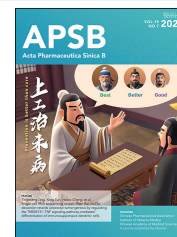




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REVIEW

Extracellular vesicles as biomarkers and drug delivery systems for tumor



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Abstract Extracellular vesicles (EVs) are crucial for facilitating intercellular communication, promoting cell migration, and orchestrating the immune response. Recently, EVs can diagnose and treat tumors. EVs can be measured as biomarkers to provide information about the type of disease and therapeutic efficacy. Furthermore, EVs with lower immunogenicity and better biocompatibility are natural carriers of chemicals and gene drugs. Herein, we review the molecular composition, biogenesis, and separation methods of EVs. We also highlight the important role of EVs from different origins as biomarkers and drug delivery systems in tumor therapy. Finally, we provide deep insights into how EVs play a role in reversing the immunosuppressive microenvironment.

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1. Introduction

The tumor microenvironment (TME), characterized by hypoxia, acidity, and high interstitial fluid pressure, is an immunosuppressive and highly complex system¹. Tumor immunotherapy, by activating the patient's immune response and reversing immunosuppressive TME to eradicate tumor cells, can lower metastatic relapse and prolong the survival time of patients². Due to the cellular specificity and immunogenicity of extracellular vesicles (EVs), they can modulate TME and are involved in biological processes of tumor development and angiogenesis³. With the natural TME modulation of EVs, it is hoped to prevent the metastasis of oncogenic molecules and inhibit tumor development and angiogenesis.

EVs encompass a diverse range of vesicles that are released by virtually all types of living cells, including both mammalian and plant cells. While mammalian-derived EVs have been extensively studied and reported, it is noteworthy that EVs derived from plants are structurally similar to mammalian-derived EVs, and also can be potentially developed as therapeutic agents⁴. Recently, EVs have become a major research hotspot as novel biomarkers and drug delivery carriers in cancer therapy⁵. Since EVs carry various bioactive molecules from donor cells, some specific loading substances may reflect the biological properties and states of the donor cells. EVs circulate stably in re-biotic fluid and can diagnose disease in its early stages⁶. For example, EVs isolated from ovarian cancer patients carry oncogenic miRNA signatures that can reliably distinguish malignant disease from benign disease.

Additionally, more than 50,000 patients have benefited from exosomal RNA for prostate cancer detection⁶. Meanwhile, with the advantages of high biocompatibility, cellular specificity, better stability, and limited immunogenicity⁷, EVs have been applied in drug delivery systems (DDSs) to transport cargo into the cytoplasm of target cells⁸. CD47, which is widely expressed on the surface of EVs, helps them avoid phagocytosis by the reticulo-endothelial system and achieve efficient immune escape. More importantly, EVs can successfully cross physiological barriers to reach target cells and efficiently transport different cargoes to target cells⁹. Rong et al.¹⁰ obtained a targeted DDS by loading CCL2-small interfering RNAs (siRNAs) into EVs *via* electroporation. To sum up, EVs have promising applications in immunotherapy as new diagnostic biomarkers and DDSs¹¹.

Overall, EVs possess a wide range of biological functions and clinical applications. They have been extensively utilized as biomarkers, therapeutic agents, and drug delivery systems to address specific clinical needs. EVs-based therapeutic regimens have been assessed in numerous preclinical studies with encouraging results. Herein, we not only introduce the molecular composition, biogenesis, separation methods, and drug loading methods of EVs but also summarize the vital role of EVs as biomarkers in tumor diagnosis and treatment. Next, we discuss the advantages of EVs when used as drug delivery systems and therapeutic agents. Of note, we provide deep insights into how EVs reverse the immunosuppressive TME, separately based on endogenous EVs, exogenous EVs, and EVs as drug delivery systems.

2. Biogenesis, molecular composition, and separation of EVs

EVs can cross biological barriers and transmit signals to other cells for intercellular communication. Initially, the term "exosome" was not accurately defined. In the mid-1990s, scientists

discovered that exosomes were secreted by dendritic cells (DCs) and B lymphocyte¹². On this basis, the potential of exosomes to regulate immune function has been widely explored¹³. Exosomes are associated with immunomodulation, angiogenesis, cell proliferation, and apoptosis¹⁴. Whereas microvesicles are produced by cell membrane outgrowth after stress¹⁵. Apoptotic bodies are formed primarily through cell membrane wrinkling and invagination in the process of cell apoptosis¹⁶. In recent years, Zhao et al.¹⁷ first proved that the chemotherapeutic agent camptothecin induces cell apoptosis and the released apoptotic bodies transport the remaining camptothecin to surrounding tumor cells and exert neighboring effects to enlarge antitumor response.

2.1. Formation, release, and uptake of EVs

Lymphocytes, nerve cells, tumor cells, and DCs release EVs with different patterns of biogenesis according to the environment in which they live¹⁸. The synthesis of exosomes mainly depends on endocytosis. EVs can fuse with early endosomes, and then begin sorting and transport to the extracellular matrix. During this period, early endosomes undergo invagination, budding, and accumulation. Finally, form multivesicular bodies (MVBs) and then integrate with lysosomes. Besides, intracellular vesicles in MVBs can be released to further secrete exosomes (Fig. 1)¹⁴. In addition, MVBs form amphisomes through interaction with autophagosomes and then merge with lysosomes^{5,19}. During this process, the ubiquitination of MVB membrane proteins influences exosome secretion by facilitating MVB fusion with lysosomes²⁰. Most organelles in the endomembrane system such as the Golgi apparatus, endoplasmic reticulum, and endosomes carry Rab GTPases. To ensure only mature endosomes are sorted, intracavitary EV formation driven by ESCRT needs to be closely combined with Rab-GTPase transformation²¹. When apoptosis-related transduction pathways are activated, apoptotic bodies will be released from apoptotic cells through plasma membrane vesiculation and dynamic membrane vesiculation¹⁶. Currently, the molecular mechanism of microvesicle formation is not clear. Some studies suggest that binding protein 1 contains an arresting domain, which integrates the endosomal sorting complex protein into the bilayer film to induce budding²². Cytoplasmic calcium influx can disrupt phospholipid asymmetry and cause membrane budding²³. The secretion process of exosomes is complex and influenced by various factors, such as long-chain noncoding hot-air (lncRNA) regulating Rab35 and SNAP to increase exosome secretion²⁴, pH value decrease promoting exosome secretion and cell uptake, P53 promoting exosome secretion under stress conditions by inhibiting activation pathways, and heparin over-expression sometimes increasing exosome secretion²⁵. In general, the synthesis and secretion mechanisms of EVs are extremely complex and require further study.

EVs can be taken by receptor cells in many ways, including ligand–receptor interactions, direct signal transduction, and direct fusion of membranes²⁶. After interacting with target cells, EVs are taken up by cells through various endocytosis pathways. These pathways such as caveolin-mediated uptake and phagocytosis are characterized by rapid and energy-intensive²⁷. Endocytosis is the main method of EV uptake, but there is almost no consensus on which type of endocytosis mechanism is the most important in the literature. This is because EVs can activate many ways to enter cells. Overall, knowledge about the formation, release, and uptake of EVs is incomplete and worthy of further exploration.

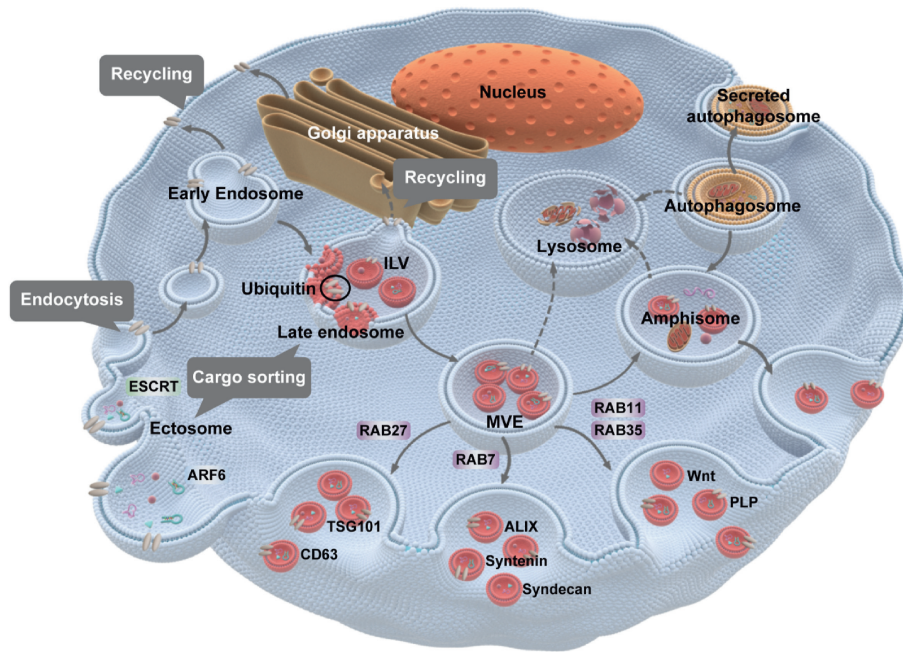


Figure 1 Vesicles derived from the plasma membrane or Golgi apparatus can fuse with early endosomes, and then begin sorting and transport to the extracellular matrix. During this period, early endosomes invaginate, bud and accumulates intraluminal vesicles to form MVBs, and finally fuse with lysosomes. MVBs can release intracellular vesicles by fusing with the plasma membrane, thus producing exosomes.

2.2. Molecular composition of EVs

EVs are vesicles derived from endocytic processes and are enclosed by a membrane. Dysregulation of EV expression is implicated in the pathogenesis and progression of numerous diseases, including cancer (Table 1)²⁸⁻⁴⁵. Exosomal lipids represent a rich reservoir of unexplored bioactive molecules, which not only contribute to the structural integrity of exosomes but also play pivotal regulatory roles in recipient cells. These lipids hold immense potential as disease biomarkers and are intricately involved in EVs biosynthesis and uptake, immune surveillance, tumor microenvironment modulation, and inflammation regulation. For example, EVs derived from tumor cells are particularly abundant in glycerolipids, sphingolipids, and sterol lipids^{46,47}. Furthermore, proteins derived from the plasma membrane and cytosol serve as the primary sources of EV proteins, which are distributed within the interior of EVs and on their membrane surface⁴⁸. The surface receptors of EVs facilitate targeting and uptake by recipient cells, allowing the binding of proteins, lipids, and genetic information carried by the vesicles to modulate their physiological state⁴⁹. However, these markers may not always be suitable as cancer markers. It is suggested that glypican-1 (GPC1) and EphA2 would serve as more effective characteristic proteins for the diagnosis of pancreatic cancer exosomes⁵⁰. Furthermore, it is now widely recognized that RNAs derived from EVs play a significant role in the transcription, post-transcriptional processing, and translational modification of genes in recipient cells. This regulation leads to the modulation of various cellular functions, including biological processes and disease progression^{51,52}.

2.3. Separation of EVs

For the complex secretion process of EVs, common techniques such as ultracentrifugation, ultrafiltration and microfluidization

are commonly used to separate EVs. We summarize the features of the mentioned separation technologies (Table 2)⁵³⁻⁵⁵. Ultracentrifugation precipitates and purifies vesicles of the same size from a sample by centrifugation at high speed⁵⁶. Although the ultracentrifugation method is well-developed, there is some drawbacks, such as the inability to guarantee purity and unstable recovery. More importantly, repeated centrifugation operations may also cause damage to vesicles. Compared with other methods, Ultrafiltration is easy to operate, possess high separation efficiency and keep the biological activity of EVs. However, when using pressure as the driving force for membrane separation in the ultrafiltration process, EVs may well be damaged. And EVs tend to clog the filter, thus affecting the separation^{57,58}.

The emerging microfluidic chip technology is a promising separation method. This technology is a high-throughput method, which separates EVs according to their physical and biochemical properties like immunoaffinity and size. In addition to this, it can also combine electromagnetic, acoustic and other external forces with microfluidic technology to realize dynamic microfluidic flow⁵⁹. Since the first application of microfluidic chips to exosome isolation by Chen et al.⁶⁰ the studies of efficient isolation of EVs based on microfluidic technology have attracted much attention. For example, Casadei et al.⁶¹ successfully isolated and captured liposarcoma-derived EVs using a micro- and nanofluidic device. This device not only reduces processing time and ensured the integrity of the EV cargo, but also greatly improves separation efficiency. In terms of quality control during the large-scale production of EVs, ensuring their efficient and high-purity separation remains a considerable challenge. The intrinsic structure of EVs determines that they differ from the intrinsic charge density or isoelectric point of impurities, which can be exploited to separate EVs by Free-flow electrophoresis (FFE). Lee et al.⁶² used light-induced electrophoresis for automatic sorting of EVs and successfully separated three kinds of EVs (100–350 nm in diameter)

Table 1 Active components and functions of EVs.

Biochemical characterization	Origin	Component	Function	Ref.
Lipid	Grape	PA, PE	Remodeling intestinal tissue, anti-inflammation	28,29
	Grapefruits	PE, PC	Anti-inflammation, anti-oxidant	30
	Ginger	PA	Anti-inflammation	31
	Ginseng	PC 28.8%, TG 16.8%, Cer 12.9%, DGMG, PE	Maintaining stability and facilitating preferential uptake by the BBB	32
	Plasma	Gb3, PEps, PS, SL, BMP, Cer, LacCer	Modulating downstream pathways associated with COVID-19 pathology	33
	Edible tea flowers	EGCG, EC, ECG	Anti-cancer	34
	Plasma	PC, SM, CE, TAG, LysoPC	Promoting exosome biogenesis and lymphocyte chemotaxis	46
Protein	Serum	GPC1, EphA2	Noninvasive monitoring and diagnosis of early pancreatic cancer	50
	Urine	EphA2	Enhancing the migration of tumor cells	35
	Plasma	CA 15-3, CA 125, CEA, HER2, EGFR, PSMA, EpCAM, VEGF	Diagnosis, monitoring and prediction of metastatic breast cancer	36
Nucleic acid	Bitter melon	microRNA	Anti-cancer, anti-inflammation	37
	Apple	miRNA	Intestinal function	38
	Watermelon	microRNA	Regulation of intestinal secretion groups	39
	Nut	miRNAs	Anti-inflammation	40
	Tartary buckwheat	miRNA	Modulation of gut bacteria	41
	Ginseng	miR396, miR159, miR166, miR156, miR164	Regulation of apoptotic c-MYC signaling pathway.	32
	Serum	miR1246, miR141-3p, miR200a-3p, miR200b-3p, miR200c-3p, miR203a-3p, miR-429	Diagnosis and prognosis of epithelial ovarian carcinoma	42
	Stem cell	let-7b-5p, miR-24-3p	Reprogramming the dermal microenvironment	43
	Osteoinductive dental pulp stem cell	miR1246	Promoting bone tissue regeneration	44
	Hypoxic lung cancer cells	miR23a	Promoting endothelial cell migration of cancer cells and blood vessel formation	45

BMP, bis(monoacylglycero)phosphate; CA 15-3, carcinoma antigen 15-3; Cer, ceramide; CE, cholesterol esters; CEA, carcinoembryonic antigen; DGMG, digalactosylmonoacylglycerol; EC, epicatechin; ECG, epicatechin gallate; EGCG, epigallocatechin gallate; EGFR, epidermal growth factor receptor; EpCAM, epithelial cell adhesion molecule; EphA2, ephrin type-A receptor 2; Gb3, globotriaosylceramide; GPC1, glypican-1; HER2, human epidermal growth factor receptor 2; LacCer, lactosylceramide; LysoPC, lysophosphatidylcholine; PA, phosphatidic acids; PC, phosphatidylcholine; PE, phosphatidylethanolamines; PEps, phosphatidylethanolamines; PS, phosphatidylserine; PSMA, prostate-specific membrane antigen; SM, sphingomyelin; SL, sulfatide; TAG, triacylglyceride; TG, triglycerides; VEGF, vascular endothelial growth factor.

Table 2 Comparison of separation technologies of EVs.

Separation technology	Advantage	Disadvantage	Ref.
Ultracentrifugation	Most commonly used	Low purity	66
Ultrafiltration	Well developed	Possible damage of EVs	57,58
	Simple and efficient	Low yield	
Size-based microfluidics	No effect on the biological activity of exosomes	Potential for pore blockage	53
	High separation efficiency	Complicated photolithography fabrication	
Microfluidic separation based on immunoaffinity	Size uniformity of yield	Isolation of only exosomes containing target proteins	54
	High purity		
	Specific separation		
Field-free separation	Fast separation		
Surface enhancement of Raman scattering	High reproducibility and purity	Inefficiency	65
	Rapid separation	Adsorption of non-target EVs	62
Single vesicle profiling	Efficient detection of biomarkers		64
	Specific separation		
Dynamic microfluidics	Identification of vesicle heterogeneity	Complexity of analytical sample handling	55
	Size uniformity	Operation requiring experienced operators	
	High purity		
	Fast separation		
	Controllable process		

with a purity as high as 86%. Notably, surface enhancement of Raman scattering (SERS) enables simultaneous rapid separation of EVs and efficient detection of biomarkers. Zhang et al.⁶³ construct six SERS tags, enabling the simultaneous detection of six breast cancer biomarkers within 1.5 h. However, the separation method may adsorb non-target EVs carrying the same biomarkers. Single vesicle profiling can accurately identify vesicle heterogeneity between different cell types⁶⁴. But the complexity of analytical sample handling limits its wide application. Bai et al.⁶⁵ developed a viscoelastic microfluidic platform DEIC to enable Field-Free Separation and downstream analysis of EVs with minimal sample volume, achieving 100% separation accuracy for selected EpCAM/PD-L1 biomarkers.

Therefore, efforts have been made to design more efficient and convenient exosome separation platforms to provide better preparation for research. Pan et al.⁶⁶ designed a fast and efficient EVs separation platform, EV-FISHER. EVs from plasma could be induced by cholesterol, followed by centrifugation to obtain. EV-FISHER is optimized for efficient plasma EVs separation compared to ultracentrifugation in terms of processing time, separation efficiency, and separation requirements. In addition, Zong et al.⁶⁷ showed a dual-switching pH-responsive peptide (referred to as D-S pHLIP) that selectively isolated and enriched cTME-EVs from plasma, which provided an additional option for selective enrichment of EVs for downstream analysis. To improve the isolation of tumor-derived EVs (TDEVs), Niu et al.⁶⁸ developed a fluidic multivalent magnetic interface (FluidmagFace). Compared with non-fluidic interfaces, FluidmagFace could significantly improve the affinity and separation rate, providing new possibilities for subsequent cancer diagnosis and biomarker discovery. Mature exosome isolation techniques are necessary for the cutting-edge research and clinical application of exosomes. Some traditional isolation methods may not meet the existing needs, so designing isolation methods to adapt to more conditions or to obtain qualified exosomes conveniently and efficiently would be hot research topics in the next few years.

3. Diagnosis of tumors by EVs as biomarkers

Biomarker, a characteristic biochemical index, which can be used for objective measurement and evaluation. Many substances such as serum and exosomal miRNAs are used as biomarkers of cancer, which can be used to diagnose the occurrence of cancer, judge the stage of cancer or assess the safety and efficacy of new therapeutic strategies⁶⁹. EVs are common intercellular signal transmission media between eukaryotic cells and prokaryotic cells⁷⁰. Nowadays, they are considered as extremely valuable biomarkers for diagnosing and predicting different diseases because the carried bioactive substance reflect the biological state of the source cells (Table 3)^{26,71-73}.

3.1. Protein as a biomarker

It is always a project worthy of intense study to find the unique proteins in EVs. With the implementation of proteomics, many potential biomarkers of proteins have been discovered. Detection of specific protein expression in EVs can diagnose cancer. EVs derived from melanoma impair the maturation of DC and the production of cytokines and promote the TME of immunosuppression. Compared with healthy controls, programmed death-ligand 1 (PD-L1) in plasma circulating EVs derived from patients

with metastatic melanoma is significantly upregulated (Fig. 2A and B)⁷⁴. The analysis of PD-L1 level enables researchers to diagnose cancer against the response and non-response of PD-1 treatment. ITGA3 and ITGB1 in exosomes were highly expressed in patients with metastatic prostate cancer⁷⁵. Moreover, detecting HSP 70 among patients with cancer can monitor whether there is a malignant solid tumor.

During the initiation and advancement of cancer, there will be alterations in the composition and types of proteins found in EVs. Marine Cordonnier et al.⁷⁶ found that the change in the level of exosomal PD-L1 in the peripheral circulatory system could be used as a relevant biomarker in melanoma treatment. Phosphorylation of AKT and ERK 1/2 in EVs of U87 glioma cells and non-small cell tumors decreased significantly after drug treatment in patients with good treatment response⁷⁷. Timothy et al.⁷⁸ used sorafenib + selective internal radiation therapy and sorafenib alone to treat patients with hepatocellular carcinoma. The proteomics results showed that high ARHGAP1 and low GPX3 expressed in plasma EVs can indicate the better efficacy of sorafenib monotherapy (Fig. 2C, D and E). Yang et al.⁷⁹ discovered that astrocyte EV markers were significantly enriched in the M7 module, and exhibited significant positive correlations with Alzheimer's disease traits (Fig. 2F). This suggests that protein markers integrin- β 1 (ITGB1) derived from astrocyte-derived EVs may serve as potential biomarkers for Alzheimer's disease (Fig. 2G). Additionally, proteins in EVs can judge cancer prognosis. Monica et al.⁸⁰ compared the proteins in EVs between healthy patients and patients with melanoma after treatment. Among 75 proteins overexpressed in patients with disease progression, PDCD 6 IP had the highest discrimination value which had prognostic significance in melanoma. In colorectal cancer, Zhang et al.⁸¹ proved that CD147 and A33, two transmembrane proteins on fecal EVs had the potential to monitor the prognosis of colorectal cancer (Fig. 3A and B). It is necessary to detect several other protein biomarkers in combination.

In conclusion, the application of proteins as biomarkers is still limited due to objective conditions. First, a single biomarker cannot accurately predict the immunotherapy response. Secondly, the difference of TIME and heterogeneity of tumor proteins make them have low predictive value in evaluating remission⁸². At the same time, sample analysis involves many protein extraction and analytical techniques, which makes it more difficult to convert into clinical application.

3.2. RNA as a biomarker

Compared with protein biomarkers, RNA biomarkers are more sensitive and specific which can be used to diagnose cancer more effectively. miRNAs are the most abundant type of foreign RNA cargo. The miRNAs are increasingly regarded as the key factors of tumor occurrence and metastasis and the transport of miRNAs promotes their evaluation as biomarkers⁸³. Zhou et al.⁸⁴ proved that exosomal miR-15a-5p in patients with endometrial cancer was downregulated and miR-106b-5p were upregulated significantly relative to healthy people. By detecting the content of these exosomes, we can judge the occurrence of cancer. Additionally, RNAs in GSC EVs were associated with angiogenesis in brain endothelial cells, and the presence of gliomas could be judged by monitoring the corresponding RNAs⁸⁵. Moreover, in patients with melanoma, the content of BRAF RNA in EVs is easier to detect than that in plasma⁸⁶. In addition, miR-1246, miR-451 and miR-

Table 3 EVs as biomarkers in clinical diagnosis, treatment and prognosis for cancer.

Disease	Biofluid	EV type	Biomarker	Biological function	Ref.
Melanoma	Plasma	Exosomes and microvesicles	PD-L1	A predictor of anti-PD-1 therapy and clinical outcomes	74
	Plasma	Exosomes	PD-L1		76
	Plasma	Small extracellular vesicles	PDCD 6 IP	Prognostic biomarkers in melanoma	80
Prostate cancer	Plasma	Exosomes	BRAF RNA		86
	Urine	Exosomes	ITGA3 and ITGB1	Diagnostics biomarkers for prostate cancer in a non-invasive manner	75
	Urine	Exosomes	Phosphatidylserine and lactosylceramide	Biomarkers in prostate cancer	101
	Urine	EVs	Steroid hormone and dehydroepiandrosterone sulphate	A non-invasive source to infer metabolic changes in prostate cancer	103
Glioma	Patient-derived glioma stem-like cultures	Microvesicles, exosomes	miR-182-5p, miR-196a-5p and miR-10b-5p	Feasibility on cerebrospinal fluid	72
Hepatocellular carcinoma	Plasma	EVs	GPX3 and ARHGAP1	A novel predictive biomarker in hepatocellular carcinoma	78
	Plasma	Exosomes	tRNA-VaTAC-3, tRNA-GlyTCC-5	Biomarkers	89
	Serum	Exosomes	circPTGRI, miR-449a-MET	Acting on tumor cell migration and invasion	92
Colorectal cancer	Fecal	EVs	CD147 and A33	Biomarkers in colorectal cancer	81
	Serum	Exosomes	miR-208b	Predictive biomarkers of response to oxaliplatin-based therapy	90
Endometrial cancer	Plasma	Exosomes	miR-15a-5p, miR-106b-5p	Diagnostic biomarkers in endometrial cancer	84
Non-small cell lung cancer	Serum	Exosomes	lncRNA TBILA, AGAP 2-AS 1 and SOX 2 OT	Promising biomarkers in non-small cell lung cancer	73
Gastric cancer	Plasma	Exosomes	let-7i-5p, miR-181a-5p, miR-1843 and miR-1307-3p, LZIC and SRSF6, lncFTH1-211 and lncPTMA-209	Identifying responders to fluorouracil-based chemotherapy	91
	Serum	Exosomes	circSHKBP 1	Diagnostic and prognostic circulating	93
Pancreatic cancer	Serum and plasma	Exosomes	GPC1 membrane protein	Biomarkers in gastric cancer	95
	Plasma	Exosomes	Phosphatidylserine	Biomarkers of early malignancy	102

122 in breast cancer MCF 7 exosomes were selectively secreted and enriched relative to other cell contents⁸⁷.

The miRNAs in EVs isolated from plasma are differentially expressed between immune checkpoint therapy responders and non-responders, which could be used to judge the therapeutic effect⁸⁸. The potential value of tRNA-derived small RNAs (tsRNAs) in cancer diagnosis has also been tapped. Zhu et al.⁸⁹ compared exosomal tsRNA levels in hepatocellular carcinoma patients with those in healthy donors and showed a significant increase in plasma exosomes of tsRNAs in hepatocellular carcinoma patients. Additionally, Ning et al.⁹⁰ found that miR-208b secreted by tumors promoted regulatory T (Treg) cell amplification, which was associated with decreased sensitivity of colorectal cancer to oxaliplatin chemotherapy. And so miR-208b is a possible biomarker for predicting the response to oxaliplatin therapy. In advanced gastric cancer (GC) patients, Guo et al.⁹¹ identified and validated the predictive ability of four miRNAs, two mRNAs, and two lncRNAs derived from plasma exosomes in NACT non-responders and responders. CircPTGRI, which could down-regulate miR 449 a-MET in recipient cells was highly

abundant in malignant cells and cells of metastatic patients, so it could provide information on the prognosis and treatment of hepatocellular carcinoma⁹². In GC patients, overexpression of circSHKBP 1 in tumors was strongly associated with advanced pathological stages. CircSHKBP 1 promoted the development of GC through uptake of miR-582-3p and induction of HSP 90⁹³. Zhao et al.⁹⁴ discovered that the expression of lncRNA HOTTIP was elevated in patients with GC and was correlated with the overall survival rate of these patients (Fig. 3C and D). Li et al.⁹⁵ discovered that the activation of specific oncogenes resulted in an increased expression of mRNA and its corresponding proteins in extracellular vesicles present in the bloodstream. The scatter plot depicting the expression of double GPC1 Exo-mRNA/tMV-mProtein demonstrated superior diagnostic performance (Fig. 3E and F). Although RNAs need to be further validated and evaluated as biomarkers, they are more suitable for use as circulating markers due to their unique biological properties. Although great progress has been achieved in identifying and characterizing circRNAs, many critical unknowns and limitations need to be addressed in further exploration.

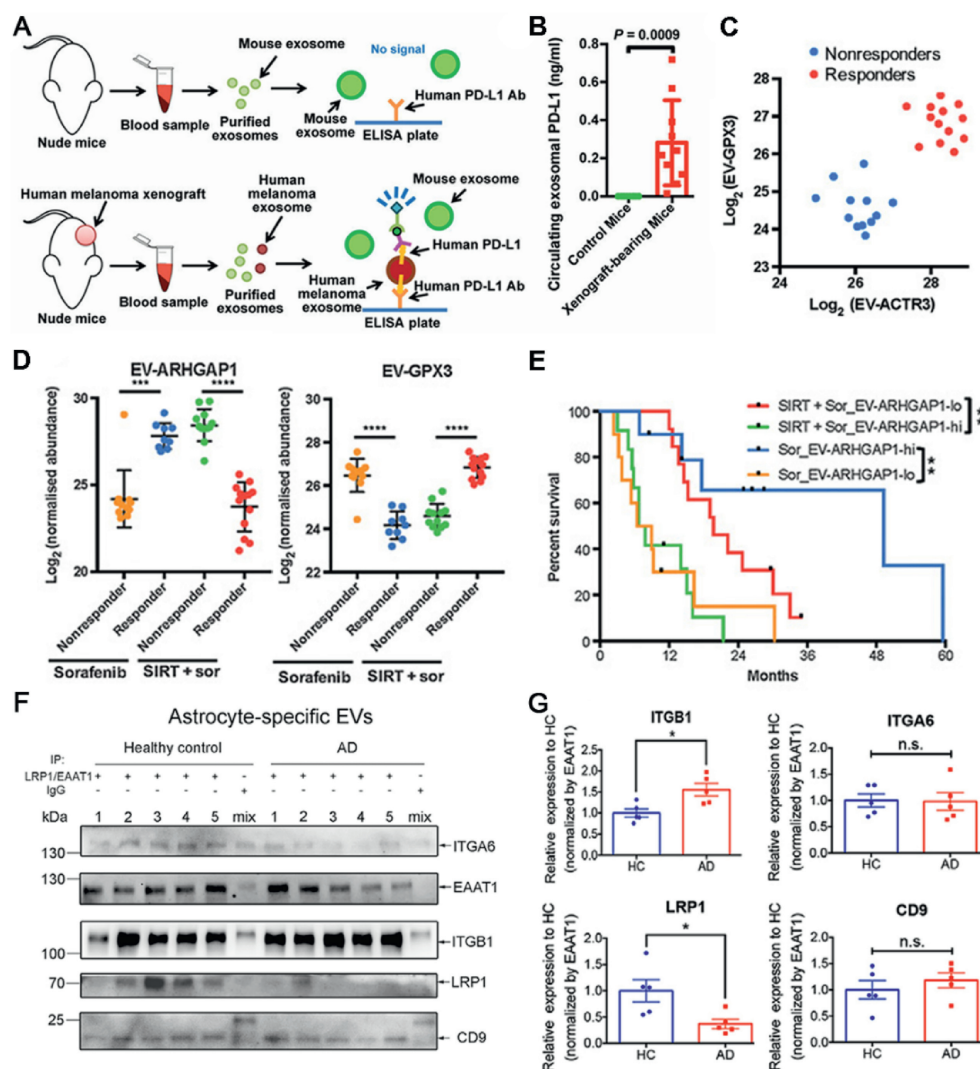


Figure 2 (A) Schematic representation of ELISA for detecting human exosomal PD-L1. (B) PD-L1 levels on exosomes isolated from plasma samples of nude mice. Reprinted with the permission from Ref. 72. Copyright © 2018 Springer Nature Limited. (C) Levels of EV-GPX3 and EV-ACR3 and corresponding protein expression in patients before SIRT + sorafenib treatment. (D) Abundance and (E) Percent survival of EV-ARHGAP1 and EV-GPX3. Reprinted with the permission from Ref. 76. Copyright © 2022 American Association for Cancer Research. (F) Western blot analysis of the astrocyte-specific EVs for the ITGB1 in the M7/astrocyte-EV module. (G) Western blot semiquantitative analysis plots. Reproduced with permission from Ref. 77. Copyright © 2021 The Authors.

3.3. Other biomarkers

The DNAs in EVs which are protected by double lipid membrane have good stability. They show great potential as cancer biomarkers. For example, Thakur BK et al.⁹⁶ proved that most DNAs related to tumor exosomes were double-stranded and had high translation value for cancer detection. Maire et al.⁹⁷ proved that the DNAs extracted from glioma EVs could not only detect tumor-specific genome-wide mutations, but also be used for subtype distribution of glioma. Additionally, liquid biopsies of EVs successfully isolated from NSCLC patients proved higher consistency than conventional tissue biopsies, and the DNAs isolated from plasma EVs of NSCLC patients could also be used to identify KRAS and the EGFR mutations, which proved that DNAs could be wonderful biomarkers⁹⁸. Cheng et al.⁹⁹ proved that the level of mtDNA was higher in patients with oral cancer. Thus, the levels of mtDNA can be taken as a biomarker in anti-PD-L1 immunotherapy and diagnosing diseases. As a biomarker

for early detection, DNA has great prospects, especially for diseases that have no screening options at present, such as ovarian cancer, pancreatic cancer and GC. When combined with protein, RNA and other biomarkers, DNA can play a deeper role¹⁰⁰. Other metabolites such as lipids, phosphatidylserine in EVs can also be used as biomarkers, but there are few studies on them. Tore Skotland et al.¹⁰¹ found the differences in lipid types between healthy people and patients by studying liposomes in urine foreign bodies from prostate cancer, which laid a foundation for liposomes as biomarkers. In addition, Raghava Sharma et al.¹⁰² revealed that the content of phosphatidylserine was higher in patients with pancreatic cancer through an ELISA-based determination system. In prostate cancer, the metabolites such as phosphatidylcholine, acyl carnitine, citrate and kynurenine had changed the urinary EVs of patients¹⁰³.

Many substances in EVs are used as biomarkers, but there are few studies on other related biomarkers except protein and RNA. Due to the limitation of technology and the heterogeneity of

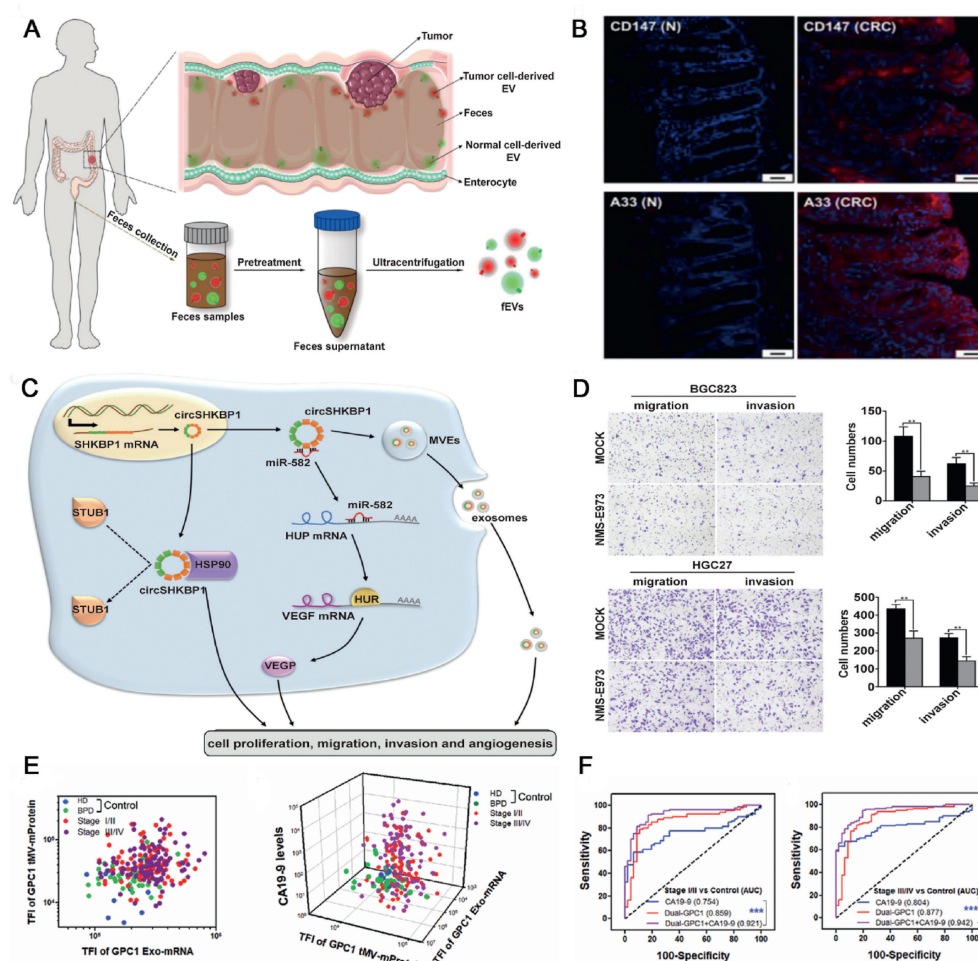


Figure 3 (A) Scheme of biogenesis and isolation of faeces-derived EVs. (B) The expression levels of CD147 and A33. Reproduced with permission from Ref. 79. Copyright © 2023 Zhang et al. (C) Mechanism of circSHKBP1 regulating the progression of GC. (D) Migration and invasion of BGC823 and HGC27 cells. Reproduced with permission from Ref. 91. Copyright © 2020 Xie et al. (E) Scatter plot of CA19-9, GPC1 Exo-mRNA and tMV-mProtein expression. Reproduced with permission from Ref. 93. Copyright © 2023 Li et al. (F) ROC curves for PDAC stage I/II and III/IV patients. Reproduced with permission from Ref. 93. Copyright 2023 Li et al.

cancer, there will be some differences in the expression of single protein and nucleic acid¹⁰⁴.

4. EVs from different sources

When used as diagnostic markers, EVs are often extracted from serum, plasma, urine, saliva, and other body fluid samples. EVs derived from tumor cells, MSCs, DCs, macrophages, and plants are often utilized in specific drug delivery systems based on their different biological properties.

4.1. EVs as diagnostic markers

In the clinical field, Tumor-derived EVs (TDEVs) are diagnostic biomarkers of tumor onsets by liquid biopsies (Fig. 4). VCAN and LGALS9, the damage-associated molecular pattern proteins, were highly enriched in pancreatic and lung adenocarcinoma-derived vesicles¹⁰⁵. Liquid biopsies are non-invasive, sensitive, and

convenient, using information in the blood to suggest tumor-specific information¹⁰⁶. Due to the large number of cells present in whole blood, blood derivatives such as plasma and serum are the most studied biofluids in liquid biopsies (Table 3). The blood components are complex, the levels of EVs in whole blood are extremely low, and the sampling procedure can affect the analysis of EVs in the sample in some way¹⁰⁷. Recently, the use of serum or plasma samples for biomarker studies has been widely discussed. Serum and plasma contain different subtypes of EVs and the presence of IV transmembrane proteins carried by platelet-derived EV is different. The concentration of EVs derived from platelets is higher in serum since platelets are activated and release substantial amounts of EVs during serum clotting *in vitro*¹⁰⁸. The current findings suggest that plasma samples may be more suitable for tumor biomarker testing, with the choice of specific samples depending on which EVs the study is interested in. In addition, the extraction of plasma from whole blood requires the use of anticoagulants, the common anticoagulants being acid-citrate-dextrose (ACD), ethylenediaminetetraacetic acid (EDTA), and

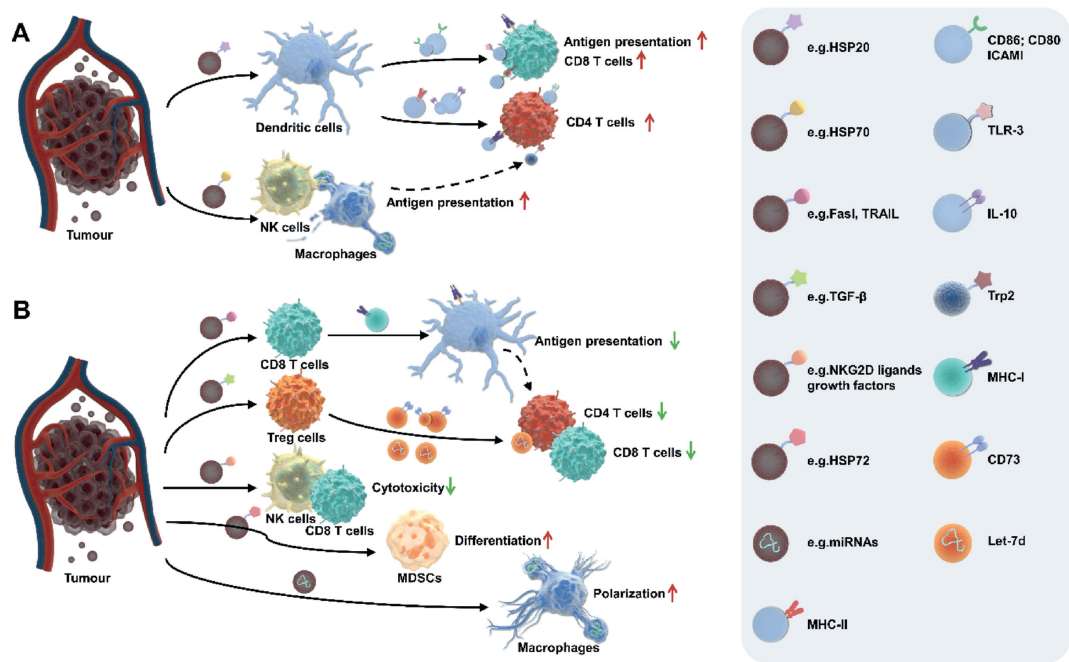


Figure 4 (A) TDEVs enhance anti-tumor immune response. (B) TDEVs inhibit anti-tumor immune response.

Table 4 Comparison of the molecular composition, and separation methods of EVs from mammalian cells and plant cells.

Type	Source	Molecular composition		Separation method	Ref.
		Lipid	Others		
Plant-derived exosome-like nanovesicles	Ginseng	DGMG, 59.4%; PE, 16.8%; Cer, 13.8%	Ginsenoside Rg3	Differential centrifugation and sucrose density gradient ultracentrifugation	156
	Ginger	PA 37.0%, DGDG 39.9%, MGMG 16.9%	6-Shogaol, 6-gingerol	Gradient ultracentrifugation	149
	<i>Catharanthus roseus(L.) Don</i>	EtherPC 16.6%; PG 15.7%; PI 14.0%; EtherPG 9.4%	Vinpocetine	Differential ultracentrifugation and enzyme digestion	150
	Edible tea flowers	PC, 26.6%; TG, 23.4%; PE, 15.2%	EGCG, ECG, EC, vitexin, myricetin-3- O-rhamnoside	Differential centrifugation and sucrose density gradient ultracentrifugation	151
	Grapefruit	PE 45.5%; PC 28.5%	Naringenin	Sucrose gradient centrifugation	152
Mammalian-derived nanovesicles	Breast cancer cells	Sphingolipids, glycerophospholipids		Differential centrifugation	153
	Plasma	Cholesterol esters, sphingolipids, PC, PE		Centrifugation and magnetic bead separation	154
	Triple-negative breast cancer cell	FA, LPC, LPE, PC, PE		Ultracentrifugation	155

Cer, ceramide; DGMG, digalactosyl monoacylglycerol; DG, diglycerols; EC, epicatechin; ECG, epicatechin gallate; EGCG, epigallocatechin-3-gallate; EtherPC, ether-phosphatidylcholines; EtherPG, ether-phosphatidylglycerols; FA, fatty acid; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; MG, monoglycerols; PA, phosphatidic acids; PC, phosphatidylcholine; PE, phosphatidyl ethanolamine; PG, phosphatidylglycerols; PI, phosphatidylinositol; TG, triglyceride.

heparin. Zhang et al.¹⁰⁹ found that anticoagulants do not influence the concentration of EVs, but the concentration of EVs decreases as time goes on. Therefore, rapid separation of the assay is required after sample extraction. However, the choice of anticoagulant affects the composition of EVs in the sample and alters EV profiles. For example, heparin and EDTA cause an immediate elevation in the number of CD15-positive myeloid EVs. Karimi

et al.¹¹⁰ found that EDTA plasma contained more CD9 EVs and residual platelets than ACD-plasma and serum.

4.2. EV as drug delivery systems

EVs of different cellular origins (e.g., tumor cells, MSCs, DCs, macrophages, and plants) differ significantly from each other and

Table 5 Communication functions of EVs from different sources.

Source of EVs	Communication object	Transmitted substance	Function
Pancreatic and lung adenocarcinoma-derived EVs	Non-tumor adjacent tissue	DAMP proteins VCAN and LGALS9	Markers to distinguish cancer <i>versus</i> non-cancer
Glioblastoma-derived EVs	PD-1	PD-L1	Stimulating NK- κ B
Metastatic prostate cancer-derived EVs	Bone cell	CDCP1 protein	Detecting bone metastasis of prostate cancer
Breast cancer-derived EVs	Tumor-associated macrophages	miR-138-5p	Promoting the polarization of tumor-associated macrophages
Tumor cell-derived EVs	Neutrophils	IL-8, MCP-1	Regulating their recruitment, activation, and apoptotic processes
Lung adenocarcinoma-derived EVs	Tumor-associated macrophages	miR-19b-3p	Facilitating M2 macrophage polarization
MSC-EVs	Mitochondrion	Mitochondrial transcription factor A mRNA	Attenuating mtDNA damage and inflammation
MSC-EVs	Brain microvascular endothelial cells	miR-612	Promoting angiogenesis
DC-EVs	Hepatoma carcinoma cell	α -Fetoprotein	Triggering antigen-specific antitumor immune responses
Tumor-associated macrophages-EVs	Pancreatic ductal adenocarcinoma	miR-365	Reducing drug sensitivity
Tumor-associated macrophages-EVs	Treg cells/Th 17	miR-21-5p and miR-29-3p	Promoting the progression and metastasis of tumor cells
M2 macrophages-EVs	Epithelial cells	Fibromodulin	Protecting the tight junction
M2 macrophages-EVs	ECM	ECM enzymes	Degrading collagens
Ginseng-derived nanoparticles	Toll-like receptors on M2 macrophages	Ceramide lipids and proteins	Promoting polarization of M2 phenotype towards M1 phenotype and producing ROS
Lemon-derived EVs	Gastric cancer cell	Substances in EVs	Inducing cell death
Citrus limon-derived nanovesicles	TNF-related apoptosis-inducing ligand-receptor	Substances in EVs	Inhibiting cancer cell proliferation

possess properties similar to their derived cells¹¹¹. This section is to separately summarize their unique biological properties when it comes to drug delivery systems (Table 5 and Fig. 5).

4.2.1. EVs derived from tumor cells

TDEVs have a natural tropism for tumor tissue, possibly because EVs acquire the same surface protein pool as their parental cells. EVs derived from melanoma cells accumulated more significantly in the tumor tissue compared to EVs from other sources¹¹². Similar results were obtained with EVs from sarcoma cells, showing preferential tumor homing¹¹³. Accumulating evidence has shown that TDEVs are double-edged swords in the growth and recurrence of tumors. On the one hand, TDEVs carry tumor-associated antigens, which further activate CD8⁺ T cells to trigger cellular immunity¹¹⁴. Besides, TDEVs can inhibit tumorigenesis by improving the TME. For example, TDEVs could bind to the inhibitory immune receptor CD300a expressed on DCs to prevent Treg cell activation in the TME, thereby preventing melanoma development¹¹⁵. In addition, TDEVs affect the anti-tumor activity of neutrophils by regulating their recruitment, activation, and apoptotic processes. TDEV produces cytokines and chemokines that lead to neutrophil recruitment at the tumor site and more effective phagocytosis and killing of tumor cells¹¹⁶.

Conversely, TDEVs harbor a range of immunosuppressive factors naturally from the donor cells that suppress the immune response and facilitate the formation of immunosuppressive TMEs. TDEVs can stimulate nuclear factor κ B (NF- κ B) or intervene in the PD-L1/PD-1 axis to support tumor immune evasion¹¹⁷. In addition, TDEVs not only lead to macrophage

polarization toward M2 phenotypes that promote tumor progression in TME but also inhibit the expression of NKG2D of natural killer (NK) cells to suppress the activity and cytotoxicity of them¹¹⁸. TDEVs have the ability to activate myeloid-derived suppressor cells (MDSCs), resulting in significant suppression of T cell function. This suppression leads to tumor proliferation, migration, immune evasion, and resistance to tumor immunotherapy^{119,120}. Taken together, TDEVs can exert a bidirectional regulatory effect on TME by modulating immune cell function.

4.2.2. EVs derived from MSCs

MSCs are adult stem cells with multidirectional differentiation potential¹²¹. MSC-derived EVs (MSC-EVs) can function as an immunomodulator by mediating the interaction of bioactive substances such as multiple cytokines, chemokines, and miRNAs with receptors expressed on target cells¹²². EVs derived from adipose MSCs, acting as immunomodulators, could reduce the level of interferon γ and transcription factors while upregulating the level of immunomodulatory cytokines, thereby inhibiting local inflammatory responses. MSC-EVs containing the inflammation inhibitor IL-1RA could block the release of pro-inflammatory cytokines to induce rapid wound healing¹²³. As for brain diseases, MSC-EVs can cross the blood-brain barrier smoothly and are enriched in cytokines related to nerve regeneration, thus promoting the survival of nerve cells and inhibiting their apoptosis¹²⁴. MSC-EVs, enriched in enkephalinase, can downregulate the expression of β -amyloid peptide when co-cultured with neuroblastoma cells, implying that MSC-EVs could be an effective treatment for Alzheimer's disease¹²⁵. However, in addition to the

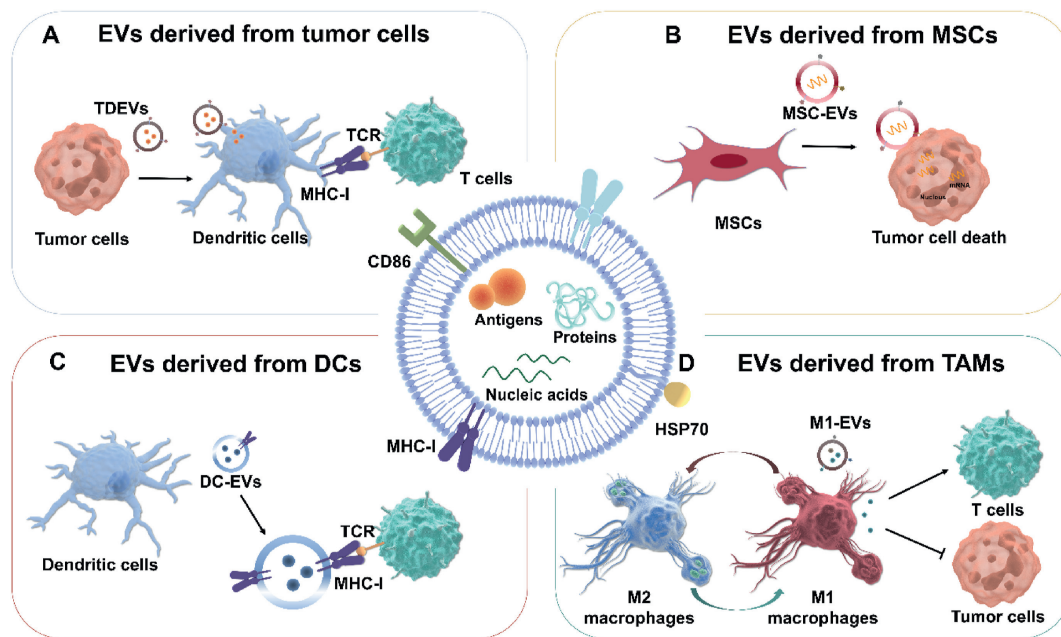


Figure 5 (A) TDEVs activate immune response by promoting T cell expansion. (B) MSC-EVs inhibits tumor cell growth through miRNA-mediated mechanisms. (C) DC-EVs stimulate immune activation by promoting T cell expansion directly. (D) M1-EVs polarize TAMs from the M2 phenotype to the M1 phenotype, thus reversing the TME.

therapeutic effects, MSC-EVs are at risk of promoting tumor growth. MSC-EVs activate the AKT signaling pathway of GC cells and enhance their invasive ability, thereby inducing epithelial–mesenchymal transition in GC cells¹²⁶. Cytokines carried by MSC-EVs could activate signal transduction downstream of ERK1/2 and then increase the release of VEGF, thereby stimulating tumor progression¹²⁷.

4.2.3. EVs derived from DCs

DCs are versatile antigen-presenting cells and build bridges between innate and adaptive immunity¹²⁸. DC-EVs express MHC peptide complexes and co-stimulatory molecules on their surface, which provides conditions for its action with other immune cells¹²⁹. DC-EVs can present antigens to initiate T-cells to induce anti-tumor immune responses and interact with antigen-specific CD4⁺ T lymphocytes to generate an adaptive immune response¹³⁰. Liu et al.¹³¹ found that DCs that migrate to the lymphatic organs can stimulate alloreactive T cells by transferring clusters of EVs containing functional MHC molecules to resident DCs. DC-EVs can also activate CD8⁺ T cell and B cell responses *in vivo* antitumor immunity¹³². Moreover, DC-EVs can induce Treg cell production by producing TGF- β and IL-10, thus inhibiting the activation of antigen-specific T cells and inducing immune tolerance¹³⁶. EVs derived from donor DCs could induce antigen-specific immune tolerance of the donor and significantly reduce expression of infiltrating leukocytes and interferons (IFN)- γ , thus markedly prolonging the survival of rat heart transplantation¹³³. In addition, DC-EVs express transmembrane on their surface, which can activate NK cells and directly mediate the apoptosis of tumor cells¹³⁴. Recently, DC-EVs have been used as an improved strategy for cell-free vaccines with specific antigens that confer a personalized anti-tumor response. DC-EVs-based immunotherapy has shown a strong safety profile to date. Li et al.¹³⁵ established a nanovaccine delivery platform using DC-EVs-based nanovaccines. Comparing DC-EVs-based

personalized antigen carriers with liposomes, they found that DC-EVs improved lymph node delivery efficiency and promoted CD8⁺ T cell-mediated cytotoxic responses.

4.2.4. EVs derived from macrophages

Macrophages can show different phenotypes under different environmental stimuli, including M1 macrophages and M2 macrophages. The balance between these two subtypes is important in fighting pathogens, regulating inflammation, and repairing tissues¹³⁶. M2-EVs can exert anti-inflammatory effects and alleviate atherosclerosis through surface-bound chemokine receptors¹³⁷. M2-EVs can promote endothelial cell proliferation, migration, and angiogenesis, with good tissue repair ability¹³⁸. Moreover, M2-EVs possessed the neuroprotective effects of macrophages and could repair damaged neurons (Fig. 6A)¹³⁹. Li et al.¹⁴⁰ demonstrated bioactive M2-EVs can accelerate skin wound healing by promoting endothelial cell angiogenesis and macrophage polarization to the M2 phenotype *in vivo* (Fig. 6B).

Since the TME is highly relevant to the inflammatory state, macrophage-derived EVs could regulate tumor development. Specifically, M1-EVs have tumor suppressor activity¹³⁶. M1-EVs can enhance immune responses and suppress tumor growth by promoting the production of Th1 cytokines. In contrast, M2-EVs are not only able to promote tumor growth and migration by secreting chemokines and cytokines that inhibit inflammatory responses, but also promote immunosuppression and immune escape¹⁴¹. Recently, Wang et al.¹⁴² verified that M2-EVs upregulate PD-L1 *via* the MISP/IQGAP1/STAT3 axis to exert immunosuppressive effects and promote immune escape from HCC cells using plasmid transfection technology (Fig. 6C). Ma et al.¹⁴³ transformed M1-type macrophage-derived exosomes into radiotherapy sensitizers, which loaded three functional molecules, namely catalase, DNA damage repair inhibitor and PD-L1 antibody, to achieve three different functions thus effectively improving the effect of radiotherapy. Since M1-type macrophage-

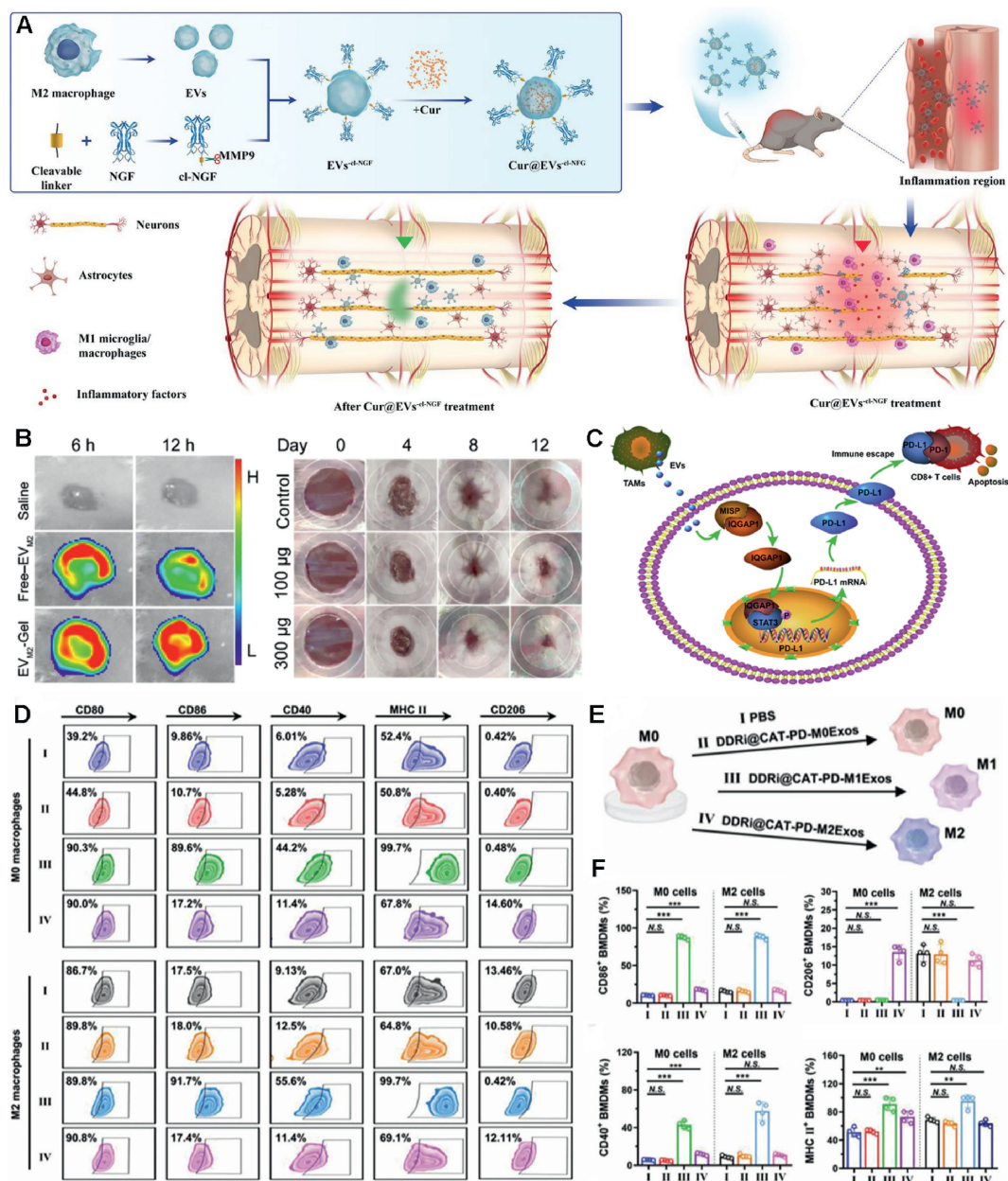


Figure 6 (A) Scheme diagram of engineered EVs derived from M2 macrophages for tospinal cord injury therapy. Reproduced with permission from Ref. 139. Copyright © 2021 The Author(s). (B) Proportion of fluorescence intensity and wound size of skin wounds extracted from different treatments. Reproduced with permission from Ref. 140. Copyright © 2023 American Chemical Society. (C) M2-EVs down-regulate MISP levels, up-regulate IQGAP1 levels and then phosphorylated STAT3 and promote PD-L1 transcript expression. Reproduced with permission from Ref. 142. Copyright © 2023 Elsevier B.V. (D–F) Flow cytometry analysis of phenotypes of macrophages. Reproduced with permission from Ref. 143. Copyright © 2022 Wiley-VCH GmbH. (E) Schematic diagram of macrophage polarization. Reproduced with permission from Ref. 143. Copyright © 2022, Wiley-VCH GmbH.

derived exosomes can inherit proteins and nucleic acids from donor cells, it is very likely to promote other types of macrophages to become anti-tumor M1-type. The authors used M1-type exosomes to be uptaken by M0 and M2 macrophages to evaluate whether the polarity changes (Fig. 6E). After incubation with M1-type exosomes, the expressions of M1-type markers CD86, CD40, and MHC-II were increased in both M0 and M2 cells. After incubation with M2-type exosomes, the expression of M2-type marker CD206 was increased in M0 cells, and the expression of

M2-type marker CD206 was decreased in M2-type cells after incubation with M1-type exosomes (Fig. 6D–F).

4.2.5. EVs derived from plants

Research on the biogenesis of plant-derived exosome-like nanovesicles (PDENs) is still in its early stages. Most plant-derived vesicles used in biomedical applications are obtained by disrupting plant tissue, and the exact biogenetic pathway remains unclear¹⁴⁴. There are three main biogenetic pathways for

plant-derived vesicles: the MVB pathway, similar to exosome production in mammalian cells, and a unique exocyst-positive organelles pathway that differs morphologically from MVB, operates independently of endosomes and autophagosomes, and releases vesicles into extracellular space¹⁴⁵. Furthermore, vacuoles are capable of fusing with the plasma membrane to release intraluminal vesicles¹⁴⁶. Lipids are essential for the stability of PDENs due to their composition being primarily responsible for the maintenance of the bilayer membrane. Lipidomics analysis has revealed that the main lipid classes present in PDENs are phosphatidyl acid, phosphatidylethanolamine, and phosphatidylcholine¹⁴⁷. In contrast, EV lipids of mammalian origin consist mainly of ceramides, cholesterol, glycolipids, sphingomyelin, and phosphatidylserine¹⁴⁸. In addition to being rich in lipids, PDENs also contain phytochemicals and exhibit unique biological characteristics (Table 4)¹⁴⁹⁻¹⁵⁵.

PDENs are membranous vesicles secreted by plants. As natural nano-agents, PDENs show unique functions in anti-tumor, immune function regulation and treatment of intestinal diseases. Studies on the use of PDENs as anticancer therapeutic agents have demonstrated their promising applications in cancer therapy¹⁴⁷. PDENs can regulate macrophage polarization and promote to affect tumor growth. Specifically, the EVs-liked ginseng-derived nanoparticles (GDNPs) could induce M1 polarization and increase apoptosis in mouse melanoma cells. This suggested that GDNPs could be involved in the mammalian immune response as immunomodulators, thus providing novel nanomedicines for cancer immunotherapy¹⁵⁶. In view of the rich pharmacological effects of citrus fruits, more in-depth studies of the vesicles isolated from them is warranted. Studies have shown that grapefruit-derived nano- and micro-vesicles could induce apoptosis¹⁵⁷. In addition, lemon-derived EVs (LDEVs) were able to promote to generate reactive oxygen species (ROS) and promote TRAIL release. The released TRAIL were bind to DR5 and induce apoptosis of tumor cells^{158,159}. Besides, LDEVs could enter tumor cells *via* caveolin-mediated endocytosis and micropinocytosis to deplete intracellular ATP to overcome multidrug resistance¹⁶⁰. Due to the different uptake capacities of different cells for exosome-like nanovesicles derived from *Asparagus cochinchinensis* (Lour.) Merr. (ACNVs), ACNVs did not significantly damage normal hepatocytes but inhibited proliferation and induced apoptosis in hepatocellular carcinoma cells. Moreover, ACNVs treated with polyethylene glycol had stronger antitumor effects due to prolonged blood accumulation and increased tumor guidance¹⁶¹. The discovery of EVs from *Pinus densiflora* with antitumor effects suggested that these EVs from plant sap were new candidates for cancer treatment¹⁶². Additionally, the specific population of ginger-derived nanoparticles (GDNPs 2) showed favorable therapeutic effects on intestinal epithelial cells. This study proved that GDNPs 2 had good anti-inflammatory properties by down-regulating inflammatory factors, which provided a feasible treatment protocol for the prevention and treatment of colitis-associated cancer¹⁶³.

5. EVs as drug delivery systems

With the advantages of high biocompatibility, cellular specificity, better stability, and limited immunogenicity compared to traditional synthetic drug delivery vehicles⁷, EVs have been applied in drug delivery systems to transport cargo into the cytoplasm of target cells⁸. EVs can protect themselves against the clearance of the immune system due to their small size and specificity of

membrane-bound proteins. More importantly, EVs can successfully cross physiological barriers to reach target cells and efficiently transport different cargoes to target cells⁹.

5.1. Drug loading methods

EVs can carry multiple types of drugs, mainly nucleic acid drugs, protein drugs, and small molecule drugs¹⁶⁴. Ways to achieve EV loading are divided into post-loading and pre-loading in this section¹⁶⁵.

5.1.1. Pre-loading

Pre-loading is the loading of drug molecules into parental cells so that the secreted EVs with membrane integrity carry drug molecules. The most common methods used are co-incubation and transfection (Fig. 7A)^{166,167}. Co-incubation refers to the simultaneous incubation of drug molecules with parent cells that produce extracellular vesicles (EVs) under specific conditions. The drug molecules, particularly hydrophobic drugs, penetrate the cell by interacting with the phospholipid bilayer of the cell membrane and are then encapsulated within the subsequently released EVs^{168,169}. The loading efficiency of curcumin co-incubated with adipose-derived mesenchymal stem cells was $82.26 \pm 5.25\%$ ⁹. Transfection refers to parental cells that overexpress therapeutic molecules (*e.g.*, miRNAs, siRNAs, proteins) and load these molecules into subsequent secretion into EVs¹⁷⁰. The binding efficiency of carbataxel/TNF-related apoptosis-inducing ligand by transfection was $93.7 \pm 1.53\%$ ¹⁷¹. Mizrak et al.¹⁷² use mRNA/protein transfection of parental cells, releasing EVs carrying suicide mRNA and protein-cytosine deaminase, inducing apoptosis in cancer cells. This loading method has high reproducibility, but the main disadvantage of transfection may be a low transfection rate due to the high dependence on cell activity and RNA species and/or sequence¹⁷³.

5.1.2. Post-loading

Post-loading refers to the passive or active loading of drug molecules directly to the surface bilayer membrane gap or cavity of the EVs after the separation of EVs. Passive loading is mainly achieved through co-incubation, and active loading requires physical or chemical methods to form many transient apertures, allowing drug molecules to enter^{168,169,174}. Co-incubation, electroporation, ultrasound, repeated freeze-thaw, and extrusion are common methods (Fig. 7B).

Electroporation can form transient pores on the membrane by a high-intensity pulsed electric field, allowing drug molecules to enter. In general, the drug loading efficiency is very unstable due to variable parameters such as pulse medium, electric field strength, and pulse interval¹⁷⁴. Notably, electroporation may decrease the zeta potential of EVs and thus destabilize the colloid. Similar to electroporation, ultrasound also forms transient pores¹⁷⁵, but the efficiency of ultrasound is often higher than electroporation and further improves the incorporation of drug molecules¹⁷⁶. However, the powerful mechanical shear force can damage the structure of the vehicles to a certain extent, resulting in a decrease in the drug-loading effect. Therefore, sonication in an ice bath is recommended. Hydrophobic drugs, such as curcumin and viola alcohol, can be directly bound to EVs by co-incubation. Experiments conducted by Kim et al.¹⁷⁵ show that co-incubation is not as efficient as electroporation, and ultrasound is the most efficient method among them.

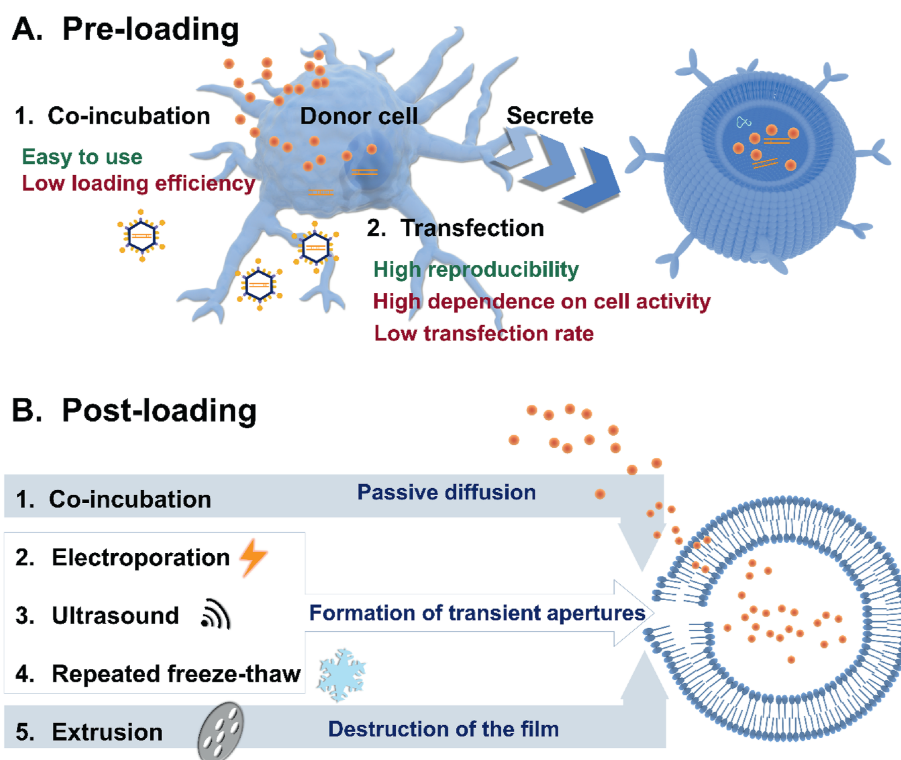


Figure 7 (A) Common methods of pre-loading. (B) Common methods of post-loading.

Alternatively, the repeated freeze-thaw and the extrusion method have been widely used in recent years. Repeated freeze-thaw is the mixing of EVs and drug molecules for a freeze-thaw cycle, during which the EVs membrane is temporarily destroyed, allowing drug molecules to enter the EVs and it is greatly affected by temperature, pH, etc.¹⁶⁸. So, it has limited loading efficiency and may change the size of EVs and lead to aggregation. The extrusion method is to mix EVs with drugs and then pass the mixture through a nanoporous membrane^{168,177,178}. However, exogenous loading techniques might lead to the accumulation of EVs and may also cause changes in their physicochemical and morphological characteristics. The utilization of different approaches has diverse effects on the drug-loading efficiency of EVs. Generally, the preloading method is simpler but requires more time. compared to ultrasonic-broken electroporation and extrusion. Moreover, while the post-loading method can enhance loading efficiency, it may also lead to decreased stability. Currently, numerous new technologies are being employed to improve drug efficiency, such as the microfluidic device, which effectively enhances DOX loading in U251-GM-derived EVs five times *ex vivo*. The acoustic fluid device simplifies drug loading. It is important to note that these advanced drug-loading methods offer simple operation, adjustability, quick encapsulation, and high loading efficiency as prominent features, providing a useful tool for efficient delivery¹⁷⁹.

5.2. Application of EVs as drug delivery systems

At present, liposomes and polymer nanoparticles are widely used in DDSs. When they carry drugs into the body, they can promote drug transport and extend the half-life of drugs, thus improving their pharmacological properties¹⁸⁰.

Firstly, due to the similarity of their biological components to cells, EVs exhibit excellent biocompatibility and are resistant to phagocytosis by macrophages in blood and other body fluids or degradation by lysosomes. Consequently, they have the capability of efficiently delivering drugs to target cells over extended distances¹⁸¹. Furthermore, due to variations in the cellular origin and nucleic acid cargo of different EVs, distinct surface molecules are displayed¹⁸². The specialized molecules present on the surface of EVs effectively inhibit immune responses *in vivo* by obstructing interactions with antibodies, opsonin, and coagulation factors¹⁸⁰. Moreover, through engineering modifications or integration with other materials, EVs can exhibit enhanced delivery capabilities. The modified EVs may possess improved cellular absorption properties and significantly enhance drug delivery efficiency¹⁸³. As gene therapies advance, the utilization of EVs will emerge as a pivotal catalyst for these therapeutic interventions. This section provides a concise overview of various applications of EVs as carriers for drug delivery (Table 6).

5.2.1. EVs as carriers of chemicals

Currently, the majority of chemical drugs available in the market exhibit poor solubility, high toxicity, and short half-life, thereby resulting in limited absorption, low bioavailability, shortened blood circulation time, and multiple side effects. Although paclitaxel (PTX) demonstrates significant advantages in targeted lung cancer treatment, its systemic toxicity imposes limitations on its utilization¹⁸⁴. Temozolomide (TMZ) is the primary pharmacological agent employed in the treatment of glioblastoma (GBM); however, a significant proportion of patients exhibit inherent resistance to this therapeutic intervention¹⁸⁵. These challenges can be overcome by leveraging EVs as drug delivery

Table 6 Summary of the application of EVs as drug delivery systems.

Drug	Type of drug	Extracellular vesicle origin	Disease	Therapeutic effect
Paclitaxel	Chemical drug	Mesenchymal stem cell line SR4987	Pancreatic adenocarcinoma	Inhibit the growth of human pancreatic cell line CFPAC-1
Paclitaxel	Chemical drug	Macrophage	Lung cancer	Decreased proliferation of Lewis lung carcinoma cell
Paclitaxel	Chemical drug	Prostate cancer cell lines	Autologous prostate cancer	Enhanced drug cytotoxicity to tumor cells
Paclitaxel	Chemical drug	Mesenchymal stem cells	Breast cancer	Targeted primary tumors and reduced side effects
Paclitaxel	Chemical drug	Macrophages	Breast cancer	Released pro-inflammatory factors and suppressed tumor growth
Doxorubicin	Chemical drug	EL4 cells	T-cell lymphoma	Improved cellular uptake and intracellular release of DOX and slowed tumor growth
Doxorubicin	Chemical drug	Bone mesenchymal stem cells	Colorectal cancer	Inhibited tumor growth
Doxorubicin	Chemical drug	Bone mesenchymal stem cells	Breast cancer	Reduced tumor growth rate
Doxorubicin	Chemical drug	Dendritic cells	Breast cancer	Decreased proliferation of breast cancer cell
Paclitaxel and doxorubicin	Chemical drug	RAW264.7 cells	Triple negative breast cancer	Inhibit the growth of the orthotopic T11 tumors and human MDA-MB-231 tumors in xenograft model
Dopamine	Chemical drug	Kunming mice blood	Parkinson's disease	Enhanced therapeutic effect resulting from drug delivery targeted to the brain
Triptolide	Chemical drug	SKOV3 cells	Ovarian cancer	Inhibited tumor cell proliferation and growth
Gemcitabine	Chemical drug	PANC-1	Pancreatic cancer	Inhibited tumor growth
Curcumin	Chemical drug	The EL-4 (mouse lymphoma cell line) and RAW 264.7 (murine macrophage cell line)	Lipopolysaccharide-induced septic shock	Improved curcumin activity and reduced off-target effects
miR-146b	Gene drug	Mesenchymal stem cells	Glioblastoma multiforme	Inhibited glioma xenograft growth
miRNA-486-5p	Gene drug	Human cord blood endothelial colony-forming cells	Ischemic kidney injury	Reduced ischemic kidney injury
miR-122	Gene drug	Adipose tissue-derived mesenchymal stem cells	Hepatocellular carcinoma	Increased the antitumor efficacy on tumor <i>in vivo</i>
miRNA-126	Gene drug	MDA-MB-231 cells	Non-small cell lung cancer	Inhibited the formulation of lung metastasis
miR-195	Gene drug	Stellate cells	Cholangiocarcinoma	Reduced tumor size of the orthotopic rat model and improved the overall animal survival
miRNA-199a	Gene drug	Mesenchymal stem cells	Hepatocellular carcinoma	Improved HCC chemosensitivity
miR-335-5p	Gene drug	Stellate cells	Hepatocellular carcinoma	Inhibited tumor cell invasion and reduced tumor size
Lamp2b	Gene drug	Dendritic cells	Alzheimer's disease	Knocked out specific genes
si-ciRS-122	Gene drug	HEK293T	Colorectal cancer	Mediated chemoresistance from drug-resistant cells to drug-sensitive cells
Spherical nucleic acids	Gene drug	PC-3 cells	Prostate cancer	Increased three thousand times the intensity of target miR-21 knockdown
miR-145-5p	Gene drug	Mesenchymal stem cells	Pancreatic ductal adenocarcinoma	Suppressed the proliferation and invasion of PDAC cells, enhanced apoptosis, and induced cell cycle arrest
miR-379	Gene drug	Mesenchymal stem cells	Breast cancer	Promoted tumor necrosis

systems to devise sophisticated delivery systems for PTX curcumin, TMZ, doxorubicin, and other therapeutic agents.

Zhu et al.¹⁸⁶ developed a hybrid vesicle for the delivery of paclitaxel (PTX), consisting of bi-specific MSLN/PD-L1 CAR-T cell-derived exosomes expressing both mesothelin and PD-L1 single-chain variable fragments and lung-targeted liposomes. This delivery system improved tumor targeting ability and enhanced PTX-induced immunogenic cell death by blocking PD-L1 on the tumor surface, thereby promoting anti-tumor immune response. Wang et al.¹⁸⁷ utilized EVs isolated from Raw264.7 cells for encapsulating PTX and subsequently engineered the EVs with RGE peptide to achieve specific targeting towards NRP-1, thereby enabling active glioma targeting of ICG/PTX@RGE-EV. The pH-responsive EVs were prepared by encapsulating sodium bicarbonate and PTX within the EVs. These pH-sensitive EVs exhibit enhanced drug release in response to acidic pH values within the intracellular/lysosomal compartments¹⁸⁸. Meanwhile, Passucci et al.¹⁶⁶ made a discovery that MSCs can secrete exosomes when loaded with PTX. These exosomes are then able to deliver PTX to human pancreatic cancer cells, thus providing an effective anti-tumor treatment. TMZ, also the widely used chemotherapy drug, resistance is mainly due to the hyperactivation of O(6)-methylguanine-DNA methyltransferase, which repairs the damaged DNA, thus offsetting the influence of TMZ¹⁸⁹. When TMZ is loaded into EVs, its chemotherapeutic resistance and off-target effects are significantly reduced. Due to the overexpression of heme oxygen-1 in TMZ-resistant GBM cells, the modification of EVs derived from bone marrow MSCs with a heme oxygen-1 specific peptide and the encapsulation of TMZ inside these EVs significantly enhanced the drug resistance of tumor cells to TMZ¹²⁴. Curcumin, a diketone compound extracted from plants of Zingiberaceae and Araceae, has multiple effects of anti-tumor, anti-inflammation, and cholagogue. Sun et al.¹⁹⁰ extracted exosomes from the EL-4 cell line and loaded them with curcumin, which significantly increased the solubility and utilization of the drug and reduced the toxicity of the drugs themselves. Exosomal doxorubicin (exoDOX) can avoid the cardiotoxicity of DOX and improve the therapeutic effect¹⁹¹. When DOX is loaded into EVs, its toxicity to the heart is significantly reduced¹⁹². Furthermore, Niu et al.¹⁹³ used natural grapefruit EVs to load DOX, which makes it easier to deliver to tumor tissues.

5.2.2. EVs as carriers of gene-drug

Gene drugs are easily degraded by various nucleases *in vivo*, while EVs naturally carry nucleic acids. Among them, miRNAs and mRNAs have been extensively studied and applied. At present, complex methods have been developed to load specific RNAs into EVs in order to protect them from extracellular endonucleases, improve their stability and invisibility, and reduce their immunogenicity. These methods also enable the transportation of RNAs to target cells, primarily for the treatment of tumor diseases. Genetic material can be exchanged between cells by RNAs in EVs. When receptor cells ingest these EVs containing RNAs, siRNAs can silence them by interfering with the translation of target genes in the receptor cells. miRNAs can target and regulate the levels of mRNAs in the receptor cells so that they can be translated into the corresponding proteins in the recipient cells.

siRNA can induce gene silencing induced to treat various diseases. Because of its hydrophilicity, negative charge, and other characteristics, naked siRNA is easily degraded by serum nuclease which has a short half-life¹⁹⁴. Poor targeting and immunogenicity also affect the efficacy of siRNA. However, as natural nucleic acid

carriers, EVs can overcome these obstacles. siRNAs were first transported by exosomes to treat diseases in 2011, and their roles in tumor treatment have gradually become significant. Chiang et al.¹⁹⁵ engineered double-targeted extracellular vesicles (dtEVs) for the delivery of therapeutic RNAs, resulting in effective inhibition of PDAC tumors (Fig. 8A). The presence of the CD64 protein on the surface of EVs, in combination with tissue-targeting peptides and humanized monoclonal antibodies, facilitates dual targeting to improve receptor-mediated tissue penetration and uptake by tumor cells. dtEVs exhibit a deeper tumor sphere penetration compared to single-targeted vectors carrying α ROR1 or CK alone (Fig. 8B). Additionally, Tian et al. developed c(RGDyK)-conjugated extracellular vesicles (RGD-EVs) loaded with siRNA targeting PD-L1, which exhibited enhanced targeted delivery to GBM in a TAM-dependent manner following short-burst radiation therapy. However, the administration of radiotherapy resulted in an upregulation of PD-L1 expression, leading to a compromised state of anti-tumor immunity. Therefore, the delivery of siPD-L1 could offer significant therapeutic benefits for GBM treatment, which significantly augmented the presence of CD8⁺ T cells within the GBM micro-environment (Fig. 8C). To achieve targeted and efficient delivery of siRNA to specific cells, Pi et al.¹⁹⁸ employed RNA nanotechnology to design ligand-expressed EVs as RNA nanoparticles, which exhibited significant tumor regression in models of prostate cancer, breast cancer, and colorectal cancer¹⁹⁶. In addition, the bacterial adventitia vesicles contain proteins derived from parental cells and carry a variety of cargos, which can also serve as targeting delivery platforms¹⁹⁷. Bioengineered bacterial outer membrane vesicles (OMVs) could deliver siRNAs to target and kill breast cancer cells overexpressing HER2. The laboratory utilized the *E. coli* K-12 W3110 strain, known for its production of a low-acylated LPS that decreases the immunogenicity of OMVs.

miRNA is most widely used in gene-drug transportation. The interaction between miRNAs and target genes leads to gene silence or translation inhibition, which regulates protein expression levels. Similar to siRNA, EVs can also protect miRNA from degradation, deliver them to target cells, promote cell uptake, and effectively improve drug delivery efficiency. For example, for gliomas, MSC-derived EVs containing miR-146b-5p reduced their expression and further reduced the invasiveness of gliomas by transporting miR-146b-5p to bind to EGFR mRNA in glial cells¹⁹⁹. During the growth and invasion of GBM, the expression of miR-1 is decreased. EVs carrying miR-1 were developed to reduce the growth, angiogenesis, and invasion of GBM²⁰⁰. Viñas et al.²⁰¹ reported that miRNA-486-5p was successfully transferred to exocrine for the treatment of acute renal injury. Let-7, as a miRNA for tumor inhibition, could be specifically delivered by exosomes to EGFR, thereby exerting an anti-breast cancer effect²⁰².

mRNA can be directly translated into the specific target proteins in cells, and proteins can interact with receptors to treat diseases. Direct delivery of mRNAs can produce an obvious protective immune response, which is easily degraded by nuclease in plasma and tissues, while its molecular weight and negative charge make it difficult to cross cell membranes. Endogenous EVs can deliver mRNA/protein specifically and protect mRNA/protein from degradation. At the same time, the bilayer lipid membranes enable them to cross the biological barriers to enhance effective cellular uptake. Mizrak et al.¹⁷² successfully loaded therapeutic mRNA and protein-cytosine deaminase fused to uracil phosphoribosyltransferase into EVs for the treatment of related tumor diseases. The prodrug 5-fluorocytosine could be locally converted into an active agent by cytosine deaminase in tumors and cause

Table 7 EVs from different sources regulate TME.

Type of EVs	Origin of extracellular vesicles	Cancer type	Mechanism	Ref.
Endogenous EVs	Prostate cancer cell	Prostate cancer	Inhibiting T cell activation in draining lymph nodes	209
	Metastatic melanoma	Metastatic melanoma	PD-L1 mediated immune escape on EVs membrane surface	74
	Non-small cell lung cancer cells	Non-small cell lung cancer	Promoting CD8 T cell dysfunction in NSCLC	210
	Plasma from hepatocellular carcinoma patients	Hepatocellular carcinoma	Inducing NK cell dysfunction in HCC	211
	Human melanoma cell lines WM9 and WM164	Melanoma	Adhesion to activated CD8 T cells <i>via</i> ICAM1-LFA-1 interaction inhibits CD8 T cell proliferation and cytotoxicity	212
Exogenous EVs	Anti-MSLN CAR-T cells	Triple-negative breast cancer	The secretion of perforin and granzyme B effectively target MSLN-positive TNBC cells	221
	Bone marrow MSC	Pancreatic ductal carcinoma	Hindering the polarization of tumor-associated macrophages	213
	Tumor antigen-stimulated dendritic cell	Melanoma	Up-regulating the expression of PD-L1 and activating endogenous T cells	214
	FAP genetically engineered tumor cells	Colon cancer, lung cancer, melanoma, breast cancer	Promoting the maturation of dendritic cells to target tumor cells	215
EVs as drug delivery systems	Serum	Glioblastoma	Facilitating the maturation of DCs and the polarization of M2 TAMs into M1 TAMs.	186
	Dendritic cells	Glioblastoma	Drainage of tumor-associated antigens to lymph nodes to activate classical antigen-presenting cell-induced immune stimulation	216
	GL261 cells	Glioblastoma	Promoting the polarization of macrophages	224
	Serum	Glioblastoma	Inhibiting tumor angiogenesis, up-regulating SHP-2 activity	217
	Bone marrow-derived dendritic cells	Melanoma	Inducing the upregulation of MHC class I and PD-L1 on tumor cells, and enhancing the invasion of total immune cells	226
	Dendritic cell	Melanoma	Activation of toll-like receptor 4 signaling stimulates macrophage and DC maturation	227

tumor immune microenvironment of diethylnitrosamine-induced native tumors, along with a decrease in immunosuppressive cytokines and regulatory T cells.

6.2. Exogenous EVs

Recently, EVs derived from CAR-T cells express all surface membrane molecules from the parental cells. This promotes the expression of perforin and granzyme B, which in turn induce cytotoxicity in target cells²²¹. By modifying the stimulation of tumor antigens on dendritic cells (tDC-Exo) with anti-CD3 and resistance to EGFR antibody, the secretion outside of the cell

is simulated to activate T cells through CAR-T endogenous therapy. This process enhances T cell infiltration in tumor tissues and ensures specific immune responses targeting cancer antigens²²². Additionally, CAR-T-derived EVs delivering RN7SL1 ultimately induced endogenous effector-memory and tumor-specific T cell expansion, generating an immune response in solid tumor cells with CAR antigen loss²²³. Inspired by CAR-T cell therapy, Fan et al.²²² developed tumor antigen-stimulated DC-derived EVs that could provide the same essential signals for T-cell activation as CAR-T cells, as well as anti-CD3 and anti-EGFR to enable precision therapy for solid tumors.

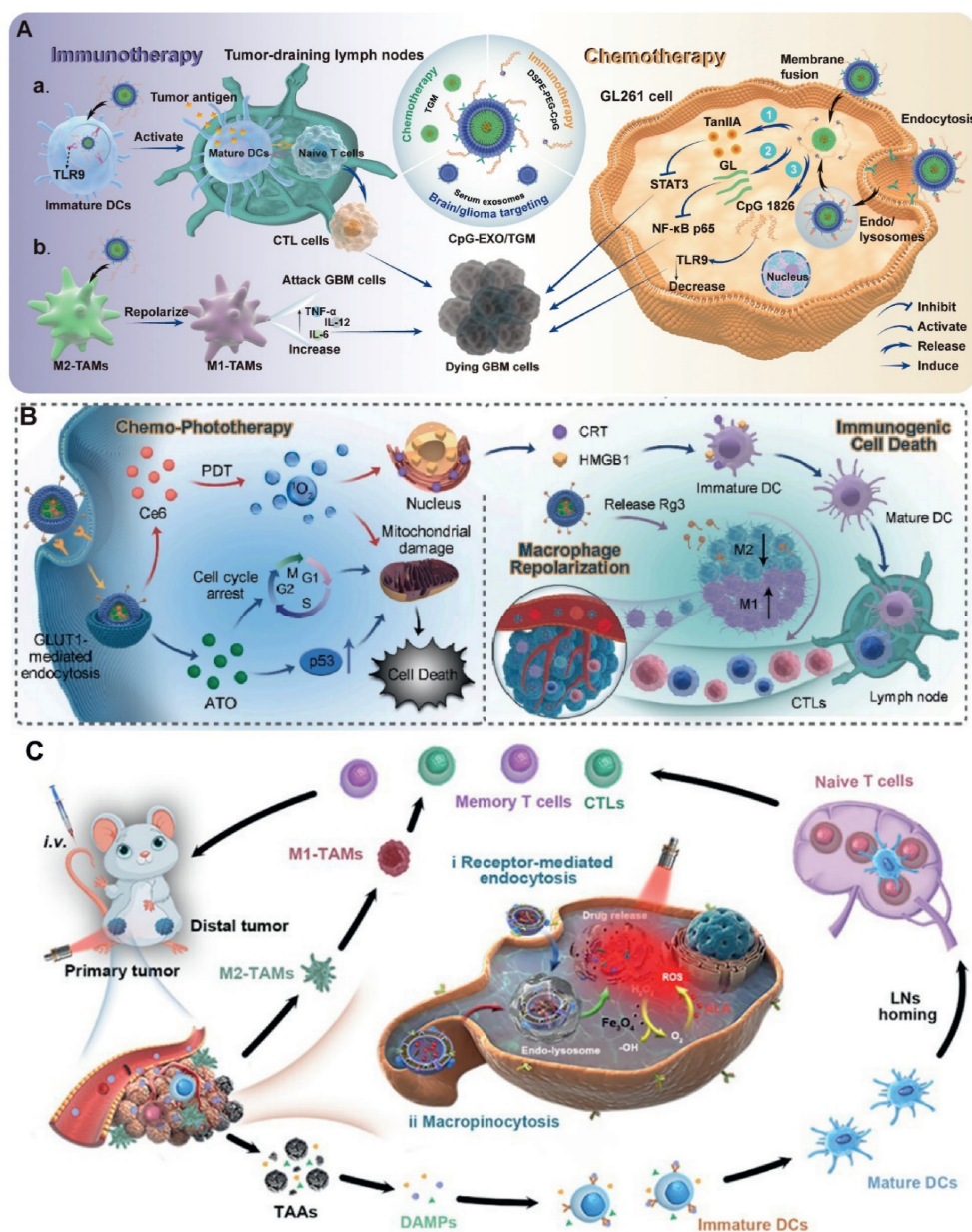


Figure 9 (A) The therapeutic mechanism of CpG-EXO/TGM. Reprinted with the permission from Ref. 227. Copyright © 2023 American Chemical Society. (B) Schematic depiction of Rg3-Exo/ATO@Ce6-mediated highly potent and orthotopic GBM-specific synergistic therapy. Reprinted with the permission from Ref. 217. Copyright © 2023 Elsevier B.V. (C) Mechanism diagram of personalized nanovaccine Dex-HDL/ALA-Fe₃O₄ enhancing lymph node aggregation and reprogramming tumor microenvironment. Reprinted with the permission from Ref. 218. Copyright © 2024 American Chemical Society.

6.3. EVs as drug delivery systems

Our research group constructed CpG-EXO/TGM and used them in combination with temozolomide to treat glioma, which proved that it can regulate the immunosuppressive microenvironment (Fig. 9A)¹⁸⁶. To kill tumor cells precisely with immunotherapy and chemotherapy, Li et al.²²⁴ modified ginsenoside Rg3 on the surface of EVs (Fig. 9B). Similarly, Wang et al.²²⁵ constructed a photodynamic therapeutically activated nanovaccine (Dex-HDL/ALA-Fe₃O₄) that co-delivers 5-aminopentulonic acid and Fe₃O₄, which achieves significant tumor site aggregation and lymph node

homing, and can induce specific and memory anti-tumor immune responses (Fig. 9C).

DC-EVs have been demonstrated to elicit an antitumor immune response through the activation of antigen-specific T cells. Veerman et al.²²⁶ further demonstrated that the combination of EVs with anti-PD-1 or anti-PD-L1 could improve the response rate to immune checkpoint inhibition therapy. Matsumoto et al.²²⁷ activated DCs with LPS and IFN- γ and added ovalbumin to the collected EVs to significantly enhance the immune activity of EVs. Zuo et al.²²⁸ also modified the surface of EVs to promote the recruitment, accumulation and activation of DCs. Hansen et al.²²⁹

found that EVs derived from CD4⁺ T cells could be used as immune adjuvants to enhance the immune response to STING agonists. Shin et al.²³⁰ found that EVs produced by IL-2-stimulated CD4⁺ T cells were more effective in inducing CD8⁺ T cell-mediated antitumor responses without activating Treg cells as IL-2 did. TDEVs contain a large number of neoantigens, which have good immunostimulant effects and can be used to develop personalized immunotherapies. Zuo et al.²³¹ found that multiple tumor-associated antigens of EVs could activate DCs and enhance tumor recognition. In addition, Kim et al.²³² loaded misfolded proteins (MPs) into RSVF-expressing EVs and found that bRSVF-EV not only induced tumor cell heterodimerization to trigger the innate immune response, but also delivered MPs directly to the cytoplasm of the cancer cells to induce ICD. Cheng et al.²³³ genetically engineered natural exosomes and modified them to create genetically engineered exosomes that display antibodies on their surface and contain immunomodulatory proteins. These genetically engineered multifunctional immunomodulatory exosomes demonstrated strong cytotoxicity against triple-negative breast cancer cells, while also exhibiting enhanced stability and biocompatibility.

7. Conclusions and perspectives

Over the past decade, EVs have emerged as valuable biomarkers and DDSs for tumor therapy. This progress is evident in the growing number of high-quality publications and citations. EVs obtained from the body fluids of tumor patients carry specially

labeled molecules such as proteins, nucleic acids and specific metabolites, which have shown great value as prognostic and diagnostic biomarkers for tumors²³⁴. These methods of detecting EVs are widely accepted by patients due to their real-time and minimally invasive sample collection, facilitating the diagnosis of disease and giving a comprehensive insight to the overall condition of the disease. Additionally, EVs not only reflect the biological properties and states of source cells, but also are common signaling mediators between cells. They can be employed to construct novel targeted DDSs to carry drugs. Compared with conventional DDSs, EVs have better biocompatibility as well as are less likely to elicit an immune response, so they have extended circulation time in the body and are able to deliver drugs to tumor tissues more efficiently⁸. Moreover, EVs have target specificity, and by altering the surface proteins of EVs can be made to bind to specific receptors on the surface of tumor cells, thus improving drug targeting and therapeutic efficacy. These engineered EVs are more plausible prospects as DDSs²³⁵. More surprisingly, EVs have a natural tumor killing ability and can be used as immunostimulation and cancer vaccine for clinical treatment (Table 8).

In the clinical settings, the issues of precise drug delivery and standardized production of EVs still exist. Whether surface membrane protein quantification and the number of EVs can be used for indirect quantification remains highly controversial. As research on various EVs continues, new surface markers continue to be discovered. And with that, it has driven the development of new extraction and assay techniques for EVs, which gives the possibility to prepare EVs products with higher purity and less

Table 8 Summary of EVs in cancer immunotherapy.

Purpose	Source of EVs	Cancer	Therapeutic effect
Delivery carrier	Red blood cells (RBCs)	Breast cancer	Inhibited tumor growth by activating the RIG-I cascade while increasing immune cell infiltration
Immunostimulation	Dendritic cells	—	Enhanced innate immunity and stimulated macrophages and DCs
Immunostimulation	Tumor cells	Breast cancer	Release of DNA-containing exosomes, activation of DCs <i>via</i> STING signaling
Immunostimulation	Tumor cells	Breast cancer	Induced DC maturation
Immunostimulation	Tumor cells	Hepatocellular carcinoma	Generation of long-term memory T cells and activation of potent anti-tumor immunity
Cancer vaccine	Dendritic cells	—	Inhibition of tumor growth and extension of survival
Cancer vaccine	Dendritic cells	Melanoma	Suppressed tumor growth, eradicated lung metastases, and extended survival
Cancer vaccine	Breast cancer	Triple negative breast cancer	Enhanced CD8 ⁺ T cell responses in tumor microenvironment
Cancer vaccine	Tumor cells and dendritic cells	Neck squamous cell carcinoma	Robust innate immune response
Cancer vaccine	Immunogenically dying tumor cells	Pancreatic ductal adenocarcinoma	Delayed tumor growth
Cancer vaccine	Melanoma cytomembrane and Salmonella outer membrane	Melanoma	Activated immune system and induced anti-tumour immune response
Cancer vaccine	Bacteria	Colon cancer	Inhibited melanoma progression
Cancer vaccine	Outer membrane	Pulmonary metastatic melanoma and subcutaneous colon tumor	Inhibited tumor growth and re-invasion
Cancer vaccine	Bacterial outer membrane	—	Efficient recognition and uptake by DCs after binding to tumor antigens
Cancer vaccine	Bacterial outer membrane	—	Promoted DC maturation and activated antigen presentation function

—Not available.

batch variation, as well as to promote precise diagnostics for EVs-based liquid biopsies. It is widely recognized that EVs exhibit a high level of biocompatibility and degradability, and none of the oncology treatments based on EVs have demonstrated significant dose toxicity. However, since some EVs are naturally immunogenic and produce immune rejection or cytokine storm syndrome²³⁶, isolating inflammatory substances in EVs by pre-blocking or knocking down their immune proteins may evade immune recognition and allow better targeting. Therefore, researchers still need to pay more attention to the safety of EVs administration, and they are trying to design DDSs with high safety, desirable efficacy, and high clinical translational potential.

EVs-based tumor immunotherapy is also emerging as a cutting-edge strategy to regulate tumor progression and TME. EVs of different origins carry bioactive molecules capable of exerting bi-directional effects of immune activation and immune suppression. For one thing, EVs can enhance the growth, maturation of targeted immune cells, thus exerting a robust anti-tumor efficacy. For another, EVs can support immune escape of tumors and induce drug resistance of tumor cells by promoting suppressive immune cells, including Treg cells, MDSCs, and M2-like TAMs, or by inducing T-cell depletion. Recently, more potential immunotherapeutic strategies are derived based on the mechanism of action of different EVs for TME. For example, TDEVs are more inclined to promote tumor progression, and inhibiting their biogenesis would significantly suppress tumor growth. Rab27a plays a role in the docking of multivesicular endosomes to the plasma membrane and is a commonly used therapeutic target in the clinic. Rab27a silencing inhibits the secretion of TDEVs²³⁷.

In the future, considering the powerful role of EVs as biomarkers and DDSs for tumor treatment, we put forward the following views on the development prospect of EVs: (1) Establish EV-TRACK knowledgebase to promote the comparison of different study methods of EVs and make guidelines for experimental design and report in EVs research to reap more vital biomarker findings²³⁸. (2) The further development of EVs as new DDSs must be based on a broader and deeper understanding of tumor immune system and the advancement of basic research. (3) The artificial intelligence and machine learning can be applied in solving the heterogeneity of EVs and transforming a large amount of information from omics methods into practical clinical applications^{239,240}. However, despite the wealth of preclinical data suggesting the positive effects of EVs, we are still waiting for comprehensive results from an accumulating number of human clinical trials²⁴¹.

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Author contributions

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Conflicts of interest

The authors declare no conflict of interest.

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