wound healing in corneal fibroblast cells

and enhance epithelialization. Ha *et al.*[101] conducted a toxicological evaluation of exosomes derived from human adipose tissue-derived mesenchymal stem cells (ADSCs), and the eye irritation test suggested that ADSC exosomes are safely used as a topical treatment.

Altogether, MSC-EV-dependent therapeutic molecules can regulate intercellular signaling pathways, and engineered EVs may be an emerging agent for corneal diseases.

Retinal diseases

Retinal ganglion cell (RGC) death is the irreversible endpoint of optic neuropathy. Glaucoma is a group of progressive optic neuropathies characterized by the gradual disappearance of RGCs. Under an *in vitro* strict culture, MSCs can induce differentiation into neuroectodermal cells, including neuronal cells[117]. MSCs used for the treatment of glaucoma mainly contribute to producing neurotrophins, differentiation into functional RGCs, and interaction with TM (trabecular meshwork), thereby reducing the intraocular pressure of glaucoma[118]. The capacity of MSC-EVs for neuroprotection and immunomodulation in the treatment of retinal diseases is mainly due to miRNA-dependent and inflammatory responses[119].

Studies have proven that miR-34a-5p, miR-126, and miR-222 affect the progression of retinal damage through diverse mechanisms[103,104,120]. For instance, in a cell model of diabetic retinopathy, MSC-derived exosomal lncRNA SNHG7 suppresses endothelial-mesenchymal transition and tube formation by negatively regulating miRNA[120]. miR-126 has been reported as an endothelial cell-restricted miRNA that mediates inflammation and vascular development[103]. HMGB1, one of the target genes of miR-126, has high expression levels in various inflammatory and autoimmune diseases[121]. Co-culture of MSC-EVs with high expressions of miR-126 and human retinal endothelial cells was found to significantly reduce the level of HMGB1 protein and improve retinal inflammation caused by hyperglycemia in diabetic rats[103]. A previous study showed that MSC-EVs are endocytosed by retinal neurons, retinal ganglion cells, and microglia as biomaterials for neuroprotective and regenerative therapy of retinal disorders[105]. Moreover, in a clinical trial, Zhang *et al.*[122] also proved that MSC-EV therapy may be an advantageous and safe method for improving visual outcomes after surgery for refractory macular holes.

With the advent of induced pluripotent stem cells (iPSCs), tremendous progress has been made in stem cell biology and regenerative medicine. Human iPSCs are widely used in animal modeling, drug discovery, and cell therapy development[123]. Besides MSC-EV-based therapies, iPSCs can also be used as an unlimited source for retinal degenerative diseases[124]. Retinal pigment epithelial cells derived from iPS (iPS-RPE) replace damaged or diseased cells and promote the healing and repairing of eye tissues[125]. Studies have shown that iPS-RPE plays an effective role in delaying photoreceptor degeneration by stably surviving in a degraded ocular environment and releasing neuroprotective factors, such as the pigment epithelium-derived factor[126]. To obtain an adequate source of cells, Reichman *et al.*[127] developed a two-step culture system to effectively differentiate iPSCs into retinal cells and achieved large-scale production and storage of hiPSCs-derived retinal cells and tissues. The development of iPSCs is expected to be another novel approach to treat retinal diseases in the future.

Other ocular diseases

The immunoregulatory effects of MSC-EVs have been reported in a variety of experimental models, such as rheumatoid arthritis[128], neurodegenerative disorders[129], and inflammatory bowel disease[130]. Studies have demonstrated that the anti-inflammatory effect of MSC-EVs is closely associated with regulating the activity of macrophages[131-133], natural killer cells[134], B cells[135,136], and T cells[137,138]. Scholars also used this positive influence in the modeling of inflammatory-related eye diseases. Uveitis, an inflammatory disorder involving the pigmented vascular coat of the eyeball, can result in blindness in the absence of timely therapy. Similar

to inflammatory eye disease, MSC-EVs suppress autoimmunity in models of experimental autoimmune uveoretinitis (EAU) by inhibiting the development of T cells[106]. Administering MSC into rodents with induced models of clinical diseases with an appropriate dose can result in the reversal of abnormalities for weeks thereafter. Zhang *et al.*[139] examined the long-term effects of BMSCs in a recurrent EAU model in rats. The results demonstrate that BMSCs significantly decreased responses of T helper 1 (Th1) and Th17 cells, suppressed the functions of antigen-presenting cells, and upregulated T regulatory cells. In the study of EAU in Lewis rats, MSCs showed an inhibitory effect on activation and maturation of dendritic cells via regulation of STAT1 and STAT6 phosphorylation[140]. In the subsequent studies using MSC-EVs on the same EAU models, it was found that administration of MSC-EVs could ameliorate uveitis similar to their parent cells[141]. Using *in vitro* experiments, the effects of MSC-EVs on immune-cell activation were assessed using allogeneic mixed lymphocyte reaction assays. Consistent with previous MSC-related findings, MSC-EVs simultaneously reduce the infiltration of T cells and the levels of inflammatory cytokines[106].

Sjögren's syndrome (SS), a chronic multi-system autoimmune disease mainly involving the exocrine gland, causes dry mouth (hyposialia or even asialia) and dry eye (xerophthalmia)[142,143]. MSCs, as a therapeutic approach to treat SS, have been assessed in preclinical trials[144,145]. In an *in vivo* study, Xu *et al.*[146] proposed a novel therapeutic approach to alleviate diseases in patients with primary SS by infusing allogeneic UMSCs. These effects are nutritive, anti-inflammatory, anti-immunologic, and associated with the healing of abnormalities. Regarding EVs secreted by MSCs, MSC-EVs may be an ideal replacement for decreasing the pathogenesis of SS. Rui *et al.*[147] found that murine olfactory ecto-MSC-derived exosomes significantly improved impaired immunosuppressive function of myeloid-derived suppressor cells by administering MSC-EVs intravenously into mice with induced models of SS. Considering the limited expandability, significant donor variations, and safety concerns of MSC sources, it is essential to optimize a protocol that can be easily scaled up to produce standardized iPSC-MSCs, showing the same potential to prevent the progression of SS[148].

Taken together, a combination of MSC and MSC-EVs with emerging technologies may provide novel insight for into the therapy of eye diseases.

CHALLENGES AND PROSPECTS

Although MSC-EVs are regarded as a new treatment strategy, their affiliated clinical challenges are worthy of further assessment.

Firstly, obtaining an appropriate cell line is a prerequisite for collecting EVs. The existing research on eye diseases is summarized in Table 2. Therapeutic vesicles are mainly sourced from cells of adipose, bone marrow, umbilical cord, and cornea. However, there is a lack of research comparing MSCs from various sources for the treatment of eye diseases. The impurity of MSCs leads to the complexity of the contents of EVs, negatively influencing their performance. Therefore, to obtain high-quality products, the tissue source, identification, and functional testing of MSCs are required. Despite that, the mechanism of MSC-EVs for other diseases has been studied in detail [Table 1], EV contents such as protein and genetic cargo for the therapy of eye diseases remain unknown. Most studies still observe the curative effect by injecting intact MSC-EVs into model animals [Table 2]. Above all, the personalized design of EVs is also essential; to date, based on the pathogenesis and pathological process, various genetic or non-genetic engineering methods have been developed for producing EVs with specific biological characteristics[148-152]. To achieve precision treatment, it is necessary to master the knowledge of EVs related to the composition, identification, purification, and function of distinct cell origins and under various physiological statuses.

Another question is how to achieve a standardized and stable production of EVs as drugs with a GMP level. Studies showed that the heterogeneity of EVs derives from their size, contents, and cell origin[153]. Proteomics analysis of EVs revealed the heterogeneity of protein profiles, suggesting that there is an urgent need to optimize and standardize the purification method to obtain high-quality EVs[154]. Despite the emergence of many novel EV preparation techniques, a consensus on manufacturing the therapeutic vesicles from cell culture needs to be reached. Referring to the recent ISEV workshop position papers, there are some issues that should be considered[155]. First, the maximum cell death rate must be less than 5% to prevent dead cells from releasing particles unrelated to the therapeutic purpose that affect EV purity. Secondly, the detection of cellular microbial contamination such as mycoplasma and viruses must be performed to meet the requirements of standard level for clinical use. In addition, the standardized protocol of MSC-EV preparation from cultured cell-conditioned-medium should be automated, timesaving, and have a high recovery efficiency. Some operational considerations need to be noted, for example, avoiding repeated freeze–thaw samples and ensuring temperature control during EV separation to prevent the destruction of functional molecules in vesicles. The EXODUS platform is a promising tool that highly satisfies all demands for the collection of EVs in a large-volume culture medium[64]. Finally, the quality of the MSC-EV preparation should be evaluated by the size, morphology, specific markers, and detection of contaminants. The scientific storage and transportation conditions of MSC-EVs are important to ensure the efficacy.

Given the security of EVs, the mechanism-dependent and safety data of MSC-EVs mainly originate from preclinical *in vitro* and animal research. However, it is important to indicate whether the results of the application of MSC-EVs in animal experiments can be reliably used in human clinical trials. This relies on conducting a large number of clinical trials. For the treatment of eye diseases, the administration routes of EVs mainly involve injection, eye drops, and dressing. MSC-EVs serve as an ideal source for drug delivery, regardless of encapsulating in biomaterials or dissolving in liquid[156], maintaining the biological activity.

CONCLUSIONS

The main limitation of MSC therapy for optic neuritis is the difficulty of reaching the site of pathology in the optic nerve and retina. In the view that EVs can cross the BBB, while MSCs cannot, and deliver various therapeutic factors to the brain, MSC-EVs have been extensively tested as a beneficial treatment for the control of chronic inflammation of the central nervous system. Adequate EVs can be produced by large-scale expanding parental cells. For precision medicine, engineering and modification of EVs can improve the targeted drug delivery efficiency by overexpressing therapeutic molecules, such as miRNAs. Although additional advanced research is still required to explore the mechanism of EVs in the therapy of various eye diseases, it is undeniable that MSC-EVs have promising prospects in ocular repairing.

DECLARATIONS

Authors' contributions

Conceptualized the manuscript: Liu F, Hu L
Wrote the first draft of the manuscript: Liu X
Supplied technical knowledge to support the manuscript throughout the revision process: Liu F, Hu L
Drew figures: Liu X
Contributed to the manuscript revision: Liu F, Hu L
All the authors read and approved the submitted version of the manuscript

Availability of data and materials
Not applicable.

Financial support and sponsorship
The work was supported by the research funds provided by the National Natural Science Foundation of China (21904098), the Zhejiang Provincial and Ministry of Health Research Fund for Medical Sciences (WKJ-ZJ-1910), the Wenzhou Basic Research Project (Y2020202), and the Wenzhou Institute of Biomaterials & Engineering (WIBEZD2017006-05).

Conflicts of interest
All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

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REFERENCES
1. Tsiapalis D, O'Driscoll L. Mesenchymal stem cell derived extracellular vesicles for tissue engineering and regenerative medicine applications. *Cells* 2020;9:991. DOI PubMed PMC
2. Hsu TW, Lu YJ, Lin YJ, et al. Transplantation of 3D MSC/HUVEC spheroids with neuroprotective and proangiogenic potentials ameliorates ischemic stroke brain injury. *Biomaterials* 2021;272:120765. DOI PubMed
3. Regmi S, Seo Y, Ahn JS, et al. Heterospheroid formation improves therapeutic efficacy of mesenchymal stem cells in murine colitis through immunomodulation and epithelial regeneration. *Biomaterials* 2021;271:120752. DOI PubMed
4. Cocucci E, Meldolesi J. Ectosomes and exosomes: shedding the confusion between extracellular vesicles. *Trends Cell Biol* 2015;25:364-72. DOI PubMed
5. Uccelli A, Moretta L, Pistoia V. Mesenchymal stem cells in health and disease. *Nat Rev Immunol* 2008;8:726-36. DOI PubMed
6. Rani S, Ritter T. The exosome-A naturally secreted nanoparticle and its application to wound healing. *Adv Mater* 2016;28:5542-52. DOI PubMed
7. Niel G, D'Angelo G, Raposo G. Shedding light on the cell biology of extracellular vesicles. *Nat Rev Mol Cell Biol* 2018;19:213-28. DOI PubMed
8. Boriachek K, Islam MN, Möller A, et al. Biological functions and current advances in isolation and detection strategies for exosome nanovesicles. *Small* 2018;14:1702153. DOI PubMed
9. Clayton A, Boilard E, Buzas EI, et al. Considerations towards a roadmap for collection, handling and storage of blood extracellular vesicles. *J Extracell Vesicles* 2019;8:1647027. DOI PubMed PMC
10. Théry C, Witwer KW, Aikawa E, et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J Extracell Vesicles* 2018;7:1535750. DOI PubMed PMC
11. Chen C, Zong S, Liu Y, et al. Profiling of exosomal biomarkers for accurate cancer identification: combining DNA-PAINT with machine- learning-based classification. *Small* 2019;15:e1901014. DOI PubMed
12. Huang T, Song C, Zheng L, Xia L, Li Y, Zhou Y. The roles of extracellular vesicles in gastric cancer development, microenvironment, anti-cancer drug resistance, and therapy. *Mol Cancer* 2019;18:62. DOI PubMed PMC
13. Zhao Y, Weber SR, Lease J, et al. Liquid biopsy of vitreous reveals an abundant vesicle population consistent with the size and morphology of exosomes. *Transl Vis Sci Technol* 2018;7:6. DOI PubMed PMC
14. Li S, Li Y, Chen B, et al. ExoRBase: a database of circRNA, lncRNA and mRNA in human blood exosomes. *Nucleic Acids Res* 2018;46:D106-12. DOI PubMed PMC
15. Thakur BK, Zhang H, Becker A, et al. Double-stranded DNA in exosomes: a novel biomarker in cancer detection. *Cell Res* 2014;24:766-9. DOI PubMed PMC
16. Skotland T, Sandvig K, Llorente A. Lipids in exosomes: current knowledge and the way forward. *Prog Lipid Res* 2017;66:30-41. DOI PubMed

17. Colombo M, Raposo G, Théry C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annu Rev Cell Dev Biol* 2014;30:255-89. DOI PubMed
18. Barile L, Moccetti T, Marbán E, Vassalli G. Roles of exosomes in cardioprotection. *Eur Heart J* 2017;38:1372-9. DOI PubMed
19. Hori J, Yamaguchi T, Keino H, Hamrah P, Maruyama K. Immune privilege in corneal transplantation. *Prog Retin Eye Res* 2019;72:100758. DOI PubMed
20. London A, Benhar I, Schwartz M. The retina as a window to the brain-from eye research to CNS disorders. *Nat Rev Neurol* 2013;9:44-53. DOI PubMed
21. Perez VL, Caspi RR. Immune mechanisms in inflammatory and degenerative eye disease. *Trends Immunol* 2015;36:354-63. DOI PubMed PMC
22. Stein-Streilein J. Immune regulation and the eye. *Trends Immunol* 2008;29:548-54. DOI PubMed
23. Robbins PD, Morelli AE. Regulation of immune responses by extracellular vesicles. *Nat Rev Immunol* 2014;14:195-208. DOI PubMed PMC
24. Xu H, Jia S, Xu H. Potential therapeutic applications of exosomes in different autoimmune diseases. *Clin Immunol* 2019;205:116-24. DOI PubMed
25. Mansoor H, Ong HS, Riau AK, Stanzel TP, Mehta JS, Yam GH. Current trends and future perspective of mesenchymal stem cells and exosomes in corneal diseases. *Int J Mol Sci* 2019;20:2853. DOI PubMed PMC
26. Klingeborn M, Dismuke WM, Bowes Rickman C, Stamer WD. Roles of exosomes in the normal and diseased eye. *Prog Retin Eye Res* 2017;59:158-77. DOI PubMed PMC
27. Liu S, Wang W, Ning Y, et al. Exosome-mediated miR-7-5p delivery enhances the anticancer effect of Everolimus via blocking MNK/eIF4E axis in non-small cell lung cancer. *Cell Death Dis* 2022;13:129. DOI PubMed PMC
28. Yu B, Shao H, Su C, et al. Exosomes derived from MSCs ameliorate retinal laser injury partially by inhibition of MCP-1. *Sci Rep* 2016;6:34562. DOI PubMed PMC
29. Batsali AK, Georgopoulou A, Mavroudi I, Matheakakis A, Pontikoglou CG, Papadaki HA. The role of bone marrow mesenchymal stem cell derived extracellular vesicles (MSC-EVs) in normal and abnormal hematopoiesis and their therapeutic potential. *J Clin Med* 2020;9:856. DOI PubMed PMC
30. Phinney DG, Pittenger MF. Concise review: MSC-derived exosomes for cell-free therapy. *Stem Cells* 2017;35:851-8. DOI PubMed
31. Mead B, Tomarev S. Extracellular vesicle therapy for retinal diseases. *Prog Retin Eye Res* 2020;79:100849. DOI PubMed
32. Anderson ME, Goldhaber J, Houser SR, Puceat M, Sussman MA. Embryonic stem cell-derived cardiac myocytes are not ready for human trials. *Circ Res* 2014;115:335-8. DOI PubMed PMC
33. Xie Y, Chen Y, Zhang L, Ge W, Tang P. The roles of bone-derived exosomes and exosomal microRNAs in regulating bone remodelling. *J Cell Mol Med* 2017;21:1033-41. DOI PubMed PMC
34. Di Pace AL, Tumino N, Besi F, et al. Characterization of human NK cell-derived exosomes: role of DNAM1 receptor in exosome-mediated cytotoxicity against tumor. *Cancers (Basel)* 2020;12:661. DOI PubMed PMC
35. Counil H, Krantic S. Synaptic activity and (neuro)inflammation in Alzheimer's disease: could exosomes be an additional link? *J Alzheimers Dis* 2020;74:1029-43. DOI PubMed
36. Xia X, Zhang L, Chi J, et al. Helicobacter pylori infection impairs endothelial function through an exosome-mediated mechanism. *J Am Heart Assoc* 2020;9:e014120. DOI PubMed PMC
37. Li C, Guo F, Wang X, et al. Exosome-based targeted RNA delivery for immune tolerance induction in skin transplantation. *J Biomed Mater Res A* 2020;108:1493-500. DOI PubMed
38. Ko KW, Park SY, Lee EH, et al. Integrated bioactive scaffold with polydeoxyribonucleotide and stem-cell-derived extracellular vesicles for kidney regeneration. *ACS Nano* 2021;15:7575-85. DOI PubMed
39. Zhang C, Shang Y, Chen X, et al. Supramolecular nanofibers containing arginine-glycine-aspartate (RGD) peptides boost therapeutic efficacy of extracellular vesicles in kidney repair. *ACS Nano* 2020;14:12133-47. DOI PubMed
40. Huang CC, Kang M, Lu Y, et al. Functionally engineered extracellular vesicles improve bone regeneration. *Acta Biomater* 2020;109:182-94. DOI PubMed PMC
41. Zhang K, Dong C, Chen M, et al. Extracellular vesicle-mediated delivery of miR-101 inhibits lung metastasis in osteosarcoma. *Theranostics* 2020;10:411-25. DOI PubMed PMC
42. You B, Jin C, Zhang J, et al. MSC-derived extracellular vesicle-delivered L-PGDS inhibit gastric cancer progression by suppressing cancer cell stemness and STAT3 phosphorylation. *Stem Cells Int* 2022;2022:9668239. DOI PubMed PMC
43. Kalimuthu S, Gangadaran P, Li XJ, et al. In vivo therapeutic potential of mesenchymal stem cell-derived extracellular vesicles with optical imaging reporter in tumor mice model. *Sci Rep* 2016;6:30418. DOI PubMed PMC
44. Ma Y, Dong L, Zhou D, et al. Extracellular vesicles from human umbilical cord mesenchymal stem cells improve nerve regeneration after sciatic nerve transection in rats. *J Cell Mol Med* 2019;23:2822-35. DOI PubMed PMC
45. Qian Y, Li Q, Chen L, et al. Mesenchymal stem cell-derived extracellular vesicles alleviate M1 microglial activation in brain injury of mice with subarachnoid hemorrhage via microRNA-140-5p delivery. *Int J Neuropsychopharmacol* 2022;25:328-38. DOI PubMed
46. Sha S, Shen X, Cao Y, Qu L. Mesenchymal stem cells-derived extracellular vesicles ameliorate Alzheimer's disease in rat models via the microRNA-29c-3p/BACE1 axis and the Wnt/β-catenin pathway. *Aging (Albany NY)* 2021;13:15285-306. DOI PubMed PMC
47. Ge L, Xun C, Li W, et al. Extracellular vesicles derived from hypoxia-preconditioned olfactory mucosa mesenchymal stem cells

enhance angiogenesis via miR-612. *J Nanobiotechnology* 2021;19:380. DOI PubMed PMC
48. Sánchez-Sánchez R, Gómez-Ferrer M, Reinal I, et al. MiR-4732-3p in extracellular vesicles from mesenchymal stromal cells is cardioprotective during myocardial ischemia. *Front Cell Dev Biol* 2021;9:734143. DOI PubMed PMC
49. Wang K, Li F, Yuan Y, et al. Synovial mesenchymal stem cell-derived EV-packaged miR-31 downregulates histone demethylase KDM2A to prevent knee osteoarthritis. *Mol Ther Nucleic Acids* 2020;22:1078-91. DOI PubMed PMC
50. Dong L, Wang Y, Zheng T, et al. Hypoxic hUCMSC-derived extracellular vesicles attenuate allergic airway inflammation and airway remodeling in chronic asthma mice. *Stem Cell Res Ther* 2021;12:4. DOI PubMed PMC
51. Haynesworth SE, Baber MA, Caplan AI. Cytokine expression by human marrow-derived mesenchymal progenitor cells in vitro: effects of dexamethasone and IL-1α. *J Cell Physiol* 1996;166:585-92. DOI
52. Bruno S, Grange C, Deregibus MC, et al. Mesenchymal stem cell-derived microvesicles protect against acute tubular injury. *J Am Soc Nephrol* 2009;20:1053-67. DOI PubMed PMC
53. Lai RC, Tan SS, Teh BJ, et al. Proteolytic potential of the MSC exosome proteome: implications for an exosome-mediated delivery of therapeutic proteasome. *Int J Proteomics* 2012;2012:971907. DOI PubMed PMC
54. Chen TS, Lai RC, Lee MM, Choo AB, Lee CN, Lim SK. Mesenchymal stem cell secretes microparticles enriched in pre-microRNAs. *Nucleic Acids Res* 2010;38:215-24. DOI PubMed PMC
55. Yu T, Zhao C, Hou S, Zhou W, Wang B, Chen Y. Exosomes secreted from miRNA-29b-modified mesenchymal stem cells repaired spinal cord injury in rats. *Braz J Med Biol Res* 2019;52:e8735. DOI PubMed PMC
56. Borderud SP, Li Y, Burkhalter JE, Sheffer CE, Ostroff JS. Electronic cigarette use among patients with cancer: characteristics of electronic cigarette users and their smoking cessation outcomes. *Cancer* 2014;120:3527-35. DOI PubMed PMC
57. Xie K, Liu L, Chen J, Liu F. Exosomal miR-1246 derived from human umbilical cord blood mesenchymal stem cells attenuates hepatic ischemia reperfusion injury by modulating T helper 17/regulatory T balance. *IUBMB Life* 2019;71:2020-30. DOI PubMed
58. Lee K, Shao H, Weissleder R, Lee H. Acoustic purification of extracellular microvesicles. *ACS Nano* 2015;9:2321-7. DOI PubMed PMC
59. Zhang H, Lyden D. Asymmetric-flow field-flow fractionation technology for exomere and small extracellular vesicle separation and characterization. *Nat Protoc* 2019;14:1027-53. DOI PubMed PMC
60. Lim J, Choi M, Lee H, et al. Direct isolation and characterization of circulating exosomes from biological samples using magnetic nanowires. *J Nanobiotechnology* 2019;17:1. DOI PubMed PMC
61. Inglis DW, Davis JA, Austin RH, Sturm JC. Critical particle size for fractionation by deterministic lateral displacement. *Lab Chip* 2006;6:655-8. DOI PubMed
62. Wunsch BH, Smith JT, Gifford SM, et al. Nanoscale lateral displacement arrays for the separation of exosomes and colloids down to 20 nm. *Nat Nanotechnol* 2016;11:936-40. DOI PubMed
63. Smith JT, Wunsch BH, Dogra N, et al. Integrated nanoscale deterministic lateral displacement arrays for separation of extracellular vesicles from clinically-relevant volumes of biological samples. *Lab Chip* 2018;18:3913-25. DOI PubMed
64. Chen Y, Zhu Q, Cheng L, et al. Exosome detection via the ultrafast-isolation system: EXODUS. *Nat Methods* 2021;18:212-8. DOI PubMed
65. Liu F, Vermesh O, Mani V, et al. The exosome total isolation chip. *ACS Nano* 2017;11:10712-23. DOI PubMed PMC
66. Panchalingam KM, Jung S, Rosenberg L, Behie LA. Bioprocessing strategies for the large-scale production of human mesenchymal stem cells: a review. *Stem Cell Res Ther* 2015;6:225. DOI PubMed PMC
67. Petry F, Smith JR, Leber J, Salzig D, Czermak P, Weiss ML. Manufacturing of human umbilical cord mesenchymal stromal cells on microcarriers in a dynamic system for clinical use. *Stem Cells Int* 2016;2016:4834616. DOI PubMed PMC
68. Rafiq QA, Coopman K, Nienow AW, Hewitt CJ. Systematic microcarrier screening and agitated culture conditions improves human mesenchymal stem cell yield in bioreactors. *Biotechnol J* 2016;11:473-86. DOI PubMed PMC
69. Huleihel L, Hussey GS, Naranjo JD, et al. Matrix-bound nanovesicles within ECM bioscaffolds. *Sci Adv* 2016;2:e1600502. DOI PubMed PMC
70. Wang W, Itaka K, Ohba S, et al. 3D spheroid culture system on micropatterned substrates for improved differentiation efficiency of multipotent mesenchymal stem cells. *Biomaterials* 2009;30:2705-15. DOI PubMed
71. Cone AS, Yuan X, Sun L, et al. Mesenchymal stem cell-derived extracellular vesicles ameliorate Alzheimer's disease-like phenotypes in a preclinical mouse model. *Theranostics* 2021;11:8129-42. DOI PubMed PMC
72. Mendt M, Kamerkar S, Sugimoto H, et al. Generation and testing of clinical-grade exosomes for pancreatic cancer. *JCI Insight* 2018;3:99263. DOI PubMed PMC
73. Cui Y, Guo Y, Kong L, et al. A bone-targeted engineered exosome platform delivering siRNA to treat osteoporosis. *Bioact Mater* 2022;10:207-21. DOI PubMed PMC
74. Song P, Song N, Li L, Wu M, Lu Z, Zhao X. Angiopep-2-modified carboxymethyl chitosan-based pH/reduction dual-stimuli-responsive nanogels for enhanced targeting glioblastoma. *Biomacromolecules* 2021;22:2921-34. DOI PubMed
75. Xu H, Li C, Wei Y, et al. Angiopep-2-modified calcium arsenite-loaded liposomes for targeted and pH-responsive delivery for anti-glioma therapy. *Biochem Biophys Res Commun* 2021;551:14-20. DOI PubMed
76. Liu X, Wu R, Li Y, et al. Angiopep-2-functionalized nanoparticles enhance transport of protein drugs across intestinal epithelia by self-regulation of targeted receptors. *Biomater Sci* 2021;9:2903-16. DOI PubMed
77. Wu JY, Li YJ, Wang J, et al. Multifunctional exosome-mimetics for targeted anti-glioblastoma therapy by manipulating protein

corona. *J Nanobiotechnology* 2021;19:405. DOI PubMed PMC
78. Tran PHL, Xiang D, Tran TTD, et al. Exosomes and nanoengineering: a match made for precision therapeutics. *Adv Mater* 2020;32:e1904040. DOI PubMed
79. Du W, Zhang K, Zhang S, et al. Enhanced proangiogenic potential of mesenchymal stem cell-derived exosomes stimulated by a nitric oxide releasing polymer. *Biomaterials* 2017;133:70-81. DOI PubMed
80. Willis GR, Fernandez-Gonzalez A, Anastas J, et al. Mesenchymal stromal cell exosomes ameliorate experimental bronchopulmonary dysplasia and restore lung function through macrophage immunomodulation. *Am J Respir Crit Care Med* 2018;197:104-16. DOI PubMed PMC
81. Ouyang X, Han X, Chen Z, Fang J, Huang X, Wei H. MSC-derived exosomes ameliorate erectile dysfunction by alleviation of corpus cavernosum smooth muscle apoptosis in a rat model of cavernous nerve injury. *Stem Cell Res Ther* 2018;9:246. DOI PubMed PMC
82. de Araujo Farias V, O'Valle F, Serrano-Saenz S, et al. Exosomes derived from mesenchymal stem cells enhance radiotherapy-induced cell death in tumor and metastatic tumor foci. *Mol Cancer* 2018;17:122. DOI PubMed PMC
83. Petrović A, Bogojević D, Korać A, et al. Oxidative stress-dependent contribution of HMGB1 to the interplay between apoptosis and autophagy in diabetic rat liver. *J Physiol Biochem* 2017;73:511-21. DOI PubMed
84. Jin Z, Ren J, Qi S. Human bone mesenchymal stem cells-derived exosomes overexpressing microRNA-26a-5p alleviate osteoarthritis via down-regulation of PTGS2. *Int Immunopharmacol* 2020;78:105946. DOI PubMed
85. Zhang X, Hsueh MF, Huebner JL, Kraus VB. TNF-α carried by Plasma extracellular vesicles predicts knee osteoarthritis progression. *Front Immunol* 2021;12:758386. DOI PubMed PMC
86. Ju C, Liu R, Zhang Y, et al. Exosomes may be the potential new direction of research in osteoarthritis management. *Biomed Res Int* 2019;2019:7695768. DOI PubMed PMC
87. Kuang MJ, Huang Y, Zhao XG, et al. Exosomes derived from Wharton's jelly of human umbilical cord mesenchymal stem cells reduce osteocyte apoptosis in glucocorticoid-induced osteonecrosis of the femoral head in rats via the miR-21-PTEN-AKT signalling pathway. *Int J Biol Sci* 2019;15:1861-71. DOI PubMed PMC
88. Hancox Z, Heidari Keshel S, Yousaf S, Saeinasab M, Shahbazi MA, Sefat F. The progress in corneal translational medicine. *Biomater Sci* 2020;8:6469-504. DOI PubMed
89. Qi X, Xie L, Cheng J, Zhai H, Zhou Q. Characteristics of immune rejection after allogeneic cultivated limbal epithelial transplantation. *Ophthalmology* 2013;120:931-6. DOI PubMed
90. Karp JM, Leng Teo GS. Mesenchymal stem cell homing: the devil is in the details. *Cell Stem Cell* 2009;4:206-16. DOI PubMed
91. Navas A, Magaña-Guerrero FS, Domínguez-López A, et al. Anti-inflammatory and anti-fibrotic effects of human amniotic membrane mesenchymal stem cells and their potential in corneal repair. *Stem Cells Transl Med* 2018;7:906-17. DOI PubMed PMC
92. Galindo S, Herreras JM, López-Paniagua M, et al. Therapeutic effect of human adipose tissue-derived mesenchymal stem cells in experimental corneal failure due to limbal stem cell niche damage. *Stem Cells* 2017;35:2160-74. DOI PubMed
93. Lu X, Ru Y, Chu C, et al. Lentivirus-mediated IL-10-expressing bone marrow mesenchymal stem cells promote corneal allograft survival via upregulating lncRNA 003946 in a rat model of corneal allograft rejection. *Theranostics* 2020;10:8446-67. DOI PubMed PMC
94. Yang YH, Hsieh TL, Ji AT, et al. Stromal tissue rigidity promotes mesenchymal stem cell-mediated corneal wound healing through the transforming growth factor β signaling pathway. *Stem Cells* 2016;34:2525-35. DOI PubMed
95. Tang Q, Luo C, Lu B, et al. Thermosensitive chitosan-based hydrogels releasing stromal cell derived factor-1 alpha recruit MSC for corneal epithelium regeneration. *Acta Biomater* 2017;61:101-13. DOI PubMed
96. Fernandes-Cunha GM, Na KS, Putra I, et al. Corneal wound healing effects of mesenchymal stem cell secretome delivered within a viscoelastic gel carrier. *Stem Cells Transl Med* 2019;8:478-89. DOI PubMed PMC
97. Nuzzi R, Buono L, Scalabrin S, De Iuliis M, Bussolati B. Effect of stem cell-derived extracellular vesicles on damaged human corneal endothelial cells. *Stem Cells Int* 2021;2021:6644463. DOI PubMed PMC
98. Park GW, Heo J, Kang JY, et al. Topical cell-free conditioned media harvested from adipose tissue-derived stem cells promote recovery from corneal epithelial defects caused by chemical burns. *Sci Rep* 2020;10:12448. DOI PubMed PMC
99. Samaeekia R, Rabiee B, Putra I, et al. Effect of human corneal mesenchymal stromal cell-derived exosomes on corneal epithelial wound healing. *Invest Ophthalmol Vis Sci* 2018;59:5194-200. DOI PubMed PMC
100. Shojaati G, Khandaker I, Funderburgh ML, et al. Mesenchymal stem cells reduce corneal fibrosis and inflammation via extracellular vesicle-mediated delivery of miRNA. *Stem Cells Transl Med* 2019;8:1192-201. DOI PubMed PMC
101. Ha DH, Kim SD, Lee J, et al. Toxicological evaluation of exosomes derived from human adipose tissue-derived mesenchymal stem/stromal cells. *Regul Toxicol Pharmacol* 2020;115:104686. DOI PubMed
102. Carter K, Lee HJ, Na KS, et al. Characterizing the impact of 2D and 3D culture conditions on the therapeutic effects of human mesenchymal stem cell secretome on corneal wound healing in vitro and ex vivo. *Acta Biomater* 2019;99:247-57. DOI PubMed PMC
103. Zhang W, Wang Y, Kong Y. Exosomes derived from mesenchymal stem cells modulate miR-126 to ameliorate hyperglycemia-induced retinal inflammation via targeting HMGB1. *Invest Ophthalmol Vis Sci* 2019;60:294-303. DOI PubMed
104. Safwat A, Sabry D, Ragiae A, Amer E, Mahmoud RH, Shamardan RM. Adipose mesenchymal stem cells-derived exosomes attenuate retina degeneration of streptozotocin-induced diabetes in rabbits. *J Circ Biomark* 2018;7:1849454418807827. DOI PubMed PMC
105. Mathew B, Ravindran S, Liu X, et al. Mesenchymal stem cell-derived extracellular vesicles and retinal ischemia-reperfusion.

Biomaterials 2019;197:146-60. DOI PubMed PMC
106. Shigemoto-Kuroda T, Oh JY, Kim DK, et al. MSC-derived extracellular vesicles attenuate immune responses in two autoimmune murine models: type 1 diabetes and uveoretinitis. *Stem Cell Reports* 2017;8:1214-25. DOI PubMed PMC
107. He GH, Zhang W, Ma YX, et al. Mesenchymal stem cells-derived exosomes ameliorate blue light stimulation in retinal pigment epithelium cells and retinal laser injury by VEGF-dependent mechanism. *Int J Ophthalmol* 2018;11:559-66. DOI PubMed PMC
108. Pan D, Chang X, Xu M, et al. UMSC-derived exosomes promote retinal ganglion cells survival in a rat model of optic nerve crush. *J Chem Neuroanat* 2019;96:134-9. DOI PubMed
109. Mead B, Ahmed Z, Tomarev S. Mesenchymal stem cell-derived small extracellular vesicles promote neuroprotection in a genetic DBA/2J mouse model of glaucoma. *Invest Ophthalmol Vis Sci* 2018;59:5473-80. DOI PubMed PMC
110. Seyedrazizadeh SZ, Poosti S, Nazari A, et al. Extracellular vesicles derived from human ES-MSCs protect retinal ganglion cells and preserve retinal function in a rodent model of optic nerve injury. *Stem Cell Res Ther* 2020;11:203. DOI PubMed PMC
111. Ferguson SW, Nguyen J. Exosomes as therapeutics: the implications of molecular composition and exosomal heterogeneity. *J Control Release* 2016;228:179-90. DOI PubMed
112. Chatterjee N, Rana S, Espinosa-Diez C, Anand S. MicroRNAs in cancer: challenges and opportunities in early detection, disease monitoring, and therapeutic agents. *Curr Pathobiol Rep* 2017;5:35-42. DOI PubMed PMC
113. Salehi M, Sharifi M. Exosomal miRNAs as novel cancer biomarkers: challenges and opportunities. *J Cell Physiol* 2018;233:6370-80. DOI PubMed
114. Zimta AA, Baru O, Badea M, Buduru SD, Berindan-Neagoe I. The role of angiogenesis and pro-angiogenic exosomes in regenerative dentistry. *Int J Mol Sci* 2019;20:406. DOI PubMed PMC
115. Reza-Zaldivar EE, Hernández-Sapiéns MA, Minjarez B, Gutiérrez-Mercado YK, Márquez-Aguirre AL, Canales-Aguirre AA. Potential effects of MSC-derived exosomes in neuroplasticity in Alzheimer's disease. *Front Cell Neurosci* 2018;12:317. DOI PubMed PMC
116. Ueno M, Asada K, Toda M, et al. Concomitant evaluation of a panel of exosome proteins and MiRs for qualification of cultured human corneal endothelial cells. *Invest Ophthalmol Vis Sci* 2016;57:4393-402. DOI PubMed
117. Petrenko Y, Vackova I, Kekulova K, et al. A comparative analysis of multipotent mesenchymal stromal cells derived from different sources, with a focus on neuroregenerative potential. *Sci Rep* 2020;10:4290. DOI PubMed PMC
118. Osborne A, Sanderson J, Martin KR. Neuroprotective effects of human mesenchymal stem cells and platelet-derived growth factor on human retinal ganglion cells. *Stem Cells* 2018;36:65-78. DOI PubMed PMC
119. Harrell CR, Fellabaum C, Arsenijevic A, Markovic BS, Djonov V, Volarevic V. Therapeutic potential of mesenchymal stem cells and their secretome in the treatment of glaucoma. *Stem Cells Int* 2019;2019:7869130. DOI PubMed PMC
120. Cao X, Xue LD, Di Y, Li T, Tian YJ, Song Y. MSC-derived exosomal lncRNA SNHG7 suppresses endothelial-mesenchymal transition and tube formation in diabetic retinopathy via miR-34a-5p/XBP1 axis. *Life Sci* 2021;272:119232. DOI PubMed
121. Tang ST, Wang F, Shao M, Wang Y, Zhu HQ. MicroRNA-126 suppresses inflammation in endothelial cells under hyperglycemic condition by targeting HMGB1. *Vascul Pharmacol* 2017;88:48-55. DOI PubMed
122. Zhang X, Liu J, Yu B, Ma F, Ren X, Li X. Effects of mesenchymal stem cells and their exosomes on the healing of large and refractory macular holes. *Graefes Arch Clin Exp Ophthalmol* 2018;256:2041-52. DOI PubMed
123. Shi Y, Inoue H, Wu JC, Yamanaka S. Induced pluripotent stem cell technology: a decade of progress. *Nat Rev Drug Discov* 2017;16:115-30. DOI PubMed PMC
124. Reichman S, Goureau O. Production of retinal cells from confluent human iPS cells. In: Turksen K, Nagy A, editors. Induced pluripotent stem (iPS) cells. New York: Springer; 2016. p. 339-51. DOI PubMed
125. Jin ZB, Okamoto S, Mandai M, Takahashi M. Induced pluripotent stem cells for retinal degenerative diseases: a new perspective on the challenges. *J Genet* 2009;88:417-24. DOI PubMed
126. Sun J, Mandai M, Kamao H, et al. Protective effects of human iPS-derived retinal pigmented epithelial cells in comparison with human mesenchymal stromal cells and human neural stem cells on the degenerating retina in rd1 mice. *Stem Cells* 2015;33:1543-53. DOI PubMed
127. Reichman S, Slembrouck A, Gagliardi G, et al. Generation of storable retinal organoids and retinal pigmented epithelium from adherent human iPS cells in xeno-free and feeder-free conditions. *Stem Cells* 2017;35:1176-88. DOI PubMed
128. Chang L, Kan L. Mesenchymal stem cell-originated exosomal circular RNA circFBXW7 attenuates cell proliferation, migration and inflammation of fibroblast-like synoviocytes by targeting miR-216a-3p/HDAC4 in rheumatoid arthritis. *J Inflamm Res* 2021;14:6157-71. DOI PubMed PMC
129. Riazifar M, Mohammadi MR, Pone EJ, et al. Stem cell-derived exosomes as nanotherapeutics for autoimmune and neurodegenerative disorders. *ACS Nano* 2019;13:6670-88. DOI PubMed PMC
130. Li Y, Ma K, Zhang L, Xu H, Zhang N. Human umbilical cord blood derived-mesenchymal stem cells alleviate dextran sulfate sodium-induced colitis by increasing regulatory T cells in mice. *Front Cell Dev Biol* 2020;8:604021. DOI PubMed PMC
131. Cao L, Xu H, Wang G, Liu M, Tian D, Yuan Z. Extracellular vesicles derived from bone marrow mesenchymal stem cells attenuate dextran sodium sulfate-induced ulcerative colitis by promoting M2 macrophage polarization. *Int Immunopharmacol* 2019;72:264-74. DOI PubMed
132. Hyvärinen K, Holopainen M, Skirdenko V, et al. Mesenchymal stromal cells and their extracellular vesicles enhance the anti-inflammatory phenotype of regulatory macrophages by downregulating the production of interleukin (IL)-23 and IL-22. *Front*

Immunol 2018;9:771. DOI PubMed PMC
133. Zhao H, Shang Q, Pan Z, et al. Exosomes from adipose-derived stem cells attenuate adipose inflammation and obesity through polarizing M2 macrophages and beiging in white adipose tissue. *Diabetes* 2018;67:235-47. DOI PubMed
134. Fan Y, Herr F, Vernochet A, Mennesson B, Oberlin E, Durrbach A. Human fetal liver mesenchymal stem cell-derived exosomes impair natural killer cell function. *Stem Cells Dev* 2019;28:44-55. DOI PubMed
135. Khare D, Or R, Resnick I, Barkatz C, Almogi-Hazan O, Avni B. Mesenchymal stromal cell-derived exosomes affect mRNA expression and function of B-lymphocytes. *Front Immunol* 2018;9:3053. DOI PubMed PMC
136. Adamo A, Brandi J, Caligola S, et al. Extracellular vesicles mediate mesenchymal stromal cell-dependent regulation of B cell PI3K-AKT signaling pathway and actin cytoskeleton. *Front Immunol* 2019;10:446. DOI PubMed PMC
137. Blazquez R, Sanchez-Margallo FM, de la Rosa O, et al. Immunomodulatory potential of human adipose mesenchymal stem cells derived exosomes on in vitro stimulated T cells. *Front Immunol* 2014;5:556. DOI PubMed PMC
138. Tian J, Zhu Q, Zhang Y, et al. Olfactory ecto-mesenchymal stem cell-derived exosomes ameliorate experimental colitis via modulating Th1/Th17 and treg cell responses. *Front Immunol* 2020;11:598322. DOI PubMed PMC
139. Zhang L, Zheng H, Shao H, et al. Long-term therapeutic effects of mesenchymal stem cells compared to dexamethasone on recurrent experimental autoimmune uveitis of rats. *Invest Ophthalmol Vis Sci* 2014;55:5561-71. DOI PubMed PMC
140. Dong L, Chen X, Shao H, Bai L, Li X, Zhang X. Mesenchymal stem cells inhibited dendritic cells via the regulation of STAT1 and STAT6 phosphorylation in experimental autoimmune uveitis. *Curr Mol Med* 2018;17:478-87. DOI PubMed
141. Bai L, Shao H, Wang H, et al. Author correction: effects of mesenchymal stem cell-derived exosomes on experimental autoimmune uveitis. *Sci Rep* 2018;8:9889. DOI PubMed PMC
142. Sandhya P, Kurien BT, Danda D, Scofield RH. Update on pathogenesis of Sjogren's syndrome. *Curr Rheumatol Rev* 2017;13:5-22. DOI PubMed PMC
143. Mavragani CP. Mechanisms and new strategies for primary Sjögren's syndrome. *Annu Rev Med* 2017;68:331-43. DOI PubMed
144. Yao G, Qi J, Liang J, et al. Mesenchymal stem cell transplantation alleviates experimental Sjögren's syndrome through IFN-β/IL-27 signaling axis. *Theranostics* 2019;9:8253-65. DOI PubMed PMC
145. Shi B, Qi J, Yao G, et al. Mesenchymal stem cell transplantation ameliorates Sjögren's syndrome via suppressing IL-12 production by dendritic cells. *Stem Cell Res Ther* 2018;9:308. DOI PubMed PMC
146. Xu J, Wang D, Liu D, et al. Allogeneic mesenchymal stem cell treatment alleviates experimental and clinical Sjögren syndrome. *Blood* 2012;120:3142-51. DOI PubMed PMC
147. Rui K, Hong Y, Zhu Q, et al. Olfactory ecto-mesenchymal stem cell-derived exosomes ameliorate murine Sjögren's syndrome by modulating the function of myeloid-derived suppressor cells. *Cell Mol Immunol* 2021;18:440-51. DOI PubMed PMC
148. Hai B, Shigemoto-Kuroda T, Zhao Q, Lee RH, Liu F. Inhibitory effects of iPSC-MSCs and their extracellular vesicles on the onset of sialadenitis in a mouse model of Sjögren's syndrome. *Stem Cells Int* 2018;2018:2092315. DOI PubMed PMC
149. Richter M, Vader P, Fuhrmann G. Approaches to surface engineering of extracellular vesicles. *Adv Drug Deliv Rev* 2021;173:416-26. DOI PubMed
150. Colao IL, Corteling R, Bracewell D, Wall I. Manufacturing exosomes: a promising therapeutic platform. *Trends Mol Med* 2018;24:242-56. DOI PubMed
151. Yao X, Wei W, Wang X, Chenglin L, Björklund M, Ouyang H. Stem cell derived exosomes: microRNA therapy for age-related musculoskeletal disorders. *Biomaterials* 2019;224:119492. DOI PubMed
152. Bei HP, Hung PM, Yeung HL, Wang S, Zhao X. Bone-a-petite: engineering exosomes towards bone, osteochondral, and cartilage repair. *Small* 2021;17:e2101741. DOI PubMed
153. Kalluri R, LeBleu VS. The biology, function, and biomedical applications of exosomes. *Science* 2020;367:eaau6977. DOI PubMed PMC
154. Kowal J, Arras G, Colombo M, et al. Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes. *Proc Natl Acad Sci U S A* 2016;113:E968-77. DOI PubMed PMC
155. Witwer KW, Buzás EI, Bemis LT, et al. Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. *J Extracell Vesicles* 2013;2:20360. DOI PubMed PMC
156. Lai RC, Yeo RW, Tan KH, Lim SK. Exosomes for drug delivery - a novel application for the mesenchymal stem cell. *Biotechnol Adv* 2013;31:543-51. DOI PubMed

Sun *et al. Extracell Vesicles Circ Nucleic Acids* 2022;3:118-32
DOI: 10.20517/evcna.2022.11

Extracellular Vesicles and Circulating Nucleic Acids

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Engineered induced-pluripotent stem cell derived monocyte extracellular vesicles alter inflammation in HIV humanized mice

Bing Sun[1], Scott Kitchen[2], Norina Tang[1], Andreas Garza[1], Sheela Jacob[3], Lynn Pulliam[1,4]

[1]San Francisco VA Health Care System, Department of Laboratory Medicine, San Francisco, CA 94121, USA.
[2]UCLA AIDS Institute, Division of Hematology and Oncology, David Geffen School of Medicine, UCLA, Los Angeles, CA 94121, USA.
[3]ATCC, Gaithersburg, MD 20877, USA.
[4]University of California, San Francisco, Departments of Laboratory Medicine and Medicine, San Francisco, CA 94121, USA.

Correspondence to: Prof. Lynn Pulliam, San Francisco VA Health Care System, Department of Laboratory Medicine, 4150 Clement St., San Francisco, CA 94121, USA. E-mail: lynn.pulliam@ucsf.edu

How to cite this article: Sun B, Kitchen S, Tang N, Garza A, Jacob S, Pulliam L. Engineered induced-pluripotent stem cell derived monocyte extracellular vesicles alter inflammation in HIV humanized mice. *Extracell Vesicles Circ Nucleic Acids* 2022;3:118-32. https://dx.doi.org/10.20517/evcna.2022.11

Received: 7 Mar 2022 **First Decision:** 30 Mar 2022 **Revised:** 10 Apr 2022 **Accepted:** 21 Apr 2022 **Published:** 24 Apr 2022

Academic Editors: Yoke Peng Loh, Shilpa Buch **Copy Editor:** Jia-Xin Zhang **Production Editor:** Jia-Xin Zhang

Abstract

Aim: A peripheral inflammatory response can drive neuroinflammation in a number of infections including human immunodeficiency virus (HIV). Monocyte/macrophage (M/Mφ) activation is a hallmark of acute HIV infection and a source of chronic inflammation in a subset of HIV-infected individuals. We sought to decrease peripheral inflammation and M/Mφ transmigration after HIV infection by engineering extracellular vesicles (EV) to antagonize a microRNA (miR) associated with inflammation. We hypothesized that induced pluripotent stem cell (iPSC)-derived monocyte EVs (mEVs), engineered to contain an antagomir to miR-155 (αmiR mEV) would target monocyte inflammation and influence neuroinflammation in an HIV-infected humanized mice.

Methods: mEVs were characterized by tetraspanins, nanoparticle tracking analysis, electron microscopy, and their preferential entry into circulating monocytes as well as testing for endogenous selected miRNAs. HIV-infected humanized mice were treated with control or antagomir155 mEVs. Plasma viral load was measured plus activation markers on lymphocytes and monocytes and the number of macrophages in the brain was quantified.

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Results: mEVs preferentially entered peripheral monocytes. HIV infection increased C-C chemokine receptor type 5 (CCR5) and major histocompatibility complex, class II, DR (HLA-DR) expression on T cells and monocytes. Treatments with mEVs did not decrease plasma HIV viral load; however, mEVs alone resulted in a decrease in %CCR5+ and %HLA-DR+ on T cells and an increase in %CCR5+ monocytes. αmiR mEVs decreased %CCR5 on M/Mφ. The mEV-treated HIV-infected mice did not show an increase in macrophage transmigration to the brain.

Conclusion: mEVs alone caused an unexpected decrease in lymphocyte activation and increase in monocyte %CCR5; however, this did not translate to an increase in macrophage transmigration to the brain.

Keywords: Monocyte, extracellular vesicles, pluripotent stem cells, miRNAs, HIV, bone marrow-liver-thymic (BLT) mouse, inflammation, neuroinflammation

INTRODUCTION

Neurocognitive impairment associated with human immunodeficiency virus (HIV) infection is initially driven by peripheral inflammation. Activated monocytes produce cytokines and monocyte-derived macrophages (M/Mφ) transmigrate into the brain, seeding the brain with HIV and contributing to neuroinflammation. Quenching peripheral inflammation with antiretroviral therapy (ART) for most HIV-infected individuals decreases impairment except for a few that have lingering chronic inflammation that perpetuates neuroinflammation and potential cognitive impairment. While ART greatly contributes to lowering the viral load to undetectable and, in most individuals decreasing chronic inflammation, cognitive impairment continues for some.

Targeting chronic inflammation remains an active area of research for many diseases including HIV. New therapeutic agents that could suppress the immune system without immunosuppression, thereby exposing someone to increased susceptibility to infection, are needed. While targeting the virus with antiretrovirals is standard care, targeting inflammation as adjunct therapy may decrease susceptible cells as well as dampen the deleterious effects of inflammation. Targeting a specific pro-inflammatory cytokine has not had widespread acceptability (Review Ref.[1,2]), although an interferon inhibitor showed promise to restore immune function in humanized HIV-infected mice[3]. Newer approaches to modulating the immune system include using noncoding microRNAs (miRs). miRNAs transcriptionally control gene expression for many targets. Our previous studies identified miRs associated with monocytes activated by interferon alpha and lipopolysaccharide (LPS) (I/L) to mimic immune activation in HIV infection[4,5]. In those studies, miR-155 was elevated in I/L-treated monocytes and their extracellular vesicles (EVs). miR-155 is highly expressed in T and B cells and M/Mφ (Review Ref.[6]).

Based on these *in vitro* studies, we set out to determine if monocyte EVs could be engineered to reduce peripheral inflammation in the setting of HIV infection, lower viral load and decrease peripheral monocyte activation markers plus neuroinflammation as defined by M/Mφ infiltration to the brain. We used an HIV-infected humanized mouse model[7,8]. This involves the implantation of immunodeficient mice with human fetal thymus, liver and hematopoietic stem cells [termed the humanized bone marrow-liver-thymus (BLT)] mouse model[8]. This mouse has a functional human immune system that can be infected with HIV. We engineered induced pluripotent stem cell (iPSC)-derived monocyte EVs (mEVs) to overexpress antagomir-155 and used these engineered EVs to target monocytes in HIV-infected BLT mice. We found that these mEVs preferentially targeted peripheral monocytes in the mouse and that the antagomir-155 silenced miR-155; however, the mEVs did not decrease HIV viral load. mEVs alone suppressed C-C chemokine receptor type 5 (CCR5) expression on T cells and increased CCR5 on M/Mφ. In spite of an increase in CCR5 expression on monocytes, there was no increase in M/Mφ transmigration to the brain. Thus, we found that

mEV treatment had distinct immunomodulatory effects *in vivo*.

METHODS

iPSC-monocyte culture and EV collection

Human iPSC differentiated monocytes (ATCC-ACS-7030) were a gift from the American Type Culture Collection (ATCC, Manassas, VA, USA). Frozen iPSC-monocytes were thawed at 37 °C and washed and cultured in RPMI-1640 with 10% exosome-depleted fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, MA, USA). A low dose of 500 pg/mL rhMCSF (RandD Systems, Minneapolis, MN) was used to maintain monocyte viability. Cells were cultured at 37 °C with 5% CO_2 for 48 hrs. Conditioned media were collected. EVs were purified with ExoQuick-TC (System Biosciences, SBI, Palo Alto, CA, USA) as described by the manufacturer [Figure 1A]. EVs were then stored at -80 °C until use.

Characterization of mEVs by NTA, electron microscopy and tetraspanins

Nanoparticle tracking analysis (NTA) was performed on the mEV samples to determine the size and particle number. Data were generated using a NanoSight LM10 instrument (Malvern Instruments, Malvern, United Kingdom) with a 405 nm laser-equipped sample chamber as previously described[4]. Results were analyzed using NTA 3.3 software. Each sample analysis consisted of three 40 s video recordings. Mode particle sizes were reported due to the skewed distributions.

mEVs were characterized by transmission electron microscopy (TEM). In brief, eluted mEVs were fixed in 4% buffered paraformaldehyde (PFA) and deposited onto Formvar carbon-coated electron microscopy nickel grids for 5 min. The excess fluid was blotted off with #1 filter paper, and the grids were stained with saturated uranyl acetate solution (Ted Pella, Inc., USA) for 5 s. Excess fluid was then blotted off again, and the grids dried overnight. Visualization of EVs was performed using a Technai 10 transmission electron microscope (Field Electron and Ion Co. USA).

The relative amount of tetraspanin proteins CD63, CD81 and CD9 on the surface of the mEVs were evaluated using a U-PLEX human tetraspanin kit (MSD, Meso Scale Diagnostics, Rockville, MD, USA) in duplicate according to the manufacturer's instructions. Analyses were done using a QuickPlex SQ 120 instrument (MSD) and Discovery Workbench® 4.0 software.

Antisense miR-155-Cy5 and transfection

Antisense miR-155-5p (αmiR) sequence (5'-/5Cy5/ AACCC CUAUC ACGAU UAGCA UUAA-3') and Control Oligo-1 miR (cmiR) (SBI) were submitted to Integrated DNA Technologies (Redwood City, CA), synthesized and labelled with Cy5 at the 5' end. EVs were transfected with antisense miR-155 or Control Oligo-1 miRs with Exofect (SBI) following the manufacturer's instructions. Transfected EVs were purified with ExoQuick-TC (SBI) and stored at -80 °C until use [Figure 1A].

Luciferase assay

The plasmid containing 3' UTR of human E2F2 (NM_004091.3, HMIT094972-MT05) was purchased from GeneCopoeia (Rockville, MD, USA). HEK293 cells were cultured in a 96-well plate at 4×10^4/well. Exchanged media every other day until 80% confluence. Then cells were transfected with 0.1ug HMIT094972-MT05 plasmid using Lipofectamine 3000 (Thermo). Media was exchanged after 24hr. Half of a microgram of cmiR or αmiR were added to the wells. Supernatants were collected after 24 h of incubation at 37 °C. Dual luminescence assays were performed on the cell culture supernatants using Secrete-Pair Dual Luminescence Assay Kit (GeneCopoeia) per manufacturer protocol. Plates were read using a SpectraMax M5 plate reader (Molecular Devices, San Jose, CA, USA). The ratio of secreted Gaussia luciferase and secreted alkaline phosphatasse was reported.

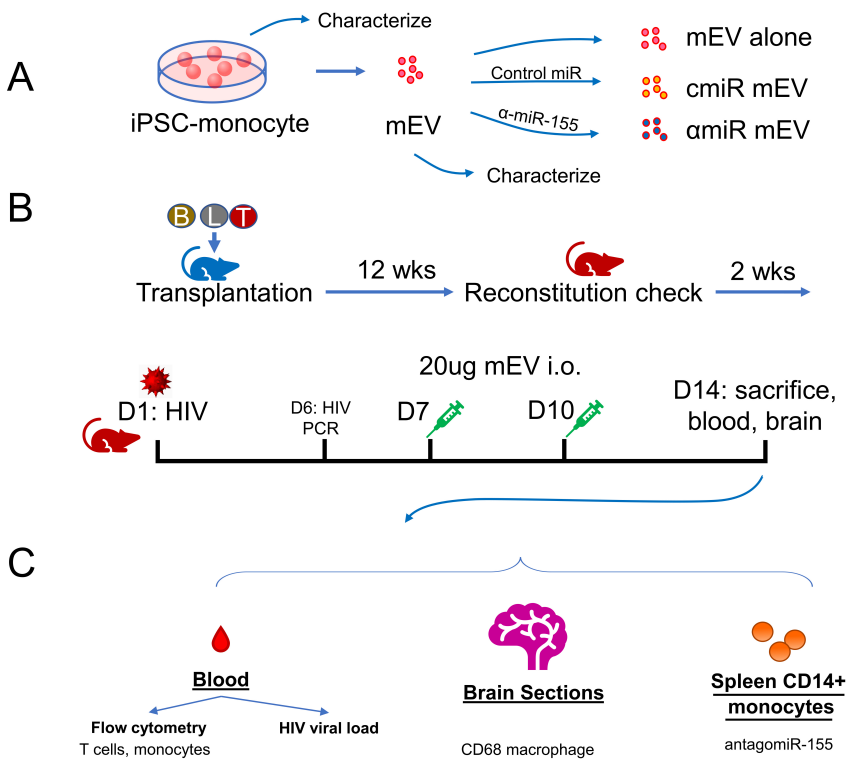

Figure 1. Experimental outline. (A) Preparation of iPSC-monocyte-derived extracellular vesicles (mEV), (B) preparation of HIV-infected Bone marrow Liver Thymic (BLT) mice, mEV treatment and (C) collection of samples. cmiR mEV: mEVs transfected with control miRNA; αmiR mEV: mEVs transfected with antagomiR-155; i.o.: intraocular injection; wks: weeks; D: day.

HIV infection of BLT mice and mEV treatment

Humanized BLT mice were constructed and housed at The University of California, Los Angeles (UCLA) Humanized Mouse Core as previously described[9]. Animal use was approved by the UCLA IACUC (Protocol ARC-2010-038). The model was validated with flow cytometry as described[10]. Briefly, human fetal CD34+ liver cells and thymus tissue were transplanted into irradiated non-obese diabetic (NOD) severe combined immunodeficient (SCID) gamma mice. Mice were monitored daily for signs of toxicity. Human immune cell engraftments were verified after 12 weeks [Figure 1B]. Thirty mice were divided into six groups: HIV-uninfected non-treated controls (HIV- NT, n = 4), HIV-uninfected antagomir-155 mEV (HIV- αmiR, n = 4), HIV-infected non-treated (HIV+ NT, n = 5), HIV-infected mEV alone (HIV+ mEV, n = 6), HIV-infected control miR mEV (HIV+ cmiR, n = 6) and HIV-infected antagomir155 mEV (HIV+ α miR, n = 5).

The mice were infected with 500 ng (approximately 6.25×10^4 infectious units) of HIV (NFN-SX, R-5 tropic) intravenously. HIV viral RNA in the peripheral blood was checked 6 days post infection (p.i.). Twenty micrograms of mEVs were injected intraocularly on day 7 and day 10 p.i. Two weeks p.i., the mice were sacrificed, and the blood was processed to evaluate HIV viral RNA, inflammatory response and the effects of EVs on the mice were analyzed. Brains were collected for immunohistochemistry staining [Figure 1B and C].

Human peripheral blood mononuclear cells isolation, mEV treatment and flow cytometry

To evaluate the propensity of mEVs to enter different cell types in the peripheral blood and particularly monocytes, we performed an mEV entrance experiment. Peripheral blood mononuclear cells (PBMCs) were

enriched from a healthy donor as previously described[11]. In an ultra-low attachment 6-well plate (Corning, Kennebunk, ME, USA), 6×10^6 PBMCs were cultured in RPMI-1640 supplemented with 10% FBS at 2×10^6 /mL. Ten μg of mEVs transfected with Cy-5 labelled antagomir-155 were added. Cells were incubated for 24 h and sampled at 2 h, 8 h and 24 h. Cells were immediately stained with CD8-FITC, CD4-PerCP-Cy5.5, CD14-PE, CD16-BV421 (all from BD, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) for 30 min at room temperature (RT). Cells were then washed and fixed in 2% PFA for 10 min at RT, followed by washing. Flow cytometry analyses were performed on a FACSAria II flow cytometer (BD) and data were analyzed with FlowJo software (BD). At least 10,000 cells were collected for each sample. Gates were set using isotype antibodies (BD).

HIV viral RNA

Blood from the HIV-infected mice was collected with EDTA anticoagulant from retro-orbital biweekly bleeding or heart puncture at sacrifice. Blood was spun at 1200 g to collect plasma supernatant. Cell-free plasma viral RNA was purified using a QIAamp Viral RNA Mini Kit (Qiagen, Germantown, MD, USA). HIV RNA was quantified by real-time reverse-transcription polymerase chain reaction (RT-PCR) using TaqMan RNA-To-Ct One-Step reagents (Thermo) with primers HIV_F: 5'-CAATG GCAGC AATTT CACCA-3' and HIV_R: 5'-GAATG CCAAA TTCCT GCTTG A-3' and a probe hybridizing to HIV1 NL4-3 HIV probe: 5'-[6FAM] CCCAC CAACA GGCGG CCTTA ACTG [Tamra-Q]-3'.

RT-PCR analysis for miRNA

Five microliters of EV solutions were lysed in 350μL Qiazol (Qiagen) and total RNA was isolated using miRNeasy Mini kit (Qiagen). Two microliters of total RNA were reversely transcribed using a Taqman MicroRNA RT kit (Thermo). Taqman assay for human miR-155-5p (Thermo) was used for RT-PCR. Taqman advanced master mix was used for PCR. Assays were performed and analyzed on a ViiA7 instrument (Thermo) in triplicate. Relative expressions to RNU6 were reported.

Antibodies and flow cytometry for mouse PBMCs

The following antibodies were used in flow cytometry: CD45-V500 (clone HI30), CD3-BV786 (clone OKT3), CD4-Vioblue (clone RPA-T4), CD8-APC780 (clone SK1), CD19-APC, CD15-PerCP-Cy5.5, CD14-ECD, CD16-AF700, CD11b-PE-Cy7, CD209-FITC, CCR5-PC5, CD163-BV605, and major histocompatibility complex, class II, DR (HLA-DR)-PE (all BD). The cells were acquired using an LSRFortessa flow cytometer and FACSDiva software (BD). Data were analyzed using FlowJo software. At least 10,000 cells were acquired for each analysis, and each representative flow plot was repeated more than three times.

Spleen monocyte isolation

Human CD14+ monocytes were sorted from splenocytes through a human CD14 microbead kit (Miltenyi, Gaithersburg, MD, USA. Cat#130-050-201). We double-checked the CD14 clone (TÜK4) in this kit, and it does not have cross-reaction with mouse species.

Immunostaining of brain sections

Mouse brains were collected and fixed in 2% paraformaldehyde. After 72 h, the tissues were washed with water and placed in 70% ethanol. Brains were embedded in paraffin and sectioned. Briefly, sections were deparaffinized and rehydrated. Antigen retrieval was performed by boiling for 30 min. Slides were treated with an endogenous peroxidase blocking solution (Vector, USA), followed by another block with 3% bovine serum albumin. Anti-CD68 rabbit monoclonal antibody (Abcam, USA, 1:500) was incubated at 4 C overnight in a humidified chamber. Anti-rabbit IgG secondary antibody was added (Vector), followed by diaminobenzidine substrate for 5 min and counterstained with hematoxylin. All slides were read blinded by

a single reader.

Statistical analysis

Data are reported as mean ± standard deviation unless otherwise specified. Wilcoxon signed-rank test was used to compare differences between two group means, analysis of variance (ANOVA) or Kruskal-Wallis rank sum test was used to compare differences between three or more group means where appropriate. Count data were compared with negative binomial regression. $P < 0.05$ was considered significant. All statistical analyses were performed with R (version 4.1.1).

RESULTS

iPSC-monocytes express CD14

iPSC-derived monocytes expressed CD14 by flow cytometry from 65.5% on day 0 to 76.9% on day 7 [Figure 2]. CD68 and CD16 were negative on day 0 and slightly increased on day 7; the majority of the cells remained CD16 and CD68 negative.

iPSC-monocyte EV characterization

NTA characterization showed the mEVs had an average mode size of 150.8 nm (standard error 19.6 nm) and a major peak at 133nm [Figure 3A]; the concentration was $1.27 \pm 0.115 \times 10^9$ particles/mL. The protein concentration was 200 μg/mL by absorbance at 280nm using a spectrophotometer measurement. Typical donut-shaped vesicles were visualized by TEM [Figure 3B]. mEVs expressed abundant CD63 ($14.4 \pm 0.17 \times 10^6$ particles/mL) with CD81 ($8.6 \pm 0.25 \times 10^6$ particles/mL) and CD9 ($7.9 \pm 0.31 \times 10^6$ particles/mL) also present [Figure 3C]. When mEVs were incubated with PBMCs, mEVs entered lymphocytes in less than 2 h followed by monocytes preferentially over time. About 84% of the monocyte population were Cy5 positive after 24 h of treatment [Figure 3D].

miRNA cargo and antagomir-155

Because the antisense sequence of miR-155 is complementary to the sense sequence, both sense and antisense miR-155 were detected by the RT-PCR assay. αmiR mEVs showed very high levels of sense / antisense miR-155 expression (5.2×10^5 fold relative to U6) compared to mEV alone (0.39 fold) and cmiR mEVs (0.19 fold) due to transfection of the antagomir-155 [Figure 4A]. The targeting of miR-155 by antagomir-155 was validated with a luciferase assay. A 58% increase in antagomir-155 treatment (31.1 ± 1.3 Gluc/SEAP ratio) compared to control miR treatment (19.7 ± 3.7 Gluc/SEAP ratio) showed that the constitutive inhibition effect from endogenous miR-155 to the downstream luciferase gene was partially reversed by antagomir-155 [Figure 4B]. To determine the entry of the αmiR mEVs into monocytes, we performed RT-PCR of miR-155 on CD14+ monocytes from the mouse spleen. The αmiR mEVs showed significant increase of miR-155 expression (1.427 ± 0.981 fold to RNU6) than HIV+ NT (0.0419 ± 0.0211, $P = 0.016$), mEV alone (0.029 ± 0.0087, $P = 0.0079$) or cmiR mEV (0.013 ± 0.0079, $P = 0.0043$) [Figure 4C].

BLT mouse construction, HIV infection and mEV treatment

After BLT mice were constructed as described previously[9], reconstitution of human blood cells was confirmed by flow cytometry after 12 weeks following transplantation [Figure 1B], and all mice showed proper reconstitution of human PBMCs [Supplementary Figure 1]. One mouse in the HIV+ αmiR group died during HIV infection and was eliminated from the group. All other mice tolerated two doses of mEV injection with no side effects. HIV viral load in the peripheral blood was measured on day 6 p.i. and after sacrifice on day 14. There was no significant difference among groups on either day 6 ($P = 0.085$) or day 14 ($P = 0.47$) [Figure 5A]. Fourteen days after HIV infection, %CCR5+ increased in CD4 T cells (7.4 ± 1.7 for HIV- *vs.* 22.5 ± 9.9 for HIV+, $P = 0.016$) and CD8 T cells (13.2 ± 3.9 for HIV- *vs.* 53.6 ± 10.0 for HIV+, $P = 0.016$). %HLA-DR+ significantly increased in CD4 T cells (7.4 ± 1.7 for HIV- *vs.* 22.5 ± 9.9 for HIV+, $P =$

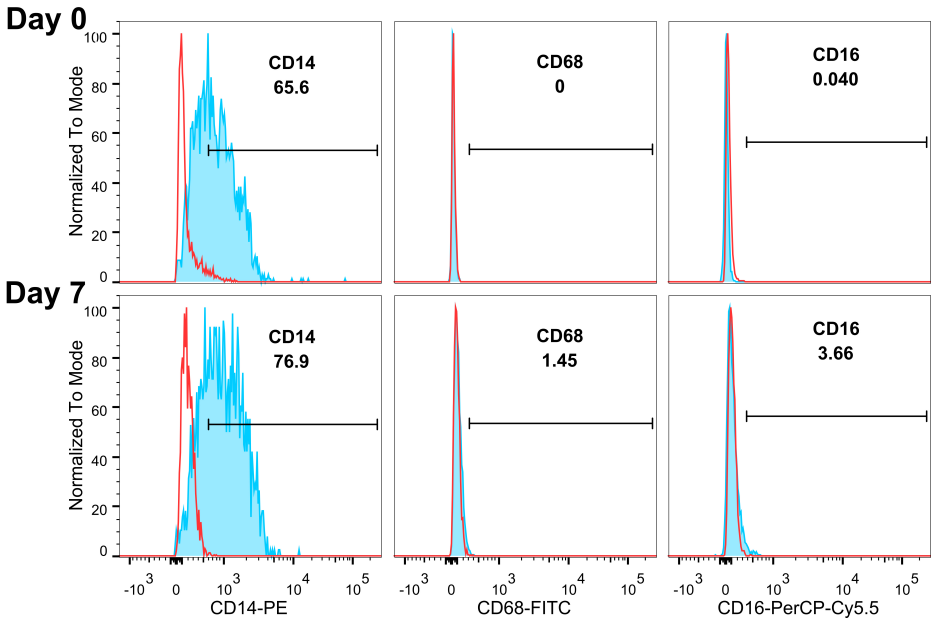

Figure 2. iPSC-monocytes remain CD14$^+$ over time. Flow cytometry analysis show monocytes continue to express CD14 and not CD68 or CD16 on Days 0 and 7. Red lines denote isotype controls, blue lines with shaded areas denote corresponding antibody staining. Gating shows in percent of the parent population.

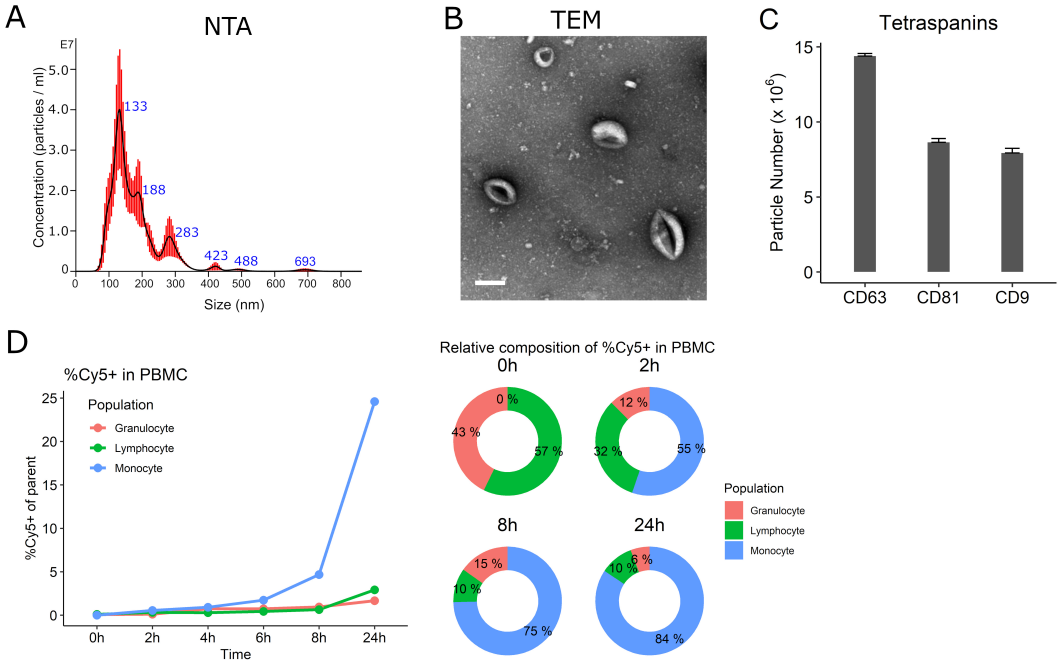

Figure 3. Characterization of iPSC-monocyte EVs (mEV). (A) Representative nanoparticle tracking analysis (NTA) showing the majority of mEVs approximately 130nm. (B) Transmission electron microscopy (TEM) of mEVs. Scale bar = 100 nm. (C) mEVs express tetraspanins CD9, CD63 and CD81. (D) Cy-5-labeled mEVs preferentially enter peripheral blood mononuclear cells (PBMC) monocytes over time.

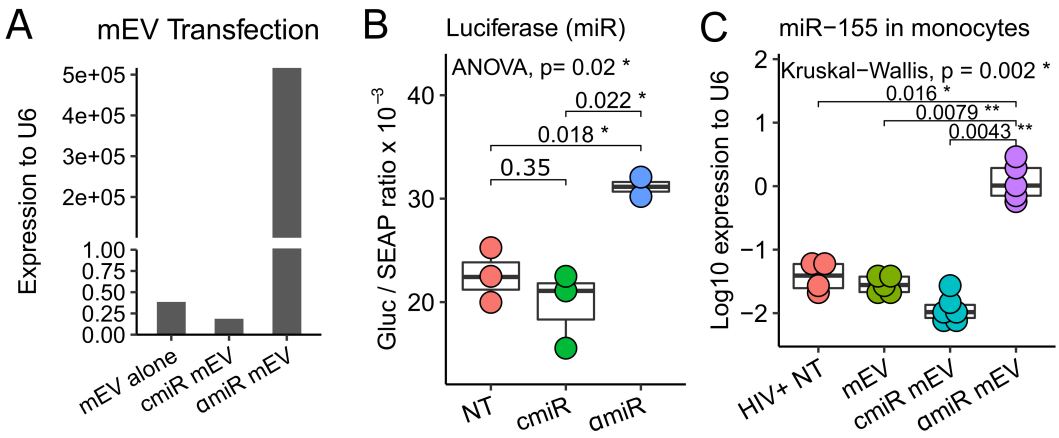

Figure 4. Antagomir-155 loading, targeting and delivery. (A) mEVs transfected with antagomiR-155 (αmiR mEV) showed expression of miR-155 compared to mEV alone and mEVs transfected with Control Oligo-1 miR (cmiR mEV). (B) αmiR, which was transfected into luciferase-plasmid-transfected HEK293 cells, successfully neutralized miR-155 and increased downstream luciferase expression. The ratio of *Gaussia* Luciferase (GLuc) and Secreted Alkaline Phosphatase (SEAP) is reported. (C) Mouse spleen CD14+ monocyte antagomiR-155 expression increases indicating successful delivery of αmiR mEV into monocytes. NT: No mEV treatment; αmiR: antagomiR-155; cmiR: control miRNA. *$P < 0.05$, **$P < 0.01$.

0.016) and CD8 T cells (27.1 ± 5.8 for HIV- *vs.* 58.0 ± 9.2 for HIV+, $P = 0.016$). The increases of CCR5 and HLA-DR on CD4+ or CD8+ T cells are consistent with previous reports[9], confirming that HIV successfully activated human PBMCs in the BLT mouse model. The %CD16+ cells increased in various cell populations indicating activation of M/Mφ, which includes the non-B non-T cells (CD3-CD19-) (2.1 ± 0.7 for HIV- *vs.* 12.4 ± 5.8 for HIV+, $P = 0.016$), CD14+ monocytes (19.1 ± 4.5 for HIV- *vs.* 37.2 ± 6.7 for HIV+, $P = 0.016$), CD11b+ macrophages (21.7 ± 5.3 for HIV- *vs.* 41.7 ± 8.9 for HIV+, $P = 0.032$) and CD163+CD11b+ activated macrophages (20.1 ± 4.4 for HIV- *vs.* 40.6 ± 8.8 for HIV+, $P = 0.016$) [Figure 5B]. Frequency of intermediate monocytes (13.5 ± 4.4 for HIV- *vs.* 32.3 ± 6.6 for HIV+, $P = 0.019$) increased while classical ($P = 0.19$) and non-classical ($P = 1$) monocytes did not change [Figure 5C]. Median fluorescent intensity (MFI) analyses of CCR5, HLA-DR and CD16 expression on the CD4 or CD8 T cell populations showed similar results [Supplementary Figure 2].

mEV altered surface marker expressions in monocytes and T cells with or without antagomir-155

The CD14 population decreased after mEV treatment in PBMCs (16.4 ± 5.3 for HIV+NT *vs.* 6.1 ± 4.1 for HIV+ mEV alone, $P = 0.017$) and partially normalized with antagomir-155 (6.0 ± 3.3 for cmiR mEV and 12.7 ± 4.5 for αmiR mEV, $P = 0.028$) [Figure 6A]. Intermediate monocytes trended lower (32.3 ± 6.6 for HIV+NT *vs.* 20.3 ± 9.5 for HIV+ mEV alone, $P = 0.082$) and were partially normalized by antagomir-155 (14.7 ± 4.4 for cmiR mEV and 27.6 ± 9.1 for αmiR mEV, $P = 0.03$). Alternatively, classical ($P = 0.77$) and non-classical ($P = 0.83$) monocytes did not show significant changes with mEV treatments [Figure 6A].

mEVs alone activated CCR5 on CD14+ monocytes (44.5 ± 11.2 for HIV+NT *vs.* 67.0 ± 10.0 for HIV+ mEV alone, $P = 0.017$), classical CD14+ CD16- monocytes (43.2 ± 13.0 for HIV+NT *vs.* 69.8 ± 11.5 for HIV+ mEV alone, $P = 0.017$), intermediate CD14++CD16+ monocytes (46.9 ± 10.6 for HIV+NT *vs.* 73.2 ± 10.7 for HIV+ mEV alone, $P = 0.017$) and was trending on non-classical CD14+CD16++ monocytes (39.9 ± 23.26 for HIV+NT *vs.* 65.5 ± 13.0 for HIV+ mEV alone, $P = 0.082$) [Figure 6B]. The treatment with antagomir-155 normalized the increase on CD14+ monocytes (67.0 ± 5.2 for cmiR mEV and 37.6 ± 14.8 for αmiR mEV, $P = 0.0043$), on classical monocytes (70.4 ± 5.0 for cmiR mEV and 37.1 ± 15.4 for αmiR mEV, $P = 0.043$), on intermediate monocytes (72.8 ± 4.3 for cmiR mEV and 40.9 ± 13.1 for αmiR mEV, $P = 0.0043$) and was

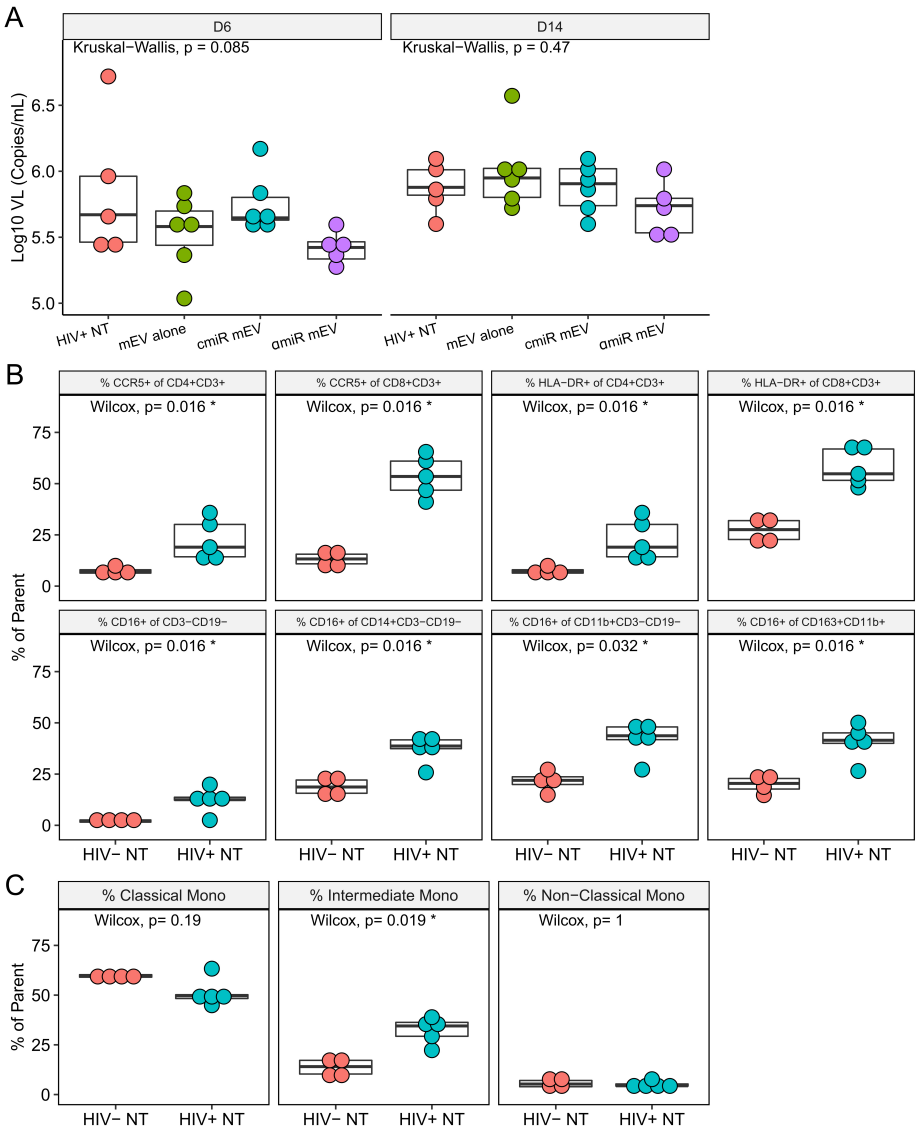

Figure 5. HIV-infected BLT mice. (A) Box plots for mouse plasma viral load on Days 6 and 14 post infection showed no difference among groups. (B) Frequencies of CCR5, HLA-DR and CD16 on PBMCs after HIV infection on Day 14. Parent populations are indicated in the titles of the panels. (C) Frequency of monocyte subsets in CD14+ monocytes. Parent population is CD14+CD3-CD19-.

trending on non-classical monocytes (68.0 ± 16.4 for cmiR mEV and 32.3 ± 28.8 for αmiR mEV, $P = 0.082$) [Figure 6B]. MFI analyses of CCR5 expression on these monocyte populations showed similar results [Supplementary Figure 3A].

The %CCR5+ increased on CD163+CD11b+ M2-type macrophages (42.6 ± 16.8 for HIV+NT *vs.* 75.1 ± 6.6 for HIV+ mEV alone, $P = 0.017$) with mEV treatment and decreased with antagomir-155 mEV (74.0 ± 7.8 for cmiR mEV and 38.1 ± 18.6 for αmiR mEV, $P = 0.0087$) in HIV-infected mice [Figure 6C]. CCR5 had no significant changes on CD163-CD11b+ M1 macrophages. CD16 did not show a significant decrease with mEV treatments alone ($P = 0.13$), but showed a decrease with cmiR mEV ($P = 0.0087$) and a partial normalization on M2 macrophages with antagomir-155 (23.3 ± 5.0 for cmiR mEV and 38.1 ± 8.3 for αmiR mEV, $P = 0.0087$) [Figure 6C]. HLA-DR expression on M/Mφ did not show significant differences between

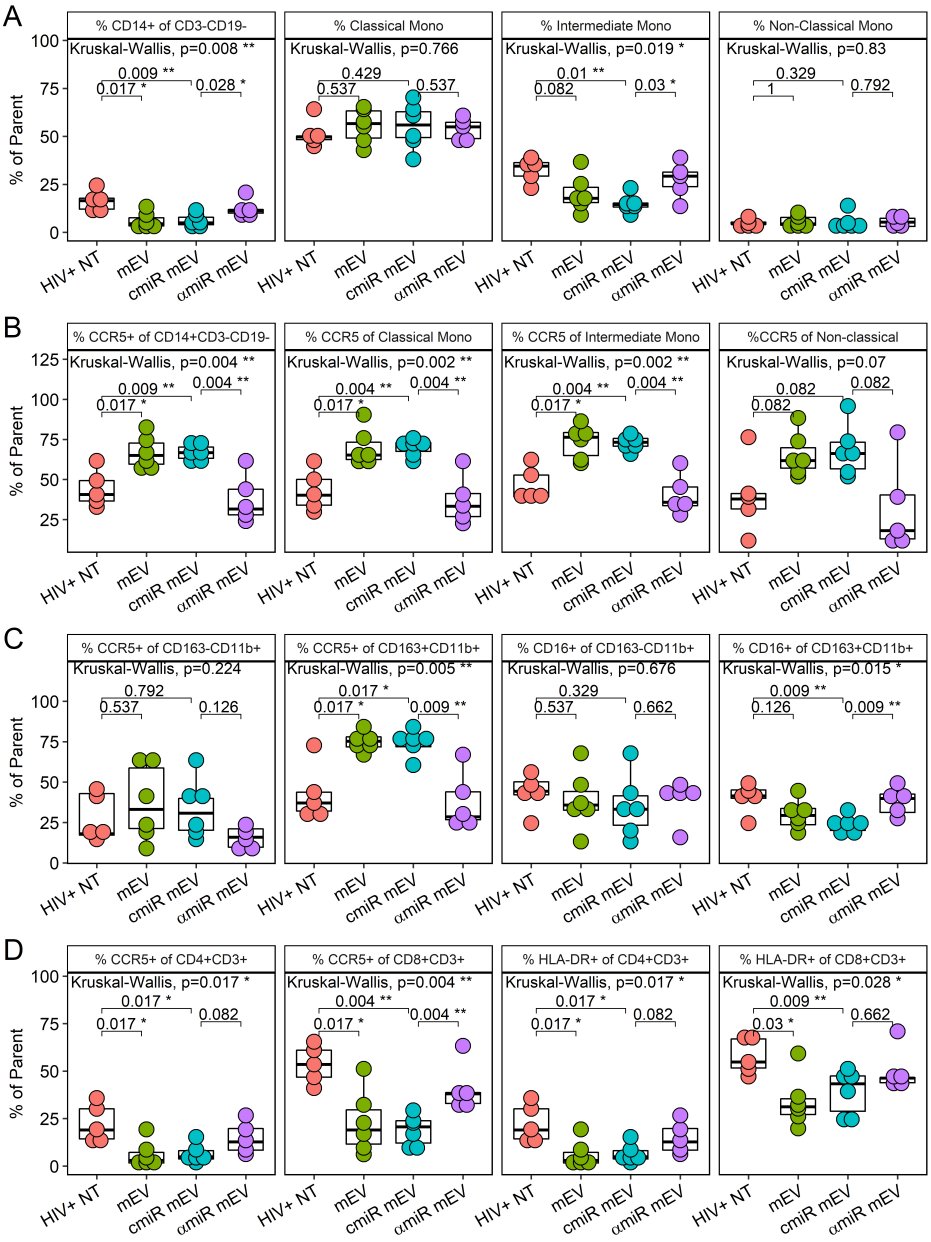

Figure 6. PBMC profile of iPSC-monocyte EV (mEV) treated HIV+ BLT mice. (A) %CD14+ of CD3-CD19-, classical monocytes (CD16-CD14++), intermediate monocytes (CD16+CD14++) and non-classical monocytes (CD16++CD14+) (B) %CCR5 expression of overall monocytes (CD14+CD3-CD19-), classical monocytes (CD16-CD14++), intermediate monocytes (CD16+CD14++) and non-classical monocytes (CD16++CD14+) (C) %CCR5 and %CD16 of macrophages including M1 macrophages (CD163-CD11b+CD3-CD19-) and M2 macrophages (CD163+CD11b+CD3-CD19-) (D) %CCR5 and %HLA-DR of CD4+ or CD8+ T cells.

groups (data not shown). MFI analyses of CCR5 and CD16 expression on the macrophage populations showed similar results except CD16 was trending low by mEV alone compared to HIV+ NT and trending increase by antagomir-155 compared to control miR [Supplementary Figure 3B].

mEVs alone also suppressed CCR5 expression in CD4+ (22.5 ± 9.9 for HIV+NT *vs.* 6.0 ± 6.8 for HIV+ mEV alone, $P = 0.017$) and CD8+ T cells (53.6 ± 10.0 for HIV+NT *vs.* 22.9 ± 16.6 for HIV+ mEV alone, $P = 0.017$) and HLA-DR expression on CD4 (22.5 ± 9.9 for HIV+NT *vs.* 6.0 ± 6.8 for HIV+ mEV alone, $P = 0.017$) and

CD8+ T cells (58.0 ± 9.2 for HIV+NT *vs.* 34.0 ± 13.6 for HIV+ mEV alone, $P = 0.03$). The decrease of CCR5 on CD8+ T cells with mEVs alone in HIV+ NT mice was normalized with αmiR mEV treatment (19.0 ± 8.1 for cmiR mEV and 40.9 ± 12.9 for αmiR mEV, $P = 0.0043$) [Figure 6D]. Trending normalizations were also observed with CCR5 ($P = 0.082$) and HLA-DR ($P = 0.082$) on CD4+ T cells but not HLA-DR on CD8 T cells ($P = 0.66$). MFI analyses showed mEV alone suppressed CCR5 on CD8 T cells and trending on CD4 T cells, while HLA-DR was trending low on both CD4 and CD8 T cells. Antagomir-155 normalized CCR5 MFI on both CD4 and CD8 T cells while HLA-DR did not [Supplementary Figure 3C].

CD68 immunohistochemistry staining

Mouse brain sections were stained for CD68 proteins [Figure 7A-D]. CD68 positive cell counts showed significant differences using negative binomial regression analysis (likelihood ratio $X^2 = 13.27$, df = 4, $P = 0.01$). CD68+ cell counts increased in HIV-infected mouse brains compared to HIV-uninfected mice (3.6 ± 1.8 for HIV- NT, 10.6 ± 3.2 for HIV+ NT, $P = 0.006$) and no significant changes with mEV, cmiR or αmiR mEV treatments [Figure 7E].

DISCUSSION

Pluripotent stem cell-derived monocytes provide a uniform cell source with the ability to scale up as well as minimize human donor variations. Extracellular vesicles generated by these stem cell monocytes can be used as a tool for transferring cargo to other cells and targeting monocytes. Using EVs instead of cells to carry agents avoids drawbacks of stem cell treatment such as malignant transformation[12,13]. iPSC-derived monocytes showed monocytic morphology and cell surface markers such as high CD14, with low CD16 and CD68. This phenotype could be maintained for at least 7 days in culture, which makes it a good source of EVs. We chose stem cell-derived monocytes to increase the chance of EVs entering and delivering cargo to peripheral monocytes.

EVs produced directly from stem cells have been used as therapeutic tools in different studies. Reports show that stem cell-derived EVs alleviated colitis[14], attenuated aging-associated vascular endothelial dysfunction[15], prevented allergic airway inflammation[16] and promoted repair of cardiac infarction[17] in mouse models. There are also various reports on engineered-parent cell generated miRNA-loaded EVs. EVs derived from stem cells transfected with miRNA mimics or antagomirs ameliorated spinal cord injury[18] and microglial activation from brain injury[19], promoted selective regeneration in ischemic hearts[20] or alleviated systemic sclerosis[21] in mouse models. We chose to transfect EVs directly due to the short lifespan of iPSC-monocytes and ease of manipulation.

In our previous studies on monocyte activation and the subsequent effect on their EVs, we found that monocytes treated with interferon/LPS released EVs that transferred functional miRs to human endothelial cells. miR-155, miR-146a, miR-146b and miR-125a-5p were significantly increased and miR-222 was significantly decreased[5]. We further determined that this activation was through the toll-like receptor 4/MYD88 innate immune signal transduction adaptor (MYD88) pathway that activates nuclear factor-κB[5]. We chose to transfect the antagomir-155 into iPSC monocyte-derived EVs to determine the effect on HIV-infected humanized mice with the hypothesis that the monocyte EVs with antagomir-155 would decrease neuroinflammation, possibly viral load and peripheral activation. miR-155 is well characterized, rapidly released in response to infections and injury, and is associated with pathological processes including inflammation and immune responses (Reviewed Ref.[6,22]). miR-155 can push myeloid cells to a pro-inflammatory phenotype[23] that can culminate in neuroinflammation. Knockout of miR-155 reduced macrophage-mediated neuron dysfunction[24] and death in mice[25]. The mEVs alone or with a scrambled control miR decreased T cell and increased monocyte activation. These divergent effects may be due to the constitutive expression of miR-155 in the EVs and its competing effects on these cell types. When an

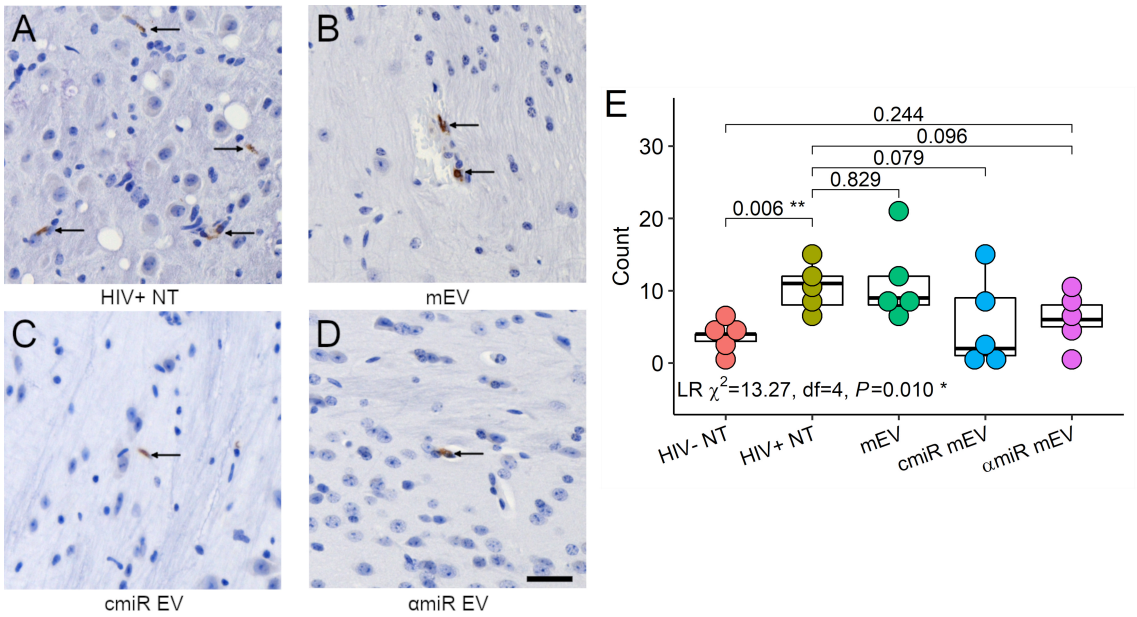

Figure 7. CD68 immunohistochemistry staining of the mouse brains. (A) HIV+ non-treated, (B) HIV+ and treated with mEV alone, (C) HIV+ and treated with cmiR mEVs, (D) HIV+ and treated with αmiR mEVs, arrows indicate positive CD68 staining. 20X. Scale bar = 25 μm. (E) CD68 positive cell counts. Group-wise counts were compared with negative binomial regression. LR: likelihood ratio test for negative binomial regression. *$P < 0.05$, **$P < 0.01$.

antagomiR-155 EV was introduced, there was a setback to HIV infection without treatment. However, there was a trend toward a decrease in the %CCR5+ on the non-classical monocytes that translated to a trend in decreased macrophage brain infiltration. These results may suggest an miR redirecting of migration[26], in this case, from the brain to other tissues. While the exosomes may have delivered the miR-155 antagomir to monocytes, there was no significant decrease in activation markers.

In this HIV-infected BLT humanized mouse model, we observed no differences in viral load before or after EV treatment suggesting that these mEVs do not act against HIV replication directly within this time period. CCR5 is a major HIV coreceptor expressed on T cells and monocytes. CCR5 also plays an important role in T cell and M/Mφ migration including infiltration of the central nervous system (CNS). Increased CCR5 in HIV infection is associated with amyloidosis, tau pathology, neurodegeneration, and blood-brain barrier alterations[27]. We observed an increase of CCR5 on monocytes after HIV infection but not on T cells. miR-155 expression in T cells regulates CCR5 and C-X-C motif chemokine receptor 4 and is central to T cell migration into organs[28]. In multiple sclerosis lesions, T cells that overexpress CCR5 migrate into lesions and the non-migratory T cells express low levels of CCR5[29]. The discrepancy between monocytes and T cells may be due to the difference in the timing of migration between monocytes and T cells. Intermediate monocytes increase and transmigrate across the blood-brain barrier after HIV infection[30]. CCR5 is associated with the CD16+ intermediate monocytes in HIV-infected individuals and a CCR5 antagonist improved neurocognitive performance in a small cohort, suggesting suppression of monocyte migration[31]. The CCR5 antagonist also blocked T cell chemotaxis *in vitro*. CD14+CD16+ monocytes are the subset of intermediate and non-classical cells that have a pro-inflammatory phenotype and migrate into tissues and the brain[32,33]. In this BLT model, the mEVs alone decreased the percent of intermediate monocytes, suggesting that treatment with mEVs had an effect on myeloid differentiation[34].

Significantly, this pilot study showed that EVs can be loaded with cargos, transported to target cells and have biological effects *in vivo* including alteration of T cell and monocyte activation markers with a trend toward decreasing M/Mφ infiltration to the brain. EVs have the advantage of crossing biological barriers, delivering anti-inflammatory cargo to recipient cells[35], thereby influencing peripheral and neuroinflammation. This would have implications for other neuroinflammatory pathologies. Alternatively, there are several limitations in this study including a small number of animals and short acute HIV infection period. mEVs were in the mice for only 7 intermediate days and this may have been an insufficient time or dose to have further effects. We used an intraorbital delivery of mEVs, and an intravenous route may have more robust effects. Future studies would require a closer examination of the timing of administration of the mEVs following infection and the optimization of the dosing (with and without antiretroviral therapy) to more closely assess the role of myeloid/T cell activation and antagomir-155 on HIV.

Our results raise a number of interesting issues. Control mEV treatments decreased the number of CD16+ or intermediate monocytes in the periphery. These monocytes are thought to preferentially transmigrate to the brain in HIV infection[33]. These results suggest that targeting these monocytes would seem more beneficial than targeting the CCR5+ monocytes. The mEV treatments also caused a significant decrease in peripheral T cell activation. Our results show a differential expression of CCR5 and CD16 on monocytes, with mEVs decreasing the percentages of CD16+ monocytes and increasing the CCR5 expression on monocytes. Since CD14++/CD16+ monocytes preferentially transmigrate and seed the brain as a reservoir, targeting this subset in the periphery would be the best approach to limit CNS seeding[33]. The mEV treatments also caused an increase in %CCR5 of CD163+CD11b+ cells and a decrease in %CD16 of CD163+CD11b cells, again suggesting a divergent beneficial effect on monocytes. We posit that the mEV treatments decreased CD16+ monocytes and increased CCR5+ monocytes that may translate to higher migration to other tissues than the brain. These studies do not show an advantage in using antagomir-155 to reduce inflammation below HIV infection alone, but the numbers are small and the outliers may obscure significance. The contents of the mEVs need to be further investigated to determine these positive effects. To our knowledge, this is the first time an EV has been engineered to suppress an inflammatory miRNA and delivered into a humanized HIV mouse to modulate neuroinflammation. In conclusion, there appears to be value in treatment with monocyte EVs in reducing CD16+ monocytes, and although CCR5 was increased on monocytes in mEV-treated mice, this did not translate to an increase in migration to the brain.

DECLARATIONS

Acknowledgments

We thank Ms. Ivy Hsieh (San Francisco VA Health Care System) for performing the electron microscopy analysis.

Authors' contributions

Conceptualized and designed study, assembled data analysis and interpretation and wrote the manuscript: Sun B, Pulliam L

Performed the experiments: Sun B, Tang N, Garza A

Contributed to design and materials: Kitchen S, Jacob S

All authors read and approved the manuscript.

Availability of data and materials

Not applicable.

Financial support and sponsorship

This work was supported by the National Institutes of Health (R21 AI138923 to LP).

Conflicts of interest

All authors declare not conflicts of interest.

Ethical approval and consent to participate

Animal use was approved by the UCLA IACUC (Protocol ARC-2010-038).

Consent for publication

Not applicable.

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REFERENCES

1. Gallitano SM, McDermott L, Brar K, Lowenstein E. Use of tumor necrosis factor (TNF) inhibitors in patients with HIV/AIDS. *J Am Acad Dermatol* 2016;74:974-80. DOI PubMed
2. Kumar A, Coquard L, Herbein G. Targeting TNF-alpha in HIV-1 infection. *Curr Drug Targets* 2016;17:15-22. DOI PubMed
3. Bochnakian A, Zhen A, Zisoulis DG, et al. Interferon-inducible microRNA miR-128 modulates HIV-1 replication by targeting TNPO3 mRNA. *J Virol* 2019;93:e00364-19. DOI PubMed PMC
4. Tang N, Sun B, Gupta A, Rempel H, Pulliam L. Monocyte exosomes induce adhesion molecules and cytokines via activation of NF-κ B in endothelial cells. *FASEB J* 2016;30:3097-106. DOI PubMed PMC
5. Dalvi P, Sun B, Tang N, Pulliam L. Immune activated monocyte exosomes alter microRNAs in brain endothelial cells and initiate an inflammatory response through the TLR4/MyD88 pathway. *Sci Rep* 2017;7:9954. DOI PubMed PMC
6. Elton TS, Selemon H, Elton SM, Parinandi NL. Regulation of the MIR155 host gene in physiological and pathological processes. *Gene* 2013;532:1-12. DOI PubMed
7. Akkina R, Allam A, Balazs AB, et al. Improvements and limitations of humanized mouse models for HIV research: NIH/NIAID "meet the experts" 2015 workshop summary. *AIDS Res Hum Retroviruses* 2016;32:109-19. DOI
8. Carrillo MA, Zhen A, Kitchen SG. The use of the humanized mouse model in gene therapy and immunotherapy for HIV and cancer. *Front Immunol* 2018;9:746. DOI PubMed PMC
9. Zhen A, Rezek V, Youn C, et al. Targeting type I interferon-mediated activation restores immune function in chronic HIV infection. *J Clin Invest* 2017;127:260-8. DOI PubMed PMC
10. Kitchen SG, Levin BR, Bristol G, et al. In vivo suppression of HIV by antigen specific T cells derived from engineered hematopoietic stem cells. *PLoS Pathog* 2012;8:e1002649. DOI PubMed PMC
11. Rempel H, Sun B, Calosing C, Pillai SK, Pulliam L. Interferon-alpha drives monocyte gene expression in chronic unsuppressed HIV-1 infection. *AIDS* 2010;24:1415-23. DOI PubMed PMC
12. Volarevic V, Markovic BS, Gazdic M, et al. Ethical and safety issues of stem cell-based therapy. *Int J Med Sci* 2018;15:36-45. DOI PubMed PMC
13. Harrell CR, Jovicic N, Djonov V, Arsenijevic N, Volarevic V. Mesenchymal stem cell-derived exosomes and other extracellular vesicles as new remedies in the therapy of inflammatory diseases. *Cells* 2019;8:1605. DOI PubMed PMC
14. Duan L, Huang H, Zhao X, et al. Extracellular vesicles derived from human placental mesenchymal stem cells alleviate experimental colitis in mice by inhibiting inflammation and oxidative stress. *Int J Mol Med* 2020;46:1551-61. DOI PubMed PMC
15. Feng R, Ullah M, Chen K, Ali Q, Lin Y, Sun Z. Stem cell-derived extracellular vesicles mitigate ageing-associated arterial stiffness and hypertension. *J Extracell Vesicles* 2020;9:1783869. DOI PubMed PMC
16. Fang SB, Zhang HY, Meng XC, et al. Small extracellular vesicles derived from human MSCs prevent allergic airway inflammation via immunomodulation on pulmonary macrophages. *Cell Death Dis* 2020;11:409. DOI PubMed PMC
17. Khan M, Nickoloff E, Abramova T, et al. Embryonic stem cell-derived exosomes promote endogenous repair mechanisms and enhance cardiac function following myocardial infarction. *Circ Res* 2015;117:52-64. DOI PubMed PMC
18. Huang T, Jia Z, Fang L, et al. Extracellular vesicle-derived miR-511-3p from hypoxia preconditioned adipose mesenchymal stem cells ameliorates spinal cord injury through the TRAF6/S1P axis. *Brain Res Bull* 2022;180:73-85. DOI PubMed
19. Qian Y, Li Q, Chen L, et al. Mesenchymal Stem cell-derived extracellular vesicles alleviate M1 microglial activation in brain injury of mice with subarachnoid hemorrhage via microRNA-140-5p delivery. *Int J Neuropsychopharmacol* 2022;25:328-38. DOI PubMed
20. Song BW, Lee CY, Kim R, et al. Multiplexed targeting of miRNA-210 in stem cell-derived extracellular vesicles promotes selective regeneration in ischemic hearts. *Exp Mol Med* 2021;53:695-708. DOI PubMed PMC
21. Rozier P, Maumus M, Maria ATJ, et al. Mesenchymal stromal cells-derived extracellular vesicles alleviate systemic sclerosis via miR-

29a-3p. *J Autoimmun* 2021;121:102660. DOI PubMed

22. Alivernini S, Gremese E, McSharry C, et al. MicroRNA-155-at the critical interface of innate and adaptive immunity in arthritis. *Front Immunol* 2017;8:1932. DOI PubMed PMC
23. Jablonski KA, Gaudet AD, Amici SA, Popovich PG, Guerau-de-Arellano M. Control of the inflammatory macrophage transcriptional signature by miR-155. *PLoS One* 2016;11:e0159724. DOI PubMed PMC
24. Gaudet AD, Mandrekar-Colucci S, Hall JC, et al. miR-155 deletion in mice overcomes neuron-intrinsic and neuron-extrinsic barriers to spinal cord repair. *J Neurosci* 2016;36:8516-32. DOI PubMed PMC
25. Gaudet AD, Fonken LK, Ayala MT, Maier SF, Watkins LR. Aging and miR-155 in mice influence survival and neuropathic pain after spinal cord injury. *Brain Behav Immun* 2021;97:365-70. DOI PubMed PMC
26. Huang Q, Chen L, Luo M, et al. HIV-1-induced miR-146a Attenuates monocyte migration by targeting CCL5 in Human primary macrophages. *AIDS Res Hum Retroviruses* 2018;34:580-9. DOI PubMed
27. Bhargavan B, Woollard SM, McMillan JE, Kanmogne GD. CCR5 antagonist reduces HIV-induced amyloidogenesis, tau pathology, neurodegeneration, and blood-brain barrier alterations in HIV-infected hu-PBL-NSG mice. *Mol Neurodegener* 2021;16:78. DOI PubMed PMC
28. Zitzer NC, Snyder K, Meng X, et al. MicroRNA-155 modulates acute graft-versus-host disease by impacting T cell expansion, migration, and effector function. *J Immunol* 2018;200:4170-9. DOI PubMed PMC
29. Zang YC, Samanta AK, Halder JB, et al. Aberrant T cell migration toward RANTES and MIP-1 alpha in patients with multiple sclerosis. Overexpression of chemokine receptor CCR5. *Brain* 2000;123:1874-82. DOI PubMed
30. Buckner CM, Calderon TM, Willams DW, Belbin TJ, Berman JW. Characterization of monocyte maturation/differentiation that facilitates their transmigration across the blood-brain barrier and infection by HIV: implications for NeuroAIDS. *Cell Immunol* 2011;267:109-23. DOI PubMed PMC
31. Ndhlovu LC, Umaki T, Chew GM, et al. Treatment intensification with maraviroc (CCR5 antagonist) leads to declines in CD16-expressing monocytes in cART-suppressed chronic HIV-infected subjects and is associated with improvements in neurocognitive test performance: implications for HIV-associated neurocognitive disease (HAND). *J Neurovirol* 2014;20:571-82. DOI PubMed PMC
32. Pulliam L, Sun B, Rempel H. Invasive chronic inflammatory monocyte phenotype in subjects with high HIV-1 viral load. *J Neuroimmunol* 2004;157:93-8. DOI PubMed
33. Veenstra M, León-Rivera R, Li M, Gama L, Clements JE, Berman JW. Mechanisms of CNS viral seeding by HIV^+ $CD14^+$ $CD16^+$ monocytes: establishment and reseeding of viral reservoirs contributing to HIV-associated neurocognitive disorders. *mBio* 2017;8:e01280-17. DOI PubMed PMC
34. Guilliams M, Mildner A, Yona S. Developmental and functional heterogeneity of monocytes. *Immunity* 2018;49:595-613. DOI PubMed
35. Zhuang X, Xiang X, Grizzle W, et al. Treatment of brain inflammatory diseases by delivering exosome encapsulated anti-inflammatory drugs from the nasal region to the brain. *Mol Ther* 2011;19:1769-79. DOI PubMed PMC

Kannan *et al. Extracell Vesicles Circ Nucleic Acids* 2022;3:133-49
DOI: 10.20517/evcna.2022.14

Extracellular Vesicles and Circulating Nucleic Acids

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HIV-1 Tat induced microglial EVs leads to neuronal synaptodendritic injury: microglia-neuron cross-talk in NeuroHIV

Muthukumar Kannan[1], Seema Singh[1], Divya T. Chemparathy[1], Abiola A. Oladapo[1], Dinesh Y. Gawande[2], Shashank M. Dravid[2], Shilpa Buch[1], Susmita Sil[1]

[1]Department of Pharmacology and Experimental Neuroscience, University of Nebraska Medical Center, Omaha, NE 68198, USA.
[2]Department of Pharmacology and Neuroscience, Creighton University, Omaha, NE 68178, USA.

Correspondence to: Prof. Shilpa Buch, Department of Pharmacology and Experimental Neuroscience, University of Nebraska Medical Center, Omaha, NE 68198, USA. E-mail: sbuch@unmc.edu; Dr. Susmita Sil, Department of Pharmacology and Experimental Neuroscience, University of Nebraska Medical Center, Omaha, NE 68198, USA. E-mail: susmita.sil@unmc.edu

How to cite this article: Kannan M, Singh S, Chemparathy DT, Oladapo AA, Gawande DY, Dravid SM, Buch S, Sil S. HIV-1 Tat induced microglial EVs leads to neuronal synaptodendritic injury: microglia-neuron cross-talk in NeuroHIV. *Extracell Vesicles Circ Nucleic Acids* 2022;3:133-49. https://dx.doi.org/10.20517/evcna.2022.14

Received: 24 Mar 2022 **First Decision:** 28 Apr 2022 **Revised:** 5 May 2022 **Accepted:** 23 May 2022 **Published:** 31 May 2022

Academic Editors: Yoke Peng Loh, Lynn Pulliam **Copy Editor:** Tiantian Shi **Production Editor:** Tiantian Shi

Abstract

Aim: Activation of microglial NLRP3 inflammasome is an essential contributor to neuroinflammation underlying HIV-associated neurological disorders (HAND). Under pathological conditions, microglia-derived-EVs (MDEVs) can affect neuronal functions by delivering neurotoxic mediators to recipient cells. However, the role of microglial NLRP3 in mediating neuronal synaptodendritic injury has remained unexplored to date. In the present study, we sought to assess the regulatory role of HIV-1 Tat induced microglial NLRP3 in neuronal synaptodendritic injury. We hypothesized that HIV-1 Tat mediated microglia EVs carrying significant levels of NLRP3 contribute to the synaptodendritic injury, thereby affecting the maturation of neurons.

Methods: To understand the cross-talk between microglia and neuron, we isolated EVs from BV2 and human primary microglia (HPM) cells with or without NLRP3 depletion using siNLRP3 RNA. EVs were isolated by differential centrifugation, characterized by ZetaView nanoparticle tracking analysis, electron microscopy, and western blot analysis for exosome markers. Purified EVs were exposed to primary rat neurons isolated from E18 rats. Along with green fluorescent protein (GFP) plasmid transfection, immunocytochemistry was performed to visualize neuronal synaptodendritic injury. Western blotting was employed to measure siRNA transfection efficiency and the extent of neuronal synaptodegeneration. Images were captured in confocal microscopy, and

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subsequently, Sholl analysis was performed for analyzing dendritic spines using neuronal reconstruction software Neurolucida 360. Electrophysiology was performed on hippocampal neurons for functional assessment.

Results: Our findings demonstrated that HIV-1 Tat induced expression of microglial NLRP3 and IL1β, and further that these were packaged in microglial exosomes (MDEV) and were also taken up by the neurons. Exposure of rat primary neurons to microglial Tat-MDEVs resulted in downregulation of synaptic proteins- PSD95, synaptophysin, excitatory vGLUT1, as well as upregulation of inhibitory proteins- Gephyrin, GAD65, thereby implicating impaired neuronal transmissibility. Our findings also showed that Tat-MDEVs not only caused loss of dendritic spines but also affected numbers of spine sub-types- mushroom and stubby. Synaptodendritic injury further affected functional impairment as evidenced by the decrease in miniature excitatory postsynaptic currents (mEPSCs). To assess the regulatory role of NLRP3 in this process, neurons were also exposed to Tat-MDEVs from NLRP3 silenced microglia. Tat-MDEVs from NLRP3 silenced microglia exerted a protective role on neuronal synaptic proteins, spine density as well as mEPSCs.

Conclusion: In summary, our study underscores the role of microglial NLRP3 as an important contributor to Tat-MDEV mediated synaptodendritic injury. While the role of NLRP3 in inflammation is well-described, its role in EV-mediated neuronal damage is an interesting finding, implicating it as a target for therapeutics in HAND.

Keywords: NLRP3, microglia-derived EVs, synaptodendritic injury, mEPSC

INTRODUCTION

Globally, 37.7 million people are living with human immunodeficiency virus (HIV) as of 2020, with greater than 50% having access to combined antiretroviral therapy (cART) as of June 2021 (UNAIDS). The introduction of cART has changed the face of HIV-1 from a death sentence to a manageable chronic disease condition. HIV enters the brain soon after infection via Trojan horse mechanisms involving the migration of infected monocytes across the blood-brain barrier (BBB)[1]. It has been demonstrated that HIV patients on cART with no detectable viral load therapy[2] continue to be inflicted with complications of the central nervous system (CNS), collectively termed HIV-associated neurocognitive disorders (HAND). This is likely due to the presence of ongoing low-level virus replication and HIV-1 viral proteins in the brain. About half of the HIV-1 infected patients go on to develop HAND regardless of the cART regimen[3], thereby implicating the effects of residual viral proteins on the CNS[4]. Among these viral proteins, the trans-activator of transcription (Tat) is an early viral regulatory protein that enhances the efficiency of viral transcription in the brain[5]. Tat is an HIV-1 protein with a variable length of 86-102 amino acids[6,7] and plays a critical role in HIV pathogenesis owing to its cytotoxic potential[8].

In the CNS, HIV does not directly infect the neurons but can efficiently infect microglia[9] and, to some extent, the astrocytes[10,11]. HIV-infected microglia or astrocytes, in turn, produce Tat protein, which then can be taken up by the uninfected bystander cells, including the neurons[12-14]. A sufficient amount of Tat is present in the CNS of HIV-infected patients to induce neurotoxicity and neuronal dysfunction *in vivo*[15] as well as *in vitro* in cultured neurons[16]. Reports have shown that Tat alters the expression of neuronal proteins such as the postsynaptic density protein 95 (PSD-95)[17], Gephyrin[17], and synaptophysin[18].

Microglia not only function as the resident immune cells of the CNS, but also communicate with various other CNS cells, including the neurons[19] and astrocytes[20], for normal functioning of the brain. In healthy cells, microglia secrete extracellular vesicles (EVs) to support the metabolic functions of neurons and to provide substrates needed for energy metabolism during synaptic activity[21-25]. Similarly, under pathological conditions, microglia-derived EVs (MDEVs) can also affect neuronal functions by delivering

proinflammatory cytokines and other neurotoxic mediators to these latter cells[26,27]. Previous reports from our laboratory have shown that HIV-1 Tat could impact the astrocyte EV cargo, which in turn, could impair the synaptic architecture of neurons[28,29]. It has also been suggested that secretion of EVs could be a necessary and compensatory pathway to eliminate damaged or toxic molecules produced due to Tat cytotoxicity[30-33]. To support this notion, we and others have shown that HIV-1 Tat inhibits the autophagy and proteasomal degradation pathways in microglia and astrocytes[30-33], which in turn, can modulate the EV biogenesis pathways[34,35]. Additionally, our previous study has demonstrated that HIV-1 Tat can induce the NLRP3 inflammasome pathway in microglia[36], resulting in their activation. Taken together, we thus hypothesized that in the context of HIV/HAND pathology EVs released from inflammasome-activated microglia cells could also carry the inflammatory mediators such as NLRP3, which, upon being taken up by the neurons, could affect their functions.

In the present study, we isolated and characterized EVs released by BV2 cells (immortalized murine microglial cells) and human primary microglia (HPM) cells in the presence or absence of HIV-1 Tat. Both the BV2 and primary microglial cells derived-MDEVs were demonstrated to carry the NLRP3 and IL1β cargoes that, upon being uptaken by the neurons, resulted in synaptodendritic injury and lowering excitatory postsynaptic currents, suggesting that the MDEVs via an NLRP3 dependent mechanism could be a contributing factor for HAND pathogenesis.

METHODS

Reagents

NLRP3 (AG-20B-0014, AdipoGen, CA, USA); IL1β (ab9722, Abcam, MA, USA); Anti-CD63 antibody (ab216130, Abcam, MA, USA); Anti-CD9 antibody (ab92726, Abcam, MA, USA), Anti-TSG101 antibody (ab125011, Abcam, MA, USA), Anti-Alix antibody (ab275377, Abcam, MA, USA), Anti-Calnexin antibody (ab133615, Abcam, MA, USA); Anti-PSD95 antibody (ab2723, Abcam, MA, USA); Anti-GAD65 antibody (ab239372, Abcam, MA, USA); Anti-Gephyrin antibody (ab181382, Abcam, MA, USA); Anti-vGLUT1 antibody (AB5905, Millipore, Burlington, MA, USA), GFP expressing plasmid (13031, Adgene, Watertown, MA, USA); β-actin (A5316, Sigma- Aldrich, MO, USA); horseradish peroxidase conjugated goat anti-rabbit (sc-2004, Santa Cruz Biotechnology, TX, USA) and horseradish peroxidase conjugated goat anti-mouse (sc-2005, Santa Cruz Biotechnology, TX, USA); human primary microglia (Cat # 1900; Sciencell research laboratory, CA, USA) and BV2 microglial cell line was received from Dr. Sanjay Maggirwar (University of Rochester Medical Center, Rochester, NY, USA).

Microglia culture

HPM cells were purchased from Celprogen (cat no:37089-01). HPM cells were grown in HPM culture complete Media with Serum (Cat. No: M37089-01, Celprogen, CA, USA). The BV2 cell lines were obtained from Dr. Sanjay Maggirwar (University of Rochester Medical Center, Rochester, NY, USA). These cultured BV2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with high glucose supplemented with 10% heated-inactivated fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 μg/mL). Both the cell types were seeded at a density of 3×10^5 (per well) in a six-well plate or 2.5×10^6 in a T150 flask for different experiments. After overnight serum starvation, cells were treated with 50 ng/mL HIV-1 Tat for 24 h in a humidified incubator at 37 °C with 5% CO_2, followed by EV isolation.

Rat primary neuron cultures

Rat primary cortical and hippocampal neurons were isolated from E18 rats as described previously[37,38]. The animal experiment is approved by the ethical committee of University of Nebraska Medical Center (IACUC# 20-057-07-FC). Briefly, the cortex and hippocampi were dissected in HBSS (14025076, Gibco™) followed by incubation with 0.25% trypsin (25200056, Gibco™) for 10 min in a 37 °C water bath. Tissue-free,

single cell suspensions were obtained by triturating, followed by passing the cell suspension through the 40 μM cell strainer. Primary neuron cultures were maintained in a complete neuronal media containing neurobasal medium (21103049, Invitrogen), with B27 supplement (17504044, Invitrogen), L-Glutamate (A2916801, Gibco™), and penicillin-streptomycin (15070063, Invitrogen). Rat primary cortical neurons were seeded at a density of 2×10^5 in poly-D-lysine (P1024, Sigma-Aldrich) coated plates for western blot analysis. For microscopy analysis, hippocampal neurons were seeded at a density of 1.5×10^5 in poly-D-lysine coated coverslips. After two weeks of culture, cells were used for further experimentation.

EV isolation

The EVs were prepared from the supernatant of BV2 cells and HPM by differential centrifugations, which was previously described[29]. Briefly, serum-starved BV2 cells and HPM were exposed to HIV-1 Tat protein (50 ng/mL) for 24 h. Then, the conditioned media from this treatment were harvested, centrifuged at 300 *g* and 2000 *g* for 10 min to eliminate cellular debris and residual cells, and the supernatant was spun at 10,000 *g* for 30 min, followed by filtration using a 0.22 μm filter. The EVs were pelleted by ultracentrifugation (Beckman Ti32 rotor, Brea, CA, USA) for 70 min at 100,000 *g*. All EV isolation protocols were performed at 4 °C. The EVs were quantified using the ZetaView nanoparticle tracking analysis system (NS300, Particle Metrix, Germany) as described previously[29]. The protein content was assessed using a BCA protein assay kit (Pierce, Rockford, IL, USA); after normalization, the EVs were used for characterization of the exosome-specific markers by Western blotting and transmission electron microscopy, as well as used for further experimentation. The total number of EVs from 2 million cells were diluted in 300 μL of PBS. Approximately 2 million BV2/HPM cells yielded 10^9/mL of EVs. All the neurons were exposed with 100, 500, and 1000 EVs/ cell for standardization, and 500 EVs/ neurons were used for further experimentation (after standardization). The neurons were exposed to these EVs for 48 h, followed by an assessment of synaptodendritic injury and electrophysiology.

Zeta view tracking analysis

Isolated EVs from BV2 or HPM supernatant were analyzed by nanoparticle tracking analysis (NTA) using ZetaView nanoparticle tracking analyzer (Particle Metrix, Germany) along with the software ZetaView 8.04.02 SP1. Prior to the analysis, the instrument was calibrated using 100 nm polystyrene nano standard particles and cell quality checking was performed before sample reading. The video was captured at a sensitivity of 85, a shutter speed of 100, and a frame rate of 30. Size (in nm) and concentration (particles/mL) for each sample were determined by injecting the diluted sample in filtered PBS, with two cycles of reading at each position.

Electron microscopy

EV pellets were subjected to negative staining. In brief, EV pellets were deposited on 200-mesh Formvar-coated copper grids and the membranes were covered for 4-5 min for the absorption. Next, for contrast staining, the grids were further transferred to uranyl acetate solution. Hereafter, the grids were washed with PBS and excess fluid was blotted with filter paper and allowed to air-dry at room temperature. Imaging was performed using a Hitachi H-7500 electron microscope (Hitachi, Tokyo, Japan) at 200kV.

NLRP3 siRNA transfection

Microglial cells were seeded at a density of 3×10^5 (per well) in a 6-well plate for siRNA transfection. At about 70% confluency, cells were transfected with NLRP3 siRNA or scrambled siRNA in Opti-MEM media (Life Technologies, 31985062) using Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific, catalog: 13778150) according to the manufacturer's instructions. Following transfection, cells were incubated for 12-18 h in a humidified incubator at 37 °C with 5% CO_2. After transfection, cells were treated with 50 ng/mL Tat in a fresh DMEM medium for 24 h. The siRNA transfection efficiency was

determined by western blotting.

GFP-plasmid transfection

Lipofectamine 3000 (2307436, Invitrogen, Carlsbad CA) was used for GFP plasmid transfection in hippocampal (0.15×10^6 cells/well) neurons according to the manufacturer's instructions. Briefly, cells were transfected with GFP plasmid (500 ng) mixed with 1μl of Lipofectamine 3000 diluted in 25 μL of Opti-MEM (31985-070, Gibco, Thermo Fisher Scientific, Amarillo, TX) media. The cells were incubated with the plasmid-lipid complex for 6 h, and the medium was changed with fresh media. Thereafter, transfected cells were used for various treatments after 72 h.

Immunocytochemistry

GFP-plasmid transfected primary rat hippocampal neurons on coverslips were exposed to MDEVs for 48 h. After the treatment, neurons were fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature and permeabilized with 0.1% Triton X-100 (BP151-1; Thermo Fisher Scientific, Grand Island, NY, USA). Blocking was performed with 5% normal goat serum in PBS for 1 h at room temperature, followed by the addition of respective primary antibodies: GAD65 (ab18258, Cambridge, MA), vGlut1 (ab5905, Cambridge, MA). After incubation with primary antibody overnight, the neurons were probed with secondary Alexa Fluor 594 goat anti-rabbit (A11012, Invitrogen, Carlsbad CA) and Alexa Fluor 647 goat anti-guinea pig (A21450, Invitrogen, Carlsbad CA) antibodies. Cells were counterstained and mounted with Prolong Gold antifade reagent with DAPI (P36935, Invitrogen, Carlsbad CA).

Image acquisition and dendritic spine quantification

Images were captured in confocal microscopy (ZEISS ELYRA PS.1 Super Resolution Microscope, Jena, Germany) with a 63× magnification with consistent contrast and brightness for each set of experiments. Dendritic spines were captured using Z-stack projection. Thereafter, Sholl analysis was performed for analyzing dendritic spines using neuronal reconstruction software Neurolucida 360 (version 2021.1.1).

Western blotting

The BV2 or HPM cells treated with HIV-1 Tat in culture were lysed using the mammalian cell lysis kit (Sigma, MCL1-1KT). Protein electrophoresis was performed using 10 μg of the lysate proteins on a 10% sodium dodecyl sulfate-polyacrylamide gel under reducing conditions, then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, IPVH00010). Then, the blots were blocked with 5% nonfat dry milk diluted in 1 × TTBS buffer. After washing with 1 × TTBS buffer three times, the membranes were probed with primary antibodies specific for the proteins of interest overnight. The secondary antibodies used to probe were HRP conjugated to goat anti-mouse/rabbit IgG. β-actin (Sigma, A5441) was used as a loading control for the study.

Electrophysiology

Whole-cell electrophysiology was performed on rat primary hippocampal neurons (DIV 19-21) as previously described[39]. Primary hippocampal neurons were seeded on coverslips. The signal was filtered at 2 kHz & digitized at 10 kHz using an Axon Digidata 1440A analog-to-digital board (Molecular Devices, San Jose, CA, USA). Recordings with a pipette access resistance of less than 20 mOhm and less than 20% changes during the duration of recording were included. The external solution contained (in mM): 150 NaCl, 3 KCl, 10 HEPES, 6 mannitol, 0.02 EDTA, 1.5 $MgCl_2$, and 2.5 mM $CaCl_2$ (pH 7.4). Glass pipettes with a resistance of 2-5 mOhm were filled with an internal solution consisting of (in mM) 110 cesium gluconate, 30 CsCl, 5 HEPES, 4 NaCl, 0.5 $CaCl_2$, 2 $MgCl_2$, 5 BAPTA, 2 Na_2ATP, and 0.3 Na_2GTP (pH 7.35). QX-314 was added in pipette solution to block voltage-gated sodium channels. Miniature excitatory postsynaptic currents (mEPSCs) were recorded in the presence of 0.5 μM tetrodotoxin and 100 μM picrotoxin at -70 mV.

The mEPSC recordings were analyzed using Minianalysis software (Synaposoft, Atlanta, GA, USA) with an amplitude threshold set at 5 pA. The frequency of the miniature currents was measured.

Statistics

The data are represented as mean ± SEM. Student t-test was employed to compare between two groups, and one-way ANOVA followed by Bonferroni post hoc test was employed to compare within multiple experimental groups, using the GraphPad Prism software (Version 5). For the *in vitro* study, three replicates per sample and six independent sets of experiments were analyzed. Statistical analysis for which probability levels were less than $P < 0.05$ was considered statistically significant.

RESULTS

HIV-1 Tat increases NLRP3 cargoes in exosomes derived from microglia

In our previous study, we demonstrated the role of HIV-1 Tat in activating NLRP3 inflammasome with subsequent maturation of caspase-1 and production and release of IL-1β from microglia[36]. NLRP3 inflammasome plays a crucial role in the development of neuroinflammation[40]. It has been shown that microglial exosomes can be transported and taken up by the recipient cells[24,25,41]; however, scanty reports are available on the functionality of these microglial exosomes, specifically in the context of HIV-1. In the current study, we investigated whether microglial NLRP3 plays a role in causing neuronal damage via microglia-neuron cross-talk involving the exosomes. We first sought to isolate and characterize EVs from conditioned media of BV2 cells and HPM with or without Tat exposure [Figure 1A]. The total number of EVs and particle size distribution were determined by NTA. Exposure of BV2 cells [Figure 1B] and HPM [Figure 2A] to HIV-1 Tat (50 ng/mL, 24h) resulted in increased release of exosomes compared to the control or heated Tat (HT) exposed cells; however, there was no significant difference among the groups. Size distribution by NTA showed that isolated EVs were in the size range of 50-150 nm in BV2 cells [Figure 1C] and HPM [Figure 2B]. The protein expression of exosomal markers such as Alix, TSG101, CD9, and CD63 was analyzed by western blotting in BV2 [Figure 1D] and HPM [Figure 2D] EVs. Additionally, immunoblotting of calnexin was also performed to demonstrate that isolated EVs were pure and enriched vesicles [Figures 1D and 2D]. Further characterization by TEM revealed the cup-shaped profile of EVs with sizes ranging from 50-150 nm in BV2 [Figure 1E] and HPM [Figure 2C]. Since we were specifically interested in the role of NLRP3 inflammasome pathway, we determined the protein expression of NLRP3 and its downstream IL1β in the exosomes derived from BV2 and HPM. HIV-1 Tat was found to increase the release of NLRP3, pro- and mature IL1β in EVs isolated from BV2 cells [Figure 1F] and HPM [Figure 2E].

BV-2 exosomes cause neuronal damage

Since excessive microglial activation damages the surrounding healthy cells, we next enquired whether factors derived from activated microglia could reach the recipient neurons and inflict neuronal damage. For this, BV2 cells were transfected with a plasmid encoding the exosome marker TSG101 fused with mCherry, followed by isolation of EVs from the conditioned media of transfected BV2 cells. As shown in Figure 3A, BV2-derived exosomes were found localized within the neurons. Having confirmed the transfer of exosomes from microglia to neurons, we next asked whether NLRP3 carried through the exosomes could be taken up by the neurons and inflict neuronal damage. For this, rat cortical neurons were exposed to exosomes isolated from conditioned media of BV2 cells with or without Tat exposure. As neuronal excitability relies precisely on excitatory and inhibitory signals, we analyzed the protein expression of postsynaptic density protein 95 (PSD95), a critical synaptic protein that controls synaptic transmission and plasticity. Figure 3B represents the immunoblotting analysis of PSD95, which showed a dose-dependent significant down-regulation of PSD95 in cortical neurons exposed to exosomes isolated from Tat treated BV2 cells ($P < 0.05$) compared with control EV exposed neurons. Interestingly, there was a significant increase in the expression of inhibitory postsynaptic markers, glutamic acid decarboxylase 65 (GAD65), in

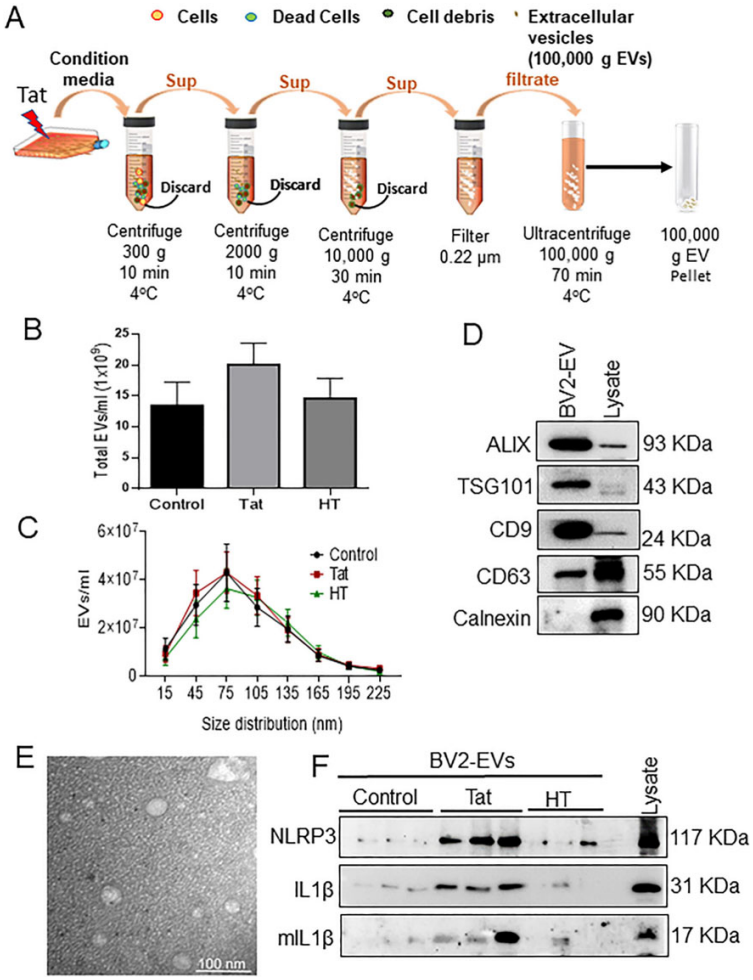

Figure 1. Characterization of microglia-derived exosomes from BV2 cells. (A) Schematic representation of the isolation protocol from BV2 cells. (B) Quantification of the total number of EVs and (C) size distribution of exosomes by NTA using ZetaView. (D) Representative western blots showing the expression of exosome-specific markers (Alix, TSG101, CD9, CD63). Calnexin used as a negative control for exosomes. (E) Representative transmission electron microscopy (TEM) image of exosome particles isolated by ultracentrifugation at 100,000 g. Scale bar 100 nm. (F) Representative western blot images showing protein expression of NLRP3, pro-IL1β, mIL-β in control, Tat (50 ng/mL) or HT treated-BV2-derived exosomes. Data are presented as mean ± SEM. NLRP3: NOD-, LRR- and pyrin domain-containing protein 3; IL: interleukin; mIL: mature interleukin; Tat: trans-activator of transcription; HT: heat inactivated Tat protein.

the neurons exposed to varying numbers of Tat-MDEVs ($P < 0.05$) compared with neurons exposed to control MDEVs [Figure 3C]. Similarly, the expression of inhibitory postsynaptic markers, Gephyrin, was also found to be significantly upregulated in neurons exposed to Tat-MDEVs ($P < 0.05$) compared with control MDEVs [Figure 3D].

While the neurotoxic effect of NLRP3 has been well documented in neurons[41], the direct role of microglial NLRP3 on neuronal synaptodendritic injury has not been shown to date. To assess this phenomenon, the expression of NLRP3 was first silenced in BV2 cells using the siRNA approach [Figure 3E]. Next, to determine the transfection efficiency, NLRP3 expression was assessed in different groups. Results showed that NLRP3 expression was significantly increased in the Tat exposed BV2 cells compared with the scrambled control group, and that NLRP3 expression was minimal in the siNLRP3 transfected groups in the presence or absence of Tat (50 ng/mL, 24 h) ($P < 0.05$) [Figure 3E]. As shown in Figure 3F, Tat-MDEVs

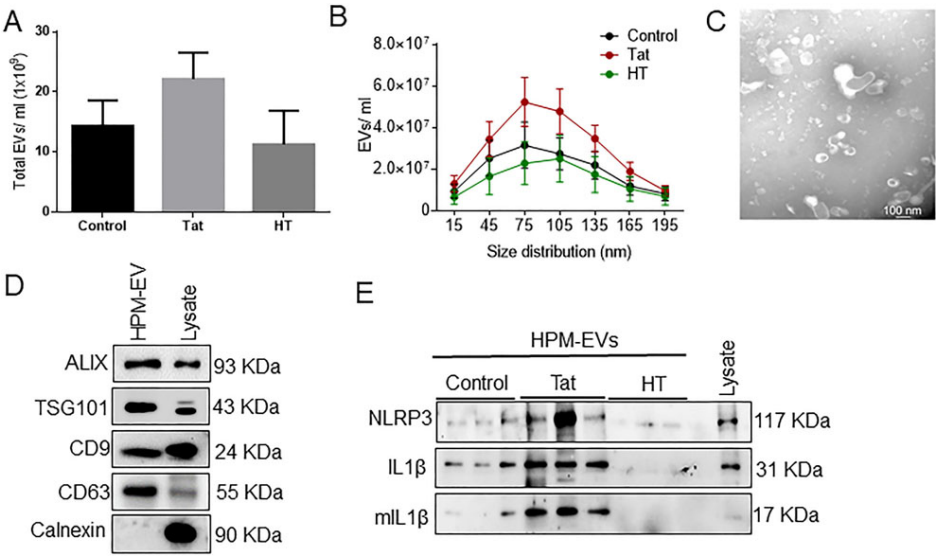

Figure 2. Characterization of microglia-derived exosomes from human primary microglia (HPM). (A) Quantification of the total number of EVs and (B) size distribution of exosomes by NTA using ZetaView. (C) Representative TEM image of exosome particles isolated by ultracentrifugation at 100,000 g. Scale bar 100 nm. (D) Representative western blots showing expression of exosome-specific markers (Alix, TSG101, CD9, CD63). Calnexin is used as a negative control for exosomes. (E) Representative western blot images showing protein expression of NLRP3, pro-IL1β, mIL-1β in control, Tat (50 ng/mL) or HT treated-HPM-derived exosomes. Data are presented as mean ± SEM. Abbreviations: similar as Figure 1.

significantly downregulated the expression of PSD95 compared with the control-MDEV group. Silencing of microglial NLRP3 significantly abrogated Tat-MDEV mediated downregulation of PSD95 in cortical neurons ($P < 0.05$). Similarly, the expression of GAD65 [Figure 3G] and Gephyrin [Figure 3H] was significantly downregulated in neurons exposed to NLRP3 silenced MDEVs exosomes compared with neurons exposed to Tat-MDEVs ($P < 0.05$).

Microglial NLRP3 leads to neuronal dendritic injury

Having demonstrated that Tat stimulated BV2-derived MDEVs induced alterations in the expression of synaptic proteins, we next wanted to assess the role of these MDEVs in mediating neuronal dendritic injury. As expected, and as demonstrated in Figure 4A, synaptic spines were abundantly present in the hippocampal neurons exposed to BV2-siControl MDEVs. There was, however, significant downregulation ($P < 0.05$) of neuronal spines in neurons exposed to BV2 Tat-MDEVs. Interestingly, neurons exposed to microglial NLRP3 silenced Tat-MDEVs showed restoration of spine numbers similar to the control group [Figure 4C]. Interestingly, the expression of the vGLUT1 was significantly ($P < 0.05$) decreased and that of the GAD65 increased in the hippocampal neurons exposed to MDEVs derived from scrambled siRNA+Tat treated BV2 cells, while both of these synaptic proteins remained unchanged in MDEV exposed neurons from NLRP3 silenced BV2 [Figure 4B]. Another important finding of this study was that the most mature spine sub-type, the mushroom type, was present in high numbers in neurons exposed to MDEVs isolated from scrambled siRNA treated BV2 cells, while they decreased significantly in the neurons exposed to MDEVs isolated from scrambled siRNA+Tat treated BV2 cells ($P < 0.05$) [Figure 4D]. A similar trend was also observed for the stubby spines [Figure 4E]. MDEVs isolated from NLRP3 silenced BV2 cells, however, showed a similar trend as that of the scrambled siRNA treated BV2 cells for mushroom and stubby spines [Figure 4D-E]. The numbers of immature filopodial and thin spines, however, did not change significantly across the different groups [Figure 4F-G].

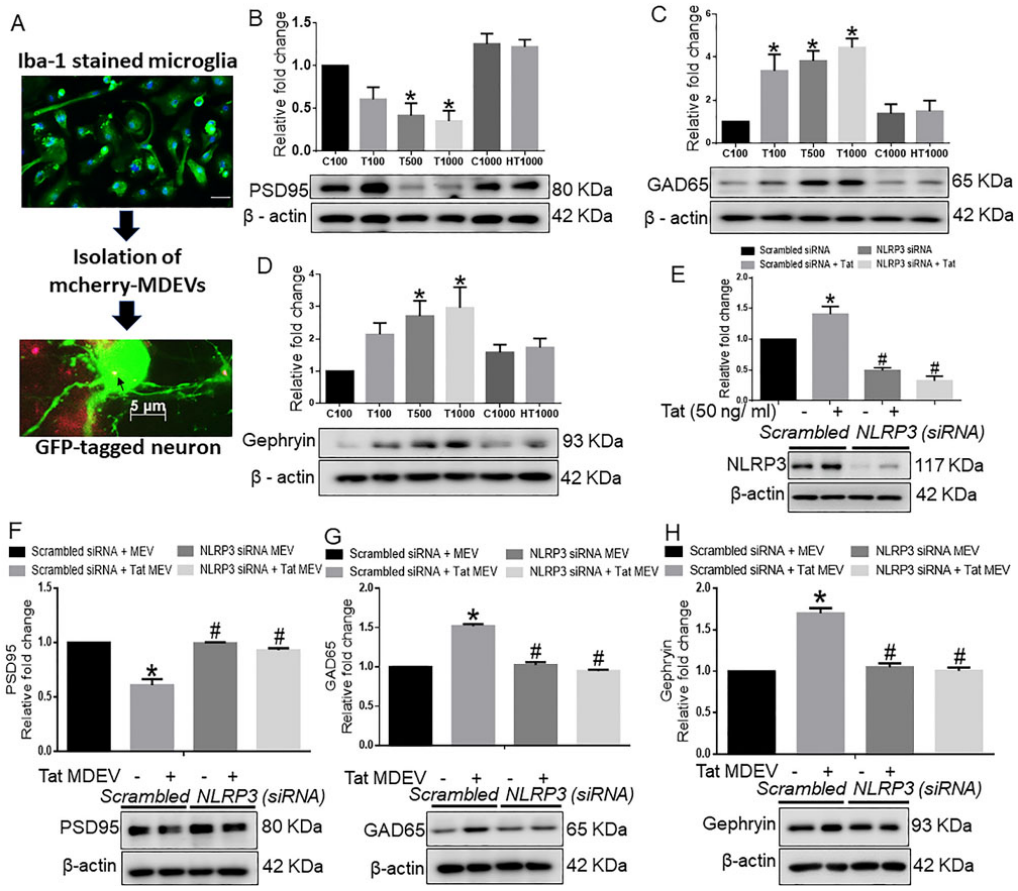

Figure 3. Role of BV2 derived exosomes from BV2 on neuronal synaptic proteins. (A) Schematic representation showing the exposure of BV2-derived exosomes to primary neurons. Representative western blot images and their quantitative analysis showing the dose-dependent effects of BV2-derived exosomes on the expression of (B) PSD95, (C) GAD65, and (D) Gephyrin in rat cortical primary neurons. (E) Representative western blot images and quantitative analysis showing the expression of NLRP3 in BV2 cells transfected with either NLRP3 or scrambled siRNA in the presence of Tat (50 ng/mL) to confirm the transfection efficiency of NLRP3. Representative western blots and their quantitative analysis showing the expression of (F) PSD95, (G) GAD65, and (H) Gephyrin in rat primary cortical neurons exposed with MDEVs from NLRP3 siRNA transfected BV2 cells in the presence of Tat (50 ng/mL). β-actin was used as a loading control. Data are presented as mean ± SEM. $^{*}P < 0.05$ *vs.* siControl MDEV, $^{\#}P < 0.05$ *vs.* Tat MDEV. One-way ANOVA followed the Bonferroni post hoc tests were used for statistical analysis. MDEV: Microglia derived exosomes' HT: heated Tat; NLRP3: NOD-, LRR- and pyrin domain-containing protein 3; PSD95: postsynaptic density protein 95; GAD65: glutamic acid decarboxylase; siRNA: small interfering RNA; Tat: trans-activator of transcription.

HPM exosomes cause neuronal synaptodendritic injury

We next wanted to validate our findings with HPM-derived EVs. We assessed the role of NLRP3 in HPM-derived EVs on a neuronal synaptodendritic injury. Similar to BV2 cells, neurons were also exposed to HPM-derived EVs for 48 h. As shown in Figure 5A, in HPMs transfected with either scrambled or NLRP3 siRNA, Tat significantly ($P < 0.05$) increased NLRP3 expression in HPM. In NLRP3 silenced cells, there was effective NLRP3 silencing. As shown in Figure 5B, HPM-Tat-EVs significantly ($P < 0.05$) downregulated the expression of PSD95 and upregulated the expression of GAD65 in neurons compared with neurons exposed to control MDEVs. In neurons exposed to MDEVs from microglial silenced NLRP3, on the other hand, Tat failed to alter the expression of synaptic proteins ($P < 0.05$). Similarly, in neurons exposed to HPM-Tat-MDEVs, significantly ($P < 0.05$) downregulated the expression of synaptophysin and upregulated the expression of Gephyrin in neurons compared to neurons exposed to control MDEVs. MDEVs from NLRP3 silenced HPM in the presence/absence of Tat showed minimal alterations in the expression of synaptic proteins ($P < 0.05$) [Figure 5C]. Having demonstrated that Tat stimulated HPM-derived EVs

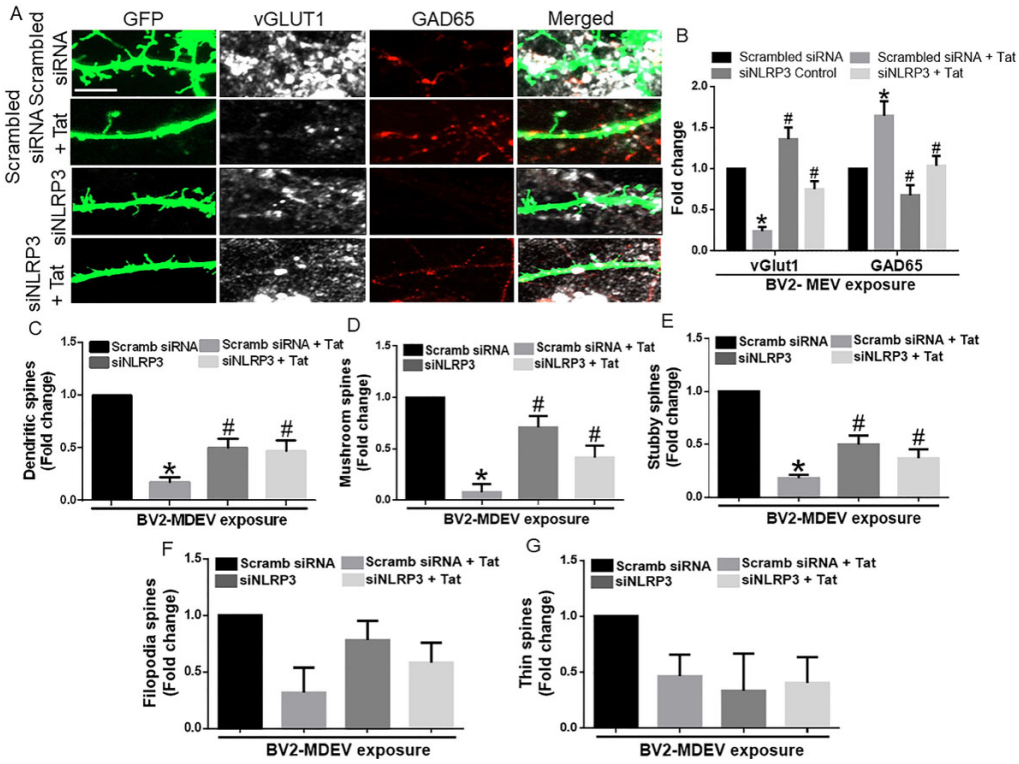

Figure 4. Role of BV2-derived exosomes on neuronal dendritic spines. (A) Representative immunofluorescence images showing hippocampal spine density & expression of vGlut1 and GAD65, after exposure of hippocampal neurons with MDEVs from control, Tat, NLRP3 silenced and NLRP3 silenced- Tat treated BV2 cells. Scale bar: 2 µm. (B) Quantitative analysis of vGlut1 and GAD65 via Image J Launcher software. (C) Quantification of spine numbers in different groups of neurons via Neurolucida software. (D-G) Quantification of spine sub-types in different groups of neurons via Neurolucida software. Data are presented as mean ± SEM. $^{*}P < 0.05$ *vs.* siControl MDEV, $^{\#}P < 0.05$ *vs.* Tat-MDEV. One-way ANOVA, followed by the Bonferroni post hoc tests, was used for statistical analysis. Scramb: scrambled siRNA (small interfering RNA); NLRP3: NOD-, LRR- and pyrin domain-containing protein 3; vGLUT1: vesicular glutamate transporters; GAD65: glutamic acid decarboxylase; Tat: trans-activator of transcription.

induced alterations in the expression of the synaptic proteins, we next wanted to assess the role of these exosomes in neuronal dendritic injury. As demonstrated in Figure 5D, there was an abundant expression of synaptic spines in hippocampal neurons exposed to MDEVs isolated from HPMs treated with scrambled siRNA. Total spine numbers, however, were significantly downregulated ($P < 0.05$) in neurons exposed to MDEVs isolated from HPM transfected with scrambled siRNA+Tat group. Neurons exposed to MDEVs from NLRP3 silenced group showed spine numbers similar to the control group [Figure 5F]. Interestingly, the expression of the vGLUT1 was significantly ($P < 0.05$) decreased, and that of the GAD65 increased in hippocampal neurons exposed to MDEVs isolated from HPM transfected with scrambled siRNA+Tat group. In neurons exposed to MDEVs from NLRP3 silenced HPMs, the expression levels of both the synaptic proteins were comparable to the neurons exposed to control MDEVs [Figure 5E]. Intriguingly, we found that the numbers of mushroom and stubby spines were significantly ($P < 0.05$) decreased ($P < 0.05$) in the neurons exposed to MDEVs isolated from the HPM+Tat group compared to that of control [Figures 5G]. As expected, the NLRP3 silenced groups showed a similar trend as that of the control group [Figure 5G]. However, the immature thin and filopodial spines did not significantly change across the different groups [Figure 5H].

HPM exosomes reduce excitatory neurotransmission

To study the functional alterations in neurons induced by the HPM-exosomes, we recorded miniature

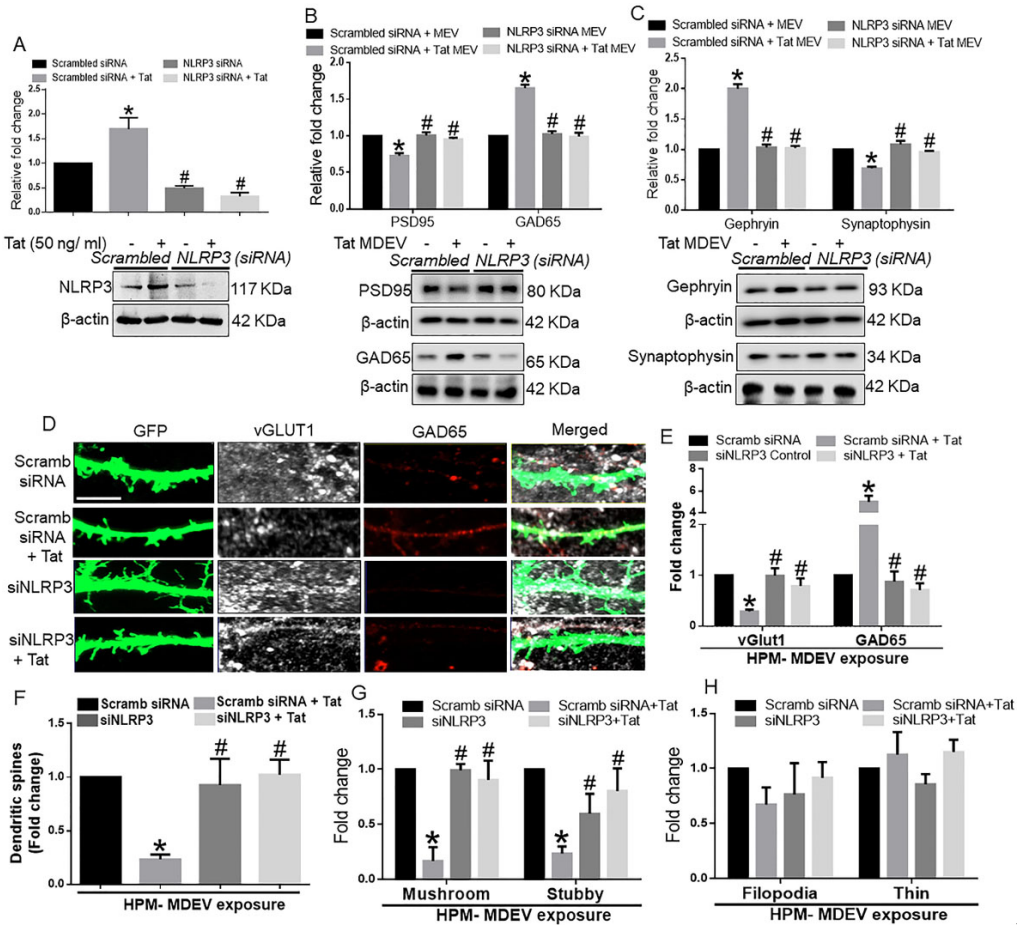

Figure 5. Role of human primary microglia-derived exosomes on neuronal miniature excitatory postsynaptic currents (mEPSC). (A) Representative traces of whole-cell voltage-clamp recording showing mEPSC, (B) mean mEPSC frequencies (Hz), and (C) amplitude (pA) in primary rat hippocampal neurons (DIV 18-22) exposed to MDEVs from control, Tat, NLRP3 silenced and NLRP3 silenced- Tat treated HPM cells. Data are presented as mean ± SEM. $^{*}P < 0.05$ *vs.* siControl MDEV, $^{\#}P < 0.05$ *vs.* Tat MDEV. One-way ANOVA, followed by the Bonferroni post hoc tests, was used for statistical analysis. Scramb: Scrambled siRNA (small interfering RNA); MDEV: microglia derived exosomes; NLRP3: NOD-, LRR- and pyrin domain-containing protein 3; PSD95: postsynaptic density protein 95; vGLUT1: vesicular glutamate transporters; GAD65: glutamic acid decarboxylase; siRNA: small interfering RNA; Tat: trans-activator of transcription.

excitatory postsynaptic currents (mEPSCs) in rat primary hippocampal neurons (DIV 18-22) treated with scrambled siRNA-MDEV, scrambled siRNA+-Tat-MDEV, NLRP3 siRNA MDEV, NLRP3 siRNA+Tat MDEVs isolated from HPM. As demonstrated in Figure 6A-C, scrambled siRNA+-Tat-MDEVs significantly ($P < 0.05$) decreased the excitatory neurotransmission (reduced frequency and amplitude) in rat primary neurons. On the other hand, in rat primary neurons exposed to NLRP3 siRNA MDEVs, NLRP3 siRNA+Tat MDEVs, the mEPSCs were comparable to the neurons exposed to scrambled siRNA-MDEV; however, the amplitudes still remained low [Figure 6C].

Schematic representation of microglia-neuronal cross-talk in synaptodendritic injury involving Tat MDEVs

In this study, we demonstrated that exposure of microglia (BV2/HPM) to HIV-1 Tat resulted in the release of MDEVs carrying NLRP3 and IL1β cargoes, which could be taken up by the neurons. Upon uptake by the neurons of the NLRP3, IL1β containing MDEV cargoes, there was a decreased expression of synaptic proteins PSD95, excitatory vGLUT1, and an increase in inhibitory synaptic proteins - GAD65 and

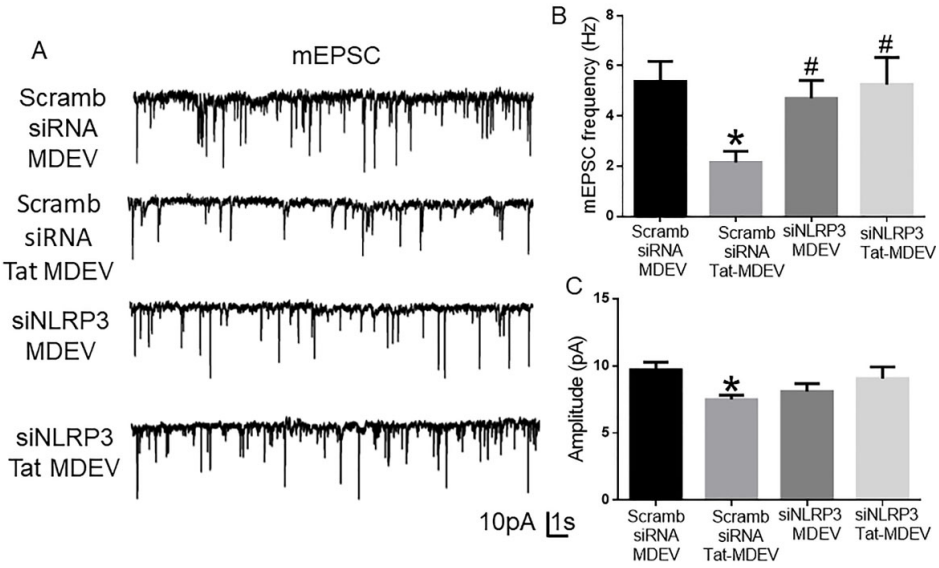

Figure 6. Schematic representation of the role of microglial NLRP3 on neuronal synaptodendritic injury via exosomes. Exposure of microglia (BV2 or HPM) with HIV-1 Tat results in the activation of NLRP3 inflammasome complex, whichleads to the production of IL-1 β and microglial activation. Thereafter, these NLRP3 and IL1β can be packaged in the exosomes and released by the microglia. These exosomes carrying NLRP3/IL1β upon uptake by the neurons result in alteration of synaptic proteins (PSD95, vGLUT1, GAD65, Gephyrin) and dendritic injury (change in the spine- numbers and sub-types). Overall, these microglial EVs carrying NLRP3 cargoes can cause synaptodendritic injury resulting in HAND in patients via microglia-neuron cross talk. NLRP3: NOD-, LRR- and pyrin domain-containing protein 3; ASC: apoptosis-associated speck-like protein; IL 1β: interleukin 1β; PSD95: postsynaptic density protein 95; vGLUT1: vesicular glutamate transporters; GAD65: glutamic acid decarboxylase; Tat: trans-activator of transcription.

Gephyrin. Further, these MDEVs also resulted in a loss of dendric spines as well as a change in the ratio of spine sub-types (mushroom, stubby, filopodia, thin). Silencing of microglial NLRP3 led to the protection of Tat MDEV mediated neuronal synaptodendritic injury [Figure 7]. Overall, the Tat-MDEVs carrying NLRP3 cargoes could mediate neuronal synaptodendritic injury underlying HAND involving the microglial-neuronal cross-talk [Figure 7].

DISCUSSION

HAND is a common cause of morbidity in HIV-1 positive individuals who are on cART[42]. The prevalence of milder forms of the disease, such as asymptomatic neurocognitive impairment (ANI) or mild-neurocognitive disorder (MND), however, continues to increase, accounting for ~70% of HAND cases[42]. As demonstrated by several investigators, people living with HIV-1 on cART continue to exhibit neuronal damage[43]. Although neurons are less susceptible to direct infection, infected microglia can mediate neuronal damage involving both the EVs and non-EV fractions[44-47]. In healthy cells, microglia secrete EVs to support the metabolic functions of neurons and to provide them with substrates needed for energy metabolism during synaptic activity[21-23]. Ample evidence suggests that EVs play a significant role in microglia-mediated neuroinflammation and the progression of several neurodegenerative disorders in the brain[27,48,49]. Recent studies indicate that EVs are key players in intercellular communication that underlies physiological processes such as synaptic plasticity and maintenance of myelination[50,51]. Similarly, MDEVs also affect neuronal functions by delivering proinflammatory cytokines and other neurotoxic mediators under pathological conditions[26,27].

As reviewed by Saylor *et al.* (2016), evidence suggests that inflammation plays a critical role in HAND[42]. HIV-1 Tat protein has been shown to be present in the brains of infected individuals and is an important

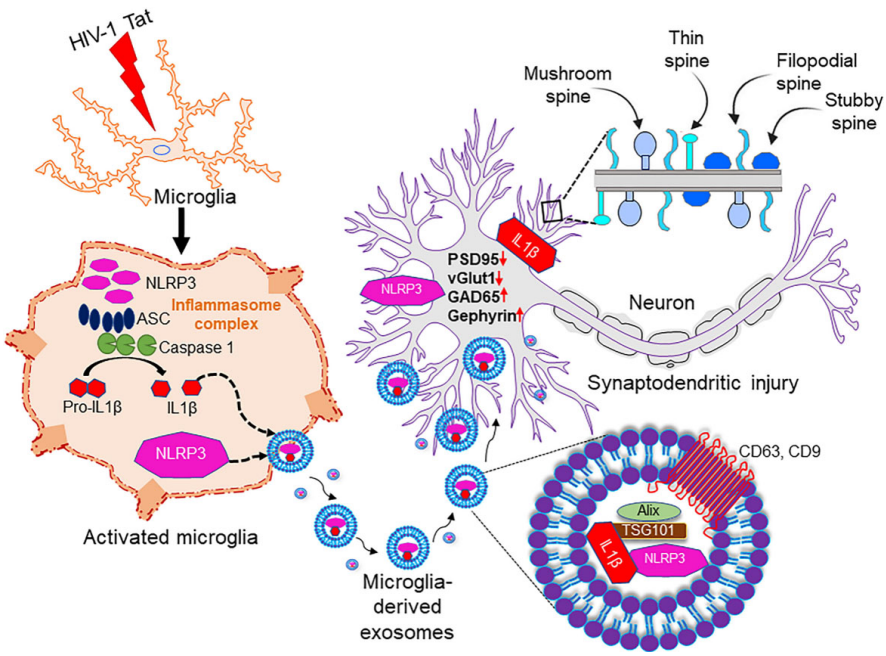

Figure 7. Schematic representation of the role of microglial NLRP3 on neuronal synaptodendritic injury via exosomes. Exposure of microglia (BV2 or HPM) with HIV-1 Tat results in the activation of NLRP3 inflammasome complex, whichleads to the production of IL-1 β and microglial activation. Thereafter, these NLRP3 and IL1β can be packaged in the exosomes and released by the microglia. These exosomes carrying NLRP3/IL1β upon uptake by the neurons result in alteration of synaptic proteins (PSD95, vGLUT1, GAD65, Gephyrin) and dendritic injury (change in the spine- numbers and sub-types). Overall, these microglial EVs carrying NLRP3 cargoes can cause synaptodendritic injury resulting in HAND in patients via microglia-neuron cross talk. NLRP3: NOD-, LRR- and pyrin domain-containing protein 3; ASC: apoptosis-associated speck-like protein; IL 1β: interleukin 1β; PSD95: postsynaptic density protein 95; vGLUT1: vesicular glutamate transporters; GAD65: glutamic acid decarboxylase; Tat: trans-activator of transcription.

contributor to the development of HAND[52-55]. In our previous study, we showed that HIV-1 Tat-mediated microglial activation involves the NLRP3 inflammasome pathway. In brief, our previous findings showed that Tat primes and activates the NLRP3 inflammasome in microglia, resulting in the release of IL-1β, a highly potent cytokine that, in turn, induces other cytokines, including IL-6 and TNF-α, to further exacerbate neuroinflammation[36]. Other investigators have also shown induction of the NLRP3 inflammasome in both microglia and monocytes during HIV-1 infection[56-58]. Interestingly, individuals who developed ANI and MND have elevated levels of NLRP3 activators such as ceramide and multiple forms of cholesterol compared with cognitively normal HIV-1-positive individuals[59]. In the present study, we demonstrate that Tat-induced NLRP3 in the microglia can be packaged in MDEVs and is released in the extracellular space. The MDEVs carrying the NLRP3 cargoes can be taken up by the neurons, in turn leading to synaptodendritic injury and functional impairment [Figure 7]. Although NLRP3 is primarily induced by microglia, recent reports have also demonstrated the role of neuronal NLRP3 in Parkinson's Disease[41]. There is, however, no evidence of activation of NLRP3 in neurons in HIV-1 to date. While the role of microglial NLRP3 in neuronal damage has been demonstrated in the presence of HIV protein gp120[60], the role of Tat in microglial NLRP3 mediated neuronal damage remains elusive.

In the present study, we demonstrated that Tat exposed MDEVs had a size distribution ranging from 50-150 nm and were found to carry the NLRP3 and IL1β cargoes. The numbers of MDEVs, however, did not change in the presence or absence of Tat. The current study was not aimed at assessing the direct role of IL1 β on neuronal injury, instead was focused on the indirect effect of activation of the microglial NLRP3 pathway. Exposure of neurons to Tat-MDEVs resulted in downregulation of synaptic proteins- PSD95,

synaptophysin, excitatory vGLUT1 and upregulation of inhibitory proteins- Gephyrin, GAD65, thus suggesting impaired neuronal transmissibility. Furthermore, Tat MDEVs exposed neurons also exhibited decreased mEPSCs, thereby implicating functional impairment of the neurons. Previous studies have demonstrated damage of the pyramidal neurons in the neocortex during HIV infection with alterations in excitatory neurotransmitters and inflammatory markers[43]. Interestingly, it was also shown that PSD95 expression was downregulated[61], and Gephyrin expression was increased in neurons following Tat exposure[17]. Other reports in HIV transgenic mice have demonstrated an increase in Gephryin, associated with inhibitory transmission and minimal dendritic pathology[62]. Dysregulation of excitatory/inhibitory proteins[62,63] could underlie functional impairment of the neurons, as evidenced by the increase in mEPSCs in our study. Previous reports from our laboratory have also shown that HIV-1 Tat could also induce alteration of EV cargoes from astrocytes, in turn leading to impairment of the synaptic architecture of neurons[28,29].

Alterations of synaptic proteins and cognitive deficits are often associated with a neuronal spine injury, as shown in HIV-Tg rats[64]. Our present study showed loss of dendritic spines, mature spine sub-types mushroom and stubby, following exposure of neurons with Tat-MDEVs. Alterations in total spine density have been demonstrated by several investigators in HAND[62-64]. In line with our study, clinical studies have shown that HIV patients exhibit loss of neurons, and aberrant sprouting, and dystrophic synaptodendritic connections in the CNS[65], with decreased expression of MAP2 and neurofilament, and markers for synaptodendritic connectivity. Intriguingly, it has also been reported that damage initiates in the synapses and dendrites and then spreads to the rest of the neuron, leading to apoptosis[66,67]. Association of alterations of spine sub-types with synaptodendritic injury has not been reported earlier in HAND. Additionally, the role of MDEVs in the process of synaptodendritic injury is a novel finding of this study. Next, to assess the role of microglial NLRP3 in this process, neurons were exposed to MDEVs from NLRP3 silenced microglia in the presence of Tat. Results showed that these MDEVs, derived from NLRP3 silenced microglia, abrogated Tat-MDEV mediated neurotoxicity as evidenced by restoration of changes in synaptic proteins- PSD95, Synaptophysin, GAD65, and Gephyrin as well as total spines and spine sub-types. The protective role was also observed in the frequency of the mEPSCs, but not in the amplitudes.

To summarize, this study demonstrated that HIV-1 Tat exposure can lead to the release of MDEVs from microglia, carrying NLRP3 cargoes. These MDEVs, upon being taken up by the neurons, resulted in synaptodendritic injury and functional impairment- as evidenced by decreased mEPSCs. The role of microglia-neuronal cross-talk via MDEVs has not been demonstrated earlier in the context of HAND; specifically, how the microglial NLRP3 plays a role in this process will open future avenues for the development of adjunctive therapeutics for HAND. Although we demonstrated the role of microglial NLRP3 in neuronal injury, further studies are warranted to assess the mechanistic underpinnings by which MDEVs mediate neuronal damage. The role of NLRP3 in inflammation is very well known; however, the role of the same NLRP3 in neuronal damage is an interesting finding that implicates the role of the therapeutic potential of NLRP3 blockers as a treatment option for HAND and other neuroinflammatory conditions.

DECLARATIONS

Acknowledgements

We are thankful to Natasha Ferguson for her technical assistance. We are also thankful to the electron microscopy core facility (UNMC).

Authors' contributions

Performed experiments, analysed data and drafted the manuscript: Kannan M, Singh S
Performed experiments and drafted manuscript: Oladapo AA, Chemparthy DT
Performed and analysed electrophysiological study: Gawande DY, Dravid SM
Conceived and conceptualized the idea, provided oversight of the experimental plan, edited the manuscript, and funded the study: Buch S
Conceptualized the idea, performed the experiments, analyzed the data, prepared the figures, drafted and edited the manuscript: Sil S

Availability of data and materials

Data and materials will be available on request.

Financial support and sponsorship

This project has been supported by NIH- R01MH112848, R01DA040397 (S. Buch).

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

The animal experiment is approved by the ethical committee of the University of Nebraska Medical Center (IACUC# 20-057-07-FC).

Consent for publication

Not applicable.

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REFERENCES

1. Joseph SB, Arrildt KT, Sturdevant CB, Swanstrom R. HIV-1 target cells in the CNS. *J Neurovirol* 2015;21:276-89. DOI PubMed PMC
2. Kranick SM, Nath A. Neurologic complications of HIV-1 infection and its treatment in the era of antiretroviral therapy. *Continuum (Minneap Minn)* 2012;18:1319-37. DOI PubMed PMC
3. Rojas-Celis V, Valiente-Echeverría F, Soto-Rifo R, Toro-Ascuy D. New challenges of HIV-1 infection: how HIV-1 attacks and resides in the central nervous system. *Cells* 2019;8:1245. DOI PubMed PMC
4. Kusdra L, McGuire D, Pulliam L. Changes in monocyte/macrophage neurotoxicity in the era of HAART: implications for HIV-associated dementia. *AIDS* 2002;16:31-8. DOI PubMed
5. Debaisieux S, Rayne F, Yezid H, Beaumelle B. The ins and outs of HIV-1 Tat. *Traffic* 2012;13:355-63. DOI PubMed
6. Schwarze SR, Ho A, Vocero-Akbani A, Dowdy SF. In vivo protein transduction: delivery of a biologically active protein into the mouse. *Science* 1999;285:1569-72. DOI PubMed
7. Roy S, Delling U, Chen CH, Rosen CA, Sonenberg N. A bulge structure in HIV-1 TAR RNA is required for Tat binding and Tat-mediated trans-activation. *Genes Dev* 1990;4:1365-73. DOI PubMed
8. Rice AP. The HIV-1 Tat protein: mechanism of action and target for HIV-1 cure strategies. *Curr Pharm Des* 2017;23:4098-102. DOI PubMed PMC
9. Garden GA. Microglia in human immunodeficiency virus-associated neurodegeneration. *Glia* 2002;40:240-51. DOI PubMed
10. Churchill MJ, Wesselingh SL, Cowley D, et al. Extensive astrocyte infection is prominent in human immunodeficiency virus-associated dementia. *Ann Neurol* 2009;66:253-8. DOI PubMed
11. Brack-Werner R. Astrocytes: HIV cellular reservoirs and important participants in neuropathogenesis. *AIDS* 1999;13:1-22. DOI PubMed
12. Sabatier JM, Vives E, Mabrouk K, et al. Evidence for neurotoxic activity of tat from human immunodeficiency virus type 1. *J Virol* 1991;65:961-7. DOI PubMed PMC
13. Conant K, Tornatore C, Atwood W, Meyers K, Traub R, Major E. In vivo and in vitro infection of the astrocyte by HIV-1. *Advances in Neuroimmunology* 1994;4:287-9. DOI PubMed

14. Tornatore C, Meyers K, Atwood W, Conant K, Major E. Temporal patterns of human immunodeficiency virus type 1 transcripts in human fetal astrocytes. *J Virol* 1994;68:93-102. DOI PubMed PMC
15. Liu Y, Jones M, Hingtgen CM, et al. Uptake of HIV-1 tat protein mediated by low-density lipoprotein receptor-related protein disrupts the neuronal metabolic balance of the receptor ligands. *Nat Med* 2000;6:1380-7. DOI PubMed
16. King JE, Eugenin EA, Buckner CM, Berman JW. HIV tat and neurotoxicity. *Microbes Infect* 2006;8:1347-57. DOI PubMed
17. Hargus NJ, Thayer SA. Human immunodeficiency virus-1 Tat protein increases the number of inhibitory synapses between hippocampal neurons in culture. *J Neurosci* 2013;33:17908-20. DOI PubMed PMC
18. Santerre M, Bagashev A, Gorecki L, et al. HIV-1 Tat protein promotes neuronal dysregulation by inhibiting E2F transcription factor 3 (E2F3). *J Biol Chem* 2019;294:3618-33. DOI PubMed PMC
19. Marinelli S, Basilico B, Marrone MC, Ragozzino D. Microglia-neuron crosstalk: signaling mechanism and control of synaptic transmission. *Semin Cell Dev Biol* 2019;94:138-51. DOI PubMed
20. Matejuk A, Ransohoff RM. Crosstalk between astrocytes and microglia: an overview. *Front Immunol* 2020;11:1416. DOI PubMed PMC
21. Potolicchio I, Carven GJ, Xu X, et al. Proteomic analysis of microglia-derived exosomes: metabolic role of the aminopeptidase CD13 in neuropeptide catabolism. *J Immunol* 2005;175:2237-43. DOI PubMed
22. Fauré J, Lachenal G, Court M, et al. Exosomes are released by cultured cortical neurones. *Mol Cell Neurosci* 2006;31:642-8. DOI PubMed
23. Upadhya R, Zingg W, Shetty S, Shetty AK. Astrocyte-derived extracellular vesicles: neuroreparative properties and role in the pathogenesis of neurodegenerative disorders. *J Control Release* 2020;323:225-39. DOI PubMed PMC
24. Delpech JC, Herron S, Botros MB, Ikezu T. Neuroimmune crosstalk through extracellular vesicles in health and disease. *Trends Neurosci* 2019;42:361-72. DOI PubMed PMC
25. You Y, Ikezu T. Emerging roles of extracellular vesicles in neurodegenerative disorders. *Neurobiol Dis* 2019;130:104512. DOI PubMed PMC
26. Yang Y, Boza-Serrano A, Dunning CJR, Clausen BH, Lambertsen KL, Deierborg T. Inflammation leads to distinct populations of extracellular vesicles from microglia. *J Neuroinflammation* 2018;15:168. DOI PubMed PMC
27. Aires ID, Ribeiro-Rodrigues T, Boia R, et al. Microglial extracellular vesicles as vehicles for neurodegeneration spreading. *Biomolecules* 2021;11:770. DOI PubMed PMC
28. Dagur RS, Liao K, Sil S, et al. Neuronal-derived extracellular vesicles are enriched in the brain and serum of HIV-1 transgenic rats. *J Extracell Vesicles* 2020;9:1703249. DOI PubMed PMC
29. Sil S, Singh S, Chemparathy DT, Chivero ET, Gordon L, Buch S. Astrocytes & astrocyte derived extracellular vesicles in morphine induced amyloidopathy: implications for cognitive deficits in opiate abusers. *Aging Dis* 2021;12:1389-408. DOI PubMed PMC
30. Apcher G, Heink S, Zantopf D, et al. Human immunodeficiency virus-1 Tat protein interacts with distinct proteasomal α and β subunits. *FEBS Letters* 2003;553:200-4. DOI PubMed
31. Sil S, Niu F, Tom E, Liao K, Periyasamy P, Buch S. Cocaine mediated neuroinflammation: role of dysregulated autophagy in pericytes. *Mol Neurobiol* 2019;56:3576-90. DOI PubMed PMC
32. McCarthy MK, Weinberg JB. The immunoproteasome and viral infection: a complex regulator of inflammation. *Front Microbiol* 2015;6:21. DOI PubMed PMC
33. Thangaraj A, Periyasamy P, Liao K, et al. HIV-1 TAT-mediated microglial activation: role of mitochondrial dysfunction and defective mitophagy. *Autophagy* 2018;14:1596-619. DOI PubMed PMC
34. Minakaki G, Menges S, Kittel A, et al. Autophagy inhibition promotes SNCA/alpha-synuclein release and transfer via extracellular vesicles with a hybrid autophagosome-exosome-like phenotype. *Autophagy* 2018;14:98-119. DOI PubMed PMC
35. Leidal AM, Debnath J. Emerging roles for the autophagy machinery in extracellular vesicle biogenesis and secretion. *FASEB Bioadv* 2021;3:377-86. DOI PubMed PMC
36. Chivero ET, Guo ML, Periyasamy P, Liao K, Callen SE, Buch S. HIV-1 Tat primes and activates microglial NLRP3 inflammasome-mediated neuroinflammation. *J Neurosci* 2017;37:3599-609. DOI PubMed PMC
37. Arikkath J, Peng IF, Ng YG, et al. Delta-catenin regulates spine and synapse morphogenesis and function in hippocampal neurons during development. *J Neurosci* 2009;29:5435-42. DOI PubMed PMC
38. Beaudoin GM 3rd, Lee SH, Singh D, et al. Culturing pyramidal neurons from the early postnatal mouse hippocampus and cortex. *Nat Protoc* 2012;7:1741-54. DOI PubMed
39. Hu G, Niu F, Liao K, et al. HIV-1 Tat-induced astrocytic extracellular vesicle miR-7 impairs synaptic architecture. *J Neuroimmune Pharmacol* 2020;15:538-53. DOI PubMed PMC
40. Song L, Pei L, Yao S, Wu Y, Shang Y. NLRP3 inflammasome in neurological diseases, from functions to therapies. *Front Cell Neurosci* 2017;11:63. DOI PubMed PMC
41. von Herrmann KM, Salas LA, Martinez EM, et al. NLRP3 expression in mesencephalic neurons and characterization of a rare NLRP3 polymorphism associated with decreased risk of Parkinson's disease. *NPJ Parkinsons Dis* 2018;4:24. DOI PubMed PMC
42. Saylor D, Dickens AM, Sacktor N, et al. HIV-associated neurocognitive disorder--pathogenesis and prospects for treatment. *Nat Rev Neurol* 2016;12:234-48. DOI PubMed PMC
43. Kovalevich J, Langford D. Neuronal toxicity in HIV CNS disease. *Future Virol* 2012;7:687-98. DOI PubMed PMC
44. Alvarez-Carbonell D, Ye F, Ramanath N, et al. Cross-talk between microglia and neurons regulates HIV latency. *PLoS Pathog*

2019;15:e1008249. DOI PubMed PMC
45. Borrajo A, Spuch C, Penedo MA, Olivares JM, Agís-Balboa RC. Important role of microglia in HIV-1 associated neurocognitive disorders and the molecular pathways implicated in its pathogenesis. *Ann Med* 2021;53:43-69. DOI PubMed PMC
46. Ru W, Tang SJ. HIV-associated synaptic degeneration. *Mol Brain* 2017;10:40. DOI PubMed PMC
47. Ru W, Liu X, Bae C, et al. Microglia mediate HIV-1 gp120-induced synaptic degeneration in spinal pain neural circuits. *J Neurosci* 2019;39:8408-21. DOI PubMed PMC
48. Guo M, Hao Y, Feng Y, et al. Microglial exosomes in neurodegenerative disease. *Front Mol Neurosci* 2021;14:630808. DOI PubMed PMC
49. Paolicelli RC, Bergamini G, Rajendran L. Cell-to-cell communication by extracellular vesicles: focus on microglia. *Neuroscience* 2019;405:148-57. DOI PubMed
50. Holm MM, Kaiser J, Schwab ME. Extracellular vesicles: multimodal envoys in neural maintenance and repair. *Trends Neurosci* 2018;41:360-72. DOI PubMed
51. Schnatz A, Müller C, Brahmer A, Krämer-Albers EM. Extracellular vesicles in neural cell interaction and CNS homeostasis. *FASEB Bioadv* 2021;3:577-92. DOI PubMed PMC
52. Valle L, Croul S, Morgello S, Amini S, Rappaport J, Khalili K. Detection of HIV-1 Tat and JCV capsid protein, VP1, in AIDS brain with progressive multifocal leukoencephalopathy. *J Neurovirol* 2000;6:221-8. DOI PubMed
53. Hudson L, Liu J, Nath A, et al. Detection of the human immunodeficiency virus regulatory protein tat in CNS tissues. *J Neurovirol* 2000;6:145-55. DOI PubMed
54. Li W, Li G, Steiner J, Nath A. Role of Tat protein in HIV neuropathogenesis. *Neurotox Res* 2009;16:205-20. DOI PubMed
55. Johnson TP, Patel K, Johnson KR, et al. Induction of IL-17 and nonclassical T-cell activation by HIV-Tat protein. *Proc Natl Acad Sci U S A* 2013;110:13588-93. DOI PubMed PMC
56. Guo H, Gao J, Taxman DJ, Ting JP, Su L. HIV-1 infection induces interleukin-1β production via TLR8 protein-dependent and NLRP3 inflammasome mechanisms in human monocytes. *J Biol Chem* 2014;289:21716-26. DOI PubMed PMC
57. Hernandez JC, Latz E, Urcuqui-Inchima S. HIV-1 induces the first signal to activate the NLRP3 inflammasome in monocyte-derived macrophages. *Intervirology* 2014;57:36-42. DOI PubMed
58. Walsh JG, Reinke SN, Mamik MK, et al. Rapid inflammasome activation in microglia contributes to brain disease in HIV/AIDS. *Retrovirology* 2014;11:35. DOI PubMed PMC
59. Bandaru VV, Mielke MM, Sacktor N, et al. A lipid storage-like disorder contributes to cognitive decline in HIV-infected subjects. *Neurology* 2013;81:1492-9. DOI PubMed PMC
60. He X, Yang W, Zeng Z, et al. NLRP3-dependent pyroptosis is required for HIV-1 gp120-induced neuropathology. *Cell Mol Immunol* 2020;17:283-99. DOI PubMed PMC
61. Kim HJ, Martemyanov KA, Thayer SA. Human immunodeficiency virus protein Tat induces synapse loss via a reversible process that is distinct from cell death. *J Neurosci* 2008;28:12604-13. DOI PubMed PMC
62. Fitting S, Ignatowska-Jankowska BM, Bull C, et al. Synaptic dysfunction in the hippocampus accompanies learning and memory deficits in human immunodeficiency virus type-1 Tat transgenic mice. *Biol Psychiatry* 2013;73:443-53. DOI PubMed PMC
63. Marks WD, Paris JJ, Schier CJ, et al. HIV-1 Tat causes cognitive deficits and selective loss of parvalbumin, somatostatin, and neuronal nitric oxide synthase expressing hippocampal CA1 interneuron subpopulations. *J Neurovirol* 2016;22:747-62. DOI PubMed PMC
64. Roscoe RF Jr, Mactutus CF, Booze RM. HIV-1 transgenic female rat: synaptodendritic alterations of medium spiny neurons in the nucleus accumbens. *J Neuroimmune Pharmacol* 2014;9:642-53. DOI PubMed PMC
65. Ellis R, Langford D, Masliah E. HIV and antiretroviral therapy in the brain: neuronal injury and repair. *Nat Rev Neurosci* 2007;8:33-44. DOI PubMed
66. Everall IP, Heaton RK, Marcotte TD, et al. Cortical synaptic density is reduced in mild to moderate human immunodeficiency virus neurocognitive disorder. HNRC Group. HIV Neurobehavioral Research Center. *Brain Pathol* 1999;9:209-17. DOI PubMed PMC
67. Masliah E, Heaton RK, Marcotte TD, et al. Dendritic injury is a pathological substrate for human immunodeficiency virus-related cognitive disorders. HNRC Group. The HIV Neurobehavioral Research Center. *Ann Neurol* 1997;42:963-72. DOI PubMed

Shinge *et al. Extracell Vesicles Circ Nucleic Acids* 2022;3:150-62
DOI: 10.20517/evcna.2021.25

Extracellular Vesicles and Circulating Nucleic Acids

Review

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New insights of engineering plant exosome-like nanovesicles as a nanoplatform for therapeutics and drug delivery

Shafiu A. Umar Shinge[1], Yin Xiao[2], Jiang Xia[3], Yujie Liang[1,4], Li Duan[1]

[1]Department of Orthopedics, Shenzhen Key Laboratory of Tissue Engineering, Guangdong Provincial Research Center for Artificial Intelligence and Digital Orthopedic Technology, The First Affiliated Hospital of Shenzhen University, Shenzhen Second People's Hospital, Shenzhen 518035, Guangdong, China.
[2]Institute of Health and Biomedical Innovation, Faculty of Science and Engineering, Queensland University of Technology, Kelvin Grove Campus, Brisbane 4000, Australia.
[3]Department of Chemistry, The Chinese University of Hong Kong, Shatin, China.
[4]Department of Child and Adolescent Psychiatry, Shenzhen Kangning Hospital, Shenzhen Mental Health Center, Shenzhen 518020, Guangdong, China.

Correspondence to: Dr. Li Duan, Department of Orthopedics, Shenzhen Key Laboratory of Tissue Engineering, Guangdong Provincial Research Center for Artificial Intelligence and Digital Orthopedic Technology, The First Affiliated Hospital of Shenzhen University, Shenzhen Second People's Hospital, No. 3002 Sungang West Road, Shenzhen 518035, Guangdong, China. E-mail: duanl@szu.edu.cn; Dr. Yujie Liang, Department of Orthopedics, Shenzhen Key Laboratory of Tissue Engineering, Guangdong Provincial Research Center for Artificial Intelligence and Digital Orthopedic Technology, The First Affiliated Hospital of Shenzhen University, Shenzhen Second People's Hospital, Shenzhen 518035, Guangdong, China. E-mail: liangyjie@126.com

How to cite this article: Shinge SAU, Xiao Y, Xia J, Liang Y, Duan L. New insights of engineering plant exosome-like nanovesicles as a nanoplatform for therapeutics and drug delivery. *Extracell Vesicles Circ Nucleic Acids* 2022;3:150-62. https://dx.doi.org/10.20517/evcna.2021.25

Received: 21 Dec 2021 **First Decision:** 7 Feb 2022 **Revised:** 8 Feb 2022 **Accepted:** 24 Jun 2022 **Published:** 29 Jun 2022

Academic Editors: Yoke Peng Loh, Cheng Jiang **Copy Editor:** Tiantian Shi **Production Editor:** Tiantian Shi

Abstract

Plant exosome-like nanovesicles (PELNVs) are membrane-encapsulated nanostructures released from cells into their surroundings. PELNVs have an important role in intercellular and interspecies communication in all three domains of life. They act as protective compartments for the long-distance transit of signal molecules like proteins, nucleic acids, lipids, and other metabolites. A range of plants and vegetables can emit PELNVs. The importance of PELNVs in interspecies communication stems from their concentration in biomolecules (lipids, proteins, and miRNAs), lack of toxicity, ease of internalization by cells, and anti-inflammatory, immune-modulatory, and regenerative characteristics. PELNVs derived from numerous fruits and vegetables are biocompatible, biodegradable, and abundant in various plant species. Moreover, their convincing physicochemical characteristics

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underpin their modulative role in physiological and pathological processes, all of which have fueled speculation that these nanovesicles could be particularly adept at developing future-generation bio-therapeutic platforms. The goal of this review was not only to present an overview of the identified roles of PELNVs in physiology and pathology, but also to provide new insight toward their engineering for effective therapeutics and drug delivery nanoplatforms, a clue for future direction to the ongoing research gaps.

Keywords: Plant exosome-like nanovesicles (PELNVs), engineering exosomes, nanotheranostics

INTRODUCTION

Exosomes are extracellular vesicles with a 30-150 nm diameter secreted by eukaryotic cells. They are vesicles released from the cells after the fusion of intracellular multivesicular bodies (MVBs) [Figure 1]. After discovering exosomes in animal cells, more and more evidence shows that MVBs and plant exosome-like nanovesicles (PELNVs) appear in plants[1,2]. PELNVs have been isolated using different methods and show similar morphology and structure to animal exosomes. They have a phospholipid bilayer structure, contain proteins and microRNAs (miRNAs), and are saucer-shaped or cup-shaped can be observed with a transmission electron microscope (TEM). PELNVs are nanoscale particles released from various plants, including broccoli[3], ginger[4], grapefruits, and lemon[5,6]. They are crucial for normal cellular homeostasis, including control of the immune system, intercellular and interkingdom communications[7], and involvement in physiological responses and pathological progression[8]. Moreover, their molecular constituents were correlated with numerous disorders and treatment responses, suggesting their potential for application as diagnostic instruments[5]. They are also employed in the development of tissue engineering and reconstructing, nucleic acids, and chemotherapeutical agent delivery, thus emerging as a novel form of nanomedicine.

Compared to synthetic nanocarriers, PELNVs are far better because they do not cause cell toxicity[5]. PELNVs also stimulate intestinal tissue renewal. Their exclusive lipid and miRNA content can modulate gut microbiota and play biological roles in fighting inflammatory disorders, including liver steatosis, colitis disease, and even cancers[9]. PELNVs can be loaded with therapeutic factors, including siRNAs, DNA expression vectors, proteins, and macromolecular therapeutics, and transferred to specific tissues in various disorders[10]. PELNVs can also be functionalized to deliver drugs into a target tissue conveniently. Despite all these promising features of PELNVs, the concept of molecular and cellular mechanisms accountable for their bio-functions is limited. However, the high pressure of interstitial fluid hinders homogeneous distributing and effective internalization of drugs in specific tissues such as a solid tumor. Functionalization of PELNVs nanocarriers could be an innovative approach for the delivery of drugs[11,12].

Furthermore, poor loading and encapsulating efficacy and problems with exogenic hydrophilic macromolecules delivery and the possible delivery of undesirable cargo substances innately exist within PELNVs. This review discusses the recent development in PELNVs research and develops mechanistic insights for advancing their engineering for innovative therapeutic and drug delivery nanoplatforms. We propose that engineering the PELNVs, through an innovative approach for developing designer PELNVs with greatly enhanced and adjustable communication efficiency, is highly necessary to address these challenges. Thus, PELNVs can be offloaded, reengineered, functionalized, and reloaded with a cargo of choice or co-loaded with their contents without distorting their structural integrity to ensure higher loading and encapsulating efficacy cargo specificity and precision[13].

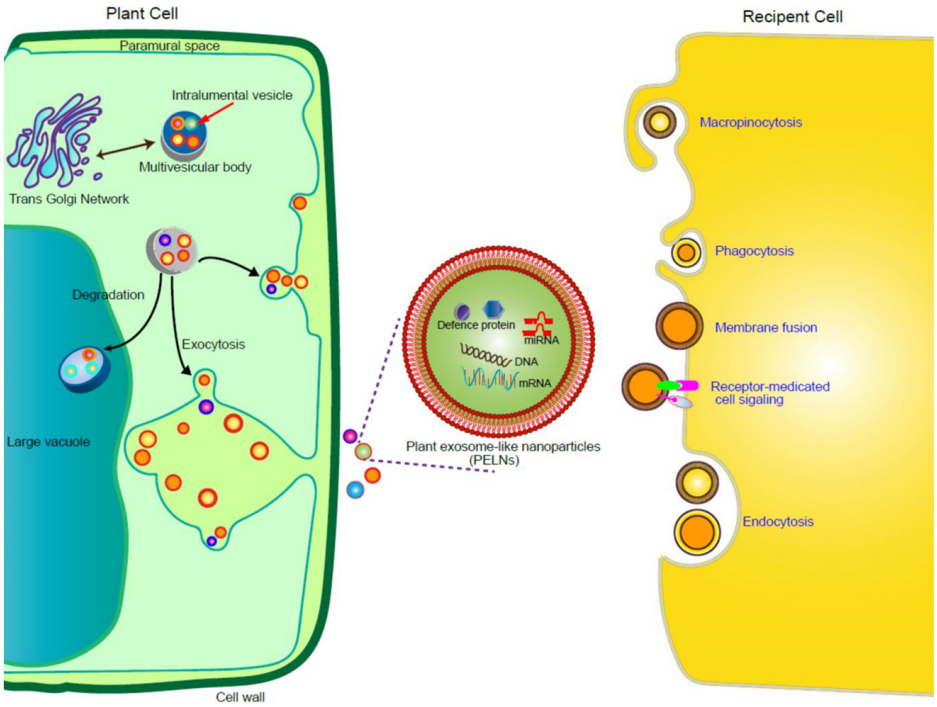

Figure 1. Schematic diagram of the biogenesis, release, structure, and uptake of PELNVs. PELNVs are formed by inward budding of the cell membrane and are produced by MVBs (also called late endosomes). The membrane of MVBs expands inwardly to fuse with the plasma membrane to release their intraluminal vesicles into the extracellular space (called exosomes) or fuse with lysosomes for degradation. In this process, proteins, nucleic acids (e.g., DNA, mRNA, miRNA), and lipid rafts are packed into PELNVs. A variety of mechanisms mediate the uptake of PELNVs, including the fusion of PELNVs with the cell membrane of recipient cells, resulting in the release of PELNVs cargo into the cytoplasm, uptaking by receptor-ligand interactions, endocytosis, and phagocytosis. PELNVs: Plant exosome-like nanovesicles.

Engineering approaches for exosomes

Multiple disciplinary technologies have been developed for exosome engineering [Figure 2] and loading therapeutic cargos, including DNAs, RNAs, pharmacological agents, lipids peptides, proteins, and nanomaterials into exosomes[14]. Incubating, transfection, physical treatments like extrusion, electroporation, sonication, freeze-thawing, surfactant treatment, and dialysis, as well as in situ synthesis, are all employed[10]. Furthermore, molecular homing with substantial receptor affinities, acidic milieu, responsivity, and magnetic features has been assembled on exosomes by transfection or chemical modification, conferring the targeting capacity to exosomes.

Indirect manipulation of exosomes

Plant-derived exosomes could be modified by genetic engineering of exosomes producing cells, including expressing fresh proteins on their membranes or increasing therapeutic proteins cargo load packing efficacy through selective peptides. In an animal model, a platelet-derived growth factor receptor transmembrane domain was fused to the GE11 peptide, which could selectively bound to EGFR on HEK293 cells. Subsequently, GE11 was found on HEK293 cell exosomes. These cells were then transfected with a synthetic let-7a miRNA. This let-7a containing exosomes could target tumor cells and efficiently distribute let-7a by interacting with EGFR and GE11 present on cancer cells. This administration method potentially affected treating EGFR-expressing breast cancer in a mouse model[15].

Direct manipulation of exosomes

Direct manipulation of exosomes is efficient for engineering exosome nanovesicles (ENVs) through direct

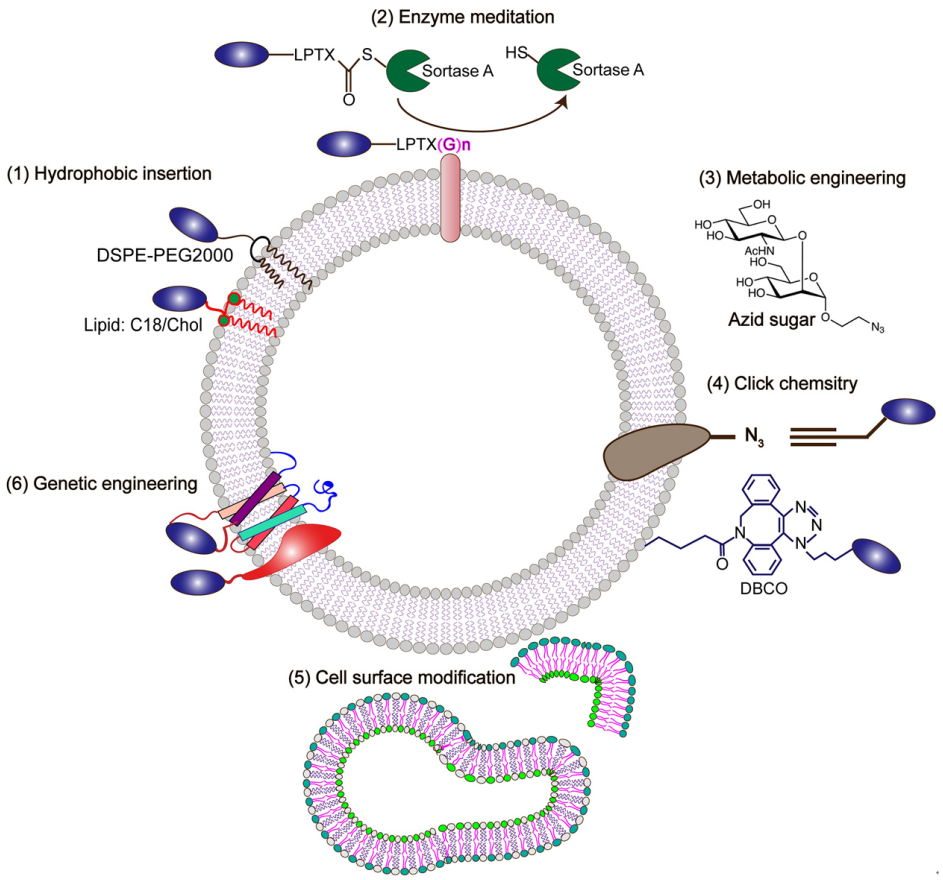

Figure 2. Reengineering PELNVs as nanoscale therapeutics. (1) Using lipophilic or amphiphilic molecules, these molecules can be directly inserted into the EV membrane through the hydrophobic interaction with the phospholipid bilayer. (2) Enzyme catalytic reaction, for example, sortase enzyme can react the sequence of LPETX with the N-terminal protein on exosomes. (3) Metabolic labeling, in which metabolite analogs are incorporated into cell biosynthesis, and functional groups (such as azide) can be introduced into EVs, thereby allowing subsequent bio-orthogonal reactions. (4) Chemical reactions can also be carried out directly on the vesicle membrane. For example, carbodiimide can modify natural amines to present azide groups for click chemistry reactions. (5) Exogenous substances can be introduced through liposomes or micelles fused with the exosomal membrane. (6) Genetic engineering can be used to fuse coding genes on exosomal membrane proteins.

modification[16]. A recent study proposed a way for directly conjugating biomolecules to the surface of ENVs using the click chemistry approach, which is a copper-catalyzed azide-alkyne cycloaddition. This work coupled alkyne groups to EVs via a copper-catalyzed azide-alkyne cycloaddition. This method did not affect EV size, adhesion, or internalization by recipient cells. Furthermore, this approach can successfully use bioconjugated micro-and macro-molecules onto the ENVs surfaces with various benefits, including excellent selectivity and compatibility to aqueous buffers. Another modified strategy for extending ENVs *in vivo* circulation period was also devised. This study transformed ENVs with EGFR conjugated to polyethylene glycol (PEG). A temperature-dependent transfer of nano-PEG-lipids to EV membranes was accomplished by mixing micelles with ENVs from neuro2A cells. The above modification method did not affect ENVs shape, size distribution, or protein content. Still, it extended EV circulation duration in an animal model, perhaps enhancing ENVs tissue-specific aggregation and cargo delivery efficacy[17].

Genetic engineering

Genetic engineering is a simple way of endowing exosomes with novel features. As in animal exosomes[18,19], in plants, ligands or homing peptides fused with transmembrane proteins were found on the surface of

exosomes. A recent study reported that the N-terminus of LAMP-2B is expressed on the surface of exosomes and may be attached with targeting sequences. Cell-specific binding peptides targeting specific organs or tissues can be screened and chosen by phage display and genetically changed at the N-terminus of LAMP-2B to achieve their targeting effects. Exosomes containing the designed peptide ligands are produced by cells that have been transfected with the plasmid. The TIWMPENPRPGTPCDIFTNSRGKRASNG peptide (TIWMPENPRPGTPCDIFTNSRGKRASNG) of the rabies virus glycoprotein (RVG) demonstrates preferential binding to acetylcholine receptors. Neuro-specific exosomes were produced to deliver pharmacological agents to the central nervous system (CNS)[20]. In the experimental mice, intravenous administered miRNA-124-loaded RVG exosomes entered ischemic cortical areas and induced neurogenesis. iRGD exosomes were also employed to precisely transport KRAS siRNA to v3-containing A549 tumors *in vivo*, resulting in specific KRAS gene knockdown and tumor growth reduction. In a recent study, we created chondrocyte affinity peptide (CAP, DWRVIIPPRPSA) modified exosomes that precisely transported miRNA-140 to chondrocytes in joints and slowed the progression of osteoarthritis[21]. Also, this genetic engineering technology was used to modify the exosomal membrane protein lamp2b to fuse with the mesenchymal stem cell affinity peptide E7, rending exosomes (E7-Exos) the ability to target mesenchymal stem cells. The modified E7-Exos can accurately deliver small molecular KGN into synovial fluid-derived MSCs (SF-MSCs), effectively promoting the differentiation of mesenchymal stem cells into chondrocytes, thus providing an advanced stem cell therapy for osteoarthritis[22].

Exosomal surface engineering

Exosomal surfaces could be efficiently manipulated, regardless of their origin. The most apparent purpose of surface engineering is to confer selective cell targeting. Genetic engineering and chemical managing are two manipulative technologies. Genetic engineering fuses gene sequencing to guided protein or polypeptide with a specified exosomal membrane protein[23,24]. This method is successful for peptide and protein surface display; however, it is restricted to targeting patterns that are genetically encoded. The chemical modification enables the presentation of a diverse spectrum of ligands, both natural and synthetic, through conjugation processes or lipid assembly. Conjugating reaction could covalently and stably transform exosomal surface protein. However, the complexity of the exosomal membrane might impair reaction efficacy and frequently limits the regulation of the response on a selective site[25]. Covalent manipulation might potentially compromise the vehicle's structure and functioning. Exosomes' lipid bilayer can also be injected with lipids or amphipathic molecules, allowing their hydrophilic sections to be exhibited on the outside. This approach, driven by lipid self-assembly, might potentially increase exosomal toxicity.

Exosome-like nanovesicles reengineering

Preparing uniform-sized plant-derived exosomes is problematic since their size differs, ranging from 50 to 500 nm and even within species, posing a significant barrier to their application in the delivery of therapeutic drugs[26]. Furthermore, effective loading of drugs is a critical challenge that is difficult to achieve in pristine form in pure plant-derived exosomes[27]. As a result, it is vital to devise a method for consistently producing uniform-sized nanoparticles with adequate drug loading[28]. Researchers have successfully used the Bligh and Dyer technique, a well-known liquid-liquid extraction technique, to extract nano-lipids from plant-derived exosomes, which are then processed via a 200-nm liposome extruder reengineers the exosomes into equal-size nano-platform called plant derive exosomes nanocarriers[29].

Production of semi-synthetic exosomes by manipulation of natural exosomes through biotechnological engineering

Despite the biocompatibility and natural targeting capacity, plant-derived exosomes can be desirably manipulated based on targeted cells for efficient and specified therapeutic targets. This modification can be achieved through a process such as the integration of pharmaceuticals and other therapeutic agents and

manipulating the surface charge for improved drug absorption. The most logical method of producing exosomes would be to harness the cellular machinery's natural processes. The composition of exosomes at all levels is known to respond to a high level of regulation at particular cellular functions[30]. As a result, purposeful manipulation of the cellular environment might regulate the exosomal composition. When using this technology, exosomes might be created with a composition profile tailored to a given function. The selection of exosome-producing cells, including their *in vitro* harvesting settings, and the exosome isolation or enrichment processes, are critical components[26]. Plant-derived exosomes from several natural origins, including vegetable juice and fruits and secretions from mammals, have been manipulated earlier by different researchers to realize their potential for biomedical benefits in nanomedicine. Plant-derived exosomes could also be driven by internal modification. As discussed earlier, the exosome's cargos morphology is influenced by surface modification, whereby the exosomes' external surface morphology is manipulated to produce semi-synthetic plant-derived exosome nanovesicles[31].

Application of bio-engineered and simulated exosomes in nanomedicine

For larger therapeutic capacity, exosomes have been manipulated. This method sometimes includes introducing additional features for targeted purposes, *in vitro* or *in vivo* traceability, or administering the item[30,32]. In other circumstances, the goal of the manipulation was to improve colloidal stability or to adjust the surface charge to boost the rate of absorption[4]. These novel methodologies have given rise to new names such as bioengineered exosomes, artificial exosomes, exosome-mimetic nanovesicles, exosome-like nanovesicles, and exosome-based semi-synthetic vesicles[33]. These expressions have been used with many meanings in the literature, but there has yet to be a definite classification criterion. "Exosome-like vesicles" is used in certain research to describe artificial vesicles created from cells using various approaches to resemble exosomes. Other scientists, however, dubbed exosome-like nanovesicles with morphological and biochemical features comparable to exosomes. Other writers using non-animal research models adopted this name to describe vesicles with exosome-like size and flotation density values. For example, a prior study confirmed the presence of plant exosome-like vesicles in sunflower fluids, whereas another work detected exosome-like vesicles during pollen germination[34]. Several isolation procedures have been used to purify PELNVs for their application in nanomedicine [Table 1].

Exosome cargo loading approaches

Several biomolecules are inherently enclosed in exosome-like nanovesicles. The content of exosomes and exosome-like particles may need to be redeployed to enhance their loading delivery and targeting efficiency[37,38]. Plant exosome-like particles contain higher biomolecules than animal exosome-like nanovesicles, although a redeployment approach could be introduced in cases where the bioactive component of interest is not present. Plant-derived exosome nanovesicles can be loaded with exogenous therapeutic molecules, including siRNAs, DNAs, proteins, and expression vectors, in addition to endogenous constituents, to ensure optimal therapeutic effects[39]. Various ways have been explored to load exosome-like nanovesicles with therapeutic compounds. These procedures begin with PELNVs from plants, which are then directly manipulated and loaded with pharmacological compounds. Active and passive cargo loading approaches are commonly utilized in the mechanical loading of exosome-like nanovesicles[39]. An incubation method is used in the passive drug loading procedure, in which plant exosome-derived nanovesicles are incubated with drug molecules at a specific temperature[40]. This drug encapsulation technique was driven by diffusion action and lipophilic contact between the drug molecule and the lipid bilayer of plant-derived exosomes. In addition to passive cargo loading, the sonication approach is applied, which disrupts the exosome-like nanovesicles membrane structure momentarily for successful cargo diffusion into exosome-like nanovesicles[41]. Following the cargo loading, the exosome-like nanovesicles' membrane morphology restores to its original state. This method was revealed to improve passive cargo loading by increasing cargo loading capability by up to 11 times. PELNVs retain a negatively charged

Table 1. Summary of the advantages and disadvantages of exosome isolation procedures

Procedures	Purity	Principles	Sources	Advantages	Disadvantages	Reference
Ultracentrifugation procedure	< 50%	Separations of constituents & solutes base on density and sizes	Ginger, grapefruit, carrot, grape, lemon, blueberry, shiitake, broccoli	Low cost & contamination risks, great yield & huge sample capacity	High cost, long run time, labor-intensive & low portability, potential damage by centrifugal speed	[35,36]
Size-based procedure	< 50%	Based on size among exosomes & constituents		Fast, direct RNA extraction, no special tool needed, good portability, high purity, maintains the integrity & biological activity, moderate sample capacity	Moderate purity, deterioration by shear stress, clogging potential and vesicle trapping, exosomes lost	[8]
Immunoaffinity capture-based procedure	99%	Fishing based on particular interaction among membrane receptors of exosomes & ligands		Best for isolation specificity, high purity, and subtyping potentials	High cost, low capacity, low yields, antigenic epitope might be blocked or masked	[27]

surface in most situations, attracting oppositely charged chemical agents like doxorubicin and eventually facilitating drugs loading into their interior chamber using the sonication approach[41]. However, negatively charged molecules like FA and neutrally surfaced pharmacological agents like curcumin have been observed to get entangled in PELNVs regardless of modulating their biological activity. According to the findings, these molecules' lipophilic features can lead to their encapsulation by overcoming the surface-related electrostatic force of nanovesicles. Because DNAs and siRNAs are very negatively-charged molecules, as shown by few reports, they may be loaded into PELNVs and perform as expected[27,42]. Nevertheless, compared to positively charged molecules, cargo loading efficiencies were deficient. The drug-loaded exosome-like nanovesicles are summarized in the table below [Table 2].

PELNVs in nanomedicine drug delivery system

The realization of exosomes like nanovesicles as natural carriers of various biomolecules, including DNA, RNA lipids, peptides, and proteins, sparked apprehension that they could be employed to deliver exogenous molecules and therapeutic drug payloads. This strategy was proven by their ability to cross the blood-brain barrier (BBB)[5,49]. The brain was used as a targeted tissue because BBB is a significant barrier to the micro and macromolecular delivery of drugs to the CNS. Exosome-like nanovesicles are devoted to transporting payloads from nearby cells to distant locations and other species via kingdoms inter-communicating[50]. Their distinct shape and delivery capacity highlight their potential for application in the delivery of therapeutic drugs[51]. For the delivery of pharmacological agents, the benefits of plant-derived exosome nanovesicles-based nanoplatforms over synthetic nanoplatforms and mammalian-derived exosome-like nanovesicles have been established[29]. Exosome-like nanovesicles were initially used as a drug delivery nano-platform[52,53]. They require careful consideration since their inherent biological functions and origin play critical roles in their immunogenic acceptability. Exosome-like nanovesicles produced from mammalian malignant cells, for example, run the danger of spreading pro-malignant features to recipient cells [Figure 3]. Plant-derived exosome nanovesicles and artificially created nanoparticles such as liposomes and micelles share fundamental characteristics such as a similar lipid bilayers morphology and the capacity to carry both hydrophilic and hydrophobic payload.

Table 2. Drug loaded PELNVs and their plant origin

Pharmacological agents	Target cell/tissue	Source of nanovesicles	Reference
miRNA, 6-shogaol & 6-gingerol	Intestinal epithelia	Ginger	[43]
siRNA-CD98	HT-29 cells & Colon-26 cells Colon-26 cells & RAW 264.7 macrophages		[44-46]
Doxorubicin	Colon tumor cell line		[10]
Sulforaphane	Colon tissues	Broccoli	
Protein	Chronic myeloid leukemia	Citrus limon	[13]
miR-18a	Hepatic Kupffer cells	Grapefruit	
Luciferase gene siRNA, Paclitaxel, JSI-124 (anti-Stat3 inhibitor)	Cancer cells (GL26, 4T1, SW620, A549 & CT26)		
Curcumin & Doxorubicin	Colon tumors		[47]
Methotrexate	Intestinal macrophages		
miR17	Brain cancer cell line (GL26)		
miRNAs	Human epithelial colorectal adenocarcinoma cell (Caco-2)	Apple	[48]

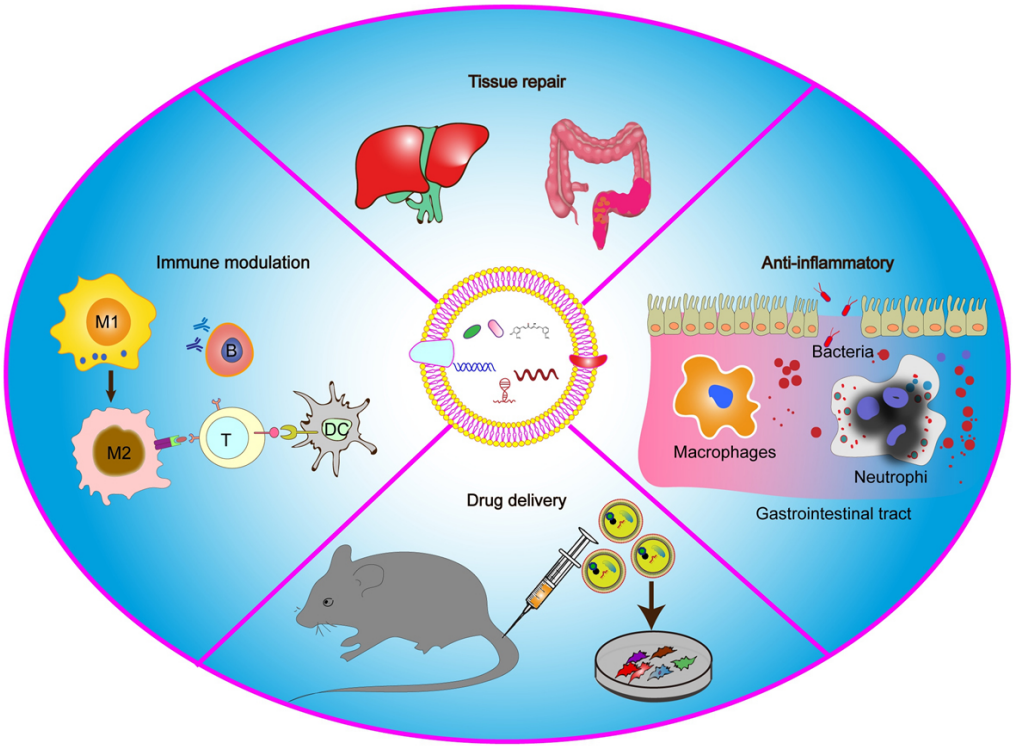

Figure 3. Therapeutic effects of PELNVs. PELNVs can regulate immunity and exert anti-inflammatory functions by inducing the functions of macrophages and dendritic cells *in vitro* and *in vivo*. PELNVs can deliver therapeutic reagents, siRNAs, and proteins to diverse cell types or *in vivo* animal models.

Nevertheless, compared to chemically produced nanoparticles, plant exosome-like nanovesicles are biocompatible and have minimal immunogenic effects, more excellent cellular absorption, higher stability in the GI tract (GIT), and selective absorption targeting ability[16]. Furthermore, PELNVs-based nanoplatforms have a less complex creation procedure, whereas artificially generated nanoparticles such as liposomes necessitate complex manufacturing methods such as membrane extrusion, micro emulsification,

etc. Furthermore, additional stringent modifications of synthetic nanoparticles for cargo loading and coating improvements, such as polyethylene glycol (PEG) coatings, are necessary to produce adequate immunological tolerance. However, PEG coating increases the circulation period and aids with immunological tolerance; nevertheless, these coatings may interfere with the interaction between the nanoparticle and targeted cells, reducing the biodistribution of the drug in the targeted tissue. Furthermore, repeated treatment with PEG-coated liposomes has been linked to the formation of anti-PEG antibodies, resulting in ineffective therapy[54].

Furthermore, chemically synthesized nanoparticles only assist in the delivery of therapeutics but lack an inherent therapeutic advantage. Lastly, the lipid bilayer structure of PELNVs secures their cargo while avoiding enzymatic decomposition by proteinases and nucleases. These outstanding characteristics, combined with the discovery of their intrinsic therapeutic actions, make PELNVs an ideal candidate for entry into the field of drug delivery applications[55-57] [Table 3].

PELNVs therapeutics application against diseases

The bioactivities of PELNVs have been demonstrated by earlier works, such as their regenerative function, lowering inflammation, promoting healing, reducing gingivitis, increasing the maturation of beneficial gut microbiota, and preventing cancer and infection[8,44]. Their biological processes in natural pristine morphological stability with intact bioactive payloads after simple separation from plants alleviate various pathologic problems in the kingdom of other species and provide different therapeutic options. Their plant origin and high safety profile of PELNVs and their various therapeutic potentials anchored in their active parent plants make them promising therapeutic candidates[48,61].

Concluding remarks

Despite the infancy of PELNVs research, they have shown various advantages, including internalization, biocompatibility, cellular uptake, bioavailability, and targeting capability compared to their synthetic counterpart. Due to the excessive curiosity about PELNVs, there has been surplus interest in their potential application in disease treatment and therapeutic drug delivery settings[62]. Since they can mediate interkingdom communication, they prioritize nanomedicine for theranostics and cargo delivery applications. Various experiments have demonstrated exosomes to involve the exchange of substances between cells in physiology and pathology. Exploiting these exosome contents transfer mechanisms may prove crucial for advancing the engineering PELNVs with improved delivery and selective targeting. For example, the synthetic drugs and RNA delivery using polymeric nanomaterial, translocation to the nucleus is a significant issue because, while passaging RNAs could come across the lysosomal pathway, which induces the lysosomal substance degradation resulting in failure, and has a substantial effect on the delivery efficiency. However, engineered PELNVs can overcome this limitation. Thus, improving various aspects of PELNVs engineering through innovative nanotechnologies is crucial[24]. For such different innovative techniques required developing a novel strategy for exosome drug delivery, the significant challenges are the complexity of the extraction, the purifications, and storage. Further work is necessary to understand their biogenesis and the loading mechanisms for addressing these challenges. Developing a novel approach for long-term storage is also required for maintaining their integrity and bioactivities after purification.

Table 3. PELNVs for drug delivery nanoplatform

Sources	Lipids	Targets	Modification	Loaded agents	Impacts	Reference
Grapefruits	PC: 28.53% PE: 45.52%	4T1 breast tumor CT26colon tumor	Inflammatory receptors	Cur DOX	DSS-induced mouse colitis Cancer suppression	[6]
Ginger	MGDG: 18.9% PC: 6.5% DGDG: 27.4% PA: 41.9%	Female mice RAW264.7 Colon-26	Passed via 200nm polycarbonate membranes	siRNA CD98	Ulcerative colitis therapeutic benefits CD98 downregulation	[43,58]
Broccoli	High ratio of monoglycerides	C57BL/6 colitis mice	Unmodified	Sulforaphane	Colitis prevention in mice	[3,5]
Lemon	-	CT 26, SW620 colon tumor A549 tumor, ML LAMA84 CML xenograft mice SW480 Colorectal adenocarcinoma	Folic acid Unmodified	PTX Proteins	Tumor suppression Suppression of different cancer cell lines Bcl-xl, survivin, Bad, Bax, Tumor suppression suppression of angiogenesis, Bax, survivin, Bcl-xl	[59]
Grape	PE: 26.09% PA: 53.17% PC: 9.03%	Intestinal stem cells in Lgr5-EGFP -IRES-CreERT2 mice	Unmodified	No agent	Protect mice against DSS-evoked colitis Proliferation of Lgr5 intestinal stem cells	[60]

DECLARATIONS

Authors' contributions

Wrote of the manuscript: Shinge SAU
Prepared the figure: Liang Y
Contributed to the manuscript review and editing: Liang Y, Duan L
Contributed to the manuscript discussion: Xiao Y, Xia J
All authors read and approved the final manuscript.

Availability of data and materials

Not applicable.

Financial support and sponsorship

This work was partially funded by National Natural Science Foundation of China (No. 81972116; No. 81772394); Shenzhen Science and Technology Projects (No. SGDX20201103095800003; GJHZ20200731095606019; No. JCYJ20170817172023838); Guangdong International Cooperation Project (No. 2021A0505030011). Shenzhen Fund for Guangdong Provincial Highlevel Clinical Key Specialties (No.SZGSP013); Shenzhen Key Medical Discipline Construction Fund (No.SZXK042).

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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REFERENCES

1. Fernandes M, Lopes I, Teixeira J, Botelho C, Gomes AC. Exosome-like nanoparticles: a new type of nanocarrier. *Curr Med Chem* 2020;27:3888-905. DOI PubMed
2. Cai Q, Qiao L, Wang M, et al. Plants send small RNAs in extracellular vesicles to fungal pathogen to silence virulence genes. *Science* 2018;360:1126-9. DOI PubMed PMC
3. Deng Z, Rong Y, Teng Y, et al. Broccoli-derived nanoparticle inhibits mouse colitis by activating dendritic cell AMP-activated protein kinase. *Mol Ther* 2017;25:1641-54. DOI PubMed PMC
4. Li Z, Wang H, Yin H, Bennett C, Zhang HG, Guo P. Arrowtail RNA for ligand display on ginger exosome-like nanovesicles to systemic deliver siRNA for cancer suppression. *Sci Rep* 2018;8:14644. DOI PubMed PMC
5. Yang C, Zhang M, Merlin D. Advances in plant-derived edible nanoparticle-based lipid nano-drug delivery systems as therapeutic nanomedicines. *J Mater Chem B* 2018;6:1312-21. DOI PubMed PMC
6. Wang Q, Ren Y, Mu J, et al. Grapefruit-derived nanovectors use an activated leukocyte trafficking pathway to deliver therapeutic agents to inflammatory tumor sites. *Cancer Res* 2015;75:2520-9. DOI PubMed PMC
7. Zieske JD, Hutcheon AEK, Guo X. Extracellular vesicles and cell-cell communication in the cornea. *Anat Rec (Hoboken)* 2020;303:1727-34. DOI PubMed PMC
8. Gioia S, Hossain MN, Conese M. Biological properties and therapeutic effects of plant-derived nanovesicles. *Open Med (Wars)* 2020;15:1096-122. DOI PubMed PMC
9. Teng Y, Ren Y, Sayed M, et al. Plant-derived exosomal microRNAs shape the gut microbiota. *Cell Host Microbe* 2018;24:637-652.e8. DOI PubMed PMC
10. You JY, Kang SJ, Rhee WJ. Isolation of cabbage exosome-like nanovesicles and investigation of their biological activities in human cells. *Bioact Mater* 2021;6:4321-32. DOI PubMed PMC
11. Kučuk N, Primožič M, Knez Ž, Leitgeb M. Exosomes engineering and their roles as therapy delivery tools, therapeutic targets, and biomarkers. *Int J Mol Sci* 2021;22:9543. DOI PubMed PMC
12. Gurung S, Perocheau D, Touramanidou L, Baruteau J. The exosome journey: from biogenesis to uptake and intracellular signalling. *Cell Commun Signal* 2021;19:47. DOI PubMed PMC
13. Regente M, Pinedo M, San Clemente H, Balliau T, Jamet E, de la Canal L. Plant extracellular vesicles are incorporated by a fungal pathogen and inhibit its growth. *J Exp Bot* 2017;68:5485-95. DOI PubMed
14. Jafari D, Shajari S, Jafari R, et al. Designer exosomes: a new platform for biotechnology therapeutics. *BioDrugs* 2020;34:567-86. DOI PubMed PMC
15. Ohno S, Takanashi M, Sudo K, et al. Systemically injected exosomes targeted to EGFR deliver antitumor microRNA to breast cancer cells. *Mol Ther* 2013;21:185-91. DOI PubMed PMC
16. Chen H, Wang L, Zeng X, et al. Exosomes, a new star for targeted delivery. *Front Cell Dev Biol* 2021;9:751079. DOI PubMed PMC
17. Kooijmans SA, Aleza CG, Roffler SR, van Solinge WW, Vader P, Schiffelers RM. Display of GPI-anchored anti-EGFR nanobodies on extracellular vesicles promotes tumour cell targeting. *J Extracell Vesicles* 2016;5:31053. DOI PubMed PMC
18. Gilligan KE, Dwyer RM. Engineering exosomes for cancer therapy. *Int J Mol Sci* 2017;18:1122. DOI PubMed PMC
19. Liang Y, Duan L, Lu J, Xia J. Engineering exosomes for targeted drug delivery. *Theranostics* 2021;11:3183-95. DOI PubMed PMC
20. Alvarez-Erviti L, Seow Y, Yin H, Betts C, Lakhal S, Wood MJ. Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. *Nat Biotechnol* 2011;29:341-5. DOI PubMed
21. Liang Y, Xu X, Li X, et al. Chondrocyte-targeted microRNA delivery by engineered exosomes toward a cell-free osteoarthritis therapy. *ACS Appl Mater Interfaces* 2020;12:36938-47. DOI PubMed
22. Xu X, Liang Y, Li X, et al. Exosome-mediated delivery of kartogenin for chondrogenesis of synovial fluid-derived mesenchymal stem cells and cartilage regeneration. *Biomaterials* 2021;269:120539. DOI PubMed
23. Vogt S, Stadlmayr G, Grillari J, Rüker F, Wozniak-knopp G. Engineering of surface proteins in extracellular vesicles for tissue-specific targeting. In: Shiomi N, editor. Current topics in biochemical engineering. IntechOpen; 2019. DOI
24. Richter M, Vader P, Fuhrmann G. Approaches to surface engineering of extracellular vesicles. *Adv Drug Deliv Rev* 2021;173:416-26. DOI PubMed
25. Richardson JJ, Ejima H. Surface engineering of extracellular vesicles through chemical and biological strategies. *Chem Mater* 2019;31:2191-201. DOI
26. Cheng Q, Shi X, Han M, Smbatyan G, Lenz HJ, Zhang Y. Reprogramming exosomes as nanoscale controllers of cellular immunity. *J Am Chem Soc* 2018;140:16413-7. DOI PubMed PMC
27. Dad HA, Gu TW, Zhu AQ, Huang LQ, Peng LH. Plant exosome-like nanovesicles: emerging therapeutics and drug delivery nanoplatforms. *Mol Ther* 2021;29:13-31. DOI PubMed PMC
28. Li P, Kaslan M, Lee SH, Yao J, Gao Z. Progress in exosome isolation techniques. *Theranostics* 2017;7:789-804. DOI PubMed PMC
29. Loureiro JA, Andrade S, Duarte A, et al. Resveratrol and grape extract-loaded solid lipid nanoparticles for the treatment of alzheimer's disease. *Molecules* 2017;22:277.
30. García-Manrique P, Matos M, Gutiérrez G, Pazos C, Blanco-López MC. Therapeutic biomaterials based on extracellular vesicles: classification of bio-engineering and mimetic preparation routes. *J Extracell Vesicles* 2018;7:1422676. DOI PubMed PMC
31. Antimisiaris SG, Mourtas S, Marazioti A. Exosomes and exosome-inspired vesicles for targeted drug delivery. *Pharmaceutics*

2018;10:218. DOI PubMed PMC
32. Li YJ, Wu JY, Liu J, et al. Artificial exosomes for translational nanomedicine. *J Nanobiotechnology* 2021;19:242. DOI PubMed PMC
33. Villarroya-Beltri C, Baixauli F, Gutiérrez-Vázquez C, Sánchez-Madrid F, Mittelbrunn M. Sorting it out: regulation of exosome loading. *Semin Cancer Biol* 2014;28:3-13. DOI PubMed PMC
34. Prado N, Cañamero M, Villalba M, Rodríguez R, Batanero E. Bystander suppression to unrelated allergen sensitization through intranasal administration of tolerogenic exosomes in mouse. *Mol Immunol* 2010;47:2148-51. DOI PubMed
35. Jeppesen DK, Hvam ML, Primdahl-Bengtson B, et al. Comparative analysis of discrete exosome fractions obtained by differential centrifugation. *J Extracell Vesicles* 2014;3:25011. DOI PubMed PMC
36. Kalra H, Adda CG, Liem M, et al. Comparative proteomics evaluation of plasma exosome isolation techniques and assessment of the stability of exosomes in normal human blood plasma. *Proteomics* 2013;13:3354-64. DOI PubMed
37. Fujita D, Arai T, Komori H, et al. Apple-derived nanoparticles modulate expression of organic-anion-transporting polypeptide (OATP) 2B1 in Caco-2 cells. *Mol Pharm* 2018;15:5772-80. DOI PubMed
38. Duan L, Xu L, Xu X, et al. Exosome-mediated delivery of gene vectors for gene therapy. *Nanoscale* 2021;13:1387-97. DOI PubMed
39. Han Y, Jones TW, Dutta S, et al. Overview and update on methods for cargo loading into extracellular vesicles. *Processes (Basel)* 2021;9:356. DOI PubMed PMC
40. György B, Hung ME, Breakefield XO, Leonard JN. Therapeutic applications of extracellular vesicles: clinical promise and open questions. *Annu Rev Pharmacol Toxicol* 2015;55:439-64. DOI PubMed PMC
41. Fan Y, Zhang Q. Development of liposomal formulations: from concept to clinical investigations. *Asian J Pharm* 2013;8:81-7. DOI
42. Theek B, Baues M, Ojha T, et al. Sonoporation enhances liposome accumulation and penetration in tumors with low EPR. *J Control Release* 2016;231:77-85. DOI PubMed PMC
43. Zhang M, Viennois E, Prasad M, et al. Edible ginger-derived nanoparticles: a novel therapeutic approach for the prevention and treatment of inflammatory bowel disease and colitis-associated cancer. *Biomaterials* 2016;101:321-40. DOI PubMed PMC
44. Zhang M, Viennois E, Xu C, Merlin D. Plant derived edible nanoparticles as a new therapeutic approach against diseases. *Tissue Barriers* 2016;4:e1134415. DOI PubMed PMC
45. Sung J, Yang C, Collins JF, Merlin D. Preparation and characterization of ginger lipid-derived nanoparticles for colon-targeted siRNA delivery. *Bio Protoc* 2020;10:e3685. DOI PubMed PMC
46. Zhang M, Wang X, Han MK, Collins JF, Merlin D. Oral administration of ginger-derived nanolipids loaded with siRNA as a novel approach for efficient siRNA drug delivery to treat ulcerative colitis. *Nanomedicine (Lond)* 2017;12:1927-43. DOI PubMed PMC
47. Woith E, Guerriero G, Hausman JF, et al. Plant extracellular vesicles and nanovesicles: focus on secondary metabolites, proteins and lipids with perspectives on their potential and sources. *Int J Mol Sci* 2021;22:3719. DOI PubMed PMC
48. Yu L, Deng Z, Liu L, Zhang W, Wang C. Plant-derived nanovesicles: a novel form of nanomedicine. *Front Bioeng Biotechnol* 2020;8:584391. DOI PubMed PMC
49. Jan AT, Rahman S, Badierah R, et al. Expedition into exosome biology: a perspective of progress from discovery to therapeutic development. *Cancers (Basel)* 2021;13:1157. DOI PubMed PMC
50. Johnsen KB, Gudbergsson JM, Skov MN, Pilgaard L, Moos T, Duroux M. A comprehensive overview of exosomes as drug delivery vehicles - endogenous nanocarriers for targeted cancer therapy. *Biochim Biophys Acta* 2014;1846:75-87. DOI PubMed
51. Zhu L, Wang H, Yuhan J, et al. Exosomes mediated the delivery of ochratoxin A-induced cytotoxicity in HEK293 cells. *Toxicology* 2021;461:152926. DOI PubMed
52. Harishkumar M, Radha M, Yuichi N, et al. Designer exosomes: smart nano-communication tools for translational medicine. *Bioengineering (Basel)* 2021;8:158. DOI PubMed PMC
53. Garaeva L, Kamyshinsky R, Kil Y, et al. Delivery of functional exogenous proteins by plant-derived vesicles to human cells in vitro. *Sci Rep* 2021;11:6489. DOI PubMed PMC
54. Nunes SS, Fernandes RS, Cavalcante CH, et al. Influence of PEG coating on the biodistribution and tumor accumulation of pH-sensitive liposomes. *Drug Deliv Transl Res* 2019;9:123-30. DOI PubMed PMC
55. Ito Y, Taniguchi K, Kuranaga Y, et al. Uptake of microRNAs from exosome-like nanovesicles of edible plant juice by rat enterocytes. *Int J Mol Sci* 2021;22:3749. DOI PubMed PMC
56. Wang MZ, Niu J, Ma HJ, et al. Transdermal siRNA delivery by pH-switchable micelles with targeting effect suppress skin melanoma progression. *J Control Release* 2020;322:95-107. DOI PubMed
57. Yang C, Zhang M, Lama S, Wang L, Merlin D. Natural-lipid nanoparticle-based therapeutic approach to deliver 6-shogaol and its metabolites M2 and M13 to the colon to treat ulcerative colitis. *J Control Release* 2020;323:293-310. DOI PubMed PMC
58. Gao C, Liu L, Zhou Y, Bian Z, Wang S, Wang Y. Novel drug delivery systems of Chinese medicine for the treatment of inflammatory bowel disease. *Chin Med* 2019;14:23. DOI PubMed PMC
59. Raimondo S, Naselli F, Fontana S, et al. Citrus limon-derived nanovesicles inhibit cancer cell proliferation and suppress CML xenograft growth by inducing TRAIL-mediated cell death. *Oncotarget* 2015;6:19514-27. DOI PubMed PMC
60. Ju S, Mu J, Dokland T, et al. Grape exosome-like nanoparticles induce intestinal stem cells and protect mice from DSS-induced colitis. *Mol Ther* 2013;21:1345-57. DOI PubMed PMC
61. Kim H, Jang H, Cho H, et al. Recent advances in exosome-based drug delivery for cancer therapy. *Cancers (Basel)* 2021;13:4435. DOI PubMed PMC

62. Sarvarian P, Samadi P, Gholipour E, et al. Application of emerging plant-derived nanoparticles as a novel approach for nano-drug delivery systems. *Immunol Invest* 2022;51:1039-59. DOI PubMed

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Review	A Review paper summarizes the literature on previous studies. It usually does not present any new information on a subject.	Unstructured abstract. No more than 250 words.	3-8 keywords	The main text may consist of several sections with unfixed section titles. We suggest that the author includes an "Introduction" section at the beginning, several sections with unfixed titles in the middle part, and a "Conclusion" section in the end.
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2.3 Manuscript Structure

2.3.1 Front Matter

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The title of the manuscript should be concise, specific and relevant, with no more than 16 words if possible. When gene or protein names are included, the abbreviated name rather than full name should be used.

2.3.1.2 Authors and Affiliations

Authors' full names should be listed. The initials of middle names can be provided. Institutional addresses and email addresses for all authors should be listed. At least one author should be designated as corresponding author. In addition, corresponding authors are suggested to provide their Open Researcher and Contributor ID upon submission. Please note that any change to authorship is not allowed after manuscript acceptance.

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