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# Role of extracellular vesicles in cancer: implications in immunotherapeutic resistance

**Edited by**
Sheila Spada, Anirban Ganguly, Zhiqing Pang and Qiang Fu

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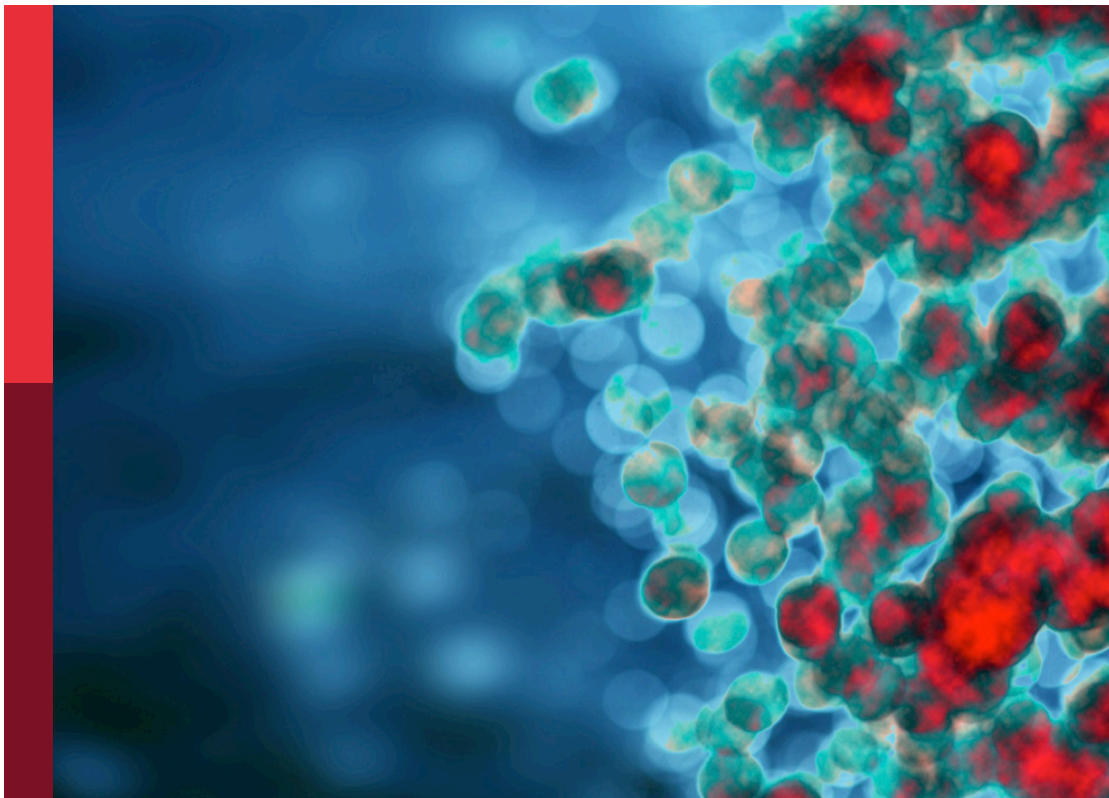

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# Role of extracellular vesicles in cancer: implications in immunotherapeutic resistance

**Topic editors**

Sheila Spada — Regina Elena National Cancer Institute, Hospital Physiotherapy Institutes (IRCCS), Italy

Anirban Ganguly — All India Institute of Medical Sciences Deoghar, India

Zhiqing Pang — Fudan University, China

Qiang Fu — Binzhou Medical University, China

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EDITED AND REVIEWED BY
Peter Brossart,
University of Bonn, Germany

*CORRESPONDENCE
Sheila Spada
sheilaspada88@gmail.com
Anirban Ganguly
anirban.biochemistry@aiimsdeoghar.edu.in

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# Editorial: Role of extracellular vesicles in cancer: implications in immunotherapeutic resistance

Sheila Spada[1]* and Anirban Ganguly[2]*

[1]Tumor Immunology and Immunotherapy Unit, Regina Elena National Cancer Institute, Rome, Italy, [2]Department of Biochemistry, All India Institute of Medical Sciences, Deoghar, Jharkhand, India

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**exosomes, nanoparticles, CD8 T cells, tumor, crosstalk, personalized medicine, onco immunology, dendritic cells**

Editorial on the Research Topic
Role of extracellular vesicles in cancer: implications in immunotherapeutic resistance

Cancer remains one of the leading causes of mortality worldwide, with therapeutic resistance representing a major challenge to long-term treatment success. Among the several factors contributing to therapy failure, the tumor microenvironment (TME) and intercellular communication have emerged as crucial features (1, 2). Extracellular vesicles (EVs) are critical mediators of cellular crosstalk within the TME. These nano-sized lipid bilayer-bound vesicles shuttle a cargo of bioactive molecules, including proteins, nucleic acids, lipids and multiple microbiota-derived metabolites, that reflects the state of their cells of origin, influencing in turn the hallmarks of cancer such as proliferation, metastasis, angiogenesis, and immune evasion (3–8).

An increasing number of studies have illuminated the pivotal role that EVs play in modulating the immune landscape of tumors, influencing the efficacy of immunotherapies (9). Indeed, EVs can contribute to the development of immunotherapeutic resistance by transferring immunosuppressive molecules or altering antigen presentation pathways. Their ability to reprogram immune cell function and promote an immune-privileged environment presents a significant obstacle to treatments such as immune checkpoint inhibitors and CAR-T cell therapies. Notably, understanding the mechanisms by which EVs modulate immune responses is therefore essential for overcoming resistance and improving clinical outcomes.

This research topic explores the multifaceted role of EVs in cancer, with a specific focus on their role in mediating resistance to immunotherapies. We aim to highlight the molecular mechanisms underpinning EV-mediated immune modulation, evaluate current evidence from preclinical and clinical studies, and discuss the therapeutic potential of targeting EVs to enhance immunotherapy efficacy.

Cancer-derived EVs are potential targets for overcoming resistance to immunotherapy and immune evasion strategies. Ahn et al. illustrated the mechanisms mediated by cancer-derived EVs to modulate the immune system and promote an immunosuppressive TME and a systemic environment that is less prone to effective anticancer immunity. EVs carry several immunomodulatory molecules, including PD-L1, TGF-β, and FasL, affecting the functions of dendritic, T and NK cells, contributing to the failure of immuno-checkpoint

blockade and CAR-T cell therapies. Being involved in intercellular communications, EVs favor metastasis and targeted therapy resistance. Therefore, targeting cancer-derived EVs might counteract EV-mediated immunosuppression and open avenues to future directions for enhancing cancer immunotherapy. Accordingly, Morini et al. described the relevant roles of tumor-derived EVs (TEVs) on immune cells, by inactivating CD8+ T cells, inducing polarization of macrophages towards M2 phenotype, inhibiting the activation and inducing the apoptosis of NK cells as well as inhibiting the secretory function of dendritic cells. These effects crucially impact immunotherapy-based treatments in neuroblastoma. In hepatocellular carcinoma (HCC), EVs derived from cancer cells as well as tumor-associated macrophages, by transferring cellular components of the TME act as mediators of immune response by modulating the macrophage polarization, thus impacting tumor growth, metastasis, glycolysis and drug resistance, as reported by Xu et al. The multinetwork regulatory mechanisms of EVs in HCC pathogenesis was elucidated by Yuan et al. They detailed the crucial roles of EVs in progression and cancer cell proliferation in HCC mediated via the modulation of PI3K/AKT and Wnt/β-catenin signaling pathways accompanied with a concomitant reprogramming of the TME cellular composition and functioning and the enhancement of malignant behaviors. The biologically potent molecular libraries comprising of proteins, lipids and nucleic acid components carried by the EVs result in systematic integration to very complex intercellular communication networks leading to a characteristic metabolic-immune cross-regulatory network regulated at multi-dimensional levels and orchestrated by cascading hubs of signal transduction pathways, highlighting the translational value of EVs as precision medicine targets. Moreover, the role of EVs in hepatoma progression was studied by Sun at al. demonstrating that hepatic stellate cells-derived EVs transfer high levels of miR-27a-3p inducing M2 macrophage polarization and promoting hepatoma progression. The phenomenon of drug resistance in cancer cells is often acquired by cell-to-cell communication mediated by the EVs, which transport the cargo of miRNAs and efflux transporters to the previously chemo-sensitive cells. This process was further highlighted by Santos et al., who elucidated the role of transcription changes modulated by EVs derived from tamoxifen- and doxorubicin-resistant breast cancer cells in sensitive cells and also studied how these EVs induced increased drug resistance with a concomitant inhibition of apoptotic pathways, resulting in increased survivability of the sensitive cells. Aligned with prior research, Wang et al. reviewed the implications of EVs in therapeutic resistance in a spectrum of neoplasms, from gynecological and breast cancer, prostate cancer, to lung and colorectal cancer. EVs drive immune evasion and therapy resistance through diverse mechanisms, including the transfer of immunosuppressive molecules (TGF-β, adenosine), antigen masking and immune evasion shedding tumor associated antigens (HER2, MUC1), that affect immune cell function and enhance tumor survival. Recent research by Liu et al. reviewed currently available scientific literature to understand the role of EVs in augmenting immunotherapy in gastric carcinoma (GC) along with highlighting the potential of EVs as therapeutic delivery carriers. These EVs provide a medium for communication between GC cells and different types of cells within the TME. In GC, EVs also act as important biomarkers for prognosis, diagnosis and treatment and act as promising vectors for targeted drug delivery rendering them very useful targets for enhancing success for immunotherapy.

Understanding the mechanisms by which EVs contribute to immunotherapeutic resistance could unlock opportunities for innovative therapeutic strategies, including the development of EV-targeted therapies or their use as biomarkers to predict and monitor responses to immunotherapy. Continued research in this field holds great promise for overcoming current therapeutic challenges and advancing the development of more precise and personalized cancer treatments.

## Author contributions

SS: Conceptualization, Writing – original draft, Writing – review & editing. AG: Conceptualization, Writing – original draft, Writing – review & editing.

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EDITED BY
Anirban Ganguly,
All India Institute of Medical Sciences Deoghar, India

REVIEWED BY
Guannan Zhou,
Karolinska Institutet (KI), Sweden
Juan Antonio Fafian Labora,
University of A Coruña, Spain

*CORRESPONDENCE
Fausto Almeida
fbralmeida@usp.br

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# Extracellular vesicles from human breast cancer-resistant cells promote acquired drug resistance and pro-inflammatory macrophage response

Patrick Santos[1], Caroline P. Rezende[1], Renan Piraine[1], Bianca Oliveira[1], Francielle B. Ferreira[1], Vinicius S. Carvalho[2], Rodrigo T. Calado[2], Matteo Pellegrini[3] and Fausto Almeida[1]*

[1]Department of Biochemistry and Immunology, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, SP, Brazil, [2]Department of Medical Imaging, Hematology, and Oncology, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, Brazil, [3]Department of Molecular, Cell, and Developmental Biology, University of California, Los Angeles, CA, United States

**Introduction:** Breast cancer is a significant public health problem around the world, ranking first in deaths due to cancer in females. The therapy to fight breast cancer involves different methods, including conventional chemotherapy. However, the acquired resistance that tumors develop during the treatment is still a central cause of cancer-associated deaths. One mechanism that induces drug resistance is cell communication via extracellular vesicles (EVs), which can carry efflux transporters and miRNA that increase sensitive cells' survivability to chemotherapy.

**Methods:** Our study investigates the transcription changes modulated by EVs from tamoxifen- and doxorubicin-resistant breast cancer cells in sensitive cells and how these changes may induce acquired drug resistance, inhibit apoptosis, and increase survivability in the sensitive cells. Additionally, we exposed human macrophages to resistant EVs to understand the influence of EVs on immune responses.

**Results:** Our results suggest that the acquired drug resistance is associated with the ability of resistant EVs to upregulate several transporter classes, which are directly related to the increase of cell viability and survival of sensitive cells exposed to EVs before a low-dose drug treatment. In addition, we show evidence that resistant EVs may downregulate immune system factors to evade detection and block cell death by apoptosis in sensitive breast cancer cells. Our data also reveals that human macrophages in contact with resistant EVs trigger a pro-inflammatory cytokine secretion profile, an effect that may be helpful for future immunotherapy studies.

**Discussion:** These findings are the first transcriptome-wide analysis of cells exposed to resistant EVs, supporting that resistant EVs are associated with the acquired drug resistance process during chemotherapy by modulating different aspects of sensitive cancer cells that coffer the chemoresistance.

KEYWORDS

chemoresistance, tamoxifen, doxorubicin, immunomodulation, membrane transporters

# 1 Introduction

Human breast cancer is the most commonly diagnosed cancer worldwide and is a primary health problem to be addressed globally for future generations (1). Breast cancer is the major cause of cancer-related deaths owing to cancer among the female population, accounting for 11.7% of all new cases and 6.9% of new deaths in 2020, according to the Global Cancer Statistics (2). The survival rate of patients with breast cancer differs across regions worldwide. Survivability is constantly increasing in developed countries owing to investments in early detection technologies and access to modern medicines (3). Currently, breast cancer therapy involves different approaches, such as tumor removal surgery, radiotherapy, and conventional drugs, including traditional chemotherapeutics in combination with hormonal or immunotherapeutic agents (4).

Nonetheless, the toxicity of many drugs toward normal tissues and resistance to chemotherapy frequently used in breast cancer treatment are critical health concerns (5). The initial innate inability to respond to drug therapy, followed by acquired drug resistance, is the central cause of cancer-associated deaths (6). Acquired drug resistance involves many factors that cancer cells can manage to reduce drug efficacy, such as the abnormal activity of membrane transporters, a decrease in cell death processes, altered cellular metabolic signaling, deregulated protein expression, and interaction with receptors (7). More recently, another mechanism by which tumor cells avoid chemotherapeutic effects was orchestrated by extracellular vesicles (EVs) (8).

What was first thought to be cell garbage became a significant mechanism of communication between cells. Almost all cell types release EVs and mediate several cellular processes under homeostasis; however, EVs play an essential role in cancer as they serve as messengers in the tumor microenvironment to send different signals, such as signals to disrupt the immune system, during the promotion and progression of cancer (9). Fundamentally, EVs are lipid-bilayered vesicles with an endosomal or cellular origin that can carry a variety of molecules, including nucleic acids, lipids, carbohydrates, and proteins, via the bloodstream and other body fluids (10). Additionally, EVs can act as nano-carriers to enhance the anticancer effect of chemotherapeutic drugs (11). In the context of drug resistance, EVs can contain the drug itself as cargo, functioning as a drug delivery method to the extracellular space. Resistant cancer cell-derived EVs can carry drug transporters, such as P-glycoprotein, to promote drug efflux in nearby sensitive cells or even carry miRNAs to induce the expression of genes related to membrane transporters (12).

In this study, we combine the evaluation of crucial cellular processes, such as viability, proliferation, and cell death, with genome-wide transcriptomic analysis of breast cancer cell lines to investigate the influence of EVs released by resistant cancer cells on acquired drug resistance mechanisms in sensitive cells, which is a topic that remains unclear in the literature. We used two different breast cancer cell lines, MCF-7 as an epithelial and primary tumor model and MDA-MB-231 as a mesenchymal/high-mobility triple-negative model, to cover different aspects of cancer progression. We managed two human macrophage cultures to identify the ability of resistant EVs to induce phenotypic polarization in these cells after exposure. Our results indicate that EVs from resistant cells may transfer the acquired drug resistance phenotype to sensitive cells by upregulating several types of membrane transporters involved in drug resistance, resulting in increased cell viability and clonogenic survival in sensitive breast cancer cells exposed to EVs released by resistant cells. Resistant cancer cell-derived EVs may modulate the immune system by downregulating different immune factors. We observed that resistant cell-derived EVs polarized human macrophages to a pro-inflammatory phenotype, which represents a potential tool for immunotherapy.

# 2 Materials and methods

## 2.1 Cancer cells, macrophages and PBMC culture

The human triple-negative breast cancer cell line MDA-MB-231 was acquired from the American Type Culture Collection (ATCC, HTB-26) and cultured in RPMI-1640 medium supplemented with 1000 IU penicillin-streptomycin and 10% fetal bovine serum (FBS) in a humidified atmosphere at 37°C with 5% CO2. The human mammary gland-derived breast cancer cell line MCF-7 was acquired from Rio de Janeiro's Cell Culture Bank and

cultured in RPMI-1640 medium supplemented with 1000 IU penicillin-streptomycin and 10% FBS in a humidified atmosphere at 37°C with 5% $CO_2$. The human monocyte cell line THP-1 was acquired from ATCC (TIB-202) and cultured in RPMI-1640 medium supplemented with 1000 IU penicillin-streptomycin and 10% FBS in a humidified atmosphere at 37°C with 5% $CO_2$. Whole blood samples (10 mL/donor) were isolated from healthy female and male donors, without a defined age range, for experimental purposes only, following the principles proposed by the National Research Ethics Commission and Research Ethics Committee under the protocol CAAE 71085023.1.0000.5440. Human peripheral blood mononuclear cells (PBMCs) were isolated from whole blood samples by density gradient centrifugation using Histopaque®-1077 (MERCK, USA). The buffy coat containing PBMCs was transferred to a different tube to isolate CD14+/CD16- monocytes by negative selection using magnetic beads from a commercial classical monocyte isolation kit (Myltenyi Biotec, USA). The purification steps and macrophage differentiation are described in Supplementary Table S1. Purified CD14+/CD16- monocytes were plated in cell culture dishes containing RPMI-1640 medium supplemented with FBS and 10000 IU of penicillin-streptomycin. To induce the differentiation of monocytes into macrophages, 10 ng/mL of granulocyte-macrophage colony-stimulating factor (Peprotech, USA) was added to the monocyte culture for five days in a 37°C and 5% $CO_2$ incubator. Tamoxifen (TAM) was acquired from AdipoGen Life Sciences (USA) and doxorubicin (DOX) was acquired from Sigma-Aldrich (USA).

## 2.2 Acquisition of the IC50 and viability of sensitive and resistant cells

The method chosen to evaluate the half-maximal inhibitory concentration (IC50) of sensitive and resistant cells was the reduction of resazurin, following the protocol described by Kumar (13). Briefly, sensitive or resistant cells of MCF-7 and MDA-MB-231 were seeded in 96-well plates, incubated for 24 h, and treated with a wide range of concentrations of TAM (0.62 to 160 µM) and DOX (0.08 to 20 µM) for 72 h. After the treatment, cells were incubated with a resazurin solution (88 µM) for 4 h, and the plates were read with 570 and 600 nm filters. Cell viability was calculated using the absorbance reads on the indicated equation according to the resazurin manufacturer's protocol, in which cell viability was expressed as a percentage of live cells in each treatment condition compared with the control. Cell viability was observed by standard staining with fluorescein diacetate (FDA) and propidium iodide (PI) to differentiate viable from dead cells. Staining solutions were prepared to a final concentration of 80 µg/mL of FDA and 200 µg/mL of PI. Cells in contact with the staining solutions were incubated for 30 min at 37°C and then washed to remove the staining excess. After washing, the cells were observed under a fluorescence microscope using the filters according to the manufacturer's protocol. The captured images were overlaid by NIS-Elements BR to create merged images.

## 2.3 Generation of drug-resistant breast cancer cell lines

TAM- and DOX-resistant breast cancer cell lines were developed following practical guidelines published by McDermott et al. (14). Using the previously calculated IC50 of each drug in MCF-7 and MDA-MB-231 sensitive cells, a high-level laboratory model of stable drug-resistant cell lines was generated by continuous exposure of sensitive cells to stepwise dose progression, following a comparative selection method based on flask cell confluence and cell viability via trypan blue staining. After obtaining the IC50 of TAM- and DOX-resistant cell lines, the fold resistance was determined as the ratio between the IC50 of the resistant cell line and the IC50 of the sensitive cell line. Cell lines with a fold resistance equal to or higher than 2 were considered sufficiently resistant to proceed with the other experiments.

## 2.4 Isolation, purification, characterization, and internalization of EVs

The differential ultracentrifugation method was used to isolate EVs, following the guidelines of Momen-Heravi (15). Briefly, the supernatants from the cell flasks or plate wells were centrifuged for 10 min at 1500 × g to remove dead cells, transferred to new tubes, and centrifuged for 30 min at 15,000 × g to remove cell debris and large particles. Finally, the supernatants were transferred to appropriate ultracentrifuge tubes and centrifuged at a high rotation speed of 100,000 × g for 70 min to isolate EVs. All centrifugations were performed at 4°C. The pellet of EVs was suspended in 100 µL of RNAse and DNAse-free ultra-pure water, following the guidelines suggested by the International Society of Extracellular Vesicles (ISEV) (16). Aliquots were prepared for characterization using transmission electron microscopy (TEM) and nanoparticle tracking analysis (NTA). The remaining suspended EVs were stored at -80°C. To observe the internalization of EVs upon contact with cancer cells, a lipophilic membrane stain was used to label the EVs following the manufacturer's instructions. Previously isolated EVs were suspended in 200 µL of phosphate-buffered saline (PBS) with 1 µM of Vybrant Dil dye (Ex, 530 nm; Em, 580 nm, Thermo Fisher Scientific, USA) for 30 min at 37°C to obtain the labeling. The labeled EVs were centrifuged at 100,000 × g for 70 min and washed using another ultracentrifugation step in PBS. Labeled EVs were incubated with sensitive breast cancer cells for 24 h at 37°C and 5% CO2. Images were then captured in a bright field and under the appropriate fluorescence measurements in a Nikon Eclipse Ts2 with an attached Digital Sight 10 camera, followed by an overlay of the bright field and fluorescence images using ImageJ software to obtain merged images (17).

## 2.5 RNA extraction, library preparation, and sequencing

Messenger RNA (mRNA) was extracted from MCF-7 and MDA-MB-231 cell lysates using a spin column supplied with the RNeasy kit (QIAGEN, Germany). Both cell lines were co-cultured with TAM- and DOX-resistant EVs for 24 h. All extractions were performed in triplicate for each cell line following the manufacturer's protocol by a single researcher to obtain high-quality mRNA. The mRNA capture and construction of 150 base pair double-stranded mRNA-Seq libraries were performed using Zymo-Seq RiboFree Total RNA (Zymo Research, USA). The protocol included the capture, elution, and fragmentation of mRNA, followed by synthesis of the first and second 150 strands. Illumina adapters were ligated to the ends of the mRNA-derived cDNA fragments, followed by library PCR amplification. The libraries were sequenced in the SP lane of an Illumina NovaSeq6000 sequencer.

## 2.6 Differential expression analysis of RNA-Seq

A quality control test was performed before alignment using FastQC software (18). All low-quality reads and Illumina adapters were removed by the 0.39 version of the Trimmomatic tool (19). Reads that passed the quality filter were aligned to the reference genome index (Homo sapiens Ensembl GRCh38, release 109) using STAR 2.7.0a alignment software (20) at the University of California, Los Angeles UNIX-based cluster. The alignment rate and total number of mapped reads are shown in Supplementary Table S1. Differential expression analysis was performed using the RStudio platform with 4.3.1 R (21). The DESeq2 package was used to detect differentially expressed genes (DEGs) by using negative binomial generalized linear models (22). The comparison between sensitive EVs vs. resistant EVs treatment for each cell line was estimated by the logarithmic fold-change $\log_2$ (sensitive vs. resistant). The lists with the DEGs of each condition were filtered using a 5% false discovery rate (FDR) cutoff and an absolute fold change > 1.5 for upregulated and downregulated genes.

## 2.7 Differential expressed genes' function enrichment

The initial gene ontology (GO) slim analysis was performed using the DEGs lists of each condition in the web-based tool WebGestAlt (23) with a 1% FDR and Benjamini-Hochberg multiple test adjustment. To investigate the signaling pathways associated with the DEGs, we performed an over-representation analysis in GSEA software 4.3.2, with a 1% FDR cutoff, using the Molecular Signatures Database (24, 25). The online tool g:Profiler was used to understand the relationship between the major enriched signaling pathways reported in the prior analysis (26). Functional interactions between potential protein expression among DEGs were performed using STRING networks with a protein-protein interaction FDR of 5% (27).

## 2.8 Quantification of apoptosis after sensitive cells were exposed to resistant EVs

The detection of apoptotic cells was based on annexin V binding and PI uptake by flow cytometry using the Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis (Thermo Fisher Scientific, USA), following the manufacturer's instructions. Briefly, MCF-7 and MDA-MB-231-sensitive cells were exposed to sensitive EVs as controls and to TAM- or DOX-resistant cells for 24 h. After exposure to EVs, the supernatant was removed from each well, and fresh media containing different concentrations of TAM or DOX, up to the previously calculated sensitive cell IC50, was incubated for another 72 h. The cells were harvested, stained with Annexin V-FITC and PI, and incubated for 15 min at room temperature. Annexin V-FITC/PI signals were assessed using a FACSCanto flow cytometer and analyzed using the FACSDiva software (BD Biosciences, USA).

## 2.9 Drug resistance-related genes expression assessment by qPCR

Total RNA was isolated from sensitive cells after 24 h of exposure to TAM- or DOX-resistant EVs using a spin column supplied in the RNeasy kit (QIAGEN, Germany), and cDNA was generated from 1 µg of RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA), according to the manufacturer's protocol. The conditions used in the qPCR reactions were performed using the Power SYBR™ Green PCR Master Mix (Applied Biosystems, USA) in an ABI 7500 FAST system (Applied Biosystems, USA) and following the manufacturer's instructions. All samples were analyzed in triplicates. Relative expression of target genes was calculated by comparing threshold cycle (Ct) values of GAPDH as the housekeeping gene, according to the 2-ΔΔCT method (28). Acquired resistance (*ABCB1*, *ABCG2*, *TGFBR1*, *TGFBR2*, *TGFBR3*) and apoptosis-related genes (*BCL2*, *CD44*, *PIK3CA*, *PTEN*) were selected as target genes. The primers used are described in Supplementary Table S2.

## 2.10 Evaluation of clonogenic survival of breast cancer cells exposed to resistant EVs

The clonogenic survival of sensitive cells previously exposed to TAM- or DOX-resistant EVs was evaluated using a colony-forming assay using an *in vitro* cell protocol published by Franken (29). Briefly, MCF-7 and MDA-MB-231 cells were cultured with

sensitive (control) or resistant EVs for 24 h. Each condition involved treatment with different concentrations of TAM or DOX for 72 h. The cells were resuspended, counted, and reseeded in a low-density manner (250 cells/well) in six-well plates for 7 days. The colonies were fixed with 6% (v/v) glutaraldehyde and stained with 0.5% (w/v) crystal violet for 30 min. Colonies containing 50 cells were counted.

## 2.11 Cytokine production of macrophages after exposure to resistant derived EVs

Interleukin (IL)-1β, IL-10, interferon-gamma (IFN-γ), and tumor necrosis factor (TNF)-α levels from macrophages supernatant after exposure of sensitive or resistant derived EVs for 24 h were measured using a commercial enzyme-linked

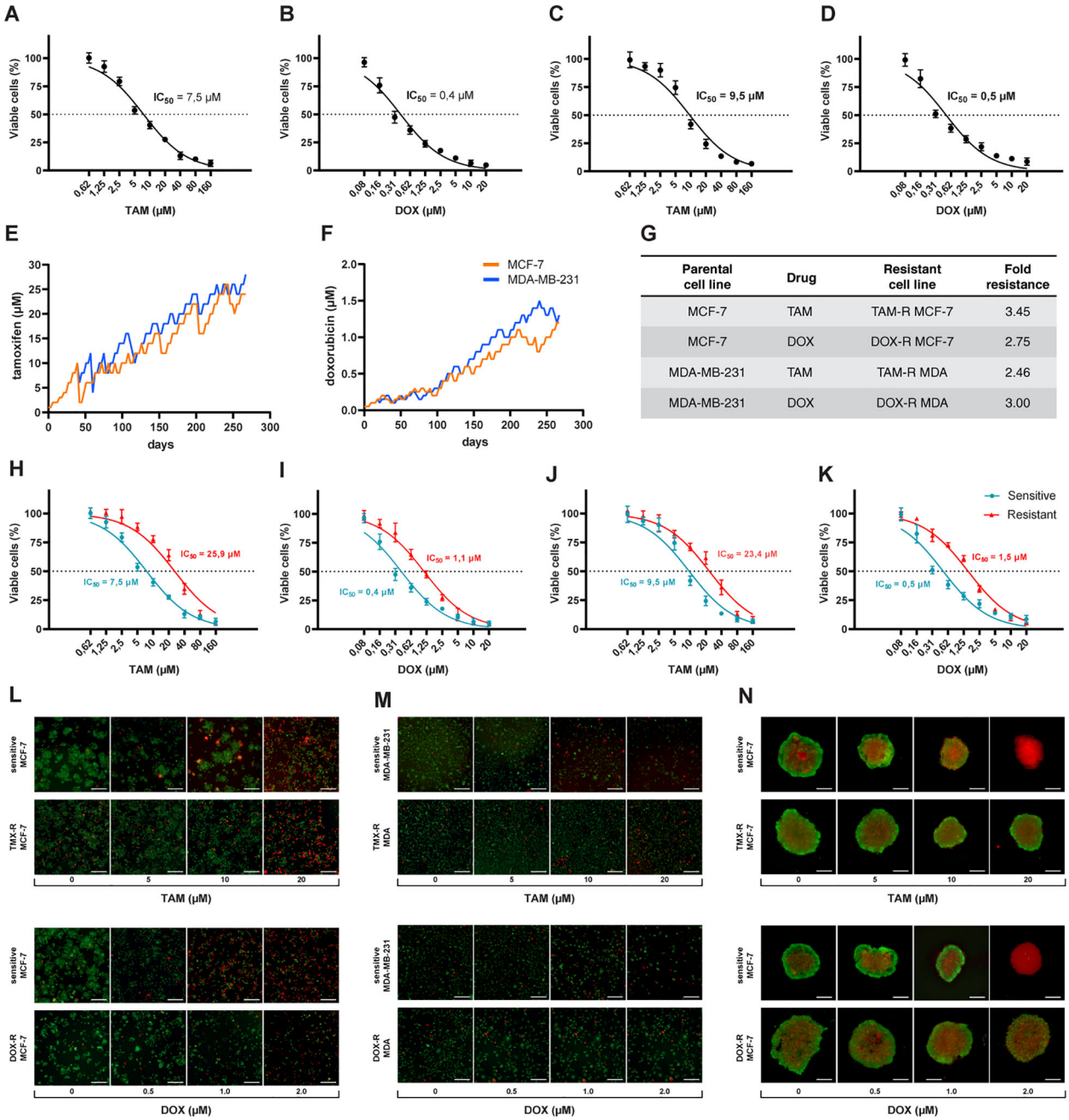


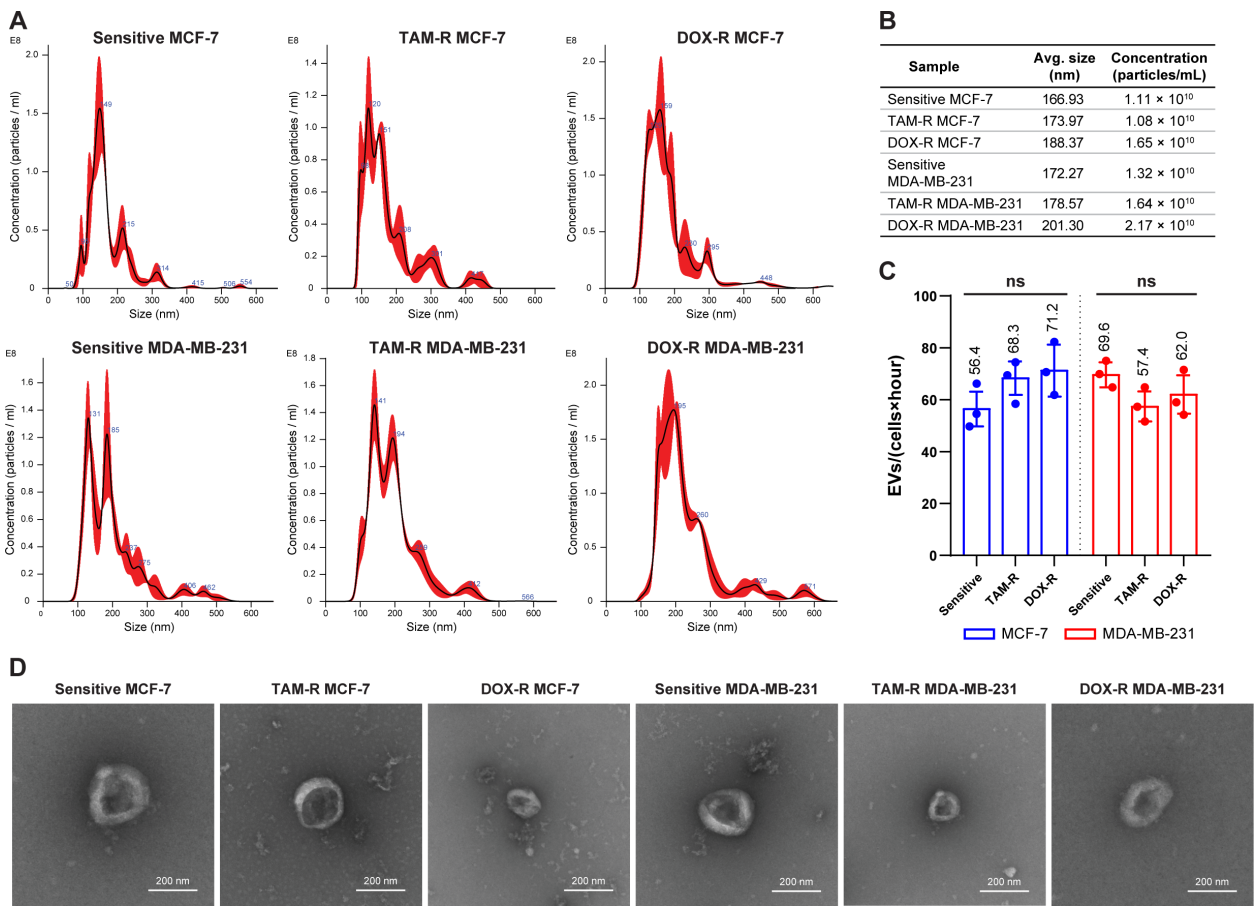

FIGURE 1
Process of generation of resistant cancer cells. Initial cell viability assay by resazurin reduction and half maximal inhibitory concentration (IC50) of sensitive MCF-7 cells treated with **(A)** tamoxifen (TAM) and **(B)** doxorubicin (DOX) and of MDA-MB-231 treated with **(C)** TAM and **(D)** DOX. Sensitive cells of MCF-7 and MDA-MB-231 exposed to **(E)** TAM and **(F)** DOX over time to generate resistant cell lines. The resistance fold calculated for each resistant cell line **(G)**, indicating the level of resistance for each drug. The IC50s of **(H)** TAM- and **(I)** DOX-resistant MCF-7 and **(J)** TAM- and **(K)** DOX-resistant MDA-MB-231 cells calculated (red line) and compared to sensitive cells (blue line). The IC50s calculated by a nonlinear regression with an adjusted confidence level of 95%. The visualization of TAM- and DOX-resistant **(L)** MCF-7 and **(M)** MDA-MB-231 cells in a two-dimensional *in vitro* setup, and **(N)** in three-dimensional spheroids of sensitive and resistant MCF-7 cells.

immunosorbent assay (ELISA) kit following the manufacturer's instructions (BD Biosciences, USA). The results were measured at an optical density of 450 nm using a SpectraMax 190 spectrophotometer (Molecular Devices). The results are reported as pg/mL of experimental and biological triplicates for each condition.

# 3 Results

## 3.1 Generation of drug resistant breast cancer cell lines

The first step was to calculate the IC50 values for sensitive cells exposed to a wide range of concentrations of TAM and DOX for 72 h. We estimated the IC50 of TAM and DOX in MCF-7 and MDA-MB-231 cells using a cell viability assay based on resazurin reduction. Nonlinear regression analysis showed that MCF-7 cells had lower TAM and DOX IC50s when compared to MDA-MB-231 cells (Figures 1A–D). The generation of drug-resistant breast cancer cell lines capable of overcoming and proliferating under exposure to higher TAM or DOX concentrations than the previously calculated IC50 of sensitive cells took nine months (Figures 1E, F). After the resistant cells reached this level of survival, we evaluated the IC50 values of these cell populations using the same resazurin protocol. We observed that the IC50 of MCF-7 exposed to TAM increased from 7.5 to 25.9 µM (Figure 1H), and the IC50 of MCF-7 exposed to DOX increased from 0.4 to 1.1 µM (Figure 1I). We observed an increase in TAM and DOX IC50 in resistant MDA-MB-231 cells, and in this case, the IC50 scaled from 9.5 to 23.4 µM in TAM-exposed cells (Figure 1J) and from 0.5 to 1.5 µM in DOX-exposed MDA-MB-231 cells (Figure 1K). TAM-resistant MCF-7 (TAM-R MCF-7) cells showed the highest fold resistance (3.45), followed by the sharp 3-fold resistance of DOX-resistant MDA-MB-231 cells (DOX-R MDA). DOX-resistant MCF-7 (DOX-R MCF-7) and TAM-resistant MDA-MB-231 (TAM-R MDA) cells displayed 2.75- and 2.46-fold resistance, respectively (Figure 1G). FDA/PI staining revealed that the resistant MCF-7 and MDA-MB-231 cell lines showed fewer PI-positive (dead) and more FDA-positive (viable) cells, even when exposed to high concentrations of TAM




| Sample | Avg. size (nm) | Concentration (particles/mL) |
|---|---|---|
| Sensitive MCF-7 | 166.93 | $1.11 \times 10^{10}$ |
| TAM-R MCF-7 | 173.97 | $1.08 \times 10^{10}$ |
| DOX-R MCF-7 | 188.37 | $1.65 \times 10^{10}$ |
| Sensitive MDA-MB-231 | 172.27 | $1.32 \times 10^{10}$ |
| TAM-R MDA-MB-231 | 178.57 | $1.64 \times 10^{10}$ |
| DOX-R MDA-MB-231 | 201.30 | $2.17 \times 10^{10}$ |

FIGURE 2
Evaluation of the production and characterization of extracellular vesicles derived from breast cancer cells. **(A)** Nanoparticle-tracking analysis showing the size distribution of extracellular vesicles (EVs) produced by sensitive and resistant MCF-7 and MDA-MB-231 cells treated with tamoxifen (TAM) and doxorubicin (DOX). **(B)** Additional information about the average size and concentration of EVs per mL. **(C)** Histogram showing the EV production of each cancer cell per hour. **(D)** Transmission electron microscopy images of sensitive and resistant EVs from breast cancer cell lines. TAM-R, tamoxifen-resistant; DOX-R, doxorubicin-resistant; ns, non-significant. The bars represent the mean with the standard deviation from three independent experiments.

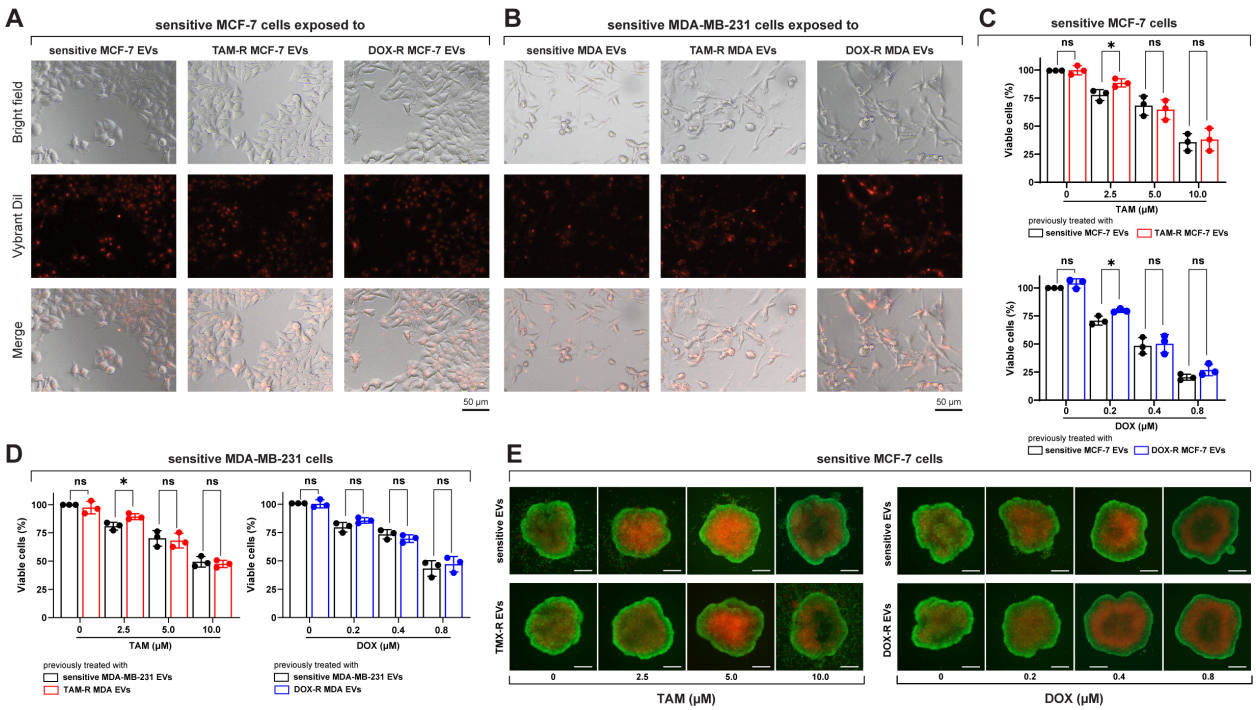



FIGURE 3
Sensitive breast cancer cells uptake resistant extracellular vesicles (EVs) and show increased cell viability. **(A)** Sensitive MCF-7 cells (Bright field) internalizing the EVs (Vybrant dil) after 24 h of exposure. **(B)** Sensitive MDA-MB-231 cells (Bright field) internalizing the EVs (Vybrant dil) after 24 h of exposure. TAM-R: tamoxifen-resistant; DOX-R: doxorubicin-resistant; ns: non-significant. A resazurin reduction assay evaluated the cell viability of sensitive **(C)** MCF-7 and **(D)** MDA-MB-231 cells exposed to resistant EVs. **(E)** Spheroids of sensitive MCF-7 cells were previously exposed to sensitive, TAM- and DOX-resistant EVs for 24 h and treated with TAM or DOX for 72h. The asterisks indicate a significant difference ($p < 0.05$) between tamoxifen or doxorubicin treatments when compared to no treatment (unpaired t-test).

and DOX (Figures 1L, M). This dynamic was observed in 3D MCF-7 spheroids, in which sensitive cells were profoundly affected by drug treatment; however, resistant-derived spheroids showed more viable cells (Figure 1N).

## 3.2 Production and characterization of EVs derived from resistant cell lines

The overall production of EVs was tracked using the supernatant from each cell line and the cells were plated for 24 h. The NTA results showed that sensitive and resistant cell populations produced approximately the same amount of EVs, averaging between 1.08 and 2.17 × $10^{10}$ EVs per mL (Figure 2A). Additionally, the average EV size in the parental and resistant cell lines ranged from 167 to 200 nm (Figure 2B). The TEM observation revealed the classic round "cup-shaped" morphology of parental and resistant breast cancer-derived EVs (Figure 2D). Furthermore, there were no differences in EV production between sensitive and resistant cells, and between cell lines. Each sensitive and resistant breast cancer cell line produced approximately 60 EVs per hour over 24 h (Figure 2C). Collectively, these results showed that there were no differences in the concentration, production, size, or shape between sensitive- and resistant-derived EVs.

## 3.3 Sensitive cells internalize drug-resistant derived EVs and resist drug treatment

After incubating sensitive breast cancer cells with fluorescently labeled EVs from parental and resistant cell lines, a fluorescent signal was observed inside the cells, indicating that these sensitive cells could take up TAM-R and DOX-r EVs (Figures 3A, B). Sensitive MCF-7 and MDA-MB-231 cells were exposed to TAM- and DOX-resistant EVs for 24 h, and subsequently treated with TAM or DOX for another 72 h. In this experiment, our results showed that the resistant-derived EVs preexposure increased the viability of sensitive cells in three different scenarios: the first and second are MCF-7 treated with 2.5 µM of TAM and 0.2 µM of DOX, and the last one is MDA-MB-231 with an increased number of viable cells after the resistant EVs preexposure and treatment with 2.5 µM of TAM (Figure 3C). Under our experimental conditions, high concentrations of TAM and DOX (near the original IC50 or above) showed no differences between sensitive cells exposed to sensitive or resistant EVs (Figure 3D). We generated MCF-7 spheroids and exposed them to sensitive or resistant EVs, which were then treated with TAM or DOX. In the images, more dead cells (red labeled) can be observed in the spheroid previously exposed to sensitive EVs and treated with 2.5 µM of TAM than the one previously exposed to TAM-R EVs.

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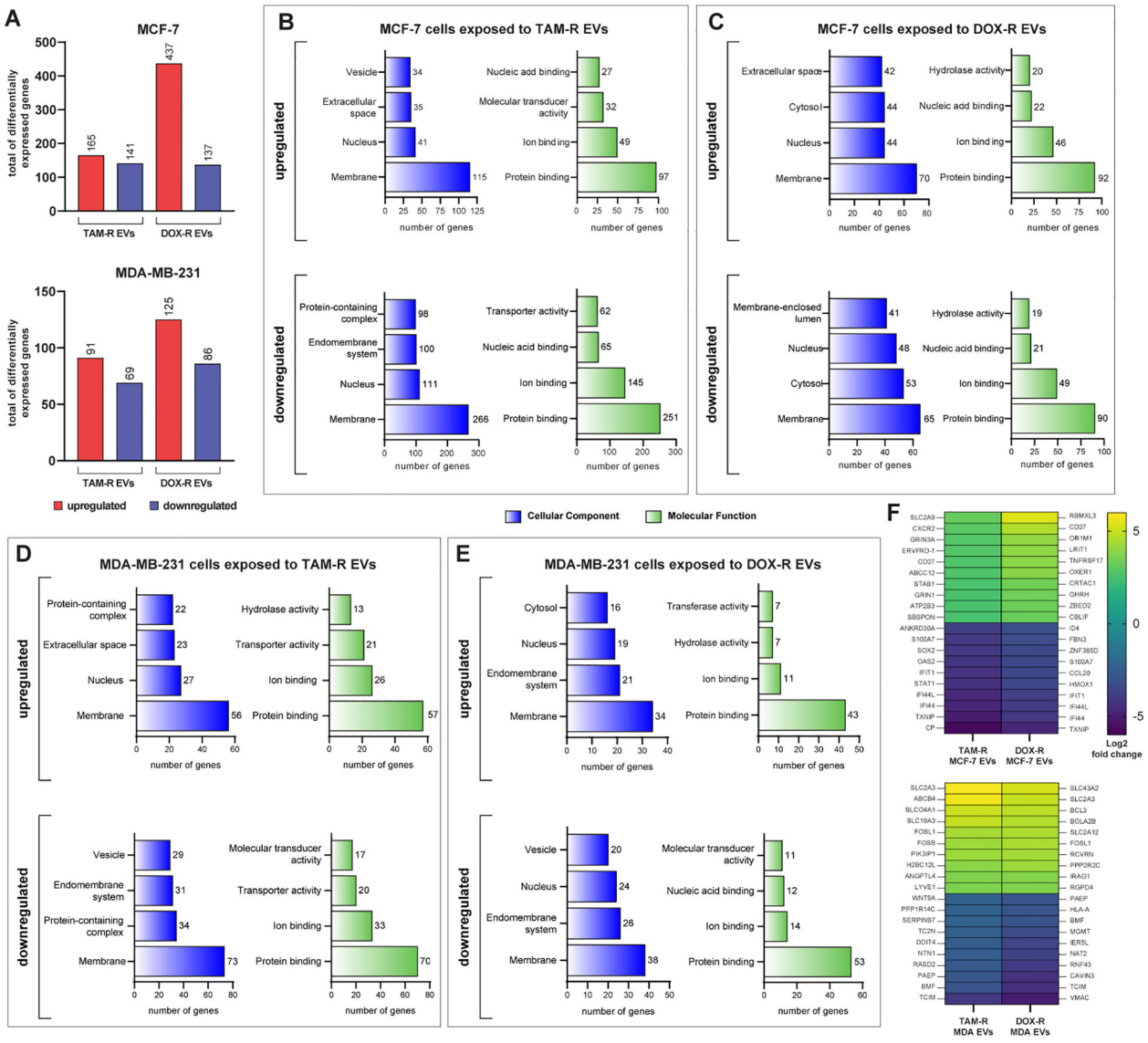


FIGURE 4
Transcriptome-wide analysis showing the differential expression in sensitive breast cancer cell lines exposed to resistant extracellular vesicles (EVs) for 24 hours. **(A)** Histogram of the total number of differentially expressed genes (DEGs) in MCF-7 and MDA-MB-231. Distribution of gene ontology (GO) slim terms associated with MCF-7 transcripts after tamoxifen-resistant EVs (TAM-R EVs) exposure **(B)** and doxorubicin-resistant EVs (DOX-R EVs) exposure **(C)**. For MDA-MB-231, the distributions of GO-slim enriched terms after TAM-R and DOX-R EV exposure are shown in **(D, E)**, respectively. **(F)** Heatmap showing each condition's top 10 up- and downregulated genes based on their log2 fold change.

Moreover, the spheroid that received 0.2 µM of DOX and was previously exposed to sensitive EVs showed more dead cells than the one exposed to DOX-R EVs (Figure 3E).

## 3.4 Transcriptome-wide analysis of drug resistant derived EVs influence on sensitive cells

To better understand the influence of drug-resistant EVs on the sensitive MCF-7 and MDA-MB-231 transcriptomes, we performed RNA sequencing (RNA-Seq) analysis in these two cell lines after exposure to TAM-R and DOX-R EVs for 24 h. After sequencing and mapping, 306 genes were considered DEGs of MCF-7 cells exposed to TAM-R EVs, of which 165 were upregulated and 141 were downregulated, and 574 genes were labeled as DEGs of MCF-7 cells exposed to DOX-EVs, separated into 437 upregulated and 137 downregulated genes, using the previously explained cutoff filtering (Figure 4A). In MDA-MB-231 cells exposed to TAM-R, we identified 160 DEGs, of which 91 were upregulated and 69 were downregulated. Additionally, 211 genes were identified as DEGs and were separated into 125 upregulated and 86 downregulated genes (Figure 4A). We analyzed the relationships to cellular components, and molecular functions using GO slim subsets to obtain a comprehensive overview of the DEG lists. This broad analysis is the first step in the transcriptome analysis pipeline, giving general information about which locations, mechanisms or cellular functions have more DEGs in breast cancer cells exposed to EVs

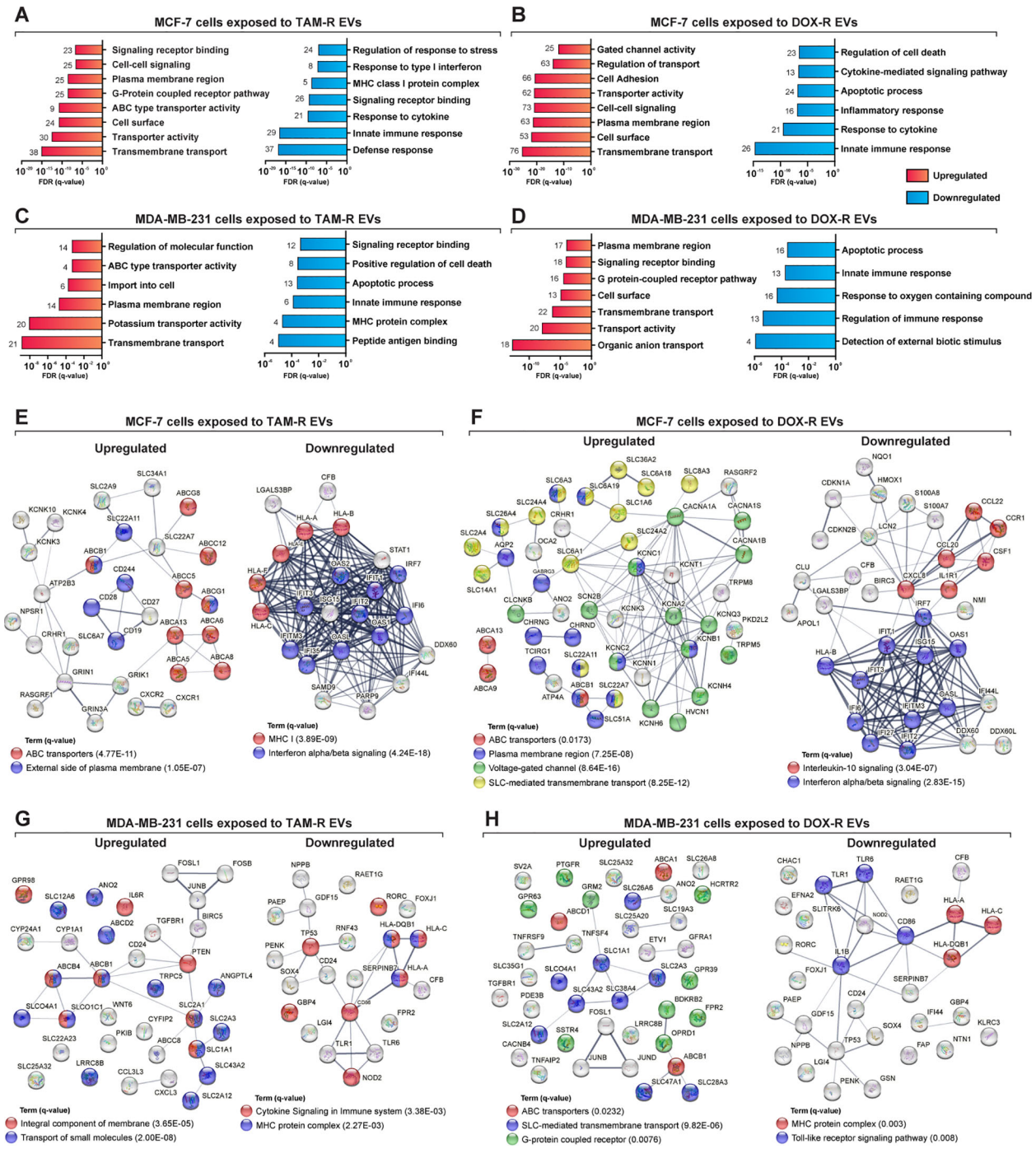


FIGURE 5
Deep over-representation analysis of the differentially expressed genes (DEGs) in breast cancer cells exposed to extracellular vesicles (EVs) released by tamoxifen-resistant (TAM-R) and doxorubicin (DOX-R) cell lines. The gene ontology (GO) analysis enriched more specific terms considering the up- and downregulated DEGs in sensitive MCF-7 cells exposed to **(A)** TAM-R EVs and **(B)** DOX-R EVs for 24 h For sensitive MDA-MB-231 cells, the enriched terms for up- and downregulated DEGs after exposure to TAM-R and DOX-R EVs are displayed in **(C, D)**, respectively. The number in front of each bar represents the total of enriched DEGs for each term. The x-axis of A to D represents the adjusted *p*-value (*q*-value) with a false discovery rate of 1%. Protein-protein interaction networks were generated using the DEG lists as references. **(E, F)** display the networks created by the MCF-7 DEGs after exposure to TAM-R and DOX-R EVs, respectively. For MDA-MB-231 cells, the exposure of TAM-R and DOX-R EVs generated the networks represented in **(G, H)**, respectively.

derived from resistant cells for further investigation. This GO analysis revealed that the most enriched term for all conditions in which MCF-7 cells were exposed to resistant EVs was related to the membrane, followed by the "nucleus" term. Additionally, in the Molecular Function category, the most enriched term in MCF-7 cells exposed to resistant EVs was related to protein binding (Figures 4B, C). We observed the same pattern of enriched GO terms in the DEG list of MDA-MB-231 cells exposed to TAM-R- or

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DOX-R-derived EVs (Figures 4D, E). We generated a heatmap containing the top 10 most upregulated and downregulated genes under each resistant EV exposure condition (Figure 4F). Inside this narrow list, we observed the upregulation of DEGs related to solute transport (solute carrier family SLC), ABC transporters (*ABCC12* and *ABCB4*), tumor growth transcription factors (*FOSL1* and *FOSB*), and the downregulation of genes induced by interferon (*IFI44L*, *IFI44*, and *IFIT1*). In addition, the tumor suppressors STAT1 and human leukocyte antigen class I (*HLA-A*) were downregulated.

To further understand the biological function of the DEGs, we performed a more in-depth GO analysis using the GSEA over-representation parameter with a 1% FDR significance level, and functionally related gene sets were enriched. The upregulated DEGs in MCF-7 cells exposed to TAM-R and DOX-R EVs were related to transport activity in the plasma membrane region via ABC transporters, gated channel activity, and G protein-coupled receptors (Figures 5A, B). The downregulated DEGs of MCF-7 cells exposed to TAM-R enriched terms associated with innate immune response and MHC I activity, especially the response to type I interferon, while the downregulated DEGs of MCF-7 cells exposed to DOX-R enriched terms associated with immune response. However, this list enriched terms related to cell death processes, including apoptosis (Figures 5A, B). The DEGs from MDA-MB-231 cells exposed to TAM-R and DOX-R EVs followed the same pattern as in MCF-7 cells; the upregulated genes were enriched in terms of the transport of molecules via ABC transporters, G-protein receptors, and potassium ion transmembrane activity on the cell surface. In contrast, the downregulated DEGs of MDA-MB-231 cells exposed to both TAM-R and DOX-R EVs showed enrichment of terms related to cell death, apoptotic processes, and innate immune responses (Figures 5C, D).

ABC transporters are involved in acquired resistance in cancer cells. Using the DEGs lists in STRING to generate physical and biological interaction networks between the proteins encoded by these genes, we observed many upregulated ABC transporters in both cell lines after exposure to resistant EVs, including *ABCB1* (P-glycoprotein); several ABC1 transporters (*ABCA1*, *ABCA5*, *ABCA6*, *ABCA8*, *ABCA9*, *ABCA13*, and *ABCB5*); and multidrug resistance-associated proteins (MRPs: *ABCC5* and *ABCC12*), especially in cells exposed to TAM-R EVs (Figures 5E, G). Our results indicate the upregulation of genes is directly related to the transport of molecules across membranes. We observed SLC genes in all

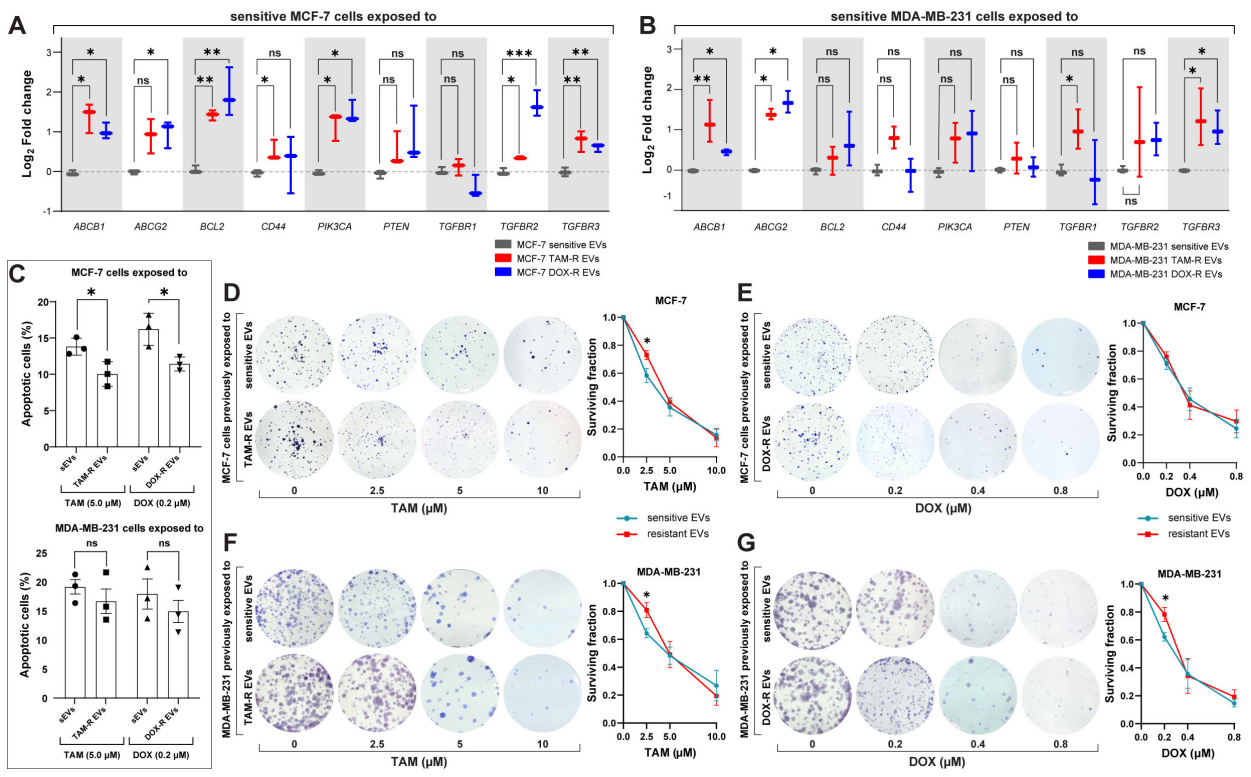


FIGURE 6
Resistant extracellular vesicles (EVs) induce the upregulation of genes associated with acquired drug resistance and increase the sensitive cells' survivability. The quantification of several genes by quantitative polymerase chain reaction (qPCR) of **(A)** sensitive MCF-7 exposed to tamoxifen-resistant (TAM-R) and doxorubicin-resistant (DOX-R) EVs. **(B)** The gene expression level of sensitive MDA-MB-231 cells exposed to TAM-R and DOX-R. **(C)** The apoptotic rate by annexin V/propidium iodide labeling in sensitive MCF-7 and sensitive MDA-MB-231 previously exposed to resistant EVs and treated with TAM and DOX. **(D)** Representative images and the clonogenic surviving fraction of MCF-7 cells previously exposed to TAM-R and **(E)** DOX-R EVs treated with the respective drug. **(F)** Representative images and the clonogenic surviving fraction of MDA-MB-231 cells previously exposed to TAM-R and **(G)** DOX-R EVs treated with the respective drug. Values are displayed as the mean ± standard deviation from three independent experiments. The asterisks indicate a significant difference between TAM-R EVs or DOX-R EVs exposure compared to sensitive EV effects (unpaired *t*-test). *p < 0.05, **p < 0.01, ***p < 0.001, ns, not significant.

upregulated DEG, especially in cancer cells exposed to DOX-R EVs (Figures 5F, H). Additionally, the networks showed the upregulation of genes associated with voltage-gated channels in MCF-7 cells that received DOX-R EVs, and the upregulation of genes related to the G-protein coupled receptor in MDA-MB-231 cells that were exposed to DOX-R EVs. When looking at the downregulated networks, we can observe the reduced expression of interferon-induced proteins, such as *IFIT1*, *IFIT2*, and *IFIT3*, in MCF-7 cells exposed to TAM-R and DOX-R EVs. The MHC protein complex was enriched in all the downregulated lists, in which we observed the downregulation of several HLA genes, including *HLA-A*, *HLA-B*, *HLA-C*, *HLA-E*, and *HLA-F*.

## 3.5 Resistant EVs modulate drug resistance-related genes and increase the survivability of cancer cells

According to our transcriptome results, resistant EVs influence several cellular mechanisms, particularly the transport of molecules and cell death-/survival-related functions. To further explore these findings, we analyzed the effects of resistant EVs on the expression of significant driver genes related to drug resistance and cell survival. The qPCR results showed that resistant EVs upregulate the expression of ABC transporters (*ABCB1* and *ABCG2*) and transforming growth factor-β receptor genes (*TGFBR1*, *TGFBR2*, and *TGFBR3*) in both sensitive cell lines, except for MCF-7 cells exposed to resistant EVs, which showed no difference in *TGFBR1* expression in comparison with sensitive EVs exposure (Figures 6A, B). We observed the upregulation of *PIK3CA* and downregulation of *BCL2* in MCF-7 cells after exposure to resistant EVs, which is an interesting result that is directly related to apoptosis. The downregulation of genes associated with apoptosis suggests that resistant EVs may inhibit this cell death mechanism. To further explore this finding, we analyzed the effects of TAM- and DOX-R EVs on sensitive cells using a cellular assay for annexin V labeling. After 24 h of initial exposure to resistant EVs, both sensitive cell lines were treated with TAM or DOX for 72 h. The results showed that TAM-R and DOX-R EVs reduced the population of apoptotic MCF-7 cells (Figure 6C). In contrast, we observed neither a difference in *PIK3CA* and *BCL2* expression nor in the apoptosis rate in MDA-MB-231 cells exposed to resistant EVs compared to the counterpart-sensitive EVs.

Taking the upregulation of transforming growth factor-β- (TGF-β) related genes as a reference, we investigate the cytostatic effects of resistant EVs to ascertain whether this upregulation of proliferation-related genes translates into increased cell growth and survivability. A clonogenic assay was performed, and the results showed that resistant EVs increased the survival rate and colony formation in the long term. We observed that MCF-7 TAM-R EVs increased the survivability and proliferation of sensitive cells after TAM treatment (2.5 μM) when compared to MCF-7 that received

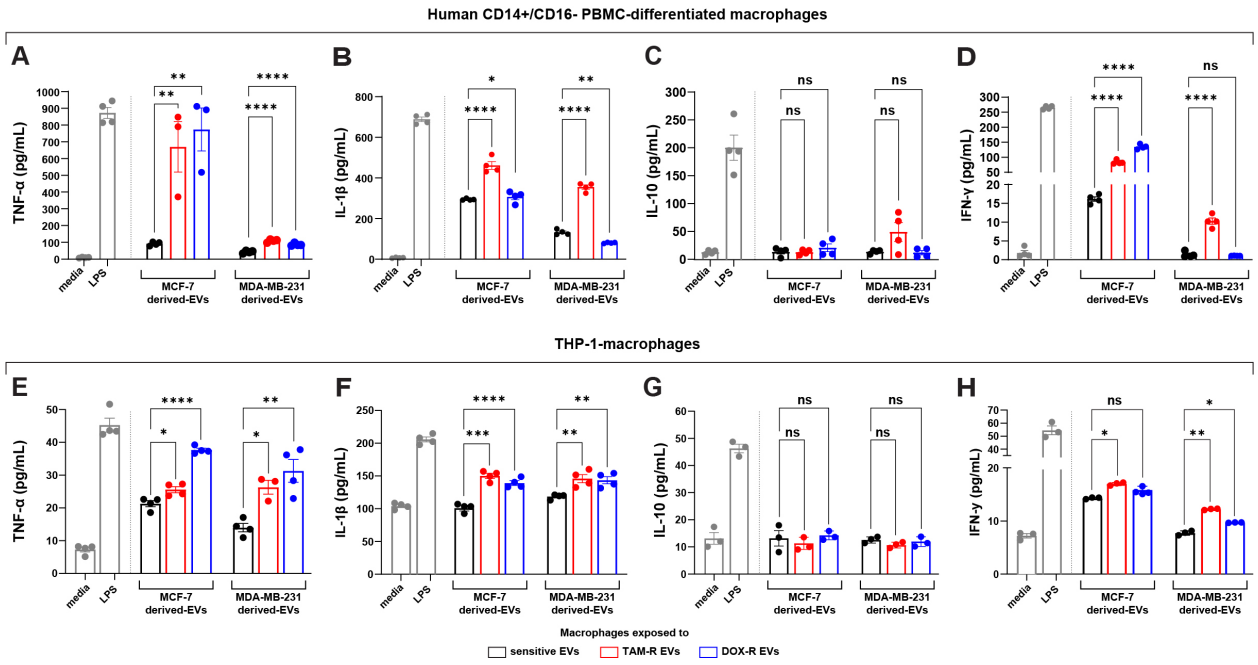


FIGURE 7
Extracellular vesicles (EVs) released by tamoxifen-resistant (TAM-R) and doxorubicin-resistant (DOX-R) breast cancer cell lines modulate the cytokine production and release of human macrophages. Human CD14+/CD16- peripheral blood mononuclear cells (PBMC)-derived macrophages were exposed to EVs released by resistant MCF-7 and MDA-MB-231, and the quantification level of **(A)** tumor necrosis factor (TNF)-α, **(B)** interleukin (IL)-1β, **(C)** IL-10, and **(D)** interferon (IFN)-γ were evaluated. Additionally, THP-1-macrophages were exposed to resistant EVs, and the secretion levels of **(E)** TNF-α, **(F)** IL-1β, **(G)** IL-10, and **(H)** IFN-γ measured by the ELISA assay. Values are displayed as the mean ± standard deviation from three independent experiments. The asterisks indicate a significant difference between TAM-R EVs or DOX-R EVs exposure compared to sensitive EV effects (unpaired *t*-test). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; ns, not significant; media, RPMI 1640; LPS, lipopolysaccharide (positive control).

sensitive EVs and the same TAM treatment (Figure 6D); however, we did not find any difference in MCF-7 exposed to DOX-R EVs (Figure 6E). MDA-MB-231 cells previously exposed to TAM-R EVs showed a significant increase in colony formation after being treated with 2.5 μM of TAM compared to the cells that received sensitive EVs (Figure 6F). Additionally, MDA-MB-231 exposed to DOX-R EVs showed a higher survival fraction when treated with 0.2 μM of DOX compared to cells that received sensitive EVs (Figure 6G).

### 3.6 Resistant EVs modulate the macrophage's cytokine release profile

Given that genes directly related to the immune response were modulated by resistant EVs in both breast cancer cell lines, we performed ELISA to detect specific cytokines released by different human macrophages when exposed to TAM-R and DOX-R EVs from MCF-7 and MDA-MB-231 resistant cell lines. The human PBMC macrophages were exposed for 24 h to resistant EVs, and then we collected the supernatant and evaluated the levels of several cytokines. In this experiment, we observed increased levels of TNF-α and IL-1β after all resistant EVs exposure (Figures 7A, B); we observed an increase in IFN-γ levels after these macrophages were exposed to TAM-R EVs from the TAM-R MDA cell line (Figure 7C); however, we did not find any difference between the sensitive and resistant EVs in the IL-10 levels in all tested conditions (Figure 7D). Additionally, using the human monocyte cell line THP-1 transformed into macrophages by PMA, we further analyzed the impact of resistant EVs on macrophage cytokine release patterns. We observed results similar to those obtained for PBMC. We observed increased levels of TNF-α and IL-1β (Figures 7E, F) and no differences in IL-10 levels (Figure 7G) in all tested conditions. The difference lies in IFN-γ level measurement. We observed a significant increase in IFN-γ concentration in the supernatant from macrophages exposed to TAM-R and DOX-R EVs from MCF-R and TAM-R EVs from MDA-R cell lines (Figure 7H).

## 4 Discussion

The two breast cancer cell lines, MCF-7 and MDA-MB-231, were selected due to their representation of distinct phenotypes and genotypes of the disease. MCF-7 cells are hormone-dependent, being positive for estrogen and progesterone receptors. This characteristic makes them more sensitive to drugs specifically designed to target hormone receptors, such as TAM, an estrogen receptor modulator. Additionally, MCF-7 cells exhibit a luminal/epithelial phenotype and form spontaneous spheroids *in vitro*. In contrast, MDA-MB-231 cells are triple negative, which contributes to their increased resistance to various drug treatments. This lack of response, combined with their mesenchymal phenotype, makes MDA-MB-231 cells more aggressive and invasive compared to MCF-7 cells (30). Given that both cell lines have demonstrated multidrug resistance and exhibit differential susceptibilities during the development of acquired drug resistance, investigating the effects of extracellular vesicle on MCF-7 and MDA-MB-231 cells in a single study encompasses multiple aspects of cancer progression.

Several large-scale studies have been performed to investigate the role of EVs in breast cancer progression, particularly in the acquisition of a drug-resistant phenotype during chemotherapy (31), including quantitative proteomics (32), epigenetic profiling (33), and miRNA expression profiles (34). Our contributions include the generation of high-level laboratory-resistant breast cancer cell lines from parental MCF-7 and MDA-MB-231 cells, followed by transcriptome-wide analysis combined with different cellular endpoints. These findings might help understand the influence of EVs from resistant breast cancer cells in sensitive cells to trigger acquired drug resistance. Initially, the generation of resistant cell lines following the guidelines of McDermott (14) resulted in a reliable tool for studying drug resistance *in vitro*. The comparison of IC50 and the resistance fold showed a disparity in resistance levels between sensitive and resistant cells. Other recent studies with TAM-resistant MCF-7 and TAM-resistant MDA-MB-231 showed similar IC50 values to our results (35, 36). Additionally, DOX-resistant MCF-7 and MDA-MB-231 cells showed an IC50 range of 0.9 to 1.5 μM, which is close to what we observed in our DOX-resistant cell lines (37, 38).

EVs are highly heterogeneous in terms of composition; however, their morphology follows the same spherical pattern (39). More studies are needed on the EV production rate per cell. According to Chiu et al. (40), MCF-7 and MDA-MB-231 cells generated approximately 60 EVs per hour. These results are similar to ours, even though the resistant cell lines did not change their EV secretion rate. Our data suggest no differences in the concentration, production, or characterization of sensitive and resistant EVs derived from breast cancer cells. The interaction between EVs and cells and the internalization of EVs are much less studied than EV formation and secretion, and information concerning whether EVs must be internalized to trigger a few effects in the cell membrane is rare (41). We observed that resistant EVs interact with and are internalized by sensitive cells in a 24-h time frame with no cytotoxic effects. Consistent with our results, EVs released by SW480 human colorectal cancer cells were internalized by THP-1 macrophages; however, SW480-derived EVs showed minor cytotoxicity towards these macrophages (42).

Considering the ability of our resistant cells to survive TAM or DOX treatment and the literature reports on the transfer of drug resistance via EVs, we hypothesized that EVs released by resistant cells may increase the number of viable cells after drug treatment in sensitive cells that have never been exposed to TAM or DOX by providing some form of drug resistance to these cells. We observed that sensitive breast cancer cells previously exposed to resistant EVs showed higher viability than cells exposed to sensitive EVs in 2D culture with MCF-7 and MDA-MB-231 cells and in 3D culture with MCF-7 spheroids. Based on these results, RNA sequencing was selected as the platform for further investigation of resistant EVs and their roles in acquired drug resistance. Bioinformatics analysis showed that resistant EVs from MCF-7-R and MDA-R cells influence transport-related mechanisms, cell death, and the innate

immune response. The most upregulated DEGs in MCF-7 and MDA-MB-231 cells were associated with transport activity.

Several studies have revealed the direct participation of transporters in drug resistance acquisition, especially ABC transporters, specifically P-glycoprotein (ABCB1). Studies have suggested that P-gp can be directly transferred to sensitive cells via EVs, or that its expression can be increased by transferring miRNAs from resistant cells to sensitive cells (43). Our results revealed an upregulation of *ABCB1*, responsible for P-gp encoding, in MCF-7 and MDA-MB-231 cells exposed to TMX-R and DOX-R EVs, suggesting that the resistant EVs in our conditions increased the expression of P-gp as a general drug resistance mechanism, independent of the cell line or drug type. This non-specific activity and transfer of ABCB1, regardless of whether the drug is a direct substrate of ABCB1 efflux transport, was observed in EVs released by KBv200 cells overexpressing ABCB1. These EVs increase ABCB1 expression in sensitive cells after treatment with drugs with different therapeutic mechanisms (44). In the context of breast cancer, there are few reports available on the influence of P-gp via EVs on drug resistance; for example, multidrug-resistant MCF-7 cells specifically transfer P-gp to EVs, which mediate resistance to several anticancer drugs (45). However, to date, there is no information on ABCB1 transfer via EV in MDA-MB-231 cells during the acquisition of drug resistance. We observed the upregulation of the *ABCG2* gene, which encodes breast cancer resistance protein (BCRP), in sensitive cells exposed to resistant EVs. Other studies have shown the accumulation of ABCG2 inside EVs from resistant cell lines (46), which induces drug resistance in sensitive cells (47). Besides P-gp and BCRP, our results showed an upregulation of several genes that are directly associated with acquired drug resistance during chemotherapy, frequently called multidrug resistance (MDR) transporters, including *ABCB4*, *ABCC5*, and *ABCG1*, to name a few. EVs containing these MDR transporters can induce a pump efflux from the cytoplasm to extracellular space in sensitive breast cancer cells (48, 49). Other studies have shown that MDR transporters contribute to chemotherapy resistance; however, do not participate in the acquired drug resistance process (50, 51). Our findings are the first transcriptome-wide study showing the influence of MDR transporters modulated by resistant EVs in sensitive breast cancer cells.

Membrane transporters are crucial for the acquisition of drug resistance, and we observed the upregulation of genes associated with SLC-mediated transmembrane transport, voltage-gated channels, and G-protein coupled receptors. While ABC transporters are active and dependent on ATP hydrolysis, SLC transporters act as secondary active transporters, using an ion gradient to handle diverse substrates (52). SLC transporters play important roles in the development of drug resistance. A recent study showed that these transporters are highly expressed in patients resistant to chemotherapy (53). In combination with high expression of P-gp, SLC transporters enhance cell proliferation in human leukemia, facilitating the acquisition of drug resistance (54). Additionally, voltage-gated channels, especially calcium and potassium channels, are often reported to be overexpressed in different types of cancer, including breast cancer, where they induce cell proliferation, tumor growth, and drug resistance (55, 56). In malignant cells, calcium mobilization and the activation of calcium signaling are directly related to EV biogenesis, facilitating plasma membrane vesiculation (57); hence, it may act as a secondary drug resistance process. Furthermore, abnormal expression of potassium voltage-gated channels contributes to breast cancer progression and drug resistance (58, 59); however, the interaction between potassium channels and EVs is unclear. Potassium channels are activated by G protein-coupled receptors (GPRs), which play an essential role in many cellular processes. In the context of cancer, they are overexpressed and associated with tumor growth and metastasis (60), and more recently, GPRs have been reported as promoters of acquired drug resistance to different drugs in breast cancer cell lines (61, 62). Our results showed the upregulation of several types of membrane transporters, including drug resistance drivers, such as *ABCB1* and *ABCG2*, in combination with SLC transporters, voltage-gated channels, and GPRs, revealing that EVs may carry a powerful setup to induce drug resistance with multiple approaches in adjacent or distant cancer cells. However, a few aspects of this puzzle are still unknown, including acquired drug resistance induced by EVs released by resistant cancer cells.

On the other side of the spectrum, we observed that many genes associated with cell death, especially apoptosis, were downregulated after exposure to resistant EVs, which was expected after the increase in viability of sensitive cells exposed to resistant EVs. Inhibition of apoptotic signaling pathways such as the p53 pathway is directly related to cell survival, proliferation, and drug resistance; the upregulation of pro-apoptotic regulators promotes drug resistance (63). We observed the upregulation of anti-apoptotic factors, such as BCL-2 and PIK3CA, and the downregulation of *TP53* (responsible for encoding p53) in MCF-7 cells after exposure to resistant EVs. These findings correlated with the decrease in the apoptotic cell population in sensitive MCF-7 cells previously exposed to resistant EVs and after treatment with TAM and DOX. Recently, EVs derived from cancer cells were shown to reduce the apoptosis rate via upregulation of *BCL2* in other cancer cells (64), and tumor-derived EVs containing extracellular matrix molecules induced mutations in *PIK3CA*, which promoted proliferation and invasion in breast cancer (65). Despite the enrichment of terms related to cell death, we did not observe changes in the apoptotic population of sensitive MDA-MB-231 cells exposed to resistant EVs and treated with TAM and DOX. Additionally, BCL-2 and PIK3CA levels showed no significant differences. Further investigation is needed to understand the anti-apoptotic events displayed in RNA-Seq, which were not observed in other experiments. Our results, especially in MCF-7 cells, showed that EVs released by resistant breast cancer cell lines can modulate the apoptotic process via the upregulation of anti-apoptotic regulators and the downregulation of pro-apoptotic factors, inhibiting cell death and therefore promoting drug resistance in sensitive cancer cells. However, the exact mechanism by which these transcriptional changes occur remains unclear. It may be related to the transfer of miRNAs, as suggested by other authors (66, 67).

Another feature that cancer cells exhibit to evade apoptosis, survive, proliferate, and resist chemotherapy is related to TGF-β signaling, and many studies showed that miRNA and proteins

associated with the TGF-β pathway, including TGF-β receptors (TGFBR), were observed in cancer-derived EVs (68). The ability of EVs to transfer TGFBR was observed in breast cancer, in which these cancer EVs transferred activated TGFBRs to CD8+ lymphocytes, exhausting them and transferring TGFBRs to MDA-MB-231 cells, promoting metastasis, paclitaxel, and DOX resistance (69). Similar results have been reported using TGFBR-rich EVs from MCF-7 cells, which induce DOX resistance. According to these studies and the upregulation of TGFBRs, we observed in sensitive cancer cells after being exposed to resistant EVs, the TGFBR transfer is crucial for cancer cells to survive and develop drug resistance in sensitive cells. Consistently, this survival mechanism supported by TGF-β signaling via EVs is linked to the clonogenic surviving fraction by the number of colonies that we observed in MDA-MB-231 cells previously exposed to TAM-R EVs and MCF-7 cells exposed to TAM-R and DOX-R EVs. In this case, resistant EV exposure was able to increase the survival rate of sensitive cells only at the lowest tested concentration, suggesting that the acquired resistance induced by EVs increases the survivability of sensitive cells. However, it cannot overcome the drug effect of high concentrations after a period without any stimuli with resistant EVs.

In the context of breast tumor, cancer cells influence the role of macrophages, which are the most abundant immune cells within the tumor microenvironment, depending on the stage of breast cancer progression (70). Tumor-associated macrophages in breast cancer exhibit a range of functions and phenotypes. Consequently, specific subsets of macrophages perform distinct and varied roles, either modulating tumor progression or exerting anticancer effects (71, 72). The downregulation of genes associated with innate immune responses observed in the RNA-seq data prompted us to investigate the influence of resistant EVs on macrophages. Cancer-derived EVs and their effects on macrophages are popular topics in cancer biology. Simultaneously, many studies reported that EVs released by cancer cells promote an anti-inflammatory phenotype polarization in macrophages as part of the immune system evasion hallmark during tumor progression, which is frequently associated with poor survival outcomes (73, 74). In the broader context of breast cancer biology, anti-inflammatory macrophages play a crucial role in the development of drug resistance by influencing various signaling pathways in cancer cells. They contribute to resistance mechanisms through the overexpression of PI3K/AKT factors, which promote tumor growth (75, 76). Several other studies have shown that cancer-derived EVs can trigger a pro-inflammatory phenotype in macrophages; consequently, this process is associated with better clinical outcomes (77, 78). When we exposed human macrophages to resistant breast cancer-derived EVs, we observed a pro-inflammatory profile with increased TNF-α, IL-1β, and IFN-γ expression, exhibiting anti-tumoral characteristics. The pro-inflammatory response involves TNF-α expression, which activates of T lymphocytes and Natural Killer cells. IFN-γ released by macrophages triggers Toll-like receptor activation and induces cell cycle arrest in cancer cells, while IL-1β acts as a significant driver of inflammation (79, 80). However, both IL-1β and TNF-α exhibit paradoxical pro-tumor effects that are associated with metastasis in breast cancer (81, 82). This potential pro-inflammatory response from macrophages contrasts with the downregulation of innate immune response-related genes in sensitive cells exposed to resistant EVs. These findings suggest that breast cancer-resistant EVs modulate immune system factors in sensitive cancer cells to evade immune detection, such as the downregulation of several MHC-related genes, including HLAs, resulting in unpaired antigen presentation. However, there is a lack of information regarding the modulation of the immune system by cancer-derived EVs in other cancer cells. Collectively, our data show that resistant EVs may play a dual role in cancer progression: they induce changes in cancer cells to evade immune surveillance, whereas these EVs may carry molecules that trigger a pro-inflammatory signature in macrophages, which represents an interesting mechanism for future immunotherapies by exploiting cancer EVs downfall.

In summary, the data discussed in this study revealed important information about EVs released by resistant breast cancer cell lines, in which initially resistant EVs reduced the toxicity of chemotherapeutic drugs, such as TAM and DOX, in sensitive MCF-7 and MDA-MB-231 cells as part of the development of acquired drug resistance. Moreover, our data highlighted that resistant EVs can modulate the transcription pattern of breast cancer cells, inducing the upregulation of transporters and downregulating immune system factors and cell death-related genes, which are the mechanisms of acquired drug resistance. Additionally, our findings shed light on a potential target for future immunotherapies related to the ability of macrophages to activate a pro-inflammatory pattern when exposed to resistant EVs. Understanding the interactions between EV released by resistant and sensitive cancer cells remains challenging. There are few such reports in the literature; however, our data provide substantial insights for future research on acquired drug resistance influenced by cancer EVs.

## Data availability statement

The data presented in the study are deposited in the GEO repository, accession number GSE273705.

## Ethics statement

The study was conducted according to the principles proposed by the Brazilian National Research Ethics Commission and approved by the Research Ethics Committee of the Ribeirao Preto Medical School at the University of São Paulo, Protocol CAAE 71085023.1.0000.5440.

## Author contributions

PS: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. CR: Formal analysis, Investigation, Methodology, Writing – review & editing. RP: Formal analysis, Investigation, Methodology, Writing – review & editing. BO: Formal analysis, Investigation, Methodology, Writing – review & editing. FF: Formal analysis, Investigation, Methodology, Writing – review & editing. VC: Investigation, Methodology, Writing – review & editing. RC: Formal analysis, Resources, Visualization, Writing – review & editing. MP: Formal analysis, Methodology, Writing – review & editing. FA: Conceptualization, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1468229/full#supplementary-material

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Anirban Ganguly,
All India Institute of Medical Sciences Deoghar, India

*CORRESPONDENCE
Fausto Almeida
fbralmeida@usp.br

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# Corrigendum: Extracellular vesicles from human breast cancer-resistant cells promote acquired drug resistance and pro-inflammatory macrophage response

Patrick Santos[1], Caroline P. Rezende[1], Renan Piraine[1], Bianca Oliveira[1], Francielle B. Ferreira[1], Vinicius S. Carvalho[2], Rodrigo T. Calado[2], Matteo Pellegrini[3] and Fausto Almeida[1]*

[1]Department of Biochemistry and Immunology, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, SP, Brazil, [2]Department of Medical Imaging, Hematology, and Oncology, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, Brazil, [3]Department of Molecular, Cell, and Developmental Biology, University of California, Los Angeles, CA, United States

KEYWORDS

**chemoresistance, tamoxifen, doxorubicin, immunomodulation, membrane transporters**

A Corrigendum on

Extracellular vesicles from human breast cancer-resistant cells promote acquired drug resistance and pro-inflammatory macrophage response

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In the published article, there was an error in **Figure 6** as published. In panel A and B of **Figure 6**, the color in the graph legend corresponding to MCF-7 and MDA-MB-231 sensitive EVs should be in grey, not in blue. The corrected **Figure 6** and its caption "Figure 6. Resistant extracellular vesicles (EVs) induce the upregulation of genes associated with acquired drug resistance and increase the sensitive cells' survivability. The quantification of several genes by quantitative polymerase chain reaction (qPCR) of (A) sensitive MCF-7 exposed to tamoxifen-resistant (TAM-R) and doxorubicin-resistant (DOX-R) EVs. (B) The gene expression level of sensitive MDA-MB-231 cells exposed to TAM-R and DOX-R. (C) The apoptotic rate by annexin V/propidium iodide labeling in sensitive MCF-7 and sensitive MDA-MB-231 previously exposed to resistant EVs and treated with TAM and DOX. (D) Representative images and the clonogenic surviving fraction of MCF-7 cells previously exposed to TAM-R and (E) DOX-R EVs treated with the respective drug. (F) Representative images and the clonogenic surviving fraction of MDA-MB-231 cells previously exposed to TAM-R and (G) DOX-R EVs treated with the

respective drug. Values are displayed as the mean ± standard deviation from three independent experiments. The asterisks indicate a significant difference between TAM-R EVs or DOX-R EVs exposure compared to sensitive EV effects (unpaired *t*-test). *p < 0.05, **p < 0.01, ***p < 0.001, ns, not significant." appear below.

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

## Publisher's note



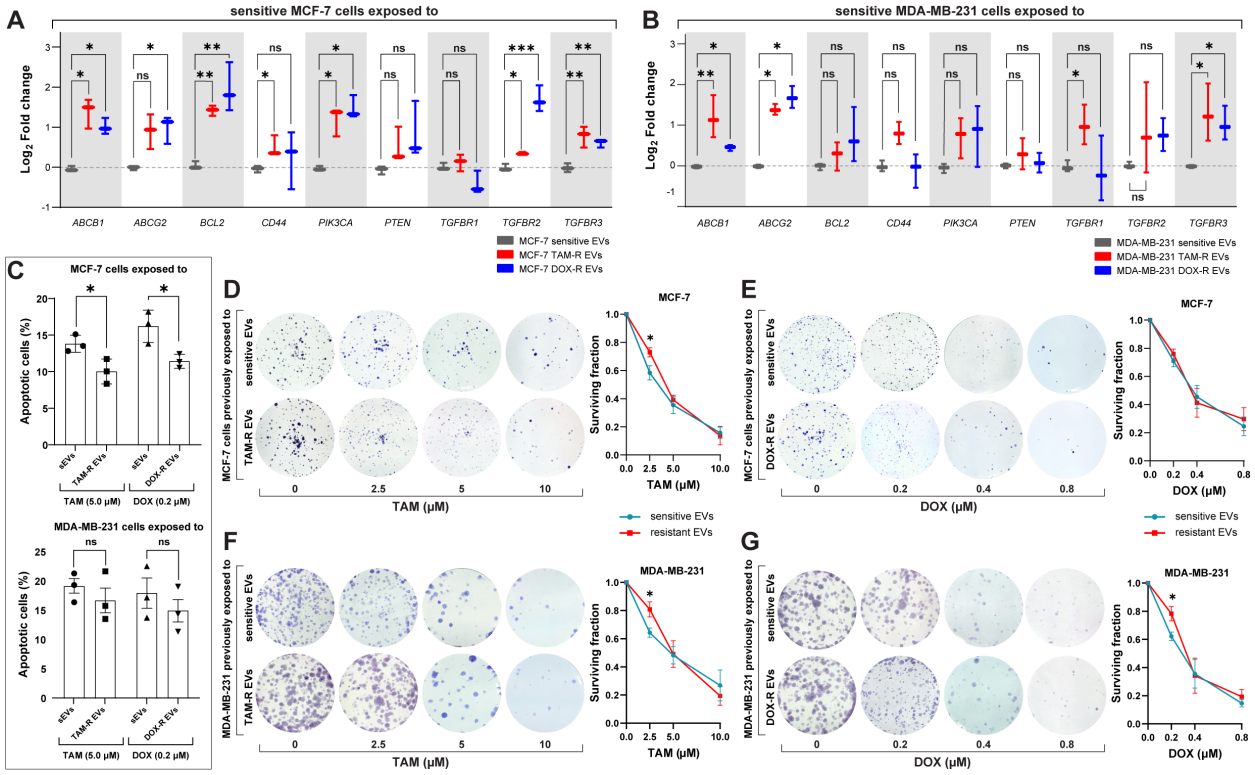


FIGURE 6
Resistant extracellular vesicles (EVs) induce the upregulation of genes associated with acquired drug resistance and increase the sensitive cells' survivability. The quantification of several genes by quantitative polymerase chain reaction (qPCR) of **(A)** sensitive MCF-7 exposed to tamoxifen-resistant (TAM-R) and doxorubicin-resistant (DOX-R) EVs. **(B)** The gene expression level of sensitive MDA-MB-231 cells exposed to TAM-R and DOX-R. **(C)** The apoptotic rate by annexin V/propidium iodide labeling in sensitive MCF-7 and sensitive MDA-MB-231 previously exposed to resistant EVs and treated with TAM and DOX. **(D)** Representative images and the clonogenic surviving fraction of MCF-7 cells previously exposed to TAM-R and **(E)** DOX-R EVs treated with the respective drug. **(F)** Representative images and the clonogenic surviving fraction of MDA-MB-231 cells previously exposed to TAM-R and **(G)** DOX-R EVs treated with the respective drug. Values are displayed as the mean ± standard deviation from three independent experiments. The asterisks indicate a significant difference between TAM-R EVs or DOX-R EVs exposure compared to sensitive EV effects (unpaired t-test). *p < 0.05, **p < 0.01, ***p < 0.001, ns, not significant..

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EDITED BY
Qiang Fu,
Binzhou Medical University, China

REVIEWED BY
Yaowu He,
The University of Queensland, Australia
Víctor Adrián Cortés-Morales,
Mexican Social Security Institute, Mexico

*CORRESPONDENCE
Juling Ji
jijuling@ntu.edu.cn;
jijuling66@126.com

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# Activated hepatic stellate cell-derived small extracellular vesicles facilitate M2 macrophage polarization and hepatoma progression via miR-27a-3p

Yufeng Sun[1,2], Xiaoqian He[1,2], Jiayi Han[1,2], Wenxuan Yin[1], Haichen Wang[1], Jing Li[1], Weiqi Liu[1], Xingwang Kuai[1,2], Jiaying Lv[1,2] and Juling Ji[1,2]*

[1]Department of Pathology, Medical School of Nantong University, Nantong, China, [2]Key Laboratory of Microenvironment and Translational Cancer Research, Nantong, China

The progression of hepatoma is heavily influenced by the microenvironment. Tumor-associated macrophages (TAMs) are considered to play a critical role in the tumor microenvironment (TME) and increase the aggressiveness of hepatoma. The activation of hepatic stellate cells (HSCs) is involved in hepatoma progression, and accumulating evidence demonstrates a change in microRNA (miRNA) expression during HSC activation. Therefore, the potential roles of HSCs-related miRNAs in macrophage differentiation and hepatoma progression deserve to be explored. The present study aimed to investigate the effects of miRNAs carried by small extracellular vesicles (sEVs) released by activated HSCs on hepatoma progression. The results indicated that miR-27a-3p was significantly upregulated in cells and corresponding sEVs during the activation of primary rat HSCs and human HSC line-LX2 cells. Furthermore, miR-27a-3p contributed to the proliferation and migration of hepatoma cells and promoted M2 polarization of macrophage. HSC-sEVs overexpressing miR-27a-3p can directly facilitate tumor progression and modulate macrophage polarization, indirectly contributing to hepatoma progression. Finally, Sprouty2 (SPRY2) was verified to be the target gene of miR-27a-3p. In conclusion, activated HSC-derived sEVs with high levels of miR-27a-3p might induce M2 macrophage polarization and promote hepatoma progression, providing new insights into the mechanism of hepatoma progression.

KEYWORDS

**hepatic stellate cell, hepatoma, miRNA-27a-3p, tumor-associated macrophages, extracellular vesicles**

# 1 Introduction

Primary liver cancer is the second leading cause of cancer-related death worldwide, with approximately 841,000 new cases and 782,000 deaths each year (1). Hepatoma is composed of a complex collection of cancer cells and nonparenchymal cells, including tumor-associated macrophages (TAMs), cancer-associated fibroblasts (CAFs), and regulatory T cells, which contribute to a supportive tumor microenvironment (TME) (2, 3). TAMs infiltrate the tumor microenvironment and may differentiate into M2 immunosuppressive type, thereby promoting the development of hepatoma (4).

About 80% of hepatoma occurs on the basis of cirrhotic liver (1). Hepatic stellate cells (HSCs), which are liver-specific mesenchymal cells residing within the perisinusoidal space of Disse, play a crucial role in hepatic fibrosis. In a healthy liver, HSCs are quiescent and constitute the body's largest reservoir of vitamin A. However, during liver injury, quiescent HSCs were activated and transformed into myofibroblasts that secrete matrix proteins, thereby leading to the development of liver cirrhosis (5). A recent study also confirmed HSCs as the major source of cancer-associated fibroblasts in hepatocellular carcinoma (HCC) (6). Considering the close relationship among liver fibrosis, cirrhosis, and hepatoma, activated HSCs may accelerate the progression of hepatoma through stroma-tumor interactions (7).

By analyzing our previous proteomic study of primary rat HSCs found that the most enriched biological function of the 200 proteins down-regulated during HSCs activation was involved in the immune response (8). We proposed that activated HSCs might affect the progression of hepatoma by participating in the immunosuppressive microenvironment. In a study later, we confirmed this hypothesis in liver cancer and para-cancer liver tissue samples from patients. Activated HSCs in para-cancer liver tissue promoted the progression of liver cancer by inducing an M2 immunosuppressive phenotype in macrophages (9, 10). However, the way in which HSCs affect the surrounding environment and the cells therein is still unclear.

Small extracellular vesicles (sEVs) are a group of nanovesicles mainly derived from endosomes with a diameter ranging from 30 to 150 nm, also known as exosomes (11, 12). They are secreted by the majority of cell types, loaded with DNAs, RNAs (including miRNA), proteins, and even metabolites from parent cells, constituting an essential intercellular communication (11, 12). Tumor cell-derived sEVs can induce the development of a tumor-promoting microenvironment by acting on surrounding stromal cells and facilitating signal transduction between cells (8, 13). There is an escalating interest in the role of stromal cell-derived sEVs in tumor progression.

MicroRNAs (miRNAs) are essential cargoes for sEVs (11, 12). They are a class of small noncoding RNAs that regulate gene expression by inhibiting the mRNA translation or the stability of targeting mRNA. They are involved in various biological and pathological processes (14). We previously reported that miR-27a-3p was upregulated in activated HSCs and promoted HSCs proliferation (15). It is worth further investigation if miR-27a-3p in sEVs derived from activated HSCs increases simultaneously, whether HSC-sEVs with higher expression of miR-27a-3p can affect the progression of hepatoma, and whether macrophages are involved.

In this study, we investigated the role of miR-27a-3p carried by activated HSC-derived sEVs in hepatoma progression, elucidating their potential and underlying mechanism in regulating tumor cells and the liver microenvironment.

# 2 Materials and methods

## 2.1 Biological materials

The human hepatic stellate cell line LX2 was provided by Feldman Laboratories. The human mononuclear cell line THP1 and human hepatoma cell lines (HepG2 and Huh7) were purchased from the Cell Bank/Stem Cell Bank of the Committee for Typical Cultures Preservation of Chinese Academy of Sciences. LX2 cells, HepG2 cells, and Huh7 cells were cultured in DMEM with 10% FBS (Gibco, USA). THP1 cells were cultured in 1640 (Invitrogen, USA) culture medium supplemented with 0.05 mM β-mercaptoethanol (Invitrogen, USA) and 10% FBS. Penicillin streptomycin (1%) was added to the above cell culture medium. Conventional culture was performed in an incubator at 37°C and 5% $CO_2$.

Male Sprague-Dawley rats (body weight 450-550 g) and 5-week-old male BALB/c nude mice were housed in the animal facility of Nantong University. All animal experimental protocols were approved by the Animal Ethics Committee of Nantong University. Animal care and experiments were performed in line with the principles of the Guide for the Care and Use of Laboratory Animals formulated by Nantong University.

## 2.2 Primary HSC isolation, purification, and identification

The nonparenchymal rat liver cell suspension was obtained using a two-step enzymatic digestion method. HSC-enriched cells were obtained from these cells by density gradient centrifugation as described previously (8). HSCs were seeded in 25 $cm^2$ culture flasks and cultured in DMEM (Gibco, USA) supplied with 10% exosome-depleted FBS (System Biosciences, Inc., USA) at 37°C in a 5% $CO_2$ atmosphere incubator. The culture medium was replenished every 3 days. The culture media from Day 3, Day 11, and Day 14 were collected for the isolation of HSC-derived sEVs. HSCs were collected and lysed in RIPA buffer (Beyotime Biotechnology, China) or TRIzol (Life Technologies, USA) for protein or RNA sample preparation on Day 3 and Day 11.

Primary HSCs grown on coverslips were incubated with monoclonal antibodies against α-SMA (Abcam, UK), which is a key marker for HSCs activation. Then, the cells were incubated with Cy3-labeled secondary antibodies (Abcam, UK) at room temperature for 45 min, and nuclei were counterstained with Hoechst 33258 (Sigma-Aldrich, USA). Images were acquired using a fluorescence microscope (Olympus, Japan) (Supplementary Figure S1).

## 2.3 LX2 activation model

Human HSCs cell line LX2 was used in the present study (16). Cells ($5\times10^5$) were seeded in a 25 $cm^2$ culture flask. After 24 h, 2% FBS DMEM culture medium was replaced, and 10 ng/mL of Recombinant Human TGF-β1 (Peprotech, USA) was added (17, 18). LX2 activation was induced, and the same volume of PBS buffer containing 5% saline solution (TGF-β1 buffer) was added as the negative control. After 24 h, the cells and supernatant were collected and verified by qRT-PCR, and western blotting was performed 48 h later.

## 2.4 Western blotting

Protein samples were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequently transferred onto PVDF membranes (Millipore). After blocking with 5% skim milk at room temperature for 1 h, the membranes were incubated with primary antibodies at 4°C overnight, and then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Total protein staining with β-actin served as the loading control. The protein bands were treated with enhanced chemiluminescent (ECL) substrate (Tanon, China) and imaged using a chemiluminescence imaging system.

## 2.5 Cell transfection

Lipofectamine 2000 Transfection Reagent (Invitrogen, USA) was used to transfect cells. Hsa-miR-27a-3p mimic (mimic-miR-27a-3p), has-miR-27a-3p inhibitor (anti-miR-27a-3p), negative controls mimic NC #22 (mimic-NC), and inhibitor NC #22 (anti-NC) were from RiboBio (RiboBio Biotechnology, China). In the present study, according to the manufacturer's recommended dosage and our preliminary experiment, a final concentration of 75 nM was used for all the chemically modified small molecular nucleic acids.

## 2.6 Nude mouse xenograft model

A total of twenty 5-week-old BALB/c male nude mice weighing 20g were divided into four groups, and each group contained 5 mice. HepG2 cells transfected with mimic-miR-27a-3p, mimic-NC, anti-miR-27a-3p, or anti-NC were subcutaneously inoculated into the forelimbs of nude mice. Each mouse was injected with $5\times10^6$ cells. The size and weight of tumors were measured at 7, 14, 21, and 28 d. After 28 days, mice were euthanized, and tumors were collected for analysis (evaluation of tumor volume and weight). Immunohistochemical staining was performed on paraffin sections of tumor samples with Envision+ kits (Dako, USA) according to the manufacturer's instructions. Monoclonal antibodies against CD206 (Boster, China), CD68 (Boster, China), Ki67 (Abcam, USA), and SPRY2 (Proteintech, China) were used. Images were acquired with a fluorescence microscope. The schematic diagram of the study design is shown in Supplementary Figure S2.

## 2.7 Purification of sEVs from the conditioned medium

The supernatant was collected and centrifuged at 3000 g at 4°C for 15 min, and then the precipitate (cells and cell debris) was removed. The supernatant was filtered through a 0.22 μm filter and centrifuged at 4000 g for 30 min at 4°C using an Ultra-15 centrifugal filter (Merck, Germany). The exosome-enriched sEV fractions were obtained from the concentrated mediums by ultra-centrifugation at 100,000 ×g for 90 min (Hitachi Ltd., Japan), then the pellet was washed with PBS, followed by a second step of ultracentrifugation at 100,000 ×g for 90 min (all steps are performed at 4°C). Afterward, the supernatant was discarded.

To increase the recovery rate, the concentrated mediums were mixed well with Tissue Culture Media SEVs Precipitation Solution (System Biosciences, Inc., USA) and incubated overnight in the dark at 4°C (19). Then, the supernatant was centrifuged at 1500 g at 4°C for 30 min, and the supernatant was discarded. The supernatant was aspirated, and the sEV precipitate was collected.

After adding 100 μl granule-free PBS to resuspended sEVs in the precipitate, nanosight NS300 (Malvern, UK) was used to measure the particle size and number of sEVs (20). The obtained sEVs were identified by transmission electron microscopy (TEM) (19). The schematic diagram of study design is shown in Supplementary Figure S3.

## 2.8 Quantitative reverse transcription polymerase chain reaction

Total cellular RNA and sEVs RNA were extracted by TRIzol Reagent and TRIzol LS Reagent (Ambion, USA), respectively. The spiking-in-cel-miR-39 was added to Trizol at the step of sEVs lysis; the final concentration of cel-miR-39 is 10 fmol. Reverse transcription of the mRNA was performed according to the PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa, Japan) reagent instructions. According to the reagent instructions of the miRNA RT–PCR Starter Kit (RiboBio, China), reverse transcription of miRNA was performed to synthesize cDNA. To quantitatively test mRNA and mature miRNAs, cDNA templates and primer sets were mixed with TB Green Premix Ex Taq ii (Takara, Japan) and corrected with ROX dye. The expression levels of mRNA and miRNAs in cells were normalized to those of GAPDH and U6 snRNA, respectively, and exosomal miRNA levels were normalized to cel-miR-39 levels.

## 2.9 Transwell migration assay

The migration ability was assessed through a transwell chamber assay (Millipore, USA). The transfected cells were serum-starved for 4 h, then seeded into the upper chamber with serum-free medium ($8\times10^4$ cells per well); the bottom of the chamber contained the mixed

culture solution with 5% FBS according to experimental groups; these plates were then cultured in a 37°C, 5% $CO_2$ incubator for 24 h. The supernatant was discarded and washed three times with 1× PBS. Then, the cells were fixed with 2% paraformaldehyde at room temperature for 10 min, stained with 0.1% crystal violet solution, and incubated at room temperature for 10 min. The cells were washed with ultrapure water 3 to 5 times after each step, and the cells in the upper chamber were removed with a medical-grade cotton swab. The number of migrated cells was counted using a microscope.

## 2.10 Luciferase assay

After 24 hours of seeding into 24-well plates for transfection, cells were transiently transfected with 2 μg psiCHECK-2/Sprouty2 (SPRY2) x3 reporter plasmid and 75 nM mimic-miR-27a-3p or mimic NC by Lipofectamine 2000 (Invitrogen, USA). A dual-luciferase reporter system (Promega, USA) was used to perform luciferase assays 42 h later. A Veritas Microplate Luminometer (Turner Biosystems, Sunnyvale, USA) was used to detect Renilla and firefly luciferase signals.

## 2.11 Transfection of SPRY2 siRNA and SPRY2 overexpression plasmid

The siRNA fragments targeting SPRY2 and negative control siRNA (Genepharma, China) were transfected into HepG2 or Huh7 cells using Lipofectamine 2000 (Invitrogen, USA) at a final concentration of 50 nM. Twenty-four hours later, these cells were subsequentially transfected with mimic-miR-27a-3p. The expression of SPRY2 was determined by western blotting. The cell function was evaluated using the CCK8 assay and transwell assay.

The SPRY2 overexpression plasmids (pLV2-SPRY2 (human)-3×FLAG-Puro, OE-SPRY2) and negative control (pLV2-MCS-3×FLAG-IRES-Puro, OE-NC) were obtained from MiaoLing Biology (MiaoLingBio, China). Plasmids were transfected into HepG2 or Huh7 cells using Lipofectamine 2000 (Invitrogen, USA) at a final concentration of 2 μg per well. Twenty-four hours later, these cells were subsequentially transfected with anti-miR-27a-3p. The expression of SPRY2 was determined by western blotting. The cell function was evaluated using the CCK8 assay and transwell assay.

## 2.12 Fluorescence *in situ* hybridization

The paraffin sections of nude mice xenograft tumors were subjected to FISH assay using an FAM-labeled miR-27a-3p probe (Genepharma, China) according to the manufacturer's instructions. Images were obtained by fluorescence microscope.

## 2.13 Statistical analysis

Quantitative data are expressed as means ± standard errors of the means (SEM). Student's t-test or one-way ANOVA (GraphPad Prism 5.0) was used to perform statistical analyses unless otherwise stated. All P values were two-sided, and statistical significance was accepted at $P < 0.05$. Unless otherwise stated, all experiments were performed three times.

# 3 Results

## 3.1 miR-27a-3p expression was significantly upregulated in cells and sEVs derived from activated HSCs vs. quiescent HSCs

To compare miRNA expression in activated and quiescent HSC-derived sEVs, we established a widely recognized *in vitro* culture activation model of primary rat HSCs (21). In accordance with the phenotype, HSCs cultured for 3 days were designated as quiescent HSCs, while those cultured for more than 10 days (11d HSCs and 14d HSCs used in the present study) were regarded as activated HSCs (8) (Supplementary Figure S1). In previous studies, we also found that the expression of miR-27a-3p in primary rat HSCs was upregulated during activation (15). Based on the hypothesis that HSCs could act on adjacent environments through sEV secretion, we hypothesized that miR-27a-3p would also be upregulated in sEVs secreted from activated HSCs.

To prove this hypothesis, we prepared RNA samples from 3-day quiescent HSCs (3d HSCs) and 14-day activated HSCs (14d HSCs) and sEVs from the corresponding culture medium. HSC-derived sEVs were identified by nanoparticle tracking analyses (NTA) to show the particle size distribution and the average diameter of the particles ranged from 90 nm to 120 nm (Figure 1A). The shape of sEVs was spherical or cup-shaped as identified by transmission electron microscopy (TEM) (Figure 1B). To further confirm that the isolated particles were indeed exosome-enriched sEVs, the expression of generally recommended exosome biomarkers was examined by western blotting (Figure 1C). The expression levels of miR-27a-3p in the cellular and supernatant sEVs of primary rat HSCs were compared by qRT-PCR. We found that miR-27a-3p was not only upregulated in activated HSCs but was also upregulated in the sEVs secreted from cells (Figure 1D).

To verify that the upregulation of miR-27a-3p in activated HSCs is a cross-species event, the human hepatic stellate cell line LX2 was selected to establish an *in vitro* activation model of HSCs. According to a previous study, α-smooth muscle actin (α-SMA), a marker for the activation of HSCs, was upregulated during the activation of HSCs (22). LX2 cells were cultured with 2% FBS DMEM culture solution supplemented with 10 ng/mL TGF-β1 cytokine, which is a robust driver of HSC activation for 24 h and 48 h (the same volume of buffer for TGF-β1 was added to the negative control group). The results showed that the expression of α-SMA was significantly higher in the activated LX2 group (TGFβ 1-treated activated group, aLX2) than in the quiescent LX2 group (negative control group, qLX2), which confirmed the success of the *in vitro* HSC activation model (Figures 1E, F). The sEVs derived from LX2 cells were isolated, purified, and then identified by TEM, western blotting, and NTA (Figures 1G–I), thereby confirming that the isolated particles were sEVs.

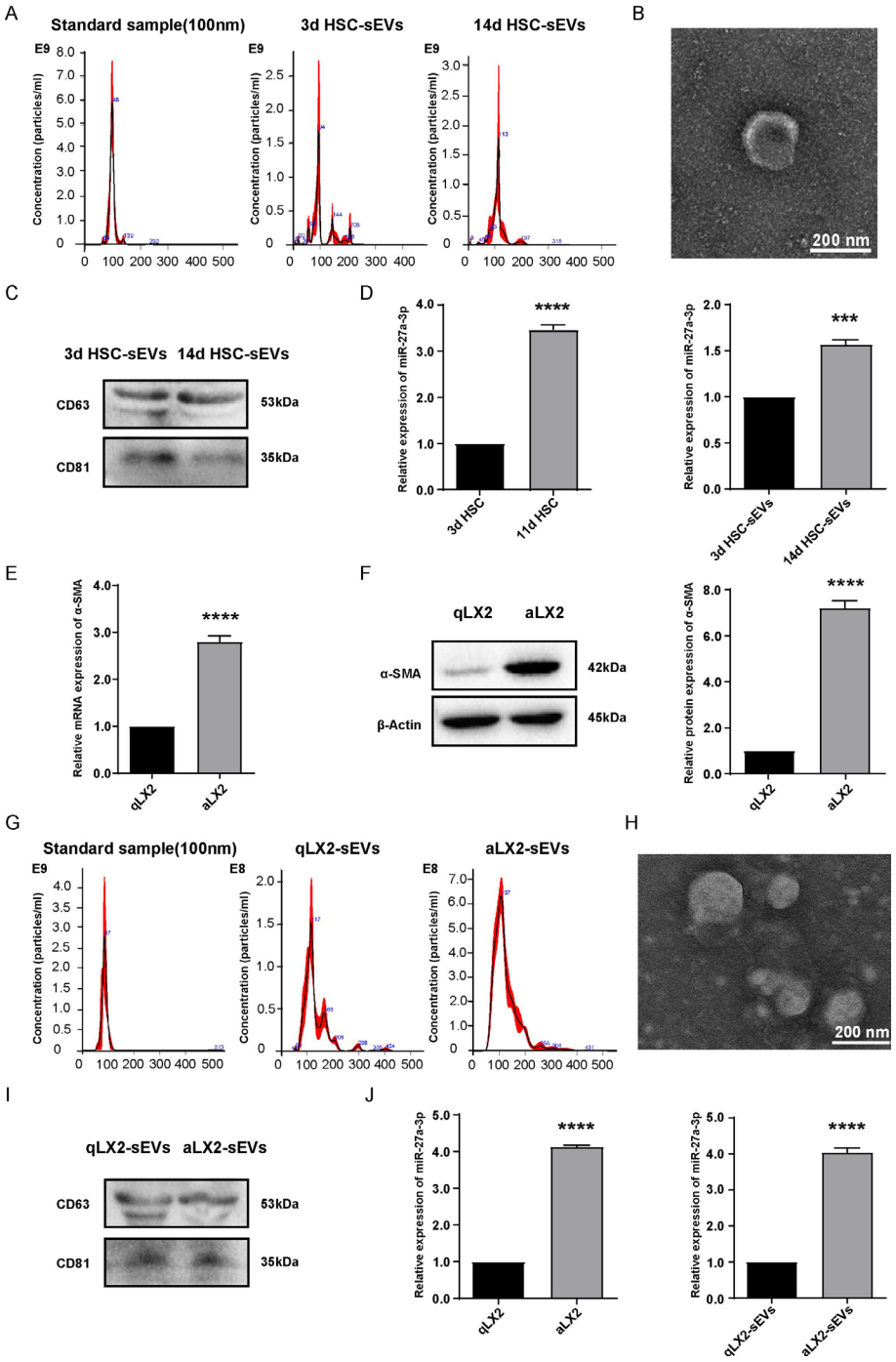


FIGURE 1
miR-27a-3p expression was increased in cells and sEVs of activated HSCs and aLX2 cells vs. their quiescent counterparts. **(A–C)** NTA, TEM, and western blotting analysis of sEVs isolated from 3 day quiescent HSCs and 14 day activated HSCs. NTA instrument (NanoSight NS300) was calibrated with 100 nm standard. The expression of the characteristic sEVs molecules CD63 and CD81 was detected by western blotting. **(D)** qRT-PCR was used to detect the expression of miR-27a-3p in cells and sEVs from quiescent (day 3) activated primary rats HSCs (day 11 and day 14). U6 snRNA served as internal reference for cell samples and cel-miR-39 served as external reference for sEVs samples (mean ± SEM). ***P < 0.001, ****P < 0.0001 vs. Control; **(E, F)** The expression of α-SMA in LX2 cells treated with 10 ng/ml TGFβ1 for 24 h for mRNA as detected by qRT-PCR normalized to GAPDH; or 48 h for protein as detected by western blotting normalized to β-actin (mean ± SEM). ****P < 0.0001 vs. Control; **(G–I)** NTA, TEM, and western blotting analysis of sEVs isolated from activated LX2 cells. NTA instrument was calibrated with 100 nm standard. The expression of the characteristic sEVs molecules CD63 and CD81 was detected by western blotting; **(J)** qRT-PCR was used to detect the expression of miR-27a-3p in qLX2 and aLX2 cells and supernatant sEVs in LX2-activation models. U6 snRNA served as internal reference for cell samples and cel-miR-39 served as external reference for sEVs samples (mean ± SEM). ****P < 0.0001 vs. control. 3d, 3 days; 11d, 11 days; 14d, 14 days; qLX2, quiescent LX2; aLX2, activated LX2. Data were from three independent tests.

The expression of miR-27a-3p was detected by qRT-PCR, and the data showed that miR-27a-3p was upregulated in both aLX2 cells and sEVs secreted from them (Figure 1J). We verified the results in primary HSCs from rats in the activation model of human HSCs.

## 3.2 Overexpression of miR-27a-3p correlated with increased proliferation and migration capability of hepatoma cells

To clarify the role of miR-27a-3p in hepatoma, the human liver cancer cell lines Huh7 (low miR-27a-3p expression) and HepG2 (high miR-27a-3p expression) were selected (Figure 2A). In our previous study, we found that the proliferation ability of HepG2 cells that highly expressed miR-27a-3p was higher than that of Huh7 cells (23). The migration ability of HepG2 cells was also significantly higher than that of Huh7 cells, as detected by transwell migration assay (Figure 2B). To study the effects of miR-27a-3p on hepatoma cell biological behaviors *in vitro*, we transfected Huh7 and HepG2 cells with mimic/anti-miR-27a-3p or controls (left of Figures 2C, D). As determined by transwell migration assays, migration of both Huh7 and HepG2 cells transfected with the mimic-miR-27a-3p was significantly enhanced (Middle of Figures 2C, D). Meanwhile, the migration of HepG2 cells was suppressed by the anti-miR-27a-3p, but the impact of anti-miR-27a-3p on the migration of Huh7 cells was insignificant. The proliferation of Huh7 and HepG2 cells was promoted by the mimic-miR-27a-3p but decreased by the anti-miR-27a-3p, as demonstrated by CCK-8 assays (right of Figures 2C, D).

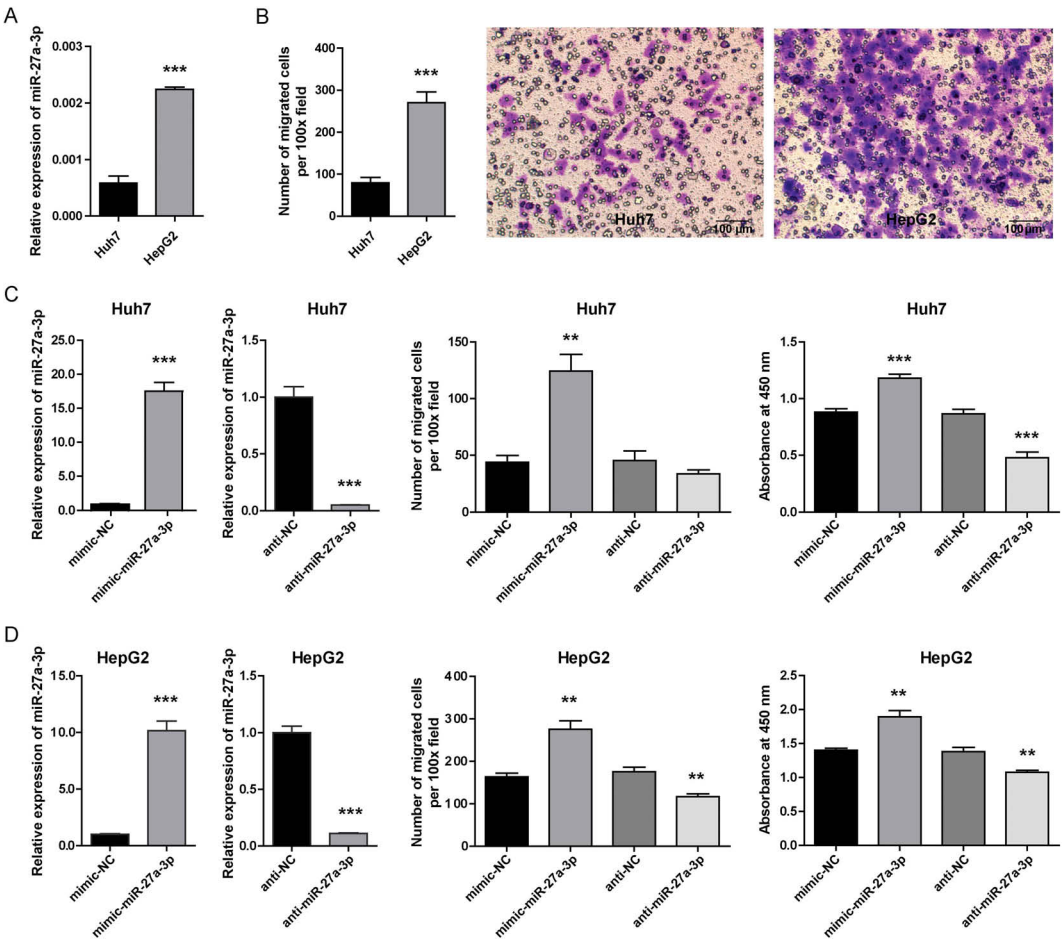


FIGURE 2
The expression of miR-27a-3p in hepatoma cells and the influence of miR-27a-3p on cellular behavior. **(A)** The expression of miR-27a-3p in the human hepatoma cell lines Huh7 and HepG2 was evaluated by qRT-PCR and normalized to that of U6 snRNA (mean ± SEM). ***P < 0.001 vs. control. **(B)** The migration ability of Huh7 and HepG2 cells. The number of migrated cells was counted manually after crystal violet staining (mean ± SEM), and representative images are provided, bar = 100 μm. ***P < 0.001 vs. control. **(C, D)** The expression of miR-27a-3p in hepatoma cells transfected with mimic-miR-27a-3p was significantly increased, and cells transfected with anti-miR-27a-3p were significantly down-regulated (left). The number of migrated cells was counted manually (middle). Cell proliferation capacity was evaluated by CCK-8 assay 36 h after mimic-miR-27a-3p and anti-miR-27a-3p transfection, and the absorbance at 450 nm is shown. (right) (mean ± SEM). **P < 0.01, ***P < 0.001 vs. control. The data were from three independent experiments.

## 3.3 miR-27a-3p promoted tumor growth in a nude mouse xenograft model

We established a nude mouse xenograft model. HepG2 cells transfected with mimic/anti-miR-27a-3p or controls were injected subcutaneously into male nude mice, which were divided into four groups (5 mice per group). After 2 weeks, 3 mice in the mimic-NC group and 3 mice in the anti-NC group formed xenograft tumors. Four xenograft tumors were formed in the mimic-miR-27a-3p group. No xenograft tumors were found in the 5 mice in the anti-miR-27a-3p group (Figures 3A, D). The tumor size was measured at 7 d, 14 d, 21 d, and 28 d. On the 28th day after injection, the tumor volume in the mimic-miR-27a-3p group was significantly larger than that in the mimic-NC group ($P = 0.004$) (Figure 3B). Correspondingly, the tumor weight of the mimic-miR-27a-3p group was approximately 195% of that of the control group. The difference in tumor volume and weight between the mimic-NC and anti-NC groups ($P = 0.345$) was not significant (Figure 3C).

To further verify the effect of miR-27a-3p on the proliferation of tumor cells, the expression of Ki67 was detected by immunohistochemistry. The Ki67-positive cells in all the 200-fold magnification fields of xenograft tumors from each group were counted. Due to the therapeutic effect of anti-miR-27a-3p, there was no tumor in the nude mice xenograft model after 28 days of anti-miR-27a-3p treatment. It turned out that the number of Ki67-positive cells was significantly increased in the mimic-miR-27a-3p group compared to the mimic-NC or anti-NC group (Figure 3E). These results suggest that miR-27a-3p promotes tumor proliferation to support a high degree of malignancy.

## 3.4 Overexpression of miR-27a-3p induced M2 polarization of macrophages

In the present study, CD68 was employed as the marker of tumor-infiltrating macrophages, while CD206 was utilized as the marker of M2-type macrophages. The result of immunohistochemistry indicated an elevated number of CD68 and CD206-positive cells in the mimic-miR-27a-3p group using the same batch of xenografts, suggesting that miR-27a-3p promotes the infiltration of liver macrophages and participates in the development of the TME. In addition, among the three groups, the mimic-miR-27a-3p group showed elevated CD206 expression, suggesting that M2-type macrophages in the tumor were dominant compared with M1-type macrophages (Figure 4A).

We further investigated whether overexpression of miR-27a-3p could polarize macrophages into the M2 type by using THP-1 cell lines. The phenotype of THP1-M0 cells transfected with mimic/anti-miR-27a-3p or controls was detected by western blotting. The transfection of mimic-miR-27a-3p effectively upregulated miR-27a-3p, and anti-miR-27a-3p effectively downregulated miR-27a-3p in THP1 cells (Figure 4B). Results showed that M2 marker (CD206) in cells transfected with mimic-miR-27a-3p were apparently upregulated (Figures 4C, D).

## 3.5 HSC sEVs highly expressed miR-27a-3p and promoted the migration of hepatoma cells

To further verify the role of miR-27a-3p carried by sEVs from activated HSCs in promoting the migration ability of hepatoma cells, we performed conditioned culture of HepG2 cells with miR-27a-3p over-expressing LX2-sEVs. Additionally, we also test the influence of miR-27a-3p over-expressing LX2-sEVs cultured macrophages on HepG2 cells (Figure 5A). LX2 cells were transfected according to groups (completely blank control group, mimic-NC group, mimic-miR-27a-3p group, anti-NC group, anti-miR-27a-3p group). After 24 h, we collected the supernatant from each group, and the sEVs were separated and purified. The particle size and particle number of sEVs were determined by NTA (Figure 5B), and the expression of miR-27a-3p in sEVs was assessed by qRT-PCR (Figure 5C).

Then, we added the sEVs highly expressed miR-27a-3p into the culture of HepG2 cells at a ratio of sEV particle number: cell number =10000: 1. Transwell assays showed that the sEVs of LX2 cells over-expressing miR-27a-3p facilitated the migration of HepG2 cells. To explore the influence of miR-27a-3p over-expressing LX2-sEVs treated macrophageson HepG2 cells, the THP1 cells were pretreated with 5 ng/ml PMA for 24 h to induce M0 macrophages. We added the miR-27a-3p over-expressing sEVs to the culture of THP1-M0 cells for 24 h, after which we collected the supernatant and added it to HepG2 cells culture. Transwell assays showed that the supernatant of THP1-M0 cells treated by miR-27a-3p over-expressing sEVs from LX2 cells further enhanced the migration of HepG2 cells (Figures 5D, E). In addition, the expression levels of surface markers in THP1 macrophages were detected using qRT-PCR, with CD86 and CD206 serving as specific markers for M1 and M2 macrophage subtypes, respectively (Figure 5F). The results showed that the expression of M1 phenotype markers (CD86) decreased after the treatment of sEVs derived from mimic-miR-27a-3p-treated LX2 while the expression of M2 phenotype markers (CD206) increased. Conversely, the opposite effect was observed after the treatment of sEVs derived from anti-miR-27a-3p-treated LX2. This indicates that sEVs released by LX2 cells overexpressing miR-27a-3p promote polarization of macrophages towards an M2 phenotype.

Based on these data, we concluded that aLX2 cell-derived sEVs containing miR-27a-3p establish a tumor-promoting microenvironment.

## 3.6 SPRY2 was a direct target of miR-27a-3p

To further elucidate the mechanisms by which miR-27a-3p is involved in the progression of hepatoma, we used an online miRNA target prediction tool TargetScan (https://www.targetscan.org), to screen the potential targets of miR-27a-3p. TargetScan predicts biological targets of miRNAs by searching for conserved sites that match the seed region of each miRNA. The miR-27a-3p belongs to a broadly conserved microRNA family. According to the website

instructions, the following criteria were adopted for its target screen: i) Species, human; ii) Context++ score percentile ≥ 99; iii) probability of conserved targeting (PCT) ≥ 0.80. Among the predicted target genes, by the gene function annotation and literature reviewing, we further identified SPRY2, which is highly conserved among species for further verification (Figure 6A).

To confirm that SPRY2 is the target of miR-27a-3p, we separately cloned the full-length wild-type and mutant SPRY2 3'-UTRs into the psiCHECK-2 vector, after which the vectors with the mimic-miR-27a-3p or the control were transfected into HEK293T cells. We found that the luciferase activity of the psiCHECK-2 vector with the wild-type SPRY2 3' UTR was inhibited by miR-27a-

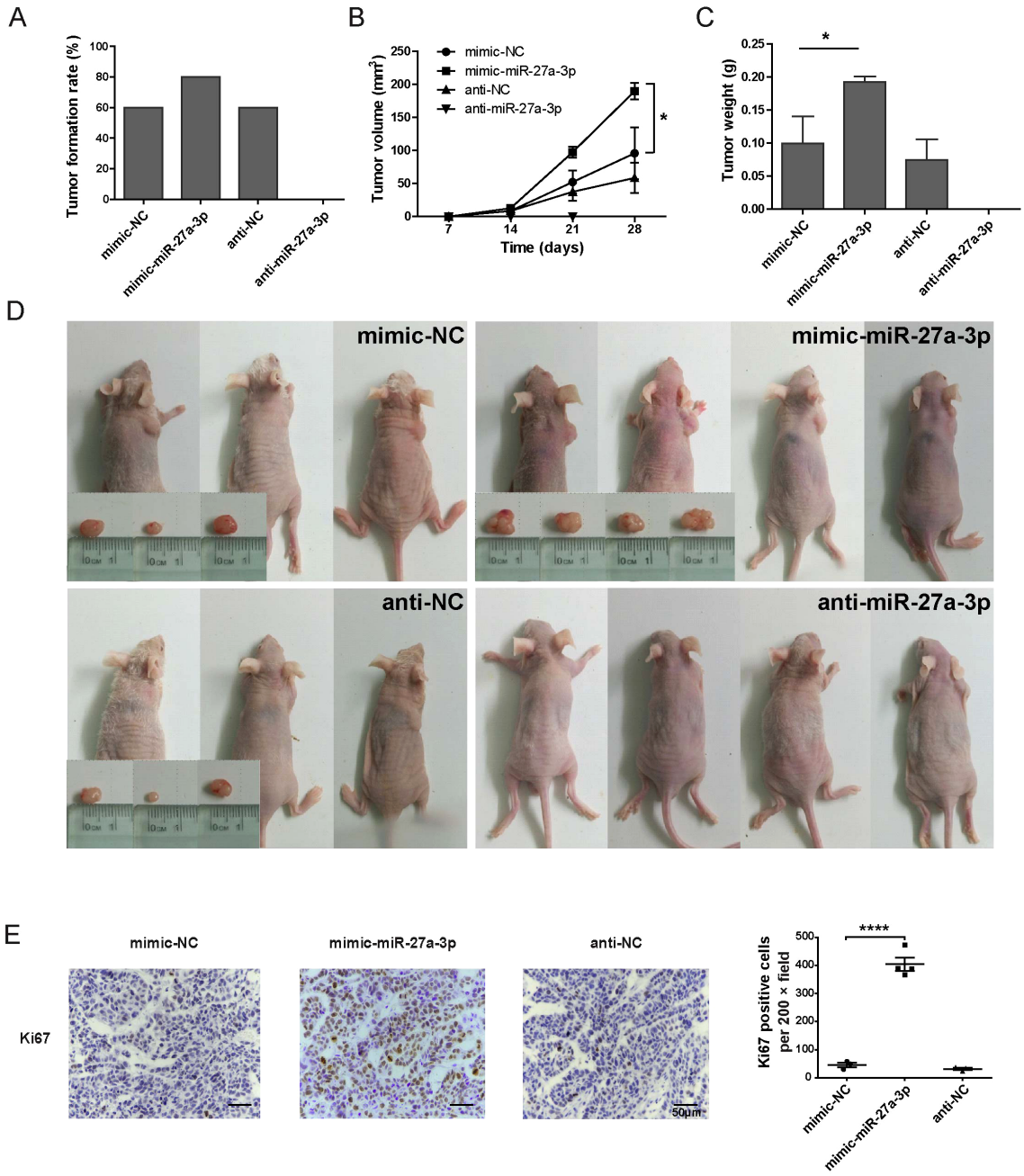


FIGURE 3
miR-27a-3p promotes hepatoma formation and growth *in vivo*. **(A)** Tumor formation rate by 2 weeks for each group of the nude mice xenograft model (n = 5). **(B)** The tumor volume by day 7, day 14, day 21, and day 28 in each group of nude mice xenograft model (mean ± SEM). Statistical analysis was conducted using Two-way Repeated-Measures ANOVA, *P < 0.05 vs. control. **(C)** The tumor weight of the nude mice xenograft model group at the time of sacrifice (mean ± SEM), *P < 0.05 vs. control. **(D)** Nude mice and xenograft tumors in each group, gross view, bar = 1 cm. **(E)** Representative images for the immunohistochemical staining of Ki67 for xenograft tumors, bar = 50 μm; and the number of Ki67 positive cells per 200 × field for each group (mean ± SEM). ****P < 0.0001 vs. control. The nude mice xenograft experiment was repeated once; the data presented were from one representative batch.

3p, but the psiCHECK-2 vector with the mutant SPRY2 3'-UTR was not affected. The results fully illustrated that SPRY2 can be directly affected by miR-27a-3p (Figure 6B).

Subsequently, the expression of SPRY2 in HepG2 cells and THP1 cells transfected with mimic/anti-miR-27a-3p was detected by western blotting at the protein level. The results revealed that SPRY2 expression in cells transfected with mimic-miR-27a-3p was dramatically reduced in comparison to that in cells transfected with mimic-NC but was increased in cells transfected with anti-miR-27a-3p (Figures 6C, D).

The expression of miR-27a-3p in hepatoma xenografts was detected by *in situ* hybridization technology. The results demonstrated that pretreatment with mimic-miR-27a-3p led to an upregulation of miR-27a-3p expression within hepatoma xenografts (Supplementary Figure S4). Subsequently, immunohistochemical staining was used to evaluate the expression level of SPRY2 in hepatoma xenografts. The results revealed that pretreatment with miR-27a-3p resulted in a downregulation of SPRY2 expression, suggesting a potential direct targeting relationship between miR-27a-3p and SPRY2 (Figure 6E).

HepG2 and Huh7 cells overexpressing SPRY2 were transfected with mimic-miR-27a-3p. The western blotting results demonstrated that the upregulation of SPRY2 in HepG2 and Huh7 cells, which were overexpressing SPRY2, was restrained by the transfection with mimic-miR-27a-3p (Figure 6F; Supplementary Figure S5A). In addition, the expression of SPRY2 in SPRY2-knockdown HepG2 and Huh7 was increased by anti-miR-27a-3p (Supplementary Figure S5B). The results of the transwell migration assay and CCK8 assay showed that the overexpression of SPRY2 in HepG2 and Huh7 partially alleviated the promoting effects of mimic-miR-

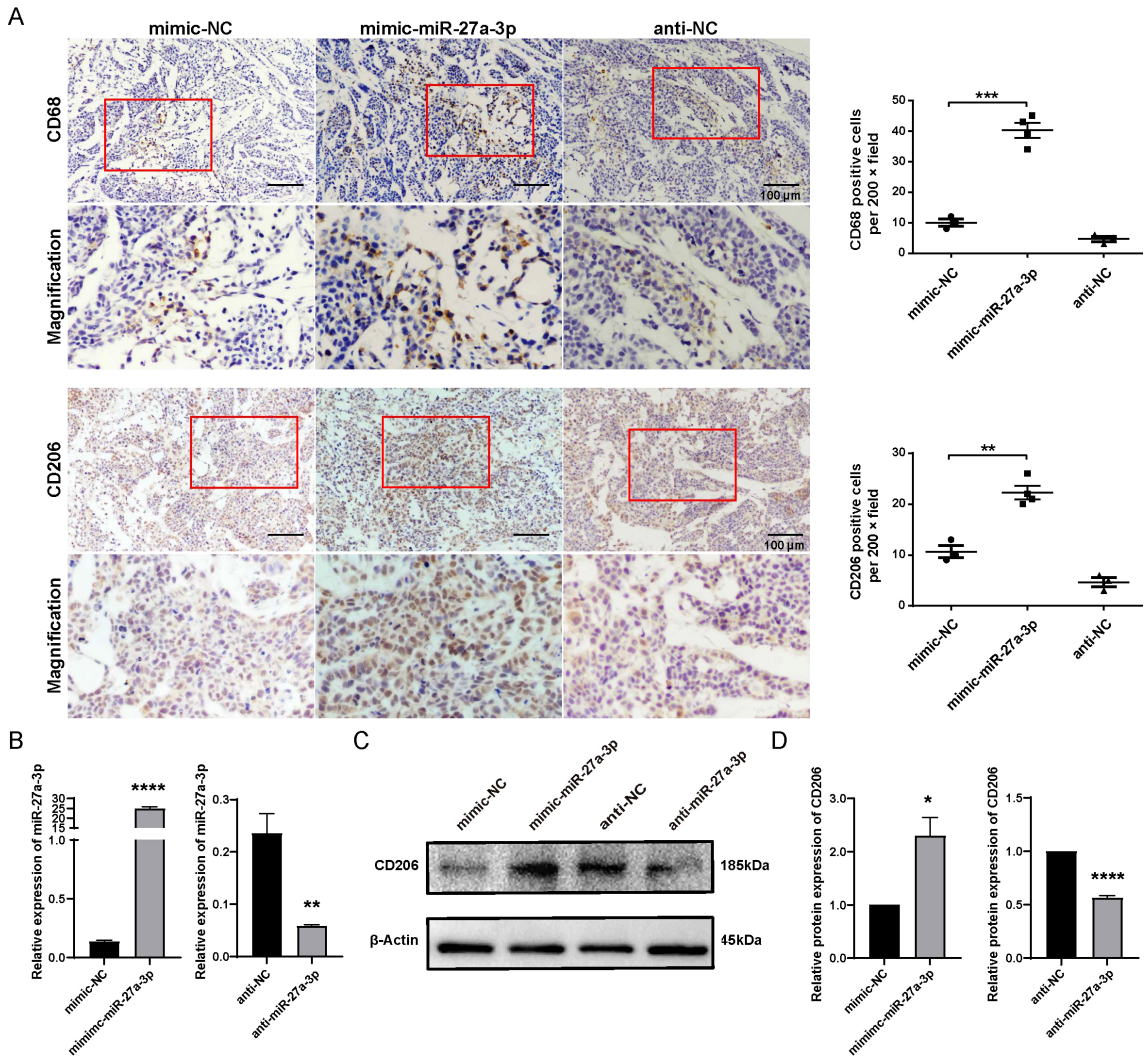


FIGURE 4
miR-27a-3p promotes the M2 polarization of macrophage. **(A)** Representative images for the immunohistochemical staining of macrophage maker CD68 and CD206 in nude mice xenografts, bar = 100 μm; and the number of CD68, CD206 positive cells per 200 × field for each group (mean ± SEM). **P < 0.01, ***P < 0.001 vs control. Below each group of images is an image showing the magnification of the corresponding box area above. **(B)** The expression of miR-27a-3p in mimic-miR-27a-3p or anti-miR-27a-3p treated THP1 cells was evaluated by qRT-PCR 24 h after transfection, normalized to U6 snRNA (mean ± SEM). **P < 0.01, ****P < 0.0001 vs. control. **(C, D)** The expression of CD206 was evaluated in mimic-miR-27a-3p or anti-miR-27a-3p treated THP1 cells by western blotting normalized to β-actin (mean ± SEM). *P < 0.05, ****P < 0.0001 vs. control. The data were from three independent experiments.

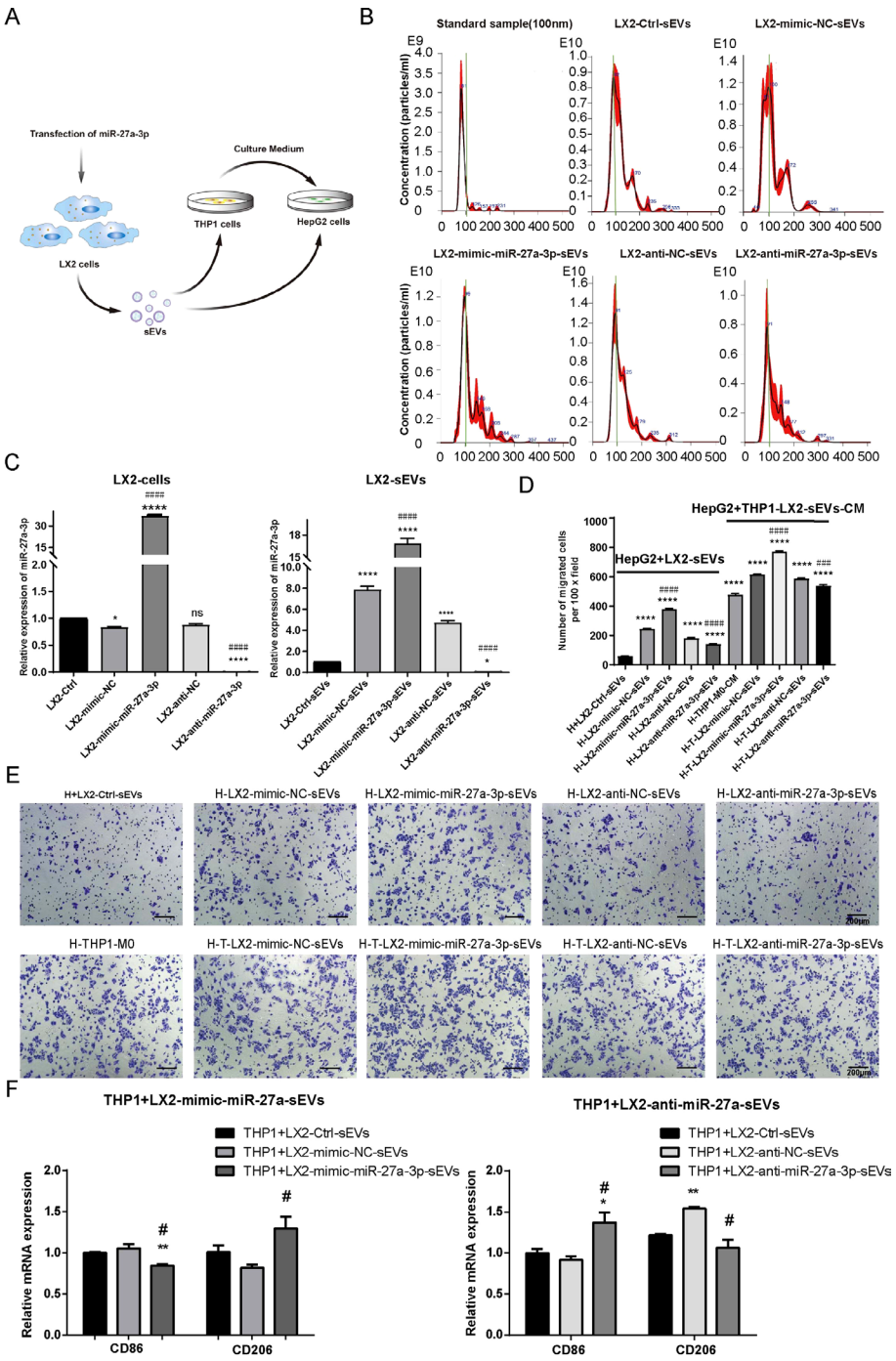


FIGURE 5
The miR-27a-3p over-expressing HSC-sEVs promote HepG2 cell migration directly or through educating macrophages. **(A)** Schematic of the conditioned culture experiments. **(B)** Representative images for the size distribution and concentration of sEV particles in the supernatant of transfected LX2 cells as detected by NTA. **(C)** The expression of miR-27a-3p in mimic-miR-27a-3p or an-ti-miR-27a-3p transfected LX2 cells and sEVs was detected by qRT-PCR, standardized to U6 snRNA for cell samples or cel-miR-39 for sEVs samples, respectively. *P < 0.05, ****P < 0.0001 vs. control. ####P < 0.0001 vs. NC. **(D, E)** Transwell migration assays for conditioned cultured. Representative images for the crystal violet staining of migrated cells, bar = 200 μm; and the number of migrated cells per 100 × field for each group (mean ± SEM). ****P < 0.0001 vs. control. ###P < 0.001, ####P < 0.0001 vs. NC. **(F)** The expression levels of surface markers in THP1 macrophages was detected using qRT-PCR. *P < 0.05, **P < 0.01 vs. control. #P < 0.05 vs. NC. H, HepG2 cells; qLX2, quiescent LX2 cells; aLX2, active LX2 cells; CM, culture medium. The data were from three independent experiments.

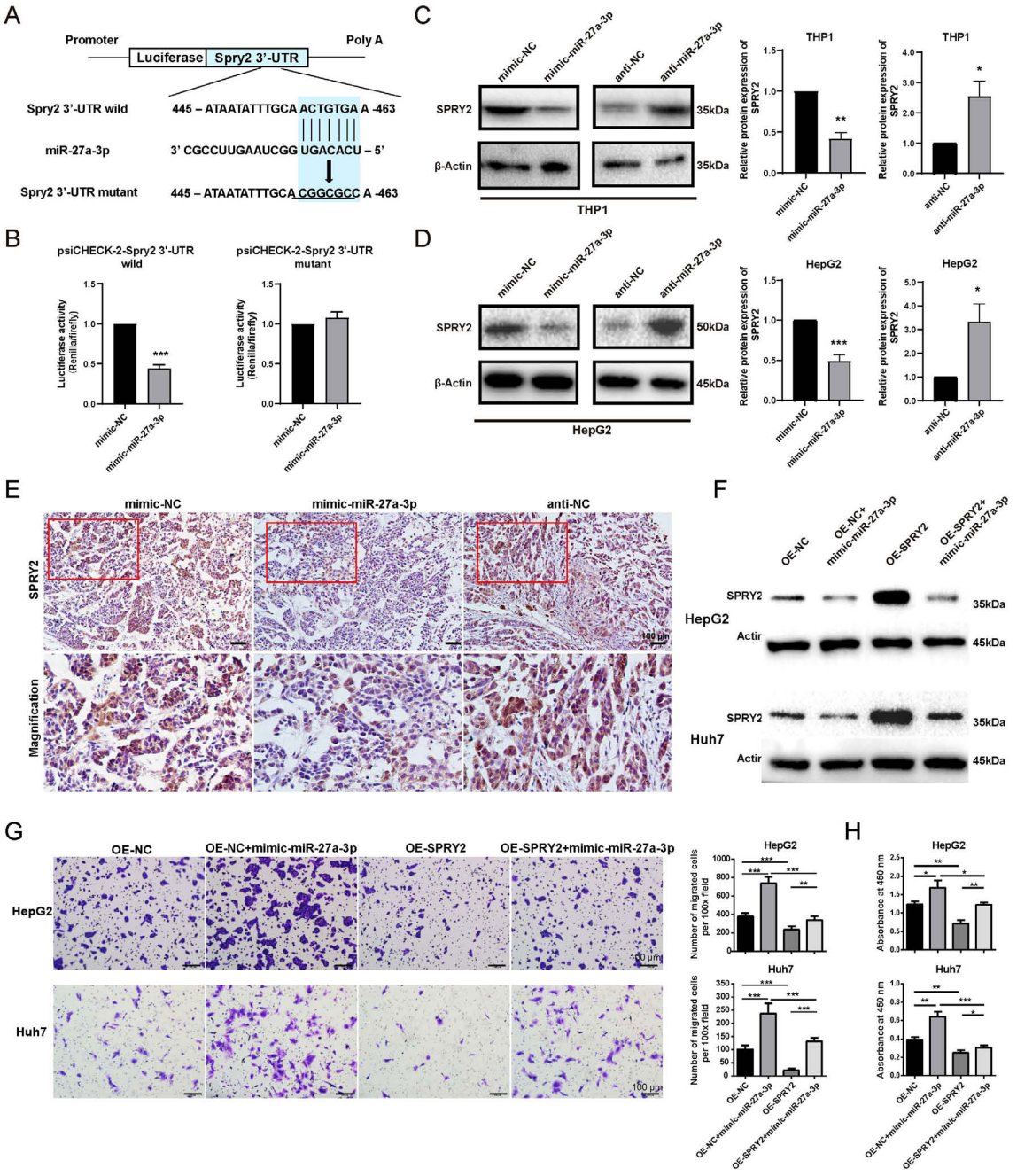


FIGURE 6
SPRY2 was a direct target of miR-27-a-3p in hepatoma. **(A)** Construct a map of psiCHECK-2/Spry2x3 used for the luciferase assay. The 3′-UTR of SPRY2 was cloned into the vector after the Renilla luciferase gene (hRluc). The firefly luciferase gene (hluc+) was expressed in the vector as an internal control. Seven nucleotides of the seed sequence were mutated, as shown in the figure, to construct the mutant vector. **(B)** miR-27a-3p directly acted on the 3′-UTR of SPRY2. HEK293T cells were cotransfected with mimic-miR-27a-3p together with a psiCHECK-2-Spry2 3′-UTR wild-type vector or psiCHECK-2-Spry2 3′-UTR mutant vector, and mimic-NC and vectors were transfected as controls (mean ± SEM). ***P < 0.001 vs. control. **(C, D)** The expression of SPRY2 in HepG2 and THP1 cells after transfection with mimic/anti-miR-27a-3p or NC was evaluated by western blotting. *P < 0.05, **P < 0.01, ***P < 0.001 vs. control. **(E)** Representative images for the immunohistochemical staining of SPRY2 in nude mice xenografts, bar = 100 μm. Below each group of images is an image showing the magnification of the corresponding box area above. **(F)** The expression of SPRY2 was evaluated by western blotting normalized to β-actin. HepG2 and Huh7 cells overexpressing SPRY2 were transfected with mimic-miR-27a-3p. **(G)** Transwell migration assay. HepG2 and Huh7 cells overexpressing SPRY2 were transfected with mimic-miR-27a-3p. **(H)** CCK8 proliferation assay. HepG2 and Huh7 cells overexpressing SPRY2 were transfected with mimic-miR-27a-3p. The absorbance at 450 nm is shown. (mean ± SEM). *P < 0.05, **P < 0.01, ***P < 0.001. The data were from three independent experiments.

27a-3p on cell migration and proliferation (Figures 6G, H). Similarly, transwell assay and CCK8 assay results indicated that the knockdown of SPRY2 in liver cancer cells relieved the inhibitory effects of anti-miR-27a-3p on cell proliferation and migration (Supplementary Figures S5C, D).

## 4 Discussion

The role of HSCs/CAF in the progression of liver cancer has received increasing attention in recent years. However, current research is primarily focused on the direct effect of HSCs on tumor cells. In quiescent HSCs, mitogen-activated protein kinase (MARK) is strongly phosphorylated, thus activating NF-kB and extracellular-regulated kinase (ERK) cascade signaling pathways (24), leading to an increased release of IL8, which promotes the invasion and metastasis of hepatoma (25, 26). Another study reported that HSCs can mediate hepatoma progression by releasing factors that promote epithelial-mesenchymal transformation and angiogenesis, such as VEGF, MMP2, MMP9, bFGF, and TGF-β (27, 28). In our previous study, we found that the activated HSCs might induce an immunosuppressive phenotype of macrophages, thus promoting the progression of hepatoma and leading to a poor prognosis (10). In the present study, focusing on the role of sEV in intercellular signaling, we further investigated the underlying mechanism.

The transportation of miRNAs mediated by sEVs is an important mechanism of genetic exchange among cells (12). The type and abundance of miRNAs loaded in sEVs changed synchronously with the functional state of the parent cells in most cases. We have reported that miR-27a-3p is upregulated in activated primary rat HSCs, which promotes the activation of primary rat HSCs by reducing fat accumulation and promoting cell proliferation (15). Several subsequent studies showed that the upregulation of miR-27a-3p can promote tumor proliferation and migration and accelerate the progression of different types of cancer, including breast carcinoma, renal cell carcinoma, cervical carcinoma, and hepatoma (17, 18, 23, 29).

According to the previous findings of our group and the relevant reports, the present study aimed to investigate the expression of miR-27a-3p in activated HSCs-derived sEVs, including culture-activated primary rat HSCs, and TGF-β1 activated human HSCs cell line-LX2. We also investigated the direct effects of miR-27a-3p over-expressing HSC-sEVs on hepatoma cell proliferation and migration and indirect effects on tumor cells through educating macrophages. We found that miR-27a-3p is upregulated in activated rat HSC-derived sEVs and TGF-β1 activated human LX2-derived sEVs. These findings confirmed that the upregulation of miR-27a-3p in activated HSCs and corresponding sEVs was a cross-species phenomenon.

We further proved that upregulation of miR-27a-3p promoted proliferation and migration *in vitro*, thus contributing to the malignancy of liver cancer cells. The tumor-promoting effect of miR-27a-3p was confirmed in the nude mouse xenograft model. Over-expressing of miR-27a-3p promoted tumor cell proliferation and increased tumor formation. The most exciting finding was that knocking down miR-27 completely suppressed tumor formation. In the xenograft tumor, we also observed an increased infiltration of CD68 macrophages and CD206 immune suppressive macrophages in the miR-27a-3p over-expressing group. These findings indicated that miR-27a-3p might affect the phenotype of TAMs and inspired us to explore the effects of miR-27a-3p on the phenotype of macrophages. It turned out that the over-expressing of miR-27a-3p induced M2 polarization, while the down-regulation of miR-27a-3p reduced the expression of the M2 marker in macrophages. These observations suggest that miR-27a-3p may promote tumor progression through direct action on tumor cells and indirect regulation of tumor-related macrophages.

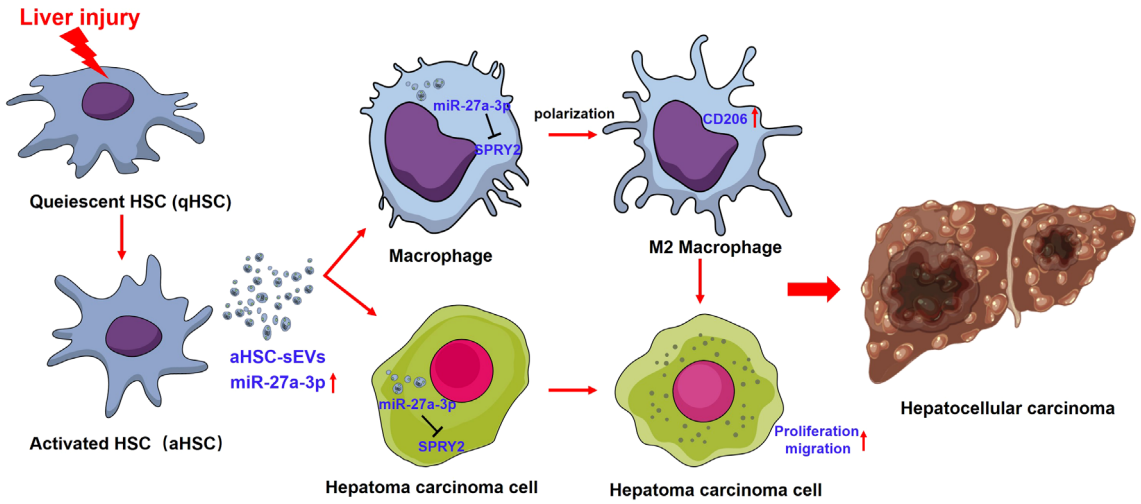


FIGURE 7
The interaction between miR-27a-3p/SPRY2 and hepatoma progression. miR-27a-3p was significantly upregulated in HSC-sEVs during the activation of HSCs. HSC-sEVs containing miR-27a-3p could promote M2 polarization of macrophage and proliferation of hepatoma cells by targeting SPRY2, leading to the progression of hepatocellular carcinoma.

The increased intracellular miR-27a-3p from activated HSCs can be passed to neighboring cells via sEVs. To test whether HSC-sEV loaded miR-27a-3p can directly promote tumor progression or indirectly by educating macrophages, we adopted the concept of engineered sEVs to obtain LX2-derived sEVs that were over-expressing or under-expressing miR-27a-3p from mimic-miR-27a-3p or anti-miR-27a-3p transfected LX2 cells. Conditioned culture of HepG2 cells with miR-27a-3p over-expressing LX2-sEVs profoundly promoted the malignant behavior of tumor cells, and miR-27a-3p over-expressing LX2-sEVs pretreated macrophage showed an even more substantial effect.

Engineered sEV is a sort of sEV modified with surface decoration and/or internal therapeutic molecules. After appropriate modification, engineered sEVs can efficiently and precisely deliver drugs to target sites with fewer adverse effects of treatment (30). In the present study, the expression of miR-27a-3p in LX2-sEVs was regulated by transfection of chemically modified small nucleic acid molecules. These LX2-sEVs showed powerful effects on tumor cells and macrophages. Studies on the effects of miRNA on the malignant biological properties of tumor cells or macrophage polarization are accumulating. For example, the expression of miR-148a-3p was found to be suppressed in activated HSC-derived sEVs and to contribute to the development of hepatoma by activating ITGA5/PI3K/Akt pathway (31). Some researchers also reported the effect of miRNAs on promoting macrophage M2 polarization, including miR-519a-3p in gastric cancer (32), miR-1246 in ovarian cancer (33), miR-3591-3p in glioma (34), and miR-452-5p in hepatocellular carcinoma (35). As for therapeutic translational application, these miRNAs could be delivered as components of engineered sEVs, providing a new strategy for targeting combined immunotherapy of tumors.

In the present study, SPRY2 was identified as a target gene for miR-27a-3p. SPRY2 is an important molecule for signal regulation *in vivo*, and the deletion of SPRY2 can lead to the activation of the PI3K/Akt/mTOR and MAPK/ERK signaling pathways, promote cell proliferation and migration, and lead to the development of hepatoma *in vivo* (36–38). Our experiments demonstrated that the increase in miR-27a-3p in both hepatoma cells and macrophages resulted in a decrease in expression of SPRY2 at the protein level. According to previous reports, the reduction in SPRY2 levels in hepatoma cells promotes the proliferation and migration of hepatoma cells, and the decrease in SPRY2 levels in macrophages leads to the polarization of macrophages toward the M2 phenotype (39). Based on the above findings, sEVs containing miR-27a-3p could promote M2 polarization of macrophage and proliferation of hepatoma cells by targeting SPRY2, leading to the progression of hepatocellular carcinoma (Figure 7).

## 5 Conclusions

In conclusion, miR-27a-3p over-expressing sEVs released by activated HSCs might shape an immunosuppressive tumor-promoting microenvironment by suppressing SPRY2, thereby promoting macrophage M2 polarization and the proliferation and migration of hepatoma cells, ultimately resulting in accelerated hepatoma progression. This is the first report of the functional role of activated HSC-derived sEVs with high miR-27a-3p expression in regulating macrophage function and the tumor immune microenvironment, which indicates dynamic interactions among cells during hepatoma progression. It is of particular interest that both macrophages and hepatoma cells could be targeted by activated HSC-derived sEVs and synergistically involved in the malignant progression of tumors. This study also highlights the possibility that engineered HSC-derived sEVs may be applied to treat hepatoma. However, in our subsequent study, we need to further verify the role of SPRY2, the target gene of miR-27a-3p, in the malignant biological behavior of tumor cells and the differentiation of macrophages which is now supported by the literature (36–39).

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

## Ethics statement

The animal study was approved by Nantong University Medical School Ethics Committee. The study was conducted in accordance with the local legislation and institutional requirements.

## Author contributions

YS: Conceptualization, Data curation, Investigation, Project administration, Visualization, Writing – original draft. XH: Methodology, Software, Validation, Investigation, Data curation, Writing – original draft, Visualization. JH: Investigation, Methodology, Software, Visualization, Writing – original draft. WY: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. HW: Data curation, Software, Writing – original draft. JLi: Investigation, Writing – original draft. WL: Formal analysis, Methodology, Writing – original draft. XK: Methodology, Software, Writing – original draft. JLv: Investigation, Software, Writing – original draft. JJ: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1489679/full#supplementary-material.

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EDITED BY
Sheila Spada,
Hospital Physiotherapy Institutes (IRCCS), Italy

REVIEWED BY
Dwijendra K. Gupta,
Allahabad University, India
Mohammed Abu El-Magd,
Kafrelsheikh University, Egypt
Mario Leonardo Squadrito,
Vita-Salute San Raffaele University, Italy

*CORRESPONDENCE
Ju Huang
huangju0777@163.com
Limei Zhang
523413618@qq.com

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# Crosstalk between exosomes and tumor-associated macrophages in hepatocellular carcinoma: implication for cancer progression and therapy

Ying Xu[1], Linyue Xu[2], Qiuyan Chen[2], Can Zou[2], Ju Huang[3]* and Limei Zhang[3]*

[1]Department of Anesthesiology Operating Room, Hospital of Chengdu University of Traditional Chinese Medicine, Chengdu, Sichuan, China, [2]School of Sports Medicine and Health, Chengdu Sport University, Chengdu, China, [3]Department of Respiratory Medicine, Hospital of Chengdu University of Traditional Chinese Medicine, Chengdu, Sichuan, China

Hepatocellular carcinoma (HCC), the most prevalent type of primary liver cancer, represents a significant cause of cancer-related mortality. While our understanding of its pathogenesis is comparatively comprehensive, the influence of the tumor microenvironment (TME) on its progression warrants additional investigation. Tumor-associated macrophages (TAMs) have significant impacts on cancer cell proliferation, migration, invasion, and immune response, facilitating a complex interaction within the TME. Exosomes, which measure between 30 and 150 nanometers in size, are categorized into small extracellular vesicles, secreted by a wide range of eukaryotic cells. They can transfer biological molecules including proteins, non-coding RNAs, and lipids, which mediates the intercellular communication within the TME. Emerging evidence has revealed that exosomes regulate macrophage polarization, thus impacting cancer progression and immune responses within the TME of HCC. Moreover, TAM-derived exosomes also play crucial roles in malignant transformation, which hold immense potential for cancer therapy. In this review, we elaborate on the crosstalk between exosomes and TAMs within TME during HCC development. Moreover, we delve into the feasible treatment approaches for exosomes in cancer therapy and emphasize the limitations and challenges for the translation of exosomes derived from TAMs into clinical courses for cancer therapy, which may provide new perspectives on further ameliorations of therapeutic regimes based on exosomes to advance their clinical applications.

KEYWORDS

**exosome, tumor-associated macrophage, tumor microenvironment, hepatocellular carcinoma, immunotherapy**

# 1 Introduction

According to the 2020 GLOBOCAN database, liver cancer ranks as the seventh most prevalent cancer with 905,677 new cases globally, accounting for approximately 4.7% of all cancer types (1). This presents a significant burden on global healthcare systems. Liver cancer primarily includes hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma, and other rare forms, with HCC accounting for approximately 85% of all liver cancer cases (2). Treatment regimes for advanced HCC include systemic chemotherapy, targeted therapy, arterial chemoembolization, and emerging strategies like immunotherapy (3, 4). However, drug resistance and limited therapeutic responses are significant challenges for improving patients' survival (5). Therefore, there is an urgent need for new scientific and technological approaches for integrated diagnosis and treatment of HCC in clinical practice.

The tumor microenvironment (TME) is a fundamental component of the tumor ecosystem, serving as the site where tumor cells interact with both other tumor cells and host cells (6). By orchestrating changes in the TME—including stromal cells, immune cells, and immune regulatory molecules—a pro-tumor TME is created, leading to tumor growth and hinder the effectiveness of anti-cancer therapies (7). Tumor-associated macrophages (TAMs) are among the most prevalent immune cell types within the TME of HCC, which secrete a diverse range of biological factors, including inflammatory molecules, chemokines, and exosomes, thereby impacting tumor cell proliferation, migration, invasion and immune response (8).

Exosomes, which measure between 30 and 150 nanometers in size, are categorized into small extracellular vesicles, secreted by a wide range of eukaryotic cells (9). While exosomes were first identified in the late 1980s, they were initially regarded merely as waste disposal mechanisms (10). However, advances in biotechnology have led to the recognition that exosomes are widely distributed in human body fluids and play an essential role in intercellular communication (11). Exosomes, arising from the endosomal pathway through the creation of late endosomes or multivesicular bodies, encapsulate a diverse array of molecules specific to their parent cells (12). These molecules can be transported over considerable distances while being shielded within a lipid bilayer-enclosed structure (13). Recently, research on the role of exosomes in cancer progression has received tremendous attention due to their ubiquitous presence and easy accessibility, they offer considerable potential for the advancement of precision medicine (14). Exosomes derived from TAMs account for a significant proportion of the blood, offering new clinical biomarkers for minimally invasive liquid biopsies in HCC patients (15). TAMs-derived exosomes transport non-coding RNAs (ncRNAs), proteins, and lipids that modulate malignant cell proliferation, metastasis, metabolic reprogramming, and immune response in the setting of HCC models (16, 17). Therefore, TAMs-derived exosomes hold immense potential for the systematic therapy of HCC. In this review, we emphasize the crosstalk between exosomes and TAMs, concentrating on the role of exosomes in macrophage polarization and their molecular functions on the cell proliferation, metastasis, and immune responses in the TME of HCC. Furthermore, novel therapeutic strategies based on exosomes and challenges faced in the clinical applications are also proposed, aiming to provide novel biomarkers and therapeutic targets in the field of HCC.

# 2 The role of macrophages in HCC

Macrophages, essential elements of the innate immune system, are widely present in the bloodstream and across multiple tissues in the body (18). Macrophages display remarkable plasticity, allowing them to adjust to a wide range of tissue microenvironments and carry out multiple functions including presenting antigens, clearance of target cells and pathogens, and immune regulation (19). They are also capable of swiftly sensing and integrating various signals from their microenvironments, thereby contributing to the maintenance of homeostasis (20). Liver macrophages can be categorized into two types based on their origin: Kupffer cells (KCs), which are tissue-resident macrophages, and macrophages derived from monocytes (21, 22). However, in the process of hepatocarcinogenesis, pro-tumorigenic molecules stimulate and activate them for phenotypic shift, resulting in the transformation into TAMs (23).

Macrophages are classified into two polarized states, M1 and M2, depending on their activation status (24). M1 macrophages are primarily characterized by their pro-inflammatory effects and the production of substantial quantities of pro-inflammatory mediators (25). Their classical activation occurs in response to various stimuli, such as 1) lipopolysaccharides, 2) interferon-γ (IFN-γ), 3) tumor necrosis factor (TNF), 4) granulocyte-macrophage colony-stimulating factor (GM-CSF), and 5) Toll-like receptor (TLR) ligands (26–28). Upon activation, M1 macrophages secrete interleukins, chemokines, and TNF-α, all of which contribute to pro-inflammatory responses (29, 30). Additionally, they are capable of exerting cytotoxic effects by producing nitric oxide (NO) and reactive oxygen species (ROS) through the enzymes NOS2 or iNOS (31, 32). M1 macrophages that express high levels of MHC-II are essential for regulating Th-1-type immune responses (33). In a subsequent phase, M1 macrophages are influenced by Th2 cytokines such as interleukin 4 (IL-4) and IL-13 to polarize at tumor sites, leading to their transformation into M2-type macrophages (34, 35). These M2 macrophages produce anti-inflammatory cytokines such as IL-10 and transforming growth factor-β (TGF-β) (36, 37). M2-type macrophages are marked by elevated expression levels of CD206, CD163, and TGFβR, primarily functioning to inhibit inflammatory responses, which can facilitate tumor growth and metastasis (38, 39). Furthermore, M2 macrophages impact various cell types within the TME, including cancer-associated fibroblasts (CAFs), endothelial cells (ECs), dendritic cells (DCs), natural killer (NK) cells, and myeloid-derived suppressor cells (MDSCs) (40, 41). Notably, the M1-M2 polarization is a highly dynamic and reversible process. Within the TME, macrophages that are designated as TAMs, predominantly of the M2 subtypes, play a pivotal role in tumor progression (40, 42).

TAMs secrete a diverse array of cytokines and inflammatory factors, enhancing interactions with other cell types in the TME, and thereby promoting tumor metastasis, angiogenesis, and mechanisms of immune evasion (43, 44).

Classically activated (M1 type) macrophages frequently display anti-tumor characteristics. In the context of HCC, Sirtuin 1 (SIRT1) was capable of enhancing the infiltration of M1-like macrophages and suppressing HCC metastasis by NF-κB pathway (45). Interleukin 12 (IL-12) facilitated the conversion of monocytes into an M1-like phenotype through the inhibition of the signal transducer and activator of transcription 3 (STAT3) pathway. This transformation markedly downregulated pro-tumoral molecules, including TGF-β, vascular endothelial growth factor (VEGF)-A, and MMP-9, resulting in the suppression of tumor cell growth and metastasis, as well as a notable reduction in xenograft tumor growth *in vivo*(46).

Alternatively activated macrophages (M2) could secrete the cytokine C-C Motif Chemokine Ligand 22 (CCL22), which enhanced tumor cell metastasis through the activation of the Smad pathway, as well as the upregulation of Snail (47). IL-25 induced M2 macrophages activation and promoted the secretion of C-X-C motif Chemokine Ligand 10 (CXCL10), leading to the facilitated HCC progression (48). Transmembrane protein 147 (TMEM147) interacted with 7-dehydrocholesterol reductase (DHCR7) and enhanced its expression by promoting the STAT2 pathway, thereby conferring ferroptosis resistance and facilitating macrophage polarization into the M2-like phenotype to promote tumor growth and invasion in HCC (49) (Figure 1).

In recent years, single-cell RNA sequencing (scRNA-seq) has been widely used to study tumor heterogeneity. Emerging studies have revealed that TAMs exhibited a combination of both canonical M1-like genes and M2-like genes (50, 51). These findings challenged the traditional polarization theory of macrophages, which posited that M1 and M2 polarization states exist at opposite ends of a spectrum. A novel framework has been proposed for categorizing macrophages that incorporates functional characteristics. For example, Yang and colleagues revealed that CK19-positive HCC possessed an inhibitory TAM niche and identifies, for the first time, a significant enrichment of specific SPP1-positive TAMs in CK19-positive HCC (52). Although $SPP1^+$ TAMs were identified as the dominant macrophage type within the immune barrier of HCC, $DAB2^+$ TAMs exhibit a higher infiltration in HCC. $DAB2^+$ TAMs primarily originate from hepatic Kupffer-like cells, whereas $SPP1^+$ TAMs are more likely derived from monocyte-like macrophages, indicating potential functional differences between these populations. While both may promote extracellular matrix remodeling through the TGF-β signaling pathway, PDGFB and ADM have been identified as specific ligands for $DAB2^+$ TAMs and $SPP1^+$ TAMs, respectively, exerting distinct exclusive functions (53). This thorough understanding of macrophage classification is paving the way for a new era of therapeutic targeting, resulting in enhanced efficacy of treatment strategies.

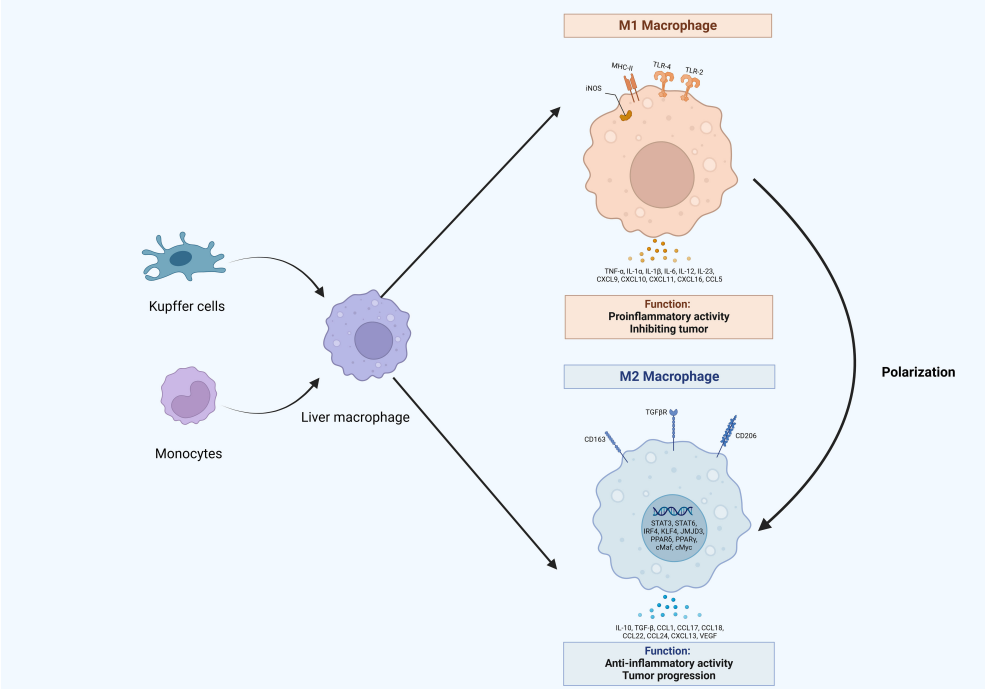


FIGURE 1
The origin and polarization of macrophages in hepatocellular carcinoma. Liver macrophages originate from Kupffer cells and monocytes, which infiltrate tumors and differentiate into tumor-associated macrophages (TAMs). TAMs would undergo different activation processes that differentiate into M1 or M2 macrophages, which release various molecules that display different functions.

## 3 Biological characteristics and properties of exosomes

Exosomes are extracellular vesicles characterized by a double-membrane structure, formed through the outward budding of the plasma membrane, and can be naturally found in blood, cerebrospinal fluid, and urine (54–56). The process of exosome biogenesis initiates with the inward invagination of the plasma membrane, leading to the formation of endosomes, which are known as multivesicular bodies (MVBs) (57). Within these endosomes, the membranes undergo further invagination to create smaller vesicles, typically ranging from 30 to 150 nm in size, referred to as intraluminal vesicles (ILVs) (58). Proteins, lipids, and nucleic acids are selectively sorted and encapsulated in ILVs (59). This process is driven by the endosomal sorting complex required for transport (ESCRT) (60). ESCRT consists of four complexes: ESCRT-0, -I, -II, and -III, which regulate the formation of ILVs and sort cargoes into specific microdomains of the limiting membrane of MVBs (61, 62). The ESCRT machinery functions sequentially. Phosphatidylinositol 3-phosphate activates ESCRT-0, which comprises hepatocyte growth factor-regulated tyrosine kinase substrate (HRS), a protein that identifies ubiquitinated proteins and associates with STAM, another member of the ESCRT-0 complex. HRS is capable of bringing tumor susceptibility gene 101 (TSG101) into the ESCRT-I complex (63). ESCRT-I then recruits ESCRT-II, activating ESCRT-III to cleave the endosomal membrane (64). Finally, ESCRT-III and the AAA ATPase Vps4, facilitate the de-ubiquitination of cargoes and the detachment of ESCRT-III from the endosomal membrane (65). Moreover, ESCRT-independent mechanisms also play an essential role in exosome biogenesis, which is facilitated by sphingomyelins (66). It promotes the formation of lipid raft microdomains, which contribute to the production of ILVs (67). Subsequently, the MVBs that contain these ILVs subsequently fuse with the plasma membrane or undergo degradation by lysosomes and autophagosomes, resulting in the release of the ILVs that encapsulate specific cargo, collectively identified as exosomes (57) (Figure 2).

Exosomes contain non-specific proteins as well as tissue-specific proteins, such as β-catenin, intercellular adhesion molecule 1 (ICAM-1) on B cells, and cytoskeletal proteins (68, 69). The lipid composition of these exosome membranes include phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin (70). The tetraspanins CD9, CD81, and CD63 are key components of exosomes; however, their roles in influencing exosome composition remain insufficiently explored. In the MCF7 breast cancer cell line, CD63 was predominantly localized within the cell as anticipated. In contrast, CD9 and CD81 showed

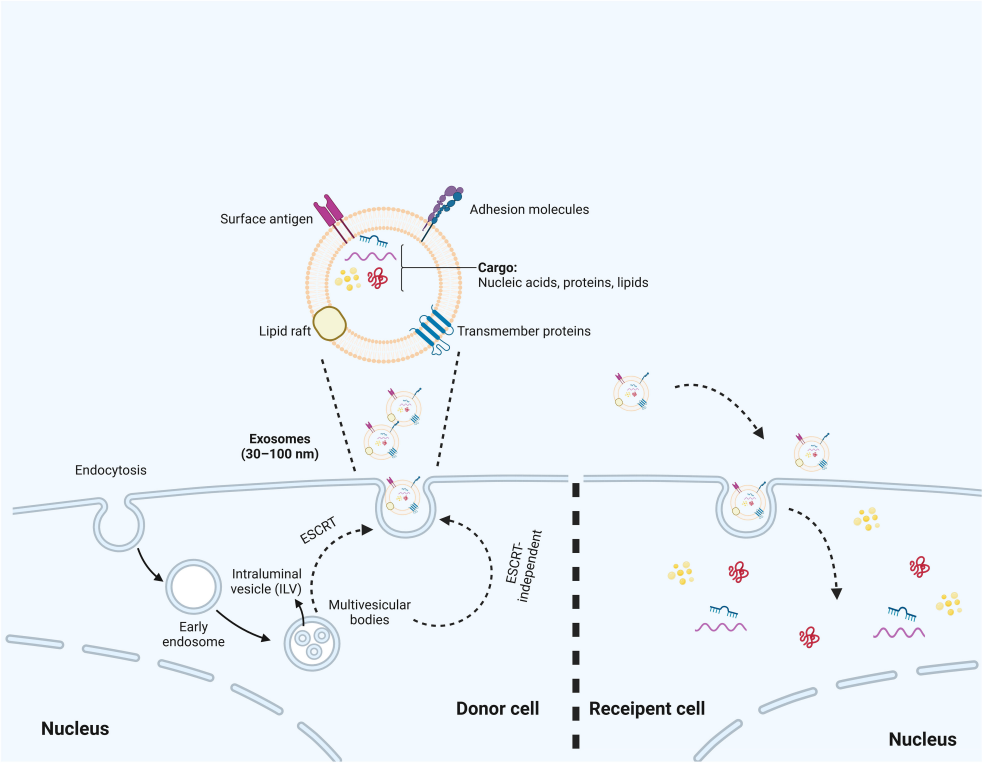


FIGURE 2
The biosynthesis process of exosomes. The biosynthesis process begins with the inward budding of the cell membrane, which leads to the formation of early endosomes. Subsequently, multivesicular bodies (MVBs) are created through additional inward budding of these endosomes, during which various miRNAs, proteins, and other selected substances are incorporated. Ultimately, MVBs can either fuse with the cell membrane, resulting in the inclusion of extracellular DNA, or merge with lysosomes, leading to the degradation of the biological information contained within the MVBs. Additionally, the production of exosomes are associated with ESCRT or ESCRT independent pathways.

significant colocalization at the plasma membrane, displaying varying ratios at different locations, which may account for the higher prevalence of CD81 in exosomes. Notably, the absence of these tetraspanins had a negligible effect on the protein composition of exosomes as assessed through quantitative mass spectrometry (71). Additionally, exosomes harbor various types of RNA, including messenger RNA and ncRNAs (72). The loading of ncRNAs into exosomes is a highly regulated and selective process that encompasses several vital steps. The initial step involves specific RNA-binding proteins (RBPs) that recognize and bind to ncRNAs intended for exosomal packaging (73). Proteins such as heterogeneous nuclear ribonucleoprotein and Ago2 identify unique motifs or secondary structures within ncRNAs, including miRNAs, thereby facilitating their selective incorporation into exosomes (10, 74).The selective nature of the packaging process guarantees that only specific ncRNAs are loaded into exosomes. This selectivity is achieved through precise regulation of ncRNA binding by RBPs and their subsequent incorporation into the developing exosomes (75, 76). Among these RNAs, microRNA (miRNA) is the most abundant RNA type found in exosomes, which can influence the transcriptome of recipient cells (77). After their release from donor cells into the extracellular environment, exosomes can modulate recipient cell functions through direct ligand-receptor interactions, fusion with the plasma membrane, or endocytosis (9, 78). Nevertheless, the underlying mechanisms of exosome uptake and their intercellular trafficking remain to be fully understood.

For an extended period, exosomes are primarily viewed as a mechanism for the transport of cellular waste. However, recent advancements in mass spectrometry and next-generation sequencing have substantially improved our understanding of exosomal contents (79, 80). In recent years, a variety of methods for exosome isolation and purification have been developed (81). These notable advancements in methodologies and experimental approaches have significantly enhanced our comprehension of the biogenesis and functions of exosomes (80). Exosomes serve as vital carriers for signaling molecules, establishing a novel system for intercellular information transfer, and playing a crucial role in various physiological and pathological processes such as cell proliferation, differentiation, migration, and communication between cells (78). For example, Qiao et al. discovered that M2-TAMs in esophageal cancer could secret exosomal LINC01592, which coordinated with E2F Transcription Factor 6 (E2F6), leading to increased degradation of major histocompatibility complex (MHC) class I on the surface of cancer cells (82). Consequently, this enabled cancer cells to evade immune attacks from cytotoxic T lymphocytes, thereby promoting tumor growth *in vivo*(82). Furthermore, circTMCO3 was delivered to ovarian cancer cells via exosomes secreted by TAMs. Exosomal circTMCO3 functioned as the molecular sponge for miR-515-5p, therefore upregulating ITGA8, which significantly promoted ovarian malignancy in mouse models (83). These findings suggested that a therapeutic approach targeting this axis could have great potential for treating malignant disease.

Exosomes can be isolated from body fluids and are capable of being stored at -80°C for extended periods, exhibiting a relatively long lifespan (84). This makes them promising candidates as diagnostic markers and prognostic indicators in bodily fluid analysis. Additionally, exosomes are naturally occurring, demonstrating good biocompatibility and low immunogenicity, making them suitable as endogenous carriers. Based on these functional characteristics, exosomes are expected to become important tools for cancer immunotherapy, and precision medicine.

In HCC, exosomes may serve as novel, noninvasive biomarkers for cancer detection. In comparison to conventional indicators, exosomes are stable in blood and other bodily fluids, providing the benefits of minimal invasiveness and easy sample collection (85). Arbelaiz et al. reported that the exosomal galectin-3 binding protein (G3BP) was significantly elevated in HCC patients when compared to healthy controls and cholangiocarcinoma patients, showcasing an area under the curve (AUC) of 0.904 and 0.894, respectively (86). Numerous exosomal proteins also demonstrate potential as prognostic indicators, enabling predictions of survival and recurrence rates in HCC patients. S100A4 is a critical component found in HCC exosomes that promoted tumor metastasis by activating STAT3 and inducing osteopontin production (87). Researchers have examined the levels of exosomal S100A4 in relation to survival and recurrence, discovering that the combination of exosomal S100A4 and osteopontin levels provides a better predictive performance than AFP alone (87). Additionally, adenylyl cyclase-associated protein 1 (CAP1) showed a correlation with HCC metastasis and was significantly valued in exosomes. Consequently, exosomal CAP1 is proposed as a potential diagnostic marker for HCC and merits further investigation (88). Researchers must remain committed to advancing this field to uncover the clinical applications of exosomal biomarkers for HCC.

## 4 Interaction of tumor cell-derived exosomes and macrophages in the microenvironment of HCC

Research has increasingly shown that not only exosomes derived from tumor cells influence the immune cells within TME, but exosomes originating from immune cells can also impact tumor cells or other immune cells, primarily targeting TAMs. Another important role of exosomes is their ability to regulate macrophage polarization within the microenvironment of HCC (Table 1; Figure 3).

MiRNAs are a highly conserved class of tissue-specific, small ncRNAs that play a crucial role in maintaining cellular homeostasis through negative regulation of gene expression. RNA sequencing analysis has revealed that miRNAs represent the predominant components in microvesicle obtained from human plasma. Exosomal miR-21-5p derived from tumor cells is associated with macrophage polarization and poor prognosis of HCC patients (89). Exosomal miR-21-5p inhibited Ras homolog family member B (RhoB) production and suppressed MAPK pathway, ultimately leading to M2-like macrophage polarization and HCC progression (89). Moreover, exosomal miR-21-5p also modulated specific protein 1 (SP1)/X-box binding protein 1 (XBP1), thus enhancing the

TABLE 1 The crosstalk between exosome and tumor-associated macrophages in impacting hepatocellular carcinoma progression.

| Exosomal cargo | Donor cell | Mechanism | Effect | Reference |
|---|---|---|---|---|
| miR-21-5p | Tumor cell | Inhibited RhoB expression and suppressed MAPK pathway | Promoted M2 macrophage polarization and cancer progression | (89) |
| miR-21-5p | Tumor cell | Modulated SP1/XBP1 axis | Enhanced M2 macrophage polarization and promoted cancer progression | (90) |
| miR-452-5p | Tumor cell | Targeted TIMP3 | Enhanced M2 macrophage polarization and promoted cancer progression | (91) |
| miR-4669 | Tumor cell | Upregulated sirtuin 1 expression | Enhanced M2 macrophage polarization and promoted acquired resistance to sorafenib. | (92) |
| miR-200b-3p | Tumor cell | Downregulated ZEB1 expression, promoted IL-4 production, and activated JAK/STAT pathway | Enhanced M2 macrophage polarization and augmented tumor growth and metastasis. | (93) |
| circUPF2 | Tumor cell | Facilitated the formation of the IGF2BP2-SLC7A11 ternary complex | Increased sorafenib resistance and inhibited ferroptosis | (95) |
| LncRNA TUC339 | Tumor cell | Regulated cytokine-cytokine receptor binding | Enhanced M2 macrophage polarization | (96) |
| LncRNA HMMR-AS1 | Tumor cell | Targeted miR-147a/ARID3A axis | Enhanced M2 macrophage polarization and promoted tumor cell proliferation | (97) |
| LncRNA HEIH | Tumor cell | Targeted the miR-98-5p/STAT3 axis | Enhanced M2 macrophage polarization and promoted cancer progression | (98) |
| FAL1 | Tumor cell | Activated the Wnt/β-catenin pathway | Induced M2 macrophage polarization and promoted cancer progression | (99) |
| SLC16A1-AS1 | Tumor cell | Enhanced the stability of SLC16A1 mRNA in macrophages | Induced M2 macrophage polarization and promoted cancer progression | (101) |
| miR4458HG | Tumor cell | Interacted with IGF2BP2, enhancing the stability of HK2 and GLUT1, | Induced M2 macrophage polarization and promoted cancer progression | (102) |
| ZFPM2-AS1 | Tumor cell | Regulated glycolysis by targeting the miRNA-18b-5p/PKM axis under hypoxia conditions | Augmented the abilities and stemness of HCC cells by contributing to M2 macrophage polarization | (103) |
| ALKBH5 | Tumor cell | Upregulated SOX4 expression, facilitated SHH pathway, promoted CCL5 secretion, upregulated IL-8 and CPT1A | Enhanced M2 macrophage polarization and promoted cancer progression | (104–107) |
| FTCD | Tumor cell | Directly promoted M1 macrophage polarization | Inhibited cancer progression | (109) |
| PSMA5 | Tumor cell | Promoting JAK2/STAT3 pathway | Enhanced M2 macrophage polarization and promoted cancer progression | (110) |
| LncMMPA | TAM | Sponged miR-548 s and upregulated ALDH1A3 expression | Enhanced M2 macrophage polarization and promoted tumor glycolysis and growth | (17) |
| hsa_circ_0004658 | TAM | Targeted miR-499b-5p/JAM3 axis | Inhibited cancer progression | (112) |
| miR-27a-3p | TAM | Inhibited TXNIP expression | Enhanced tumorigenicity, stemness, and drug resistance of cancer cells | (113) |
| MNDA | TAM | Promoted exosomal proteins secretion including MMP14, and TIMP | Enhanced cancer progression | (117) |
| miR-660-5p | TAM | Decreased expression of KLF3 | Enhanced cancer progression | (118) |
| miR-6876-5p | TAM | Promoted EMT by targeting PTEN and activated the AKT signaling pathway. | Enhanced tumor metastasis | (119) |
| miR-375 | TAM | miR-375 was found to be upregulated in ExoIL2-TAM-exosomes- | Inhibited cancer progression | (16) |
| miR-92a-2-5p | TAM | Reduced AR expression and regulated PHLPP/p-AKT/β-catenin signaling pathway | Enhanced cancer progression | (122) |

*(Continued)*

TABLE 1 Continued

| Exosomal cargo | Donor cell | Mechanism | Effect | Reference |
|---|---|---|---|---|
| miR-23a-3p | TAM | Increased VEGF and IL-4, which in turn led to further recruitment of M2 macrophages | Enhanced tumor angiogenesis | (124) |
| miR-200c-3p | TAM | Activated PI3K/AKT signaling pathway | Promoted Sorafenib resistance | (127) |
| circTMEM181 | Tumor cell | Sponged miR-488-3p and promoted adenosine pathway. | Promoted T cell exhaustion and resistance to anti-PD-1 therapy | (131) |
| miR-1246 | Tumor cell | miR-1246 was transferred by exosomes | Enhanced M2 macrophage polarization and inhibited the function of T cells | (132) |
| miR-146a-5p | Tumor cell | miR-146a-5p was induced by SALL4 and activated NF-κB pathway | Enhanced M2 macrophage polarization and inhibited the function of T cells | (133) |
| miR-23a-3p | Tumor cell | ER stress facilitated the release of exosomal miR-23a-3p and enhanced the expression of PD-L1 by regulating PTEN/PI3K signaling pathway | Inhibited T-cell function | (134) |

polarization states of M2 macrophages and affecting the progression of HCC (90). Moreover, exosomal miR-452-5p directly targeted tissue inhibitors of metalloproteinases 3 (TIMP3) to induce M2 phenotype TAMs proliferation and polarization, representing a promising miR-452-5p/TIMP3 axis in HCC therapy (91). Additionally, exosomal miR-4669 contributed to the polarization of M2 macrophages by increasing sirtuin 1, which led to acquired resistance to sorafenib, promoted tumor aggressiveness and immunosuppressive tumor microenvironment, thus influencing the recurrence of HCC (92). In line with this, Xu et al. reported that miR-200b-3p exosomes downregulated zinc finger E-box binding homeobox 1 (ZEB1) expression and promoted IL-4 production, which trained macrophage polarization into M2-like phenotype and activated JAK/STAT pathway (93). ZEB1 functions as an essential transcriptional factor that is implicated in the epithelial-mesenchymal transition (EMT) (94). M2-like TAMs significantly upregulated the proviral Integration site for Moloney murine leukemia virus 1 (PIM1) and VEGFα expression, resulting in

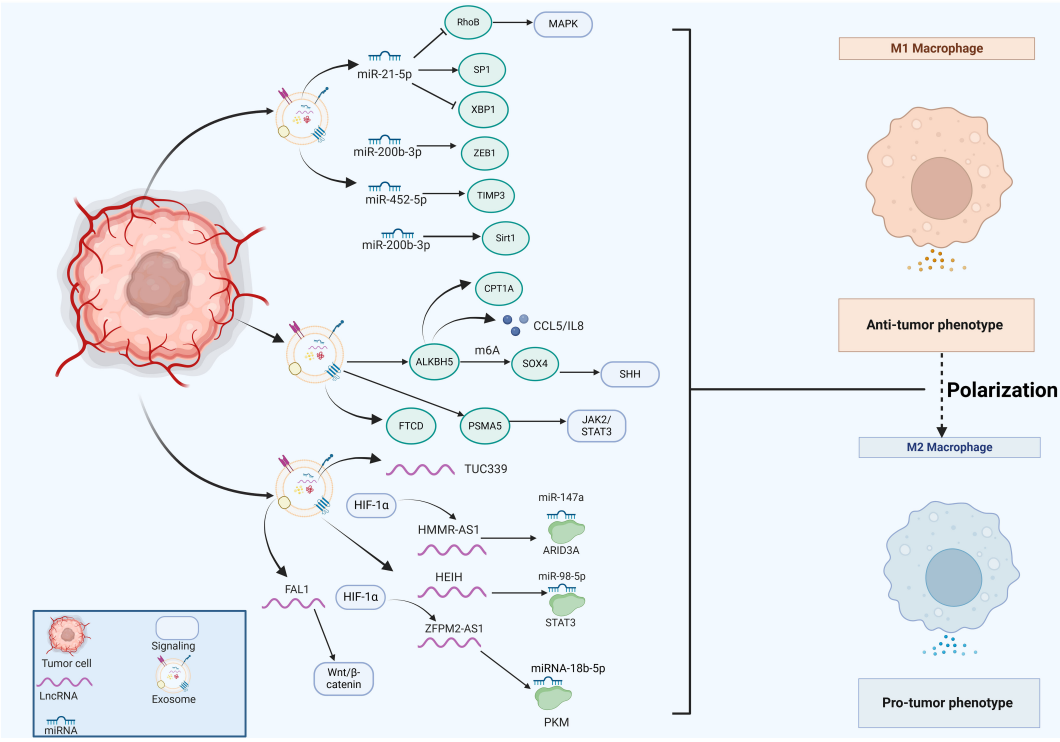


FIGURE 3
The emerging role of tumor cell-derived exosomes in HCC progression by regulating macrophage polarization. Tumor cells secret exosomes that contain miRNAs (miR-21-5p, miR-200b-3p, miR-452-5p), lncRNAs (TUC339, HMMR-AS1, HEIH, ZFPM2-AS1, FAL1) and various proteins (ALKBH5, FTCD, PSMA5), which significantly impact macrophage polarization, thus regulating HCC progression.

the activation of MEK/ERK signaling pathway and augmented cell EMT and metastasis in the setting of HCC (93). In line with this, exosomes enriched with circUPF2 from HCC cells facilitated the formation of the IGF2BP2-SLC7A11 ternary complex, which stabilized SLC7A11 mRNA, leading to increased sorafenib resistance and inhibited ferroptosis (95). Therefore, targeting exosomal circUPF2 may present a novel strategy for treating HCC.

Long non-coding RNAs (lncRNAs) are a class of ncRNAs that exceed 200 nucleotides in length and possess diverse functions both in the nucleus and the cytoplasm. Exosomal lncRNAs have been identified as signaling mediators that coordinate cellular functions. Li et al. reported that exosomal lncRNA TUC339 contributed to M1/M2 polarization by regulating cytokine-cytokine receptor binding (96). LncRNA HMMR-AS1 was notably induced by hypoxia-inducible factor-1 alpha (HIF-1α) and was associated with poor prognosis (97). Exosomes that carried HMMR-AS1 facilitated the M2 polarization of macrophages through sponging miR-147a and abrogating the degradation of ARID3A, thereby promoting HCC cell proliferation and growth (97). Furthermore, HCC cells secreted exosomal lncRNA HEIH that triggered macrophage polarization by targeting the miR-98-5p/STAT3 axis, which might shed light on the HCC treatment (98). Highly expressed lncRNA FAL1 in serum exosomes were observed in HCC patients and could promote tumor progression *in vivo*(99). It significantly induced M2 polarization of macrophages and subsequently activated the Wnt/β-catenin pathway, thus holding immense potential for novel strategies against HCC (99).

Metabolic reprogramming is a defining characteristic of cancer cells, promoting their growth and survival (100). LncRNA SLC16A1-AS1, derived from HCC exosomes, promoted the malignant progression of HCC by modulating macrophage polarization toward the M2 phenotype. Mechanistically, SLC16A1-AS1 enhanced the stability of SLC16A1 mRNA in macrophages (101). As a lactate transporter, SLC16A1 facilitated lactate influx, activating the c-Raf/ERK signaling pathway, which driven M2 polarization. In turn, M2 macrophages secreted IL-6, which activated the STAT3 pathway in HCC cells, inducing METTL3 transcription. This process increased m6A methylation and stability of SLC16A1-AS1. The reciprocal signaling between SLC16A1-AS1 and IL-6 in HCC cells and M2 macrophages promoted the proliferation, invasion, and glycolysis of HCC cells (101). Additionally, miR4458HG activated the glycolytic pathway, and promoted the polarization of TAMs in experimental models. Mechanistically, miR4458HG interacted with IGF2BP2, a key m6A RNA reader, enhancing the stability of target mRNAs such as HK2 and GLUT1, thereby impacting HCC glycolysis. Additionally, miR4458HG derived from HCC could be encapsulated in exosomes, further promoting the polarization of TAMs by increasing ARG1 expression (102). Similarly, Ji and colleagues revealed that lncRNA ZFPM2-AS1 was enriched in tumor cell-derived exosomes, which augmented the abilities and stemness of HCC cells by contributing to macrophage polarization (103). Further mechanistic studies have demonstrated that exosomal ZFPM2-AS1 regulated glycolysis by targeting the miRNA-18b-5p/PKM axis in a manner dependent on HIF-1α (103). These findings emphasized that exosomes serve as a signaling molecule that regulated metabolic regulation and macrophage polarization, suggesting that exosome could be a viable target for therapeutic intervention in HCC.

Exosomal proteins play an essential role in tumor development and progression. The expression of human AlkB homolog H5 (ALKBH5) was found to be enriched in liver cancer stem cells (LCSCs), potentially enhancing tumor growth and metastasis. Mechanistic studies have demonstrated that ALKBH5 significantly upregulated the expression of SPY-related high mobility group box 4 (SOX4) by inhibiting its $N^6$-methyladenosine (m6A) modification, which in turn facilitated the transcriptional activation of sonic hedgehog (SHH) expression, thereby stimulating the SHH signaling pathway (104). Additionally, the exosomal ALKBH5 secreted by $CD133^+$ HCC cells enhanced macrophage M2 polarization by promoting CCL5 secretion, upregulating IL-8 and mediating the upregulation of CPT1A (105–107). But in a recurrent spontaneous abortion model, overexpressed ALKBH5 reduced stromal VEGF secretion and impaired M2 macrophage differentiation and recruitment (108), thus the molecular mechanisms underlying macrophage polarization mediated by ALKBH5 awaited further investigation. Liu et al. examined the relationship between FTCD expression and immune cell infiltration using The Cancer Genome Atlas Program (TCGA) dataset and discovered that FTCD demonstrated a significant positive correlation with macrophage infiltration (109). Moreover, FTCD was considered a key potential exosome-related biomarker by stimulated macrophages exhibiting polarization towards the M1 type, leading to inhibited HCC growth (109). Knockdown of exosomal proteasome subunit alpha 5 (PSMA5) derived from HCC cells impeded M2 macrophage polarization via abrogating JAK2/STAT3 signaling pathway, leading to inhibited tumor cell proliferation, invasion, and migration (110).

## 5 TAMs-derived exosomes impact cellular functions in HCC

Exosomes derived from macrophages have demonstrated promise in targeting HCC cells. Increasing evidence suggests that these TAM-derived exosomes play a critical role in regulating cell proliferation, invasion, metastasis, metabolic reprogramming, and immune response (Figure 4).

LncMMPA was a myeloid-derived lncRNA that has been identified as a regulator for M2 macrophage polarization based on the scRNA-seq method (17). Further investigations have reported that the majority of extracellular lncMMPA existed within exosomes and the transfer of exosomal lncMMPA might take place between TAMs and Hep3B cells. The validation experiments indicated that exosomal lncMMPA significantly promoted the glycolytic pathway and cell proliferation by sponging miR-548 s and upregulating ALDH1A3 expression (17). The recombination signal binding protein-Jκ (RBPJ) functioned as a transcriptional regulator in the Notch signaling pathway, which has been implicated in various subsets of TAMs in HCC (111). Zhang et al. examined RBPJ overexpression in macrophages and its

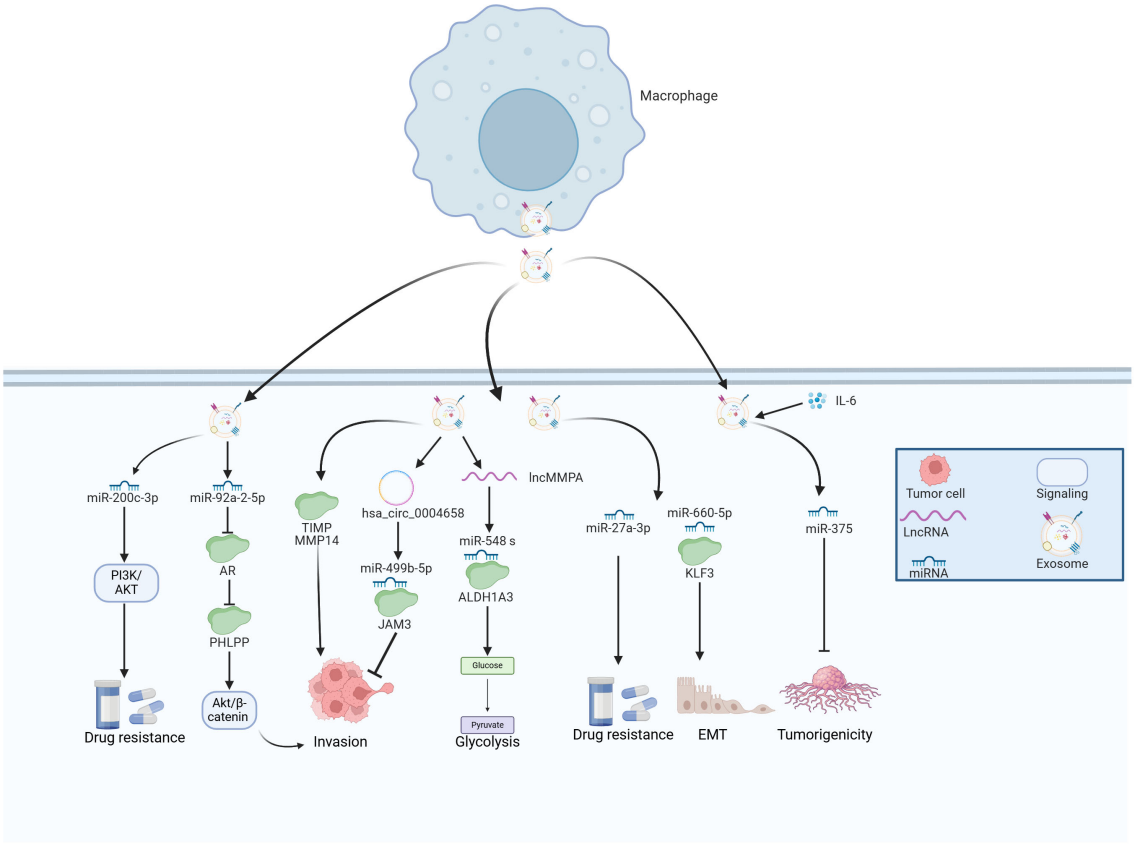


FIGURE 4
The emerging role of TAM-derived exosomes in HCC progression. TAMs secret various exosomes that contain multiple molecules, which play essential roles in the tumor growth, metastasis, glycolysis and drug resistance of HCC.

effects on HCC cells. Using circRNA microarray analysis, exosomal hsa_circ_0004658 was the most differentially regulated exosomal ncRNA in RBPJ overexpressed TAMs. Exosomal hsa_circ_0004658 demonstrated the ability to inhibit proliferation and migration while promoting apoptosis in HCC cells by targeting miR-499b-5p/JAM3 axis (112), which could function as a promising biomarker and therapeutic target for treating HCC. MiR-27a-3p was found to be upregulated while thioredoxin-interacting protein (TXNIP) was downregulated in HCC cells (113). Moreover, exosomes secreted by M2 macrophages were shown to further increase the levels of miR-27a-3p, which significantly enhanced tumorigenicity, stemness, and drug resistance of HCC cells (113).

Myeloid cell nuclear differentiation antigen (MNDA) belongs to the family of hematopoietic interferon-inducible nuclear proteins characterized by a pyrin domain (114). This protein is capable of regulating programmed cell death and inducing inflammatory responses (115, 116). MNDA acted as an independent prognostic factor and was predominantly expressed in M2-like TAMs, where it enhanced their polarization. Furthermore, MNDA-stimulated M2 TAMs secreted multiple exosomal proteins including MMP14, and TIMP, which promoted cell invasion, migration, and metastasis in HCC models (117). Tian et al. sought to explore the effects of miR-660-5p-modified M2-derived exosomes on the progression of HCC by regulating Kruppel-like factor 3 (KLF3). They observed elevated levels of miR-660-5p and decreased levels of KLF3 in HCC tissues, where increased levels of exosomal miR-660-5p facilitated the growth and EMT of HCC cells, an effect that could be reversed by overexpressing KLF3 (118). Furthermore, miR-660-5p-loaded M2 TAM exosomes bolstered the tumor-forming capacity in HCC mouse models, indicating that exosomal miR-660-5p from M2 TAMs significantly contributed to HCC tumorigenesis via modulating KLF3 (118). Moreover, miR-6876-5p within CD63-high macrophage was recognized as a key mediator, promoting EMT by targeting PTEN and activating the AKT signaling pathway. Additionally, exosomal miR-6876-5p accelerated tumor growth and metastasis in the setting of HCC (119). Chen and colleagues isolated TAM-derived exosomes from HCC tissues, and their exosomes were either treated with IL-2 (ExoIL2-TAM) or left untreated (ExoTAM). Among them, miR-375 was found to be upregulated in ExoIL2-TAM-exosomes and markedly decreased HCC cell tumorigenicity. These findings shed light on the mechanisms through which IL-2 inhibits HCC progression and underscores the potential clinical significance of exosomal miR-375 released by TAMs (16).

Androgen (AR) signaling plays a crucial role in the initiation and progression of HCC (120). Hypoxia could induce the phenotype of cancer stem cells by regulating the Androgen receptor (AR)-miR-520f-3p-SOX9 cascade, which resulted in acquired resistance to sorafenib (121). However, the relationship

between AR and the TAMs during HCC development remains ambiguous. One recent study performed by Liu et al. reported that TAMs modified the expression of miR-92a-2-5p in exosomes, which reduced AR expression and subsequently regulated the pleckstrin homology domain leucine-rich repeat protein phosphatases (PHLPP)/p-AKT/β-catenin signaling pathway, leading to enhanced the invasive capabilities of HCC cells in preclinical models (122).

Cancer cells depend on oxygen and nutrients for survival and proliferation, necessitating their proximity to blood vessels to gain access to the circulatory system, which termed angiogenesis that could promote tumor progression (123). Exosomes derived from M2 macrophages were taken up by both HCC cells, enhancing vascular permeability, and promoting angiogenesis. Importantly, levels of miR-23a-3p were significantly elevated in M2-derived exosomes, with hnRNPA1 playing a key role in the packaging of miR-23a-3p into these exosomes. Moreover, HCC cells co-cultured with M2-derived exosomes released increased amounts of VEGF and IL-4, which in turn led to further recruitment of M2 macrophages and enhanced tumor angiogenesis (124).

Sorafenib serves as a first-generation multi-targeted tyrosine kinase inhibitor, demonstrated remarkable antiangiogenic and antiproliferative effects on tumor cells, leading to extended survival rates in advanced HCC patients (125). Treatment with sorafenib led to a reduction of tumor vessels formation and the exhaustion of pericytes, which may promote the recruitment of TAMs (126). In HCC patients, a positive correlation was observed between M2 macrophage scores and sorafenib efficiencies. Moreover, exosomes from M2 macrophages containing miR-200c-3p were found to promote acquired resistance to sorafenib by activating the PI3K/AKT signaling pathway (127). The study offers valuable insights into the role of M2 macrophages and their exosomes in sorafenib resistance and underscores the therapeutic potential of targeting this molecular pathway.

Recent studies have revealed the association between GPI anchored proteins and exosomes. Adiponectin bound to T-cadherin, a unique GPI-anchored cadherin on MSCs, promoting exosome biogenesis and secretion. Furthermore, increasing plasma adiponectin levels through pharmacological or adenovirus-mediated genetic approaches significantly enhanced the therapeutic effects of MSCs (128). These findings highlight the critical role of adiponectin in mesenchymal progenitor cell-mediated organ protection. Direct studies on the relationship between GPI-anchored proteins and TAM-derived exosomes remain scarce. However, given their essential roles in signal transduction and immune regulation, this area offers significant potential for future research and practical applications.

## 6 Crosstalk between exosomes and TAMs in HCC immune microenvironment

Immunotherapy, particularly through immune checkpoint inhibitors (ICIs), constitutes a major advancement in the development of oncology therapeutics over the past few years, which has the potential to provide significant advantages in clinical management of HCC (129). However, therapeutic resistance to ICIs including antibodies blocking programmed cell death 1 protein (PD-1)/programmed cell death 1 ligand 1 (PD-L1) pathways are emerging, leading to treatment failure and progressive disease in HCC patients (130). Mechanistic studies have revealed that immunosuppressive TME with exhaustion of T cells has been recognized as a critical factor that contributes to immunotherapy resistance in HCC. Exhausted T cell phenotype partially stems from excessive accumulation of TAMs, and the activated adenosine signaling with upregulated expression of CD73 and CD39, in which exosomes might play an essential role in immune regulation. High levels of exosomal circTMEM181 sponged miR-488-3p and upregulated CD39 expression in macrophages, synergistically promoting the activation of the adenosine pathway by cooperating with CD73 expression, thereby leading to T cell exhaustion and resistance to anti-PD-1 therapy in HCC patients (131).

Moreover, exosomes play a significant role in modulating tumor progression by educating immune cells within the microenvironment. Specifically, miR-1246 was transferred to macrophages through exosomes, guiding them toward a tumor-supporting phenotype and consequently establishing the immunosuppressive TME (132). Exosomal miR-146a-5p originated from HCC cells significantly promoted M2 macrophage polarization by activating the NF-κB pathway and subsequently inducing inflammatory factors, which induced immunosuppressive microenvironment by upregulating the expression of inhibitory receptors in T cells (133). Further investigations demonstrated that Sal-like protein-4 (SALL4) mediated the transcriptional activation of miR-146a-5p, and promoted its cellular delivery via exosomes. Blocking the SALL4/miR-146a-5p axis reversed the T cell exhaustion, which provided a promising therapeutic target for HCC patients (133).

Endoplasmic reticulum (ER) stress plays a crucial role in preserving cell survival. Moreover, the activation of ER stress in immune cells is thought to influence the functionality of infiltrating immune cells, subsequently facilitating tumor growth. For example, Liu et al. reported that ER stress facilitated the release of exosomal miR-23a-3p and enhanced the expression of PD-L1 in TAMs by regulating the PTEN/PI3K signaling pathway, which subsequently inhibited T-cell function (134). Additionally, glycosylphosphatidylinositol (GPI) is a complex glycolipid broadly expressed across eukaryotic species (128). These findings have elucidated the essential of the crosstalk between exosomes and TAMs in the modulation of HCC TME (Figure 5).

## 7 Emerging role of exosomes in cancer therapy

Exosomes derived from macrophages possess the capability to transfer cargo to recipient cells, positioning them as promising candidates for targeted drug delivery and nanomaterial transport.

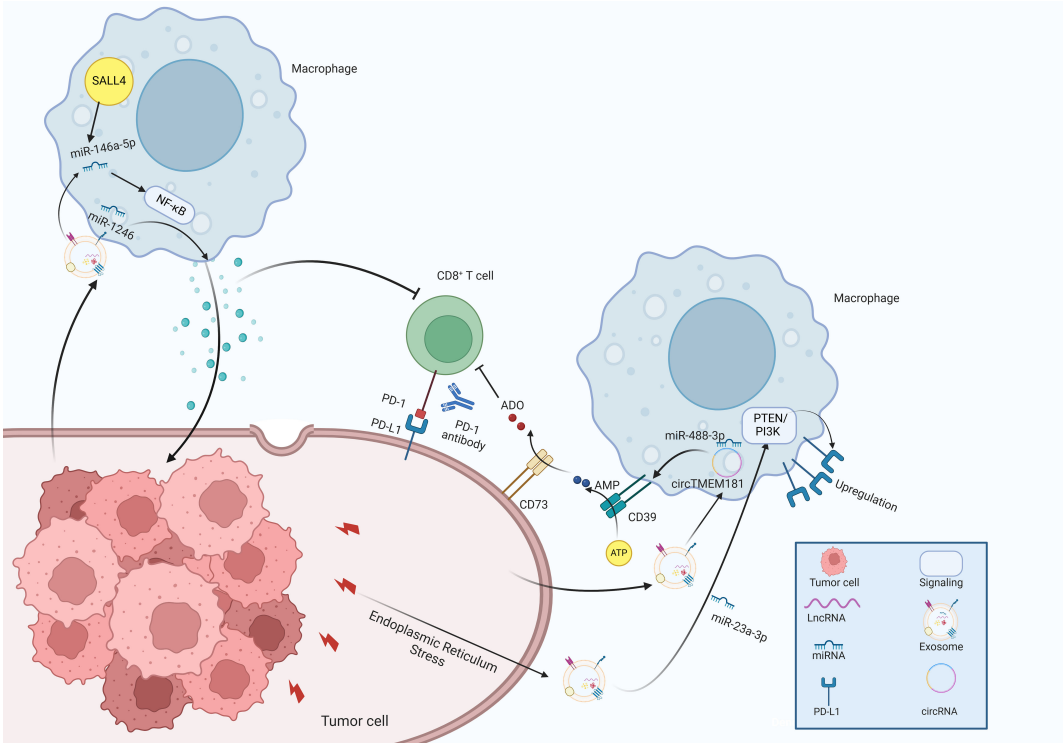


FIGURE 5
The molecular mechanisms of exosomes in immune regulation within the tumor microenvironment of HCC.

These exosomes demonstrate outstanding biocompatibility, which enhance the ability of drugs to traverse natural barriers. The significant example was the engineering of exosomes to produce a fusion protein comprising the iRGD peptide (CRGDKGPDC), which targeted the αγ integrin, along with LAMP-2B. These engineered exosomes were capable of selectively delivering KRAS siRNA to non-small cell lung cancer cells that expressed the αvβ3 integrin, effectively downregulating the KRAS gene, inhibiting tumor proliferation, and demonstrating negligible toxicity. Sonodynamic therapy (SDT) presents a potential approach for tumor ablation through the activation of sonosensitizers in conjunction with ultrasound irradiation, making it promising for glioblastoma (GBM) therapy. Wu et al. developed a biodegradable nanoplatform (CSI), encapsulating catalase in silica nanoparticles (NPs) (135). They subsequently modified CSI with AS1411 aptamer-coated macrophage exosomes (CSI@Ex-A), which remarkably enhanced blood-brain barrier penetration and promoted specifically targeting tumor cells. Tumor cell endocytosis triggered the biodegradation of CSI@Ex-A, alleviating hypoxic TME and boosting SDT effectiveness with long circulation time, presenting a promising nanoplatform for clinical application (135). Yan et al. isolated exosome-like nanovesicles from B. javanica (designated as BF-Exos) and examined their effects and underlying molecular mechanisms in triple-negative breast cancer (TNBC). BF-Exos successfully transferred ten functional miRNAs to tumor cells, significantly hindering both the growth and metastasis of these cells by modulating the PI3K/Akt/mTOR signaling pathway and promoting ROS/caspase-mediated apoptosis (136). Moreover, cancer stem cells and MSCs-derived exosomes influenced signaling pathways associated with tumor progression *in vivo*, suggesting that they can sever as potential targets in HCC therapy (137). Xu et al. encapsulated doxorubicin (Dox) within Exos derived from human placental and modified these Exos with carboxylated $Fe^3O^4$ NPs to develop an Exo-Dox-NP delivery system. As a drug delivery vehicle, Exo-Dox-NPs significantly enhanced Dox uptake by tumor cells, exhibiting strong targeting specificity. Furthermore, Exo-Dox-NPs effectively inhibited the migration of cancer cells, with this formulation showing the highest anti-tumor activity (138). Yim et al. introduced a new tool for intracellular delivery of target proteins termed EXPLORs. By incorporating a blue light-controlled reversible protein-protein interaction module into the natural process of exosome biogenesis, they could effectively load cargo proteins into newly formed exosomes. Treatment with protein-loaded EXPLORs significantly enhanced the intracellular levels and functional capacity of these proteins in recipient cells, both *in vitro* and *in vivo*, which underscored the potential of EXPLORs as an effective mechanism for the intracellular transfer of protein-based therapeutics into target cells and tissues (139). Furthermore, a lipid-like prodrug of docetaxel (DSTG) featuring a reactive oxygen species (ROS)-cleavable linker, along with a lipid-conjugated photosensitizer (PPLA), spontaneously co-assemble into nanoparticles that acted as the lipid cores of the hybrid exosomes (HEMPs and NEMPs). These nanoparticles were subsequently encapsulated within membranes derived from adipocytes, enhancing their affinity for HCC cancer cells. Experimental studies demonstrated that HEMPs not only improved the

bioactivity of the prodrug and prolonged its circulation time in the bloodstream but also effectively inhibited tumor growth by selectively targeting cancer cells. The self-facilitated synergistic drug release further enhanced the antitumor efficacy, leading to significant tumor growth inhibition with minimal side effects, suggesting a promising avenue for the development of targeted therapeutics for HCC (140).

Monoclonal antibodies (mAbs) that target specific molecules can be incorporated onto the surface of exosomes, functioning as potent "tools" to stimulate antitumor immune responses. For example, Nie et al. synthesized nano-bioconjugates by utilizing pH-sensitive linkers to conjugate Azide-modified M1 macrophage-derived exosomes with dibenzocyclooctyne-modified antibodies targeting CD47 and SIRPα, which regulated the "don't eat me" pathway in macrophages. In the acidic TME, the linkers underwent cleavage, resulting in the release of specific antibodies, which significantly enhanced macrophage phagocytosis (47). Recently, a novel type of engineered exosome, inspired by chimeric antigen receptor macrophage cells (CAR-M), has garnered attention due to its superior antitumor efficacy and reduced incidence of adverse events. Jiang et al. utilized exosomes derived from CAR-M cells as the targeted drug carrier, which were enriched with a high concentration of CXCL10. Subsequently, CAR-exosomes were covalently loaded with the chemotherapeutic agent SN-38, establishing a novel antibody-drug conjugates (ADCs), which markedly promoted the immunological activation and enhanced the recruitments of TAMs, outperforming traditional ADCs in antitumor effects, providing novel insights into future drug development (141). The above findings highlight the essential role of exosomes in cancer therapy in the preclinical settings. Additionally, more investigations are focused on the clinical utilization of exosomes in cancer therapy. For example, an ongoing clinical trial (NCT05575622) performs the detection of exosomal PD-L1 and LAG-3 proteins. The goal is to characterize the functional marker profiles associated with immunotherapy in the peripheral blood of HCC patients and to provide a comprehensive assessment of their responsiveness to such treatments. Another clinical trial (NCT06342414) aims to develop and validate a liquid biopsy that assesses circulating exosomal miRNAs for indirect sampling of tumor tissue present in the bloodstream, aiming to create a cost-effective, non-invasive assay suitable for clinical application, enhancing the sensitivity and specificity for diagnosing HCC. Moreover, camel milk contains various exosomes that hold immense potential for anti-cancer treatment (55, 142). Camel milk-derived exosomes exhibited a stronger anti-cancer effect on HCC cells by the induction of apoptosis and the suppression of inflammation and angiogenesis (143). Thus, these exosomes could act as safe adjuvants or carriers for the delivery of chemotherapeutics, enhancing their anti-cancer effects on HCC cells.

In addition, exosomes derived from macrophages may play an important role in reversing tumor resistance (Table 2). For example, exosomes from M1 macrophages loaded with cisplatin have been shown to enhance anticancer efficacy, specifically by inhibiting cancer cell growth, and increasing drug sensitivity (144). The expression of exosomal miR-301a-3p was elevated in the lenvatinib-resistant HCC cells, activating the PTEN/PI3K cascade in TAMs, which increased cell resistance to lenvatinib (145).

Moreover, exosomes isolated from M2 macrophages could transfer circ 0008253 to cancer cells, which possessed the ability to decrease oxaliplatin sensitivity and promote cell proliferation by regulating ABCG2 levels (146). Furthermore, Guo et al. performed a study that aimed to elucidate the downstream mechanisms by which exosomal miR-222-3p, delivered via exosomes derived from M2 macrophages, contributed to drug resistance (147). Both *in vivo* and *in vitro*, exosomal miR-222-3p from M2-polarized macrophages potentiated chemoresistance through the downregulation of TSC1 and the activation of the PI3K/AKT signaling pathway (147). In HCC, as we have discussed above, exosomal miR-4669, and miR-200c-3p also promoted sorafenib resistance, presenting promising targets for precision medicine (92, 127). In xenograft and liver metastasis models, the sequential administration of folic acid-modified milk exosomes loaded with c-kit siRNA (FA-mExo-siRNA-c-kit) followed by gefitinib resulted in decreased tumor growth and improved survival rates. Mechanistically, c-kit was identified as a regulator of the AKT/mTOR/4EBP1/eIF4E pathway, promoting both stemness and resistance to gefitinib in lung cancer cells. The utilization of FA-mExo-siRNA-c-kit might enhance patient outcomes by overcoming gefitinib resistance, warranting further investigation into this approach (148).

## 8 Challenges in the therapeutic application of exosomes

Despite considerable progress, various challenges continue to hinder the therapeutic application of exosomes. Firstly, exosomes are diverse and widely found entities; however, their complexities

TABLE 2 The role of exosomes in the drug resistance of HCC.

| Exosomal cargo | Donor cell | Mechanism | Effect | Reference |
|---|---|---|---|---|
| miR-301a-3p | Tumor cell | Activated the PTEN/PI3K/GSK3β/Nrf2 signaling pathway | Promoted lenvatinib resistance | (145) |
| circ 0008253 | M2 macrophages | Promoted cell proliferation by regulating ABCG2 levels | Decrease oxaliplatin sensitivity | (146) |
| miR-222-3p | M2 macrophages | Downregulated TSC1 and activated the PI3K/AKT signaling pathway | Promoted chemoresistance | (147) |
| miR-4669 | Tumor cell | Induced M2 macrophage polarization. | Promoted sorafenib resistance | (92) |
| miR-200c-3p | M2 macrophages | Activated PI3K/AKT pathway | Promoted sorafenib resistance | (127) |

remain incompletely understood, especially regarding the mechanisms of cargo sorting into exosomes and the release of that cargo into cells after exosome internalization (149). While recent studies have largely concentrated on protein sorting, it appears that the primary functions of exosomes are more associated with RNA delivery (150). Thus, understanding the mechanisms behind RNA sorting hold considerable promises for the development of various applications.

In this field, there is currently no standardized protocol for exosome isolation. Ultracentrifugation remains the most common technique for separating exosomes; it is essential to recognize that while ultracentrifugation can effectively concentrate substances with similar density and size, it does not allow for precise differentiation of exosomes (151). Ultracentrifugation has several advantages, including established technology, compatibility with a wide range of samples, and low operational costs (152). However, it suffers from low reproducibility and the potential to damage the exosomes, rendering it inappropriate for clinical applications (153). Additionally, efficient isolation of tumor-derived exosomes can be achieved by targeting specific proteins found in these exosomes, such as EpCAM and anti-A33 (154). Currently, immunomagnetic beads are commonly used; these antibody-coated beads selectively capture the corresponding exosomes, enabling their differentiation from unbound impurities via magnetic separation (155). Moreover, microfluidics, capable of manipulating small fluid volumes (microliters), offers advantages such as rapid separation, high throughput, and minimal sample requirements, making it ideal for isolating exosomes from limited biological samples (156).

Other significant challenges involve the scalability of exosome production. Typically, exosomes are produced in limited quantities, and the processes for their isolation and purification can be time-consuming and costly (157). To enhance the clinical application of exosomes, it is essential to develop scalable production methods that can generate substantial amounts of exosomes in a cost-effective manner (158). Moreover, each stage of exosome biogenesis is mediated by various mechanisms that exhibit high variability, leading to the observed heterogeneity of exosomes (159). The heterogeneity of exosomes and the complexity of the *in vivo* environment limit their precise delivery and expected outcomes (160). The source and composition of exosomes can vary due to different cell types, disease states, and microenvironmental factors, making the strategy of using exosomes as drug delivery systems in cancer treatment relatively complex (161). The TME contains various cell types, signaling molecules, and intercellular interactions, all of which collectively influence tumor progression and treatment response. This complexity means that even if a treatment is effective in preclinical stages, the actual application to patients may lead to different therapeutic outcomes due to various individual differences. Therefore, when developing personalized treatment plans, it is essential to systematically consider these complex factors to determine the most suitable therapeutic targets and strategies. To tackle this problem, autologous exosomes obtained from cancer patients have surfaced as a promising delivery system, owing to their exceptional ability to specifically target cancer cells (162). Autologous plasma-derived exosomes are readily accessible and can circumvent the immune responses often elicited by exogenous exosomes (163). Ran et al. constructed a biological scaffold based on autologous plasma exosomes, which were loaded with neuron-targeting peptides and growth-promoting peptides (162). By integrating both efficacy and safety, the autologous plasma exosome-based personalized treatment has exhibited significant potential for biomedical applications, which aided in broadening the utilization of combinatory peptides and autologous exosomes derived from human plasma in the context of human disease treatment (162). Jiang et al. loaded gemcitabine into autologous exosomes to facilitate cellular uptake and enhance the cytotoxicity of gemcitabine, leading to significant inhibition of tumor growth and reduction of tumor recurrence in mice. This approach may offer important implications for personalized therapy in cancer (163).

Moreover, a comprehensive assessment of the safety of exosome-based therapies is necessary. The *in vivo* function and safety of exosomes continue to be a subject of controversy. Given their biological activity, it is important to assess the safety of exosomes when utilized as delivery vehicles. For example, exosomes derived from TAMs may carry components that facilitate cancer cell growth and metastasis, which can pose risks of enhancing tumorigenesis (112). Additionally, potential off-target effects and unintended consequences associated with exosome therapy warrant careful investigation (164). Standardized production processes ensure high purity and consistency of exosomes, thereby reducing the presence of potential contaminants and improving the potential toxicities (80). Moreover, conducting comprehensive characterization of exosomes, including their size, surface markers, and proteomic analysis, to ensure they meet therapeutic standards (165). Consequently, it is essential to conduct more preclinical evaluations on exosomes that encompass assessments of pharmacokinetics, and toxicity profiles to minimize any potential adverse effects, which can promote the translation into clinical course.

## 9 Conclusion

Based on these emerging studies, the prospects for HCC therapy based on exosomes hold immense potential for clinical application. Exosomes derived from TAMs or tumor cells are skilled mediators of immune response, and their relatively straightforward manipulation of TME provides notable advantages, laying the foundation for future therapeutic uses in HCC. While our current comprehension of the specific mechanisms and functions of exosomes is still limited, there is a progressive unveiling of the "mysterious veil" that envelops the TME of HCC. Moreover, there are several challenges for hampering the clinical application of exosomes so far. Developing efficient strategies for exosome isolation, as well as establishing the safety and efficacy of cancer therapy based on exosome, are crucial research areas that need further attention. Further research should explore innovative engineering approaches for exosomes, such as genetic or surface modifications, to enhance their targeting capabilities and therapeutic potential. Furthermore, exploring the potential of

integrating exosomes with established chemotherapy or immunotherapy agents could enhance treatment efficacy and reduce side effects, especially in certain cancer contexts. Recent advancements in nanotechnology may be crucial in this regard, offering substantial benefits for clinical translation and holding considerable promise for HCC treatment.

## Author contributions

YX: Conceptualization, Data curation, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. LX: Conceptualization, Data curation, Investigation, Methodology, Project administration, Resources, Writing – review & editing. QC: Formal analysis, Methodology, Project administration, Software, Writing – review & editing. CZ: Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Visualization, Writing – review & editing. JH: Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Validation, Visualization, Writing – review & editing. LZ: Conceptualization, Methodology, Supervision, Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

## Publisher's note



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EDITED BY
Zhiqing Pang,
Fudan University, China

REVIEWED BY
Ephraim Ansa-Addo,
The Ohio State University, United States
Yan Wei,
Shanghai University, China

*CORRESPONDENCE
Xiyang Sun
732003356@shsmu.edu.cn
Min Liu
lm4104@shtrhospital.com

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# Role of extracellular vesicles in cancer: implications in immunotherapeutic resistance

Shuai Wang[1], Zerui Wang[2], Min Liu[3]* and Xiyang Sun[4]*

[1]Emergency Department, Tongren Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China, [2]Wuxi Medical College, Jiangnan University, Wuxi, Jiangsu, China, [3]Urology Department, Tongren Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China, [4]Hongqiao International Institute of Medicine, Tongren Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

Extracellular vesicles (EVs) are lipid membrane-bound vesicles involved in cell-cell communication, particularly in the context of cancer. Immunotherapy, a rapidly evolving field in oncology, is a type of cancer treatment relying on the body's own immune system to fight mutated cancer cells. Recently, the significance of immunotherapeutic resistance has been increasingly acknowledged owing to the heightened prevalence of cancer and its commonly advanced stage upon diagnosis. However, the complexity and heterogeneity of tumor cells pose challenges to immunotherapy, and the role of EVs in immunotherapeutic resistance remains unclear. Recent studies focused on the role of EVs as heterogeneous groups of nanoparticles in intercellular communication, particularly within the tumor microenvironment (TME). EVs, which include exosomes, shed microvesicles, while apoptotic bodies carry a diverse range of molecular cargo, including proteins, nucleic acids, lipids, and other bioactive molecules. The complexity and versatility of EVs make them a fascinating area of study, with promising implications for the future of immunology and medicine. This brief review highlights the involvement of EVs in immunotherapeutic resistance (e.g., PD-L1 transfer, miRNA-mediated pathways) with a focus on their biogenesis, secretion, and functional roles in cancer, underscoring their potential as diagnostic and therapeutic tools.

KEYWORDS

**extracellular vesicles, immunotherapeutic resistance, tumor microenvironment, cell communication, cancer progression**

## 1 Introduction

Extracellular vesicles (EVs) are membranous particles released by various cell types including cancer cells; they play a pivotal role in intercellular communication (1, 2). EVs mediate cellular signaling by transferring bioactive molecules between cells, thereby modulating cellular behavior and contributing to tumor progression, survival, and metastasis (3, 4). Moreover, cancer cells exploit EVs as vehicles for (5–7) ting drug-

resistant proteins or genetic material, thus facilitating the dissemination of resistance mechanisms within the tumor microenvironment and neighboring cells, ultimately leading to therapeutic failure. Furthermore, cancer-derived EVs can modulate the immune response by suppressing the immune cell activity, thereby establishing an immunosuppressive tumor microenvironment that facilitates tumor evasion from immune surveillance (8, 9). Consequently, these effects have garnered increasing attention with respect to immunotherapeutic resistance.

Cancer is a multifaceted disease characterized by dysregulated cellular proliferation and invasive behavior (10). A pivotal hallmark of cancer cells is their capacity to engage in intricate crosstalk with the surrounding microenvironment, including immune cells, stromal cells, and the extracellular matrix (11). This dynamic communication primarily occurs through the secretion of EVs, which exert regulatory effects on recipient cells, thereby modulating and shaping immune response (12, 13). Over the recent decades, extensive research has revealed diverse roles of EVs in cancer biology, particularly in immunotherapeutic resistance (14, 15).

Recent reports offer a comprehensive overview of the complex interplay between EVs and resistance to cancer immunotherapy (16). This highlights the multifaceted nature of this relationship, emphasizing the intricate mechanisms by which EVs influence immune responses and contribute to therapeutic resistance (17).

EVs play sophisticated roles in immune evasion and creation of an immunosuppressive microenvironment (18). Notably, cancer cells utilize EVs to export proteins or antigens, thereby evading immune detection and reducing their susceptibility to immunotherapies that target these specific antigens (19). This insight underscores the cunning tactics employed by cancer cells to subvert the body's natural defense mechanisms (20). Furthermore, the review points out the significant role of EVs in shaping the tumor microenvironment by delivering immunosuppressive molecules like TGF-β and PD-L1, which inhibits T cell activity and highlights the strategic deployment of EVs to dampen the immune response (21). Additionally, this review delves into the ability of EVs to transfer drug-resistance genes or proteins, leading to the emergence of drug-resistant cancer cell subpopulations. This aspect of EV function is particularly alarming as it suggests a mechanism for the spread of resistance within tumors, complicating the efficacy of immunotherapeutic strategies (22).

In addition, this review discusses the potential of EVs to modulate immune checkpoint molecule expression, which is a critical factor in resistance to immunes (23). This modulation can be further discussed in terms of its implications for the success of immunotherapies that rely on these checkpoints (24). Finally, the review emphasizes the dual role of EVs in instructing immune cells, such as dendritic cells, to either enhance or suppress anti-tumor immune responses. This duality is crucial for understanding how EVs influence the outcomes of adoptive cell transfer therapies to engineer immune cells for the effective targeting of cancer cells (14, 25).

Overall, this article provides a robust foundation for understanding the intricate relationship between EVs and immunotherapeutic (26). A more detailed review could further explore the nuances of this relationship, strategic maneuvers of cancer cells, and challenges posed to the development of effective immunotherapies.

## 2 Subtypes of EVs involved in immunotherapy resistance

Based on their biogenesis and biofunctions, EVs are primarily classified into exosomes, shed microvesicles, and apoptotic bodies (Figure 1).

### 2.1 Exosomes

Exosomes are small EVs (30–150 nm in diameter) that originate from the endosomal compartment and are generated by the inward budding of the plasma membrane, followed by the formation of multivesicular bodies (MVBs). Subsequently, MVBs merge with the plasma membrane to release exosomes into the extracellular space (27). Exosomes contain several types of specific surface markers, such as tetraspanins (CD9, CD63, and CD81), heat shock proteins (Hsp70 and Hsp90), MVB synthesis proteins (ALG-2-interacting protein X [Alix] and tumor susceptibility gene 101 [Tsg101]), and membrane transporters and fusion proteins (annexins and flotillin) (28). The maturation of MVBs involves the recruitment of specific proteins such as the endosomal sorting complex required for transport, which plays a crucial role in the scission of intraluminal vesicles (ILVs) into the MVB lumen. Exosomes are then released into the extracellular space upon the fusion of MVBs with the plasma membrane (29, 30).

In the intricate tapestry of cancer therapeutics, exosomes have emerged as formidable contributors to immunotherapy resistance in a spectrum of malignancies. Regarding their roles in gynecological cancers, exosomes harvested from cisplatin-resistant ovarian cancer cells encapsulate higher concentrations of cisplatin than their cisplatin-sensitive counterparts, thereby fortifying their resistance to chemotherapy (31). These vesicles are laden with an abundance of drug efflux pumps such as MRP2, ATP7A, and ATP7B, which are instrumental in chemoresistance (32). In breast cancer, adriamycin-resistant cells segregate the drug into exosomes, circumventing the anticipated nuclear accumulation. Moreover, exosomal conveyance of P-glycoprotein (P-gp) induces a chemoresistant phenotype in breast cancer cells (33). Docetaxel-resistant prostate cancer cells release exosomes with increased P-gp levels, outstripping them from their sensitive kin. Exosomes originating from gemcitabine-resistant triple-negative breast cancer cells demonstrate an uncanny ability to impart resistance to more susceptible cells (34). hey also play a role in the development of drug resistance by shuttling proteins, microRNAs, and a plethora of biomolecules capable of modulating therapeutic responses (35).

Engineered exosomes, designed to express the hepatocellular carcinoma antigen α-fetoprotein, have unveiled a potent anti-tumor response by dampening immunosuppressive cytokines and

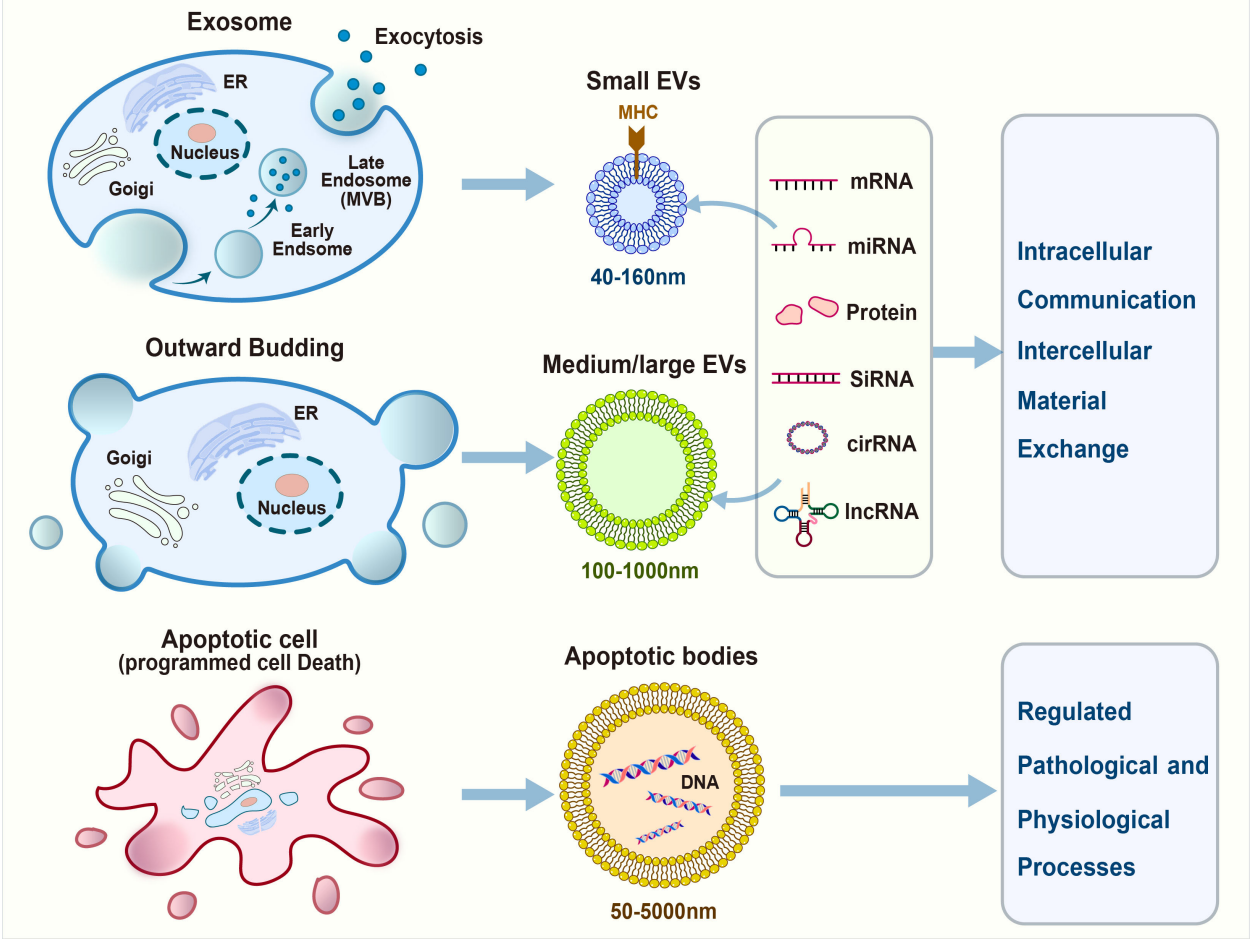


FIGURE 1
Extracellular vesicles (EVs) communicate with the immune cells in tumor microenvironment.

amplifying the presence of IFN-γ-expressing $CD8^+$ T cells (36). Exosomes derived from bone marrow mesenchymal stem cells augment chemosensitivity to cisplatin by delivering miR-199a-3p (37), which targets LRRC1 and mitigates drug resistance. Lung cancer cell-derived exosomes orchestrate drug resistance by conveying resistance-associated proteins and RNA. The presence of androgen receptor splice variant 7 within exosomal RNA has been correlated with resistance to hormonal therapy in prostate cancer, underscoring the potential of exosomal biomarkers for predicting treatment outcomes (38–40). Exosomes emanating from cancer-associated fibroblasts (CAFs) play a pivotal role in chemoresistance by fostering colorectal cancer cell stemness and epithelial-mesenchymal transition, which are pivotal in resistance to therapy. The transfer of exosomal miR-92a-3p from CAFs to colorectal cancer cells enhances stemness and epithelial-mesenchymal transition, thereby contributing to chemoresistance to 5-FU/L-OHP (41).

In the discourse on exosomes and their multifaceted roles in cancer, the majority of research underscores their pro-resistance functions; however, few studies have illuminated their contrasting effects. For instance, exosomes from A549 cells, a cisplatin-resistant human lung adenocarcinoma cell line, induce resistance to therapy by upregulating mTOR expression (42). This suggests that targeting the mTOR pathway is a potential strategy to overcome this resistance. However, it's important to recognize that these effects are not always simultaneous or reversible. In colorectal cancer, exosomes secreted by cancer cells induce resistance to 5-FU and oxaliplatin by activating the Wnt/β-catenin pathway, which promotes the stabilization and nuclear translocation of β-catenin (43). The inhibition of this pathway may be instrumental in reducing drug resistance. Furthermore, triple-negative breast cancer cells release exosomes that induce resistance to docetaxel and gemcitabine in nontumorigenic breast cancer cells (MCF10A). This resistance is mediated by the upregulation of the PI3K/AKT, MAPK, and HIF1A pathways in MCF10A cells (44). In hepatocellular carcinoma, the interaction between the high-mobility group box 1 gene, the RICTOR molecule in the mTOR pathway, and members of the miR-200 family promote glutamine metabolism and tumorigenesis (45). This interaction can reduce the efficacy of anti-PD-L1 immunotherapy in hepatocellular carcinoma. The ORAI1 calcium channel regulates intracellular calcium concentration and affects the secretion of exosomes carrying PD-L1 immune molecules. Silencing the ORAI1 channel in tumor cells inhibits the secretion of PD-L1 exosomes, increases $CD8^+$ cells, and impedes tumor progression (46).

These studies highlight the intricate roles of exosomes in modulating immune responses and drug resistance in cancer and shed light on the complexity of exosomal functions and the need for a nuanced understanding of their mechanisms which may pave the way for the development of novel therapeutic interventions.

## 2.2 Shed microvesicles

Shed microvesicles (SMVs), ranging from 100 nm to 1 μm in diameter, directly bud from the plasma membrane. Microvesicles, a subtype of EVs, unlike exosomes, originate without involvement of the endosomal compartment. They are also referred to as the ectosome and originate from outward protrusions of the plasma membrane. Several proteins have been identified as microvesicle-specific, including CD40, ADP-ribosylation factor 6 (ARF6), selectins, phosphatidylserine, and Rho family members.

CAFs secrete EVs rich in annexin A6, which, in gastric cancer cells, stabilize β1 integrin and upregulate the expression of focal adhesion kinase (FAK)-Yes-associated protein (YAP), thereby enhancing cell survival post-cisplatin treatment (47, 48). This suggests a strategic role for EVs in modulating the tumor microenvironment. Furthermore, exosomal transfer of miR-21 from adipocytes to ovarian cancer cells mitigated paclitaxel-induced apoptosis by downregulating apoptotic peptidase activating factor mRNA. This highlights the potential role of EVs in the intercellular communication that influences chemosensitivity. In hepatocellular carcinoma (HCC), sorafenib resistance is induced by the delivery of hepatocyte growth factor (HGF) through EVs, which activates the HGF/c-MET/PI3K/AKT signaling pathway. This pathway activation underscores the significance of EVs in resistance mechanisms and suggests that inhibiting them could be a strategy to mitigate resistance (42, 49).

Macrophage-derived EVs transmit miR-365, which confers gemcitabine resistance to pancreatic adenocarcinoma cells both *in vitro* and *in vivo* (50). This raises the possibility that modulating the cargo of EVs could be a viable approach to overcome drug resistance. CAF-derived exosomal miR-92 significantly promoted T-cell apoptosis and conferred immunotherapy resistance in breast cancer cells. miRNA-92 binds to LATS2, which interacts with YAP1. Chromatin immunoprecipitation confirmed that YAP1 binds to the enhancer region of PD-L1 after nuclear translocation, thereby promoting its transcriptional activity. This revealed a complex regulatory network involving EVs in immune evasion. Hypoxic conditions induce the production and secretion of circEIF3K-containing exosomes from CAFs, which reduces miR-214 expression and upregulates PD-L1 expression, leading to enhanced colorectal cancer cell proliferation, migration, metastasis, and immune escape. This further emphasizes the role of EVs in tumor progression and immune modulation (51, 52).

The following examples highlight the complex interactions between CAFs, EVs, and the tumor microenvironment, and how they contribute to immunosuppression and drug resistance. In EOC, the CAF-derived protein FMO2 facilitates lymphocyte infiltration; higher levels of FMO2 are associated with worse prognosis, suggesting its potential as a biomarker for predicting immunotherapy sensitivity. Hypoxia also induces the secretion of immunosuppressive factors such as TGF-β, VEGF, and PD-L1 from CAFs, which exert an inhibitory effect on T cell-mediated cytotoxicity. This implies the generation of an immunosuppressive microenvironment fostered by CAFs and the potential for targeting these factors to enhance immunotherapeutic outcomes. In colorectal cancer, CD133-containing microvesicles have been identified as promoters of cancer progression by inducing the M2-like polarization of tumor-associated macrophages, a process that could be targeted to combat resistance to immunotherapy. Resistance to sorafenib in invasive HCC cell lines can be attributed to the delivery of HGF via extracellular EVs, which activate the HGF/c-MET/PI3K/AKT signaling pathway. Inhibition of this pathway can potentially mitigate sorafenib resistance (53–55).

Acknowledging the contribution of microvesicles in conferring resistance to immunotherapies is crucial. However, they also present an exceptional opportunity for therapeutic interventions. EVs play a pivotal role in modulating the immune system functions by transporting pro-survival molecules with the potential to induce immune tolerance, thereby facilitating the evasion of immune surveillance by cancer cells (56). Targeting the pro-survival molecules encapsulated within EVs could significantly augment the efficacy of immunotherapeutic approaches. By inhibiting the immunosuppressive effects of microvesicles and these pro-survival molecules, we aim to enhance the effectiveness of immunotherapies and advance personalized and potent cancer treatments.

## 2.3 Apoptotic bodies

Apoptotic bodies, the largest among EVs with sizes ranging from 50 nm to 5 μm, are generated during the twilight hours of apoptosis (57). As the cell undergoes its final stages, its membrane blebs and fragments, releasing bodies laden with cellular remnants. Apoptotic bodies stand out because of their substantial girth compared with their smaller counterparts, exosomes, and microvesicles. Annexin V and histones are apoptotic body-specific proteins (58, 59).

# 3 Biofunction of EVs in cancer

EVs play a pivotal role in the intricate interplay between the immune system and cancer, particularly in their relationship with immunotherapeutic resistance. EVs are small messengers that can influence how our immune cells behave, which is a major concern in cancer growth and spread. They carry various molecules that can either calm the immune response against tumors or create a welcoming environment for cancer cells to thrive. Cancer cells use EVs for their advantage through sending out signals that promote their own growth and spread (15, 60, 61).

The potential of EVs in cancer treatment is an exciting research area. Similar to the creation of a vaccine, EVs can be trained to carry cancer-fighting agents. There is ongoing research to determine whether

EVs from dendritic cells can boost the immune response to cancer (62–64). The relationship between EVs and cancer resistance is complex. Cancer cells use EVs to dodge immune attacks, create an environment that suppresses the immune system, and confer resistance to other cancer cells. EVs can also instruct immune cells how to respond to cancer, sometimes by downregulating them (65).

## 4 EVs and immune cells

EVs are multifaceted players in the immune system and have the potential to serve as biomarkers and therapeutic agents (66). The influence of EVs on immune cells is a topic of profound interest because these vesicles have a significant impact on both the innate and adaptive arms of the immune system through a myriad mechanisms. EVs are not merely passive bystanders but also active participants in the modulation of immune responses, through activating, suppressing, or even facilitating communication between various immune cells (Figure 2). This multifaceted role is particularly evident in the cargo they carry, which reflects their cell of origin and functional state, and encompasses proteins, lipids, miRNAs, and other bioactive molecules that can profoundly influence the activation, differentiation, and effector functions of immune cells (67, 68). EVs should be recognized as pivotal messengers in the immune system, conveying crucial information among immune cells, and thereby fine-tuning immune responses while maintaining delicate homeostasis. The diversity of proteins, nucleic acids, and other bioactive molecules they transport underscores their indispensable role in orchestrating the intricate and tightly regulated processes that underpin immune defenses (69, 70).

Dendritic cells (DCs), which are integral to innate immunity, primarily function as antigen-presenting cells. EVs transfer immune signals between DCs and tumor cells, with potential implications for vaccines targeting tumor immune escape. The presence of phosphatidylserine on the membranes of tumor-derived EVs can bind to CD300a on DCs, reducing IFN-β production and influencing the regulatory T cell (Treg) population. Tumor-associated macrophages and neutrophils (TAMs and TANs), which are prominent scavengers of tumor immunity, communicate with tumor cells through EVs, guide tumor progression, and present promising therapeutic targets. TDEVs facilitate TAM infiltration and participate in establishing an inflammatory immune environment. Finally, EVs act as messengers between tumor cells and natural killer cells, potentially serving as steppingstones in novel therapies (71, 72).

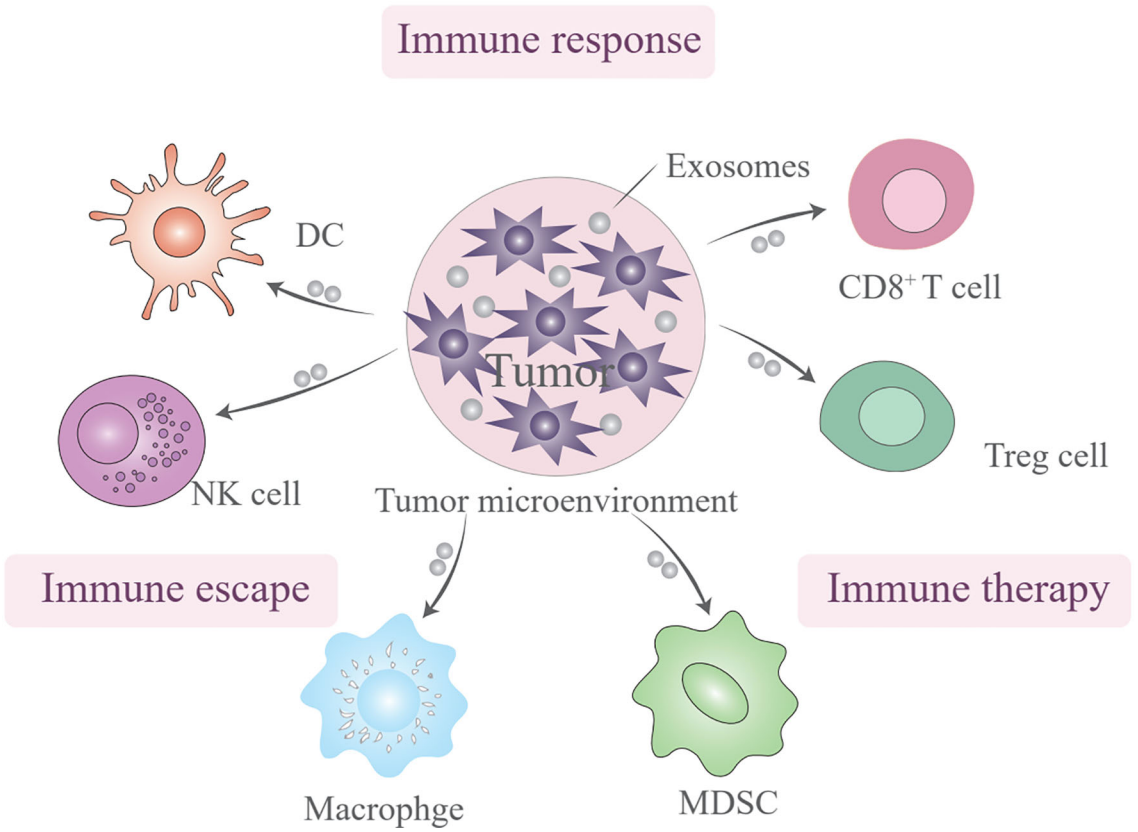


FIGURE 2
The subtypes of extracellular vesicles (EVs) involved in immunotherapy resistance.

EVs play a multifaceted role in the tumor microenvironment (TME), influencing cancer cell behavior and immune response within the tumor microenvironment.

In the realm of immune functions, EVs, especially those derived from antigen-presenting cells, harbor major histocompatibility complex (MHC) molecules that are instrumental in presenting antigens to T cells and modulating adaptive immune responses. This mechanism is of paramount importance for initiating specific immune responses against pathogens or tumors (73, 74). Furthermore, EVs can display immune checkpoint molecules such as programmed death ligand 1 and cytotoxic T lymphocyte antigen 4 on their surface, interacting with receptors on T cells and natural killer cells to inhibit their activity or induce apoptosis, thus playing a critical role in immune evasion in some cancers. It is important to highlight the immunosuppressive properties of certain immune cell-derived EVs. For instance, regulatory T cell-derived EVs (Treg-EVs) carry immunosuppressive molecules like CTLA-4 and TGF-β, which contribute to maintaining immune tolerance and preventing autoimmune reactions, thereby highlighting their role in immune regulation. EVs released by neutrophils, macrophages, and other immune cells contain bioactive molecules such as cytokines, chemokines, and lipid mediators, which can either promote or resolve inflammatory reactions. This implicates them in the pathophysiology of various diseases and positions them as potential therapeutic targets for modulating inflammation (75, 76).

In the context of cancer immunotherapy, EVs have demonstrated their potential to deliver antigens and immunomodulatory molecules to enhance anti-tumor immune responses. A striking example is the release of EVs by chimeric antigen receptor (CAR) T-cells carrying surface CARs, which recognize and induce the death of tumor cells expressing CAR-specific tumor antigens (77).This innovative approach demonstrates the therapeutic potential of EVs in cancer. Moreover, studies have revealed that EVs render target cells more susceptible to inflammatory signals and induce systemic immune responses. They can even render non-responsive cells susceptible to inflammatory agonists, with their inflammatory activity remaining unaffected by soluble receptor antagonists, which is a significant finding for understanding the role of EVs in inflammation (78). A deeper understanding of their biogenesis, cargo, and functional roles is essential to gain insights into the immune system functions and uncover novel therapeutic avenues.

## 5 Immunotherapeutic resistance and EVs

Current immunotherapies mainly focus on the effector arm of the immune system, such as reactivating T cell responses by blocking immune checkpoints, blocking immune checkpoints in cancer immunotherapy, activating adaptive immune responses using tumor vaccines, or directly transferring engineered T cells to tumors (79). For example, Universal immunotherapeutic strategy for hepatocellular carcinoma with exosome vaccines that engage adaptive and innate immune responses (80). Understanding and overcoming this resistance are crucial for improving patient outcomes and the overall success of immunotherapies (81, 82).

The primary reason for overcoming immunotherapeutic resistance is the limited effectiveness of the current immunotherapies. Despite the achieved significant progress in cancer treatment, a substantial number of patients do not respond to immune checkpoint inhibitors or eventually develop resistance, leading to disease progression. First, EVs act as diligent postmen of the TME, shuttling genetic information and various bioactive cargos between different cell types within this microenvironment. This communication network is crucial for maintaining the malignant capacity of tumor cells and plays a significant role in tumor progression and immunotherapeutic dysfunction. For instance, colorectal cancer-derived EVs containing miRNAs can modulate the behavior of recipient cells, thereby influencing the tumor fate. CAFs, a predominant component of stromal cells in the TME, contribute to tumor progression and chemoresistance through their metabolic patterns and secretion of cytokines and chemokines. EVs are indispensable in this reciprocal symbiotic dialogue between tumor cells and CAFs, often converting normal fibroblasts into CAFs through TGF-β or STAT pathways. When we consider the interaction between tumors and endothelial cells, EVs have emerged as initiators of metastasis. They facilitate distant metastasis by affecting the proliferation, migration, and permeability of endothelial cells. For example, in prostate cancer, PGAM1 is transported to human umbilical vein endothelial cells through EVs, thus influencing their metastatic potential (83, 84).

EVs play a role in the resistance to various anticancer therapies. They increase drug efflux, decrease drug toxicity, and enhance DNA repair, contributing to chemoresistance. EVs derived from mesenchymal stem cells contribute to the development of therapeutic resistance to chemoresistance, targeted therapy, and immunotherapy. CAR-T cell activity is suppressed by EVs from cancer-associated fibroblasts (CAFs) that deliver immunosuppressive cytokines (e.g., TGF-β, IL-10), fostering a TME enriched with regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) (85).EVs act as double-edged swords in these therapies. Tumor-derived EVs (TDEVs) carry functional PD-L1, which binds PD-1 on T cells, mimicking immune checkpoint interactions and blunting ICI efficacy (86). EVs also scavenge tumor-associated antigens (TAAs), reducing antigen availability for dendritic cell (DC) priming and adaptive immune activation. Additionally, The role of the TME in resistance is further complicated by its heterogeneity, with different regions within the same tumor potentially having distinct immune landscapes (87, 88). This heterogeneity can lead to differential responses to therapy and outgrowth of resistant clones.

EVs drive resistance through diverse mechanisms that compromise immune cell function and enhance tumor survival: (1) Immune suppression via cargo transfer, TDEVs deliver immuno suppressive molecules (e.g., TGF-β, adenosine) to expand Tregs and MDSCs, creating an inhibitory TME. Exosomal miR-21 from adipocytes downregulates apoptotic pathways in ovarian cancer cells, conferring resistance to paclitaxel (89). (2) Antigen masking

and immune evasion EVs shed TAAs (e.g., HER2, MUC1), reducing antigen visibility and limiting T cell recognition. CAF-derived EVs transfer miR-92a-3p to colorectal cancer cells, promoting stemness and resistance to 5-FU/oxaliplatin by activating Wnt/β-catenin signaling. Exosomal PD-L1 suppresses $CD8^+$ T cell activity, while ORAI1 calcium channel inhibition reduces PD-L1 exosome secretion, restoring T cell infiltration (90, 91). (3) Activation of pro-survival pathways: Hepatocyte growth factor (HGF) in EVs activates the HGF/c-MET/PI3K/AKT axis in hepatocellular carcinoma (HCC), driving resistance to sorafenib. (4) Drug Efflux and Metabolic Reprogramming: EVs export chemotherapeutics (e.g., cisplatin) via drug efflux pumps (e.g., MRP2, P-gp). Exosomal circEIF3K from hypoxic CAFs reduces miR-214 levels, upregulating PD-L1 and promoting immune escape in colorectal cancer (92).

Despite their role in resistance, EVs hold promise for improving immunotherapy outcomes through strategic engineering: (1) EV-Based Immune Activation: Dendritic Cell-Derived EVs Loaded with TAAs, these EVs act as vaccines to prime cytotoxic T cells and enhance antigen presentation (93). CAR-T Cell-Derived EVs display CARs on their surface, enabling bystander killing of antigen-negative tumor cells and overcoming tumor heterogeneity (94, 95). (2) Reversing Resistance Mechanisms: silencing exosomal PD-L1 or miR-92a-3p restores T cell activity and checkpoint inhibitor sensitivity (96–98). Engineered EVs carrying CRISPR-Cas9 can knockout resistance genes (e.g., β-catenin) in recipient cells (99). (3) Targeted Delivery Systems: EVs loaded with immunostimulatory molecules (e.g., IFN-γ, IL-12) reprogram the TME to support anti-tumor immunity. Hybrid EVs fused with liposomes enhance drug delivery to tumors while minimizing off-target effects (100).

In summary, the interplay between EVs and immunotherapeutic resistance underscores their dual role as both adversaries and allies in cancer treatment. While EVs facilitate immune evasion and therapy resistance through immunosuppressive cargo and pathway activation, their engineering potential offers innovative strategies to enhance ICIs, CAR-T therapies, and personalized medicine. Future research should focus on deciphering EV heterogeneity and optimizing delivery platforms to fully exploit their therapeutic capabilities (101).

## 6 Conclusion

EVs are multifaceted players in the cancer landscape that influence tumor biology, immune responses, and therapeutic outcomes. The realm of cancer immunotherapy has been invigorated by the advent of EVs, which have emerged as potent vehicles for the delivery of antigens and immunomodulatory molecules, thereby amplifying anti-tumor immune responses. This development is particularly noteworthy, given the remarkable capacity of EVs released by CAR-T cells to carry surface CARs, enabling them to specifically target and induce the death of tumor cells bearing CAR-matching tumor antigens. This innovative strategy not only underscores the therapeutic potential of EVs in oncology but also represents a significant stride in the field of targeted cancer therapies.

Furthermore, the sensitization of target cells to inflammatory signals and eliciting systemic immune responses by EVs are crucial. The ability of EVs to convert nonresponsive cells into inflammatory agonist-susceptible cells, with their inflammatory activity remaining impervious to soluble receptor antagonists, is a groundbreaking finding. This aspect of EV biology is particularly intriguing as it sheds light on its role in inflammation and suggests that it may play a key role in modulating immune responses in a variety of pathological contexts.

Further study should confront the following three understudied dimensions to advance EV-based therapeutics: a. Spatiotemporal Heterogeneity of EV Cargo. Current studies predominantly focus on bulk EV analysis, neglecting subpopulation-specific functions. For example, apoptotic bodies versus exosomes exhibit divergent roles in mediating radiation resistance versus chemotherapy tolerance. Single-vesicle profiling technologies could unravel this complexity. b. EV-Driven Metabolic Reprogramming: Emerging evidence suggests that CAF-EVs transfer lactate dehydrogenase A (LDHA) and glutamine synthetase to tumor cells, fostering an acidic, nutrient-depleted microenvironment that impairs T-cell glycolysis and cytotoxicity. c. Host-Microbiota-EV Axis: Gut microbiota-derived EVs modulate systemic immunity by regulating PD-L1 expression on dendritic cells. However, their impact on ICB resistance remains unexplored—a critical omission given the clinical correlation between dysbiosis and immunotherapy failure.

As we delve deeper into the understanding of EV biogenesis, cargo, and functional roles, it becomes increasingly clear that these vesicles are not just passive participants but also active mediators in the complex interplay of the immune system. This deeper understanding is vital for deciphering the intricacies of immune function and paving the way for innovative therapeutic strategies. Harnessing EVs for cancer treatment holds immense promise, and continued research on their mechanisms of action will undoubtedly yield valuable insights and potential clinical applications.

## Author contributions

SW: Writing – original draft. ZW: Writing – review & editing. XS: Funding acquisition, Project administration, Writing – original draft. ML: Funding acquisition, Resources, Supervision, Writing – original draft.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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EDITED BY
Sheila Spada,
Hospital Physiotherapy Institutes (IRCCS), Italy

REVIEWED BY
Peng Xu,
Yale University, United States
Guo Zhao,
Chinese Academy of Medical Sciences and Peking Union Medical College, China

*CORRESPONDENCE
Cristina Bottino
cristina.bottino@unige.it

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# Exosomes and immune modulation: implications for neuroblastoma immunotherapy

Martina Morini[1], Chiara Vitale[2], Martina Ardito[1], Alessandra Dondero[2], Katia Cortese[2], Cristina Bottino[2,3]* and Roberta Castriconi[2,3]

[1]Laboratory of Experimental Therapies in Oncology, IRCCS Istituto Giannina Gaslini, Genova, Italy, [2]Department of Experimental Medicine, University of Genova, Genova, Italy, [3]Laboratory of Clinical and Experimental Immunology, IRCCS Istituto Giannina Gaslini, Genova, Italy

Exosomes are nano-sized extracellular vesicles involved in cell homeostasis. Tumor-derived exosomes (TDEs) promote tumor progression by creating an immunosuppressive tumor microenvironment (TME), inhibiting T and NK cell activity, preventing dendritic cell maturation, and expanding immunosuppressive cell populations. Cancer Stem Cell (CSC)-derived exosomes further trigger functional changes in immune cells subsets, enhancing immune suppression. Consequently, blocking the release or the uptake of TDEs significantly impact immunotherapy efficacy, making them potential therapeutic targets. On the other hand, NK cell-derived exosomes can be engineered to carry immune-activating molecules or inhibitors of immune checkpoint molecules to elicit immune responses. This review highlights the interplay between TDEs and immune cells, particularly NK cells, in different tumors, with a focus on neuroblastoma, and explores exosome-based strategies to improve immunotherapy efficacy.

KEYWORDS

**exosomes, immunotherapy, tumor microenvironment, neuroblastoma, NK cells, cancer stem cells**

## 1 Introduction

Exosomes are nano-sized extracellular vesicles released by different cell types. They mediate intercellular communication by carrying a variety of biomolecules including proteins, lipids, and nucleic acids (1). Their function affects physiological and pathological processes, particularly in cancer biology. Tumor-derived exosomes (TDEs) interact with tumor and stromal tissues, reprogramming cells and facilitating tumor initiation and progression (2). Specifically, TDEs induce the establishment of an

immunosuppressive tumor microenvironment (TME) by different mechanisms, including the suppression of T and Natural Killer (NK) cell activation and proliferation, the inhibition of maturation of Dendritic Cells (DCs), and the functional reprogramming of macrophages (3).

An immunosuppressive TME represent the main barrier to the efficacy of immunotherapeutic approaches that are often included in combined treatment against aggressive neoplastic diseases.

Neuroblastoma (NB) - the most common extracranial cancer diagnosed during infancy and originating from neural crest cells - has heterogeneous clinical manifestations, ranging from localized low risk disease to metastatic, high-risk (HR) tumors. The current therapeutic strategy for HR-NB patients includes immunotherapy with a monoclonal antibody targeting the disialoganglioside GD2, a marker expressed on NB cell surface (4). The crosstalk between tumor associated macrophages (TAMs) and NK cells play a major role in favoring tumor progression and immunotherapy resistance (5).

Moreover, solid tumors including NB are characterized by the presence of Cancer Stem Cells (CSCs), a rare cellular population with highly malignant features (6). CSCs release exosomes carrying stemness markers and regulators, which can trigger functional changes in immune cells, further contributing to the suppression of the immune response (7).

The multiple interactions occurring between tumor and immune cells via TDEs bring forward the impact of the released vesicles on the efficacy of immunotherapies. Reducing the immune suppression by blocking the release or the uptake of TDEs could have therapeutic benefits. A deeper knowledge of TDEs-mediated effects on the immune system would allow us to develop new treatments.

The present review will discuss the most relevant results on the crosstalk between TDEs and immune cells with a focus on NB. We will report how such interaction can shape the TME and, consequently, the response to immunotherapy-based treatments and how exosomes could be employed for developing novel therapeutic strategies aimed at improving immunotherapy efficacy.

## 2 The role of tumor-derived exosomes in immune suppression

Different components of the TME, including endothelial cells, extracellular matrix, fibroblasts, and immune cells, strongly influence cancer initiation and progression. The crosstalk between these components and tumor cells occurs mainly through the secretion of specific molecules exerting pro or antitumor activity. Over the last years, it has been shown that cellular communication occurs also through the release of TDEs, and extracellular vesicles, especially exosomes, that help cancer cells communicate also with distant cells. TDEs promote tumor growth and metastasis but also shape an immunosuppressive TME interfering with the activity of different immune cell populations (Figure 1).

### 2.1 TDEs modulate T cell immunity and macrophage function through PD-L1 and TGF-β

TDEs can suppress T cell activation and proliferation by carrying molecules such as programmed death-ligand 1 (PD-L1), transforming growth factor-β (TGF-β), and specific microRNAs. PD-L1 is a checkpoint molecule that binds to PD-1 on T cells, preventing their activation and enabling immune evasion. It has been observed in metastatic melanoma that exosomes carry surface PD-L1 (exo-PD-L1). Its expression is upregulated by Interferon-γ (IFN-γ), and in patients the levels of circulating exo-PD-L1 positively correlated with IFN-γ expression (8). Exo-PD-L1 was shown to inhibit the proliferation and the cytotoxic activity of CD8 T cells, and higher levels of exo-PD-L1 were associated with poorer response to treatment and clinical outcomes. High levels of exo-PD-L1 in pre-treated melanoma patients may reflect the exhaustion of T cells, making anti-PD1 therapy ineffective. For on-treatment patients, exo-PD-L1 levels increased early during treatment, correlating with T-cell reinvigoration and better clinical outcomes. This could reflect the attempt of tumor cells to inactivate CD8 T cells, which is though prevented by the ongoing anti-PD1 treatment that block the PD-L1/PD1 axis (8). These findings suggest that exo-PD-L1 may serve as a potential biomarker for melanoma progression and response to immunotherapy.

Within the TME, tumor-associated macrophages (TAMs) also express PD-L1, allowing them to contribute to anti-tumor response impairment. Exosomes mediate the crosstalk between TAMs and tumor cells, favoring tumor growth and progression. TDEs can induce TAMs to polarize towards the M2 phenotype, which is associated with increased PD-L1 expression and the release of exosomes inducing PD-L1 expression in tumor cells, a phenomenon mediated by exosomal molecules including microRNAs and cytokines (9). In gliomas, M2-polarized TAMs release exosomes enriched with LINC01232, a long non-coding RNA (lncRNA) that interacts with E2F2, promoting its nuclear entry and the subsequent transcription of the autophagy transport receptor NBR1 (10). This causes an increased degradation of MHC-I, reducing MHC-I expression on the tumor cell surface, and enabling the evasion from CD8+ T cell-mediated recognition.

The plasma of patients with head and neck squamous cell carcinoma is characterized by the presence of exosomes containing TGF-β (11). Importantly, the exosome concentration of exosome-associated TGF-β (exo-TGF-β) reflects tumor size and decreases after therapy. These findings suggest the potential use of exosome-associated TGF-β as a biomarker. Importantly, exo-TGF-β are also involved in different pro-tumor pathways including angiogenesis (11), the formation of cancer-associated fibroblasts (CAF) (12), and the initiation of the pre-metastatic niche formation (13). It has also been reported that exo-TGF-β can drive the remodeling of the pulmonary vascular niche, facilitating the lung colonization of triple-negative breast cancer cells (14). In pancreatic cancer cells, TGF-β mRNA is transferred via exosomes in recipient

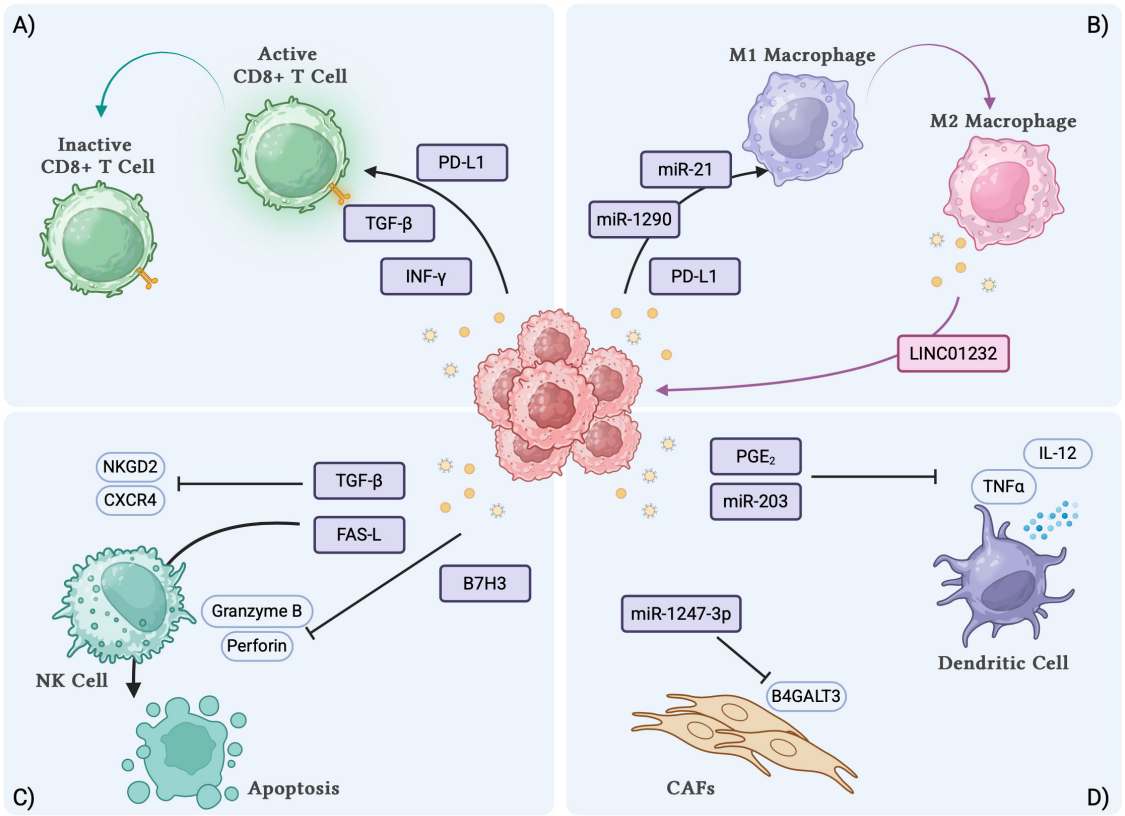


FIGURE 1
TDEs interactions with immune cell subsets and biological effects. **(A)** TDEs induce the inactivation of CD8+ T cells. **(B)** TDEs induce the polarization of macrophages towards M2 phenotype. **(C)** TDEs can inhibit the activation and induce the apoptosis of NK cells. **(D)** TDEs inhibit the secretory function of DCs and induce the transformation of fibroblasts to CAFs. TDEs, tumor derived exosomes; DCs, dendritic cells; CAFs, cancer associated fibroblasts. Created with BioRender.com.

cancer cells, leading to increased TGF-β protein and activation of epithelial to mesenchymal transition (EMT), promoting early-stage metastasis (15).

## 2.2 TDEs carry immunomodulatory non-coding RNAs

### 2.2.1 Exosomal microRNAs

TDEs immunomodulatory function is achieved also through the transfer of specific microRNAs, small non-coding RNA molecules regulating protein translation. The exosomal miR-1290 has been identified as a key player in antitumor immunity. Hypoxic lung adenocarcinoma cells release exosomes containing high levels of miR-1290, which targets and inhibits the suppressor of cytokine signaling 3 (SOCS3) (16). SOCS3 is involved in the maintenance of M1-type macrophages, thus its inhibition promotes the polarization toward the M2 phenotype. In hypoxic hepatocellular carcinoma, exo-miR-1290 induces the M2 polarization of macrophages by inhibiting Akt2 and upregulating PD-L1, stimulating CD8+ T cells apoptosis and triggering the EMT process, which promotes metastasis formation (17). In hepatoblastoma, the most frequent pediatric liver cancer, miR-126 delivered via exosomes promotes the differentiation of bone marrow mesenchymal stem cells into cancer stem cells, sustaining cancer growth, invasion and metastasis (18). Ewing sarcoma (EWs) cells expressing IGF2BP3 protein can release extracellular vesicles with a specific miRNA cargo that can positively regulate the migration rate of recipient cells, promoting metastasis formation (19). Moreover, EWs cells in hypoxic conditions produce miR-210-enriched exosomes, which downregulates the pro-apoptotic protein CASP8AP2, thus inhibiting apoptosis in recipient cancer cells (20).

The pro-metastatic effect of exosomes has been also reported in rhabdomyosarcoma (RMS), a rare soft tissue cancer affecting children. Exosomes released by metastatic RMS carry the transmembrane protein CD147, which facilitates the crosstalk between tumor and stromal cells, enhancing tumor invasive properties and modulating the TME (21).

MiR-21 is also known for its oncogenic function in different cancer types, including non-small cell lung cancer, breast cancer, and head and neck squamous cell carcinoma (HNSCC) (22–24). HNSCC cancer cells overexpress miR-21 and release TDEs abundant in miR-21. Such miR-21-enriched TDEs are taken up by TAMs that polarize toward M2, favoring the establishment of an immunosuppressive TME (25). TDEs enriched in miR-21 have also been detected in osteosarcoma (OS), a malignant pediatric tumor of

the bone. Exosomes released by human-derived OS cell lines with different metastatic potential showed the upregulation also of miR-143-3p, miR-181a-5p, and miR-148-5p. These miRNAs are actively involved in in promoting cell motility and invasion and regulating apoptotic processes (26). Importantly, OS-derived exosomes carry miR-25-3p that can promote capillary formation and, thus, induce angiogenesis (27).

Within the TME, cancer-associated fibroblasts (CAFs) actively contribute to tumor spread and response to treatment through the release of growth factors and proinflammatory cytokines attracting immunosuppressive cells and facilitating immune evasion (28). Tumor cells can shape the TME by communicating with CAFs via exosomes. It has been reported that tumor-derived exosomal miR-1247-3p in lung pre-metastatic niche induce the fibroblast to CAFs conversion by activating the β1-integrin–NF-κB signaling pathway. Specifically, exo-miR-1247-3p inhibits the expression of B4GALT3, which glycosylates β1-integrin, resulting in the activation of the NF-κB signaling pathway (29). CAF-released exosomes can also shape the TME by communicating with cancer cells. CAF-derived exosomes containing miR-1228 promote the migration of OS cells through the downregulation of the *SCAI* gene, which negatively regulates cancer cell invasion (30).

TDEs can hinder DC maturation and the production of pro-inflammatory molecules but also suppress the differentiation of myeloid precursors and induce cell apoptosis (31). In prostate cancer, TDEs carry prostaglandin $E_2$ ($PGE_2$), which is responsible for inducing the expression of the CD73 ecto-5-nucleotidase on the DC surface. The expression of CD73 resulted in the inhibition of TNFα- and IL-12 production by DCs (32). Similarly, in pancreatic cancer, TDEs contain miR-203, which downregulates the expression of TLR4 on the DCs surface leading to a significant decrease of TNFα and IL-12 release (33).

### 2.2.2 Exosomal long non-coding RNAs

Long non-coding RNAs (lncRNAs) are included among the immunomodulatory molecules transferred by TDEs. A list of the most relevant exosomal lnc-RNAs (exo-lnc-RNAs) is reported in Table 1. Exosomal lncRNAs (exo-lncRNAs) mediate the communication between cancer cells and immune cell subsets, contributing to the establishment of a tumor-favorable microenvironment. To this end, exo-lncRNAs play a major role in metabolic reprogramming. It is known that cancer cells show an altered metabolic activity compared to normal cells; they rely on aerobic glycolysis, also known as the Warburg effect, which ensures high energy availability to sustain a high proliferation rate (34).

Hypoxic non-small-cell-lung cancer (NSCLC)-derived exosomes are enriched in lncRNA-p21, which promotes angiogenesis. The knockdown of lncRNA-p21 results in the downregulation of metabolism-related genes, negatively affecting tumor growth and invasive properties (35). Moreover, higher levels of exo-lncRNA HOTAIR are associated with poor prognosis in breast cancer patients, and it can induce EMT of breast cancer cells and metastasis formation (36, 37). This effect is likely due to the mTOR-dependent stimulation of the glycolytic pathway, as it has been reported in hepatocellular carcinoma (38).

TABLE 1 List of exo-lnc-RNAs exerting immunomodulatory functions in different tumors.

| LncRNA | Tumor | Cell of origin | Function |
|---|---|---|---|
| lncRNA-p21 | NSCLC | Hypoxic tumor cells | Induce angiogenesis |
| HOTAIR | Breast cancer | Tumor cells | Induce EMT and metastasis |
| SNHG3 | Breast cancer | CAFs | Induce aerobic glycolysis |
| HISLA | Breast cancer | TAMs | Induce glycolysis and chemoresistance |
| FLJ22447 | OSCC | CAFs | Induce fibroblast malignant transformation |

NSCLC, non-small cell lung cancer; EMT, epithelial to mesenchymal transition; CAFs, cancer associated fibroblasts; TAMs, tumor associated macrophages; OSCC, oral squamous cell carcinoma.

In breast cancer, another key lnc-RNAs, SNHG3 (small nucleolar RNA host gene 3), is released via exosomes by CAFs. SNHG3 can sponge miR-330-5p, leading to a positive regulation of pyruvate kinase M2 (PKM2). PKM2 activation triggers aerobic glycolysis and enhances tumor cell proliferation (39). Additionally, the aerobic glycolysis in breast cancer is further enhanced by TAM-derived exosomes containing the lncRNA HISLA (HIF-1α-stabilizing lncRNA). Besides favoring the proliferation of tumor cells, HISLA can also protect them from chemotherapy-induced apoptosis (40). Tumor cell resistance to chemotherapy can also occur through autophagy. Autophagy is a physiological catabolic process required to remove damaged organelles, aberrant proteins or intracellular pathogens, which can also be used by cancer cells to protect themselves from stress and to replenish their energy supply. Importantly, its regulation can affect the efficacy of immunotherapies (41). It has been reported that in oral squamous cell carcinoma CAFs secrete exosomes containing lncRNAs responsible for the malignant transformation of normal stromal fibroblasts, supporting tumor growth (42).

Taken together, these results point out the key role of exo-lncRNAs in the communication between cancer cells and the TME. This crosstalk aims at promoting tumor cell metabolic reprogramming, proliferation, migration and chemoresistance.

## 2.3 TDEs impair NK cell function

NK cells represent the front-line defense against the malignant neoplastic transformation of cells (43). NK cell function is ensured by several receptors; the inhibitory ones interact with self-molecules whereas the activating receptors specifically recognize ligands up-regulated or *de novo* expressed at the surface of malignant cells, without the need for previous antigen exposure (44). It has been reported that many tumors can release TDEs causing NK cell dysfunction favoring cancer progression. TDEs lead to the downregulation of the expression of activating receptors such as NKGD2 and NKp30, impairing NK cell recognition and cytotoxic

effects (45, 46). The interplay between TDEs and NK cells occurs mainly through surface receptor-ligand interaction rather than internalization. In acute myeloid leukemia, TDEs act via the TGFβ/TGFβRI/II pathway to suppress the migration and the cytotoxicity of NK-92 cells, altering cytokine production and reducing the expression of surface receptors (NKGD2, CXCR4) also involved in migration. TDEs prevent NK-92 cells from targeting leukemia cells effectively, hindering the adoptive cellular therapy based on infused NK-92 cells (47).

The incubation of NK cells with TDEs showed that these vesicles trigger an initial upregulation of the expression of activating receptors, whereas continuous exposure to TDEs caused a remarkable decrease of the same receptors, with a consequent dysfunction of NK cells. This may mimic what occurs within the TME, where the prolonged exposure of NK cells to TDEs released by cancer cells may lead to the loss of NK cell cytotoxic activity (48).

The cytotoxic effect of NK cells mainly depends on the release of granzyme B- and perforin-containing granules and on the activation of the FAS/FAS ligand pathway. Katsiougiannis S. et al. showed that saliva samples from mice with pancreatic ductal carcinoma contain exosomes that, besides downregulating NKGD2, also significantly reduced the expression of granzyme B and perforin (49). Moreover, TDEs released by glioblastoma multiforme (GBM) express Fas-L on their surface, which can bind to Fas receptors on the NK cell surface, triggering the caspase cascade and NK cell apoptosis (50). GBM-derived exosomes also carry B7-H3 protein (CD276), which can impair NK cell cytotoxicity, providing tumor cells with an additional mechanism of immune escape (51). Despite the B7-H3 receptor and the molecular mechanism underlying its inhibitory effect on NK and other immune cells must still be elucidated, scientific evidence points out the B7-H3 axis as a novel druggable target for checkpoint inhibitors (52). It has been reported that B7-H3 expression is a negative prognostic factor in NB (53), as it can inhibit the antitumor activity of NK cells (54). B7-H3 has been detected also in the exosomes released by medulloblastoma, a pediatric cancer of the central nervous system. B7-H3 expression characterizes exosomes, which are enriched with other tumorigenic proteins such as STAT3, and that can be transferred to distant endothelial cells. Moreover, exosomes can be transferred to other cancer cells, increasing the intracellular levels of B7-H3 that, in turn, reduces the phosphorylation of STAT1. These results highlight a receptor-independent B7-H3-mediated mechanism modulating TME through distal and proximal effects (55).

Interestingly, several pre-clinical studies showed the potential application of B7-H3 targeted immunotherapy also in pediatric solid tumors other than NB, fostering the possibility of translating such results into a clinical setting (56).

# 3 Cancer stem cells and exosome-mediated immune modulation

Tumors are characterized by the presence of Cancer Stem Cells (CSCs) that have self-renewal capacity, multipotency, enhanced tumorigenicity, and resistance to therapy. Neuroblastoma (NB) CSCs are characterized by surface stemness-associated markers. Among them CD133 is the most common, CD44 mediates cell-cell interactions and attachment to ECM, CD24 is found in bone marrow metastases and is associated with a higher tumorigenic potential, CD117 regulates the tumor cell fate in hematopoietic stem cell niches, and ABCG2, an efflux pump for chemotherapeutic drugs that increase CSC resistance to treatment (57). Several biological pathways are involved in CSC maintenance. A hallmark of CSCs is the enhanced glycolytic metabolism, achieved through the increased expression of glycolysis-related transporters and enzymes. This metabolic flexibility enables CSCs to switch between glycolysis and oxidative phosphorylation, optimizing their survival with respect to nutrient availability. Moreover, the nuclear-erythroid 2-related factor 2 (NRF2) sustains CSC proliferation and self-renewal by regulating the antioxidant systems that protect CSCs from reactive oxygen species present within the TME. Also, oncogenic signaling pathways such as PI3K/Akt and MAPK and HIF-regulated genes significantly contribute to the maintenance of CSCs and their adaptation to hypoxic tumor niches, respectively.

## 3.1 Functions of CSC-derived exosomes in shaping the TME

### 3.1.1 Stemness maintenance, metastasis development and angiogenesis

CSCs acquire aggressive tumorigenic features also through the release of exosomes that act on neighboring cells of the TME (Figure 2). It has been shown that exosomes produced by glioblastoma CSCs can reprogram non-CSCs to a stem-like phenotype by transferring Notch1 protein and, thus, triggering the associated signaling pathway responsible for an enhanced tumor potential (58). CSCs-derived exosomes can also carry RNA molecules resulting in the activation of stemness properties in recipient cells. For example, esophageal CSCs-derived exosomes contain the lnc-RNA FMR1-AS1, which activates NF-kB signaling that triggers the expression of the stemness-related gene c-Myc, promoting the malignant phenotype (59). Similarly, thyroid CSCs release exosomes containing the lnc-RNA DOCK9-AS2, responsible for the activation of the Wnt/β-catenin pathway, which enhances stemness and cancer cell proliferation (60). Interestingly, liver CSCs produce exosomes containing circular RNAs, circ-ZEB1 and circ-AFAP1, that can increase the expression of the stemness marker CD133 and decrease the levels of the EMT-related proteins E-cadherin and epithelial cell adhesion molecule (EpCAM) (61). These findings highlight another effect of CSC-derived exosomes, which can increase the metastatic potential of cancer cells by enhancing cell migration rate. Similar results have also been reported for exo-miR-19b-3p in renal carcinoma (62), for exo-miR-210-3p in lung cancer (63), and for pancreatic tumors (64). CSC-derived exosomes can also promote tumor angiogenesis by carrying pro-angiogenic factors that facilitate the crosstalk between endothelial and stromal cells within the TME. For

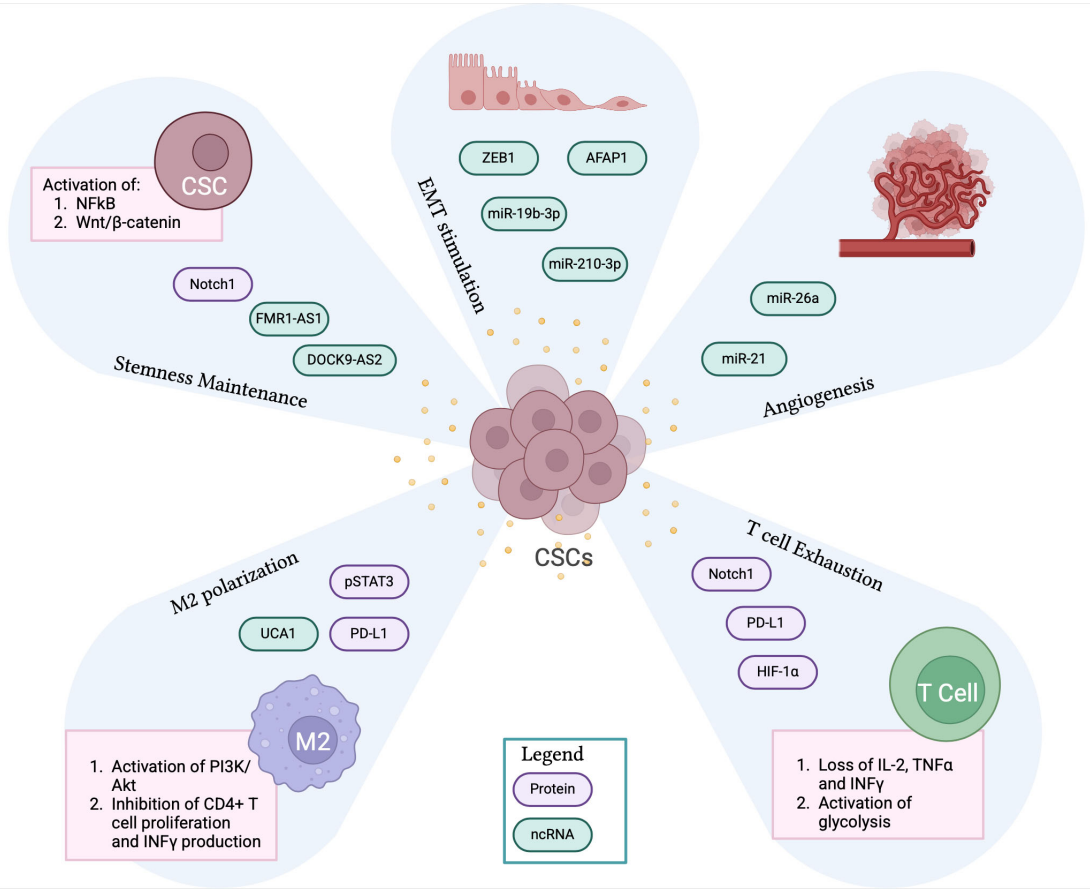


FIGURE 2
CSC-derived exosomes contribute to the immunosuppressive TME. The panel shows CSCs-derived exosomes containing proteins and non-coding RNA molecules that significantly contribute to the creation of an immunosuppressive TME by promoting stemness maintenance, EMT process, angiogenesis, M2 macrophage polarization and T cell exhaustion. CSCs, cancer stem cells; TME, tumor microenvironment; EMT, epithelial to mesenchymal transition. Created with BioRender.com.

example, glioma tumors release CSCs carrying miR-26a and miR-21, which exert a pro-angiogenic function by activating PI3K/Akt and VEGF signaling pathways, respectively (65, 66).

### 3.1.2 Crosstalk with immune cell subsets

Emerging studies indicate that CSCs can promote immunosuppression by targeting specific subsets of immune cells, such as TAMs and T cells, via exosomes (Figure 2).

It has been reported that glioblastoma-derived CSCs release exosomes inducing the polarization of macrophages towards the pro-tumor M2 phenotype. These vesicles are enriched with phosphorylated STAT3, which is transferred to monocytes, leading to the overexpression of PD-L1 and creating an immunosuppressive TME (67). CSC-derived exosomes can promote the M2 polarization of macrophages also through the transfer of lncRNA UCA1, which targets the PI3K/Akt signaling pathway while inhibiting the proliferation of CD4+ T cells and INF-γ production (68).

CSC-derived exosomes can directly target T cells, leading to their exhaustion, through different mechanisms. Indeed, CSC-derived exosomes express PD-L1 that, interacting with PD-1 on CD8+ T cells, leads to their exhaustion and hyporesponsive state (69). This result can be further exacerbated with the transfer of Notch1 via CSC-exosomes, which increases the expression of PD-1 on CD8+ T cells, enhancing the inhibitory signal mediated by the PD-L1/PD-1 axis (70). Moreover, CSC-derived exosomes can induce T cell exhaustion leading to the loss of secretion of effector cytokines such as IL-2, TNF-α, and IFN-γ. CSC-derived exosomes can also impair the activity of T cells by metabolic reprogramming, specifically inducing glycolysis through the activation of HIF-1α (71).

## 4 Exosomes as barriers to immunotherapy in neuroblastoma

A few studies investigated the role of exosomes in NB patients. It has been reported that downregulation of exosomal let-7b, miR-29c, and miR-342-3p characterizes high-risk (HR)-NB patients with a poor response to the front-line induction chemotherapy. Moreover, the exosomal microRNA expression profile can be used for the calculation of a "chemoresistance index" that predicts the

sensitivity/resistance of HR-NB patients to specific chemotherapeutic drugs (72). Similarly, the exosomal protein content has been shown to include molecules with a strong prognostic and diagnostic value, discriminating NB patients from control subjects, and differentiating between high-risk and low-risk NB patients (73). These findings highlight the contribution of exosomes to tumor progression and the acquisition of aggressive NB tumor phenotype. A hallmark of HR-NB patients is the infiltration of tumor cells in the bone marrow (BM) compartment. It has been demonstrated that PD-L1 expression can limit the immune surveillance in metastatic NB (74). A recent study showed that extracellular vesicles in the BM metastatic niche of HR-NB patients express on their surface both PD-L1 and HLA-G, a non-classical MHC class I molecule involved in immune tolerance by interacting with inhibitory receptors on T and NK lymphocytes. Authors point out a synergistic effect of PD-L1 and HLA-G in promoting immune evasion by enhancing the secretion of immunosuppressive cytokines and inhibiting pro-inflammatory cytokines, thus dampening T-cell response (75). These results provide direct evidence that NB-derived exosomes provide a mechanism of immune evasion by actively engaging NK cell inhibitory pathways. Despite the mechanism of immunotherapy resistance mediated by exosomal-PD-L1 is still under investigation, studies suggest that it can significantly inhibit the activation of cytotoxic T cells in melanoma, breast cancer and head and neck cancer patients, negatively affecting the response to anti-PD1 antibody (76). The same has been reported for patients affected by Wilms tumor, who showed higher levels of extracellular vesicles expressing PD-L1, that caused a severe inhibition of CD8+ T cells. Indeed, CD8+ T cell function was impaired, as demonstrated by the reduced production of TNFα and the decreased intracellular levels of INFγ (77). In addition, exosomes expressing PD-L1 were detected in OS patients: their presence correlate with clinical outcome and they are actively involved in the development of pulmonary metastasis (78, 79). HLA-G is a non-classical major histocompatibility complex class I molecule exerting immune inhibitory activity and its high expression has been associated with worse overall survival in patients with solid tumors (80). HLA-G promotes immunosuppression by binding the inhibitory receptors ILT-2 and ILT-4, reducing the proliferation, migration and cytotoxicity of T cells and NK cells, while favoring the expansion of myeloid-derived suppressive cells and regulatory T cells (81, 82). HLA-G upregulation has been detected in Ewing sarcoma xenografts who did not respond to NK cell activated treatment, providing a mechanism of immune evasion (83). HLA-G expressing exosomes in the BM of NB patients contribute to the establishment of an immunosuppressive TME, being functionally involved in the inhibition of NK cell cytotoxicity. These data corroborate previous results showing that higher soluble HLA-G levels in the BM were detected in metastatic disease (84). Furthermore, it has been reported that microvesicles expressing specific ectoenzymes play a key role in the dynamics of NB cells within the BM metastatic niche. Specifically, the presence of vesicles expressing CD38 and CD73 was detected in NB patients with a worse survival, having a prognostic value. CD73 upregulation caused a higher production of adenosine, which creates an immunosuppressive environment in the BM by inhibiting T cell activation and the production of pro-inflammatory cytokines (85). Vesicles detected in the BM do not derive exclusively from infiltrating NB cells, but also from other resident cells, underlining the possible contribution of normal BM components to the establishment of an immunosuppressive TME (75).

*In vitro* studies demonstrated that the immunosuppressive TME in NB results in the inhibition of NK cell response, leading to a reduced efficacy of immunotherapeutic approaches based on antibody-dependent cellular cytotoxicity (ADCC) mediated by NK cells. TGF-β exerts a major immunomodulatory role in the TME of NB by strongly inhibiting NK cell response (86, 87). Neviani et al. demonstrated that NK cells secrete exosomes enriched with the onco-suppressor miR-186, inhibiting tumor growth and regulating TGF-β-dependent escape mechanisms in NB (88). The silencing of miR-186 within NK cell-derived exosomes significantly reduced their cytotoxicity. On the other hand, NB-derived exosomes lack miR-186 expression and a downregulation of miR-186 was observed in high-risk NB patients, thus associating with poor survival. In silico analysis showed that miR-186 could directly target molecules known to be involved in NB progression such as MYCN, AURKA, TGFBR1, and TGFBR2. The authors demonstrated that the ectopic expression of miR-186 inhibited the proliferation and migration of MYCN-amplified cell lines. Indeed, the miR-186 effect on the TGF-β pathway could lead to the downregulation of Vimentin, thus interfering with EMT transition (88). Importantly, authors showed that lipid nanoparticles containing miR-186 specifically targeting NK cells restore their cytotoxicity by preventing the TGF-β-mediated inactivation. *In vivo* studies in orthotopic mouse models of NB showed also that GD2-expressing nanoparticles loaded with miR-186 effectively increased intratumoral miR-186 expression, reducing tumor burden (88). Overall, these results demonstrated that a reduced miR-186 expression in the TME of patients with NB significantly contributes to the immune escape mechanism promoted by TGF-β pathways on NK cells. Together, these findings support a model in which NB-derived exosomes either fail to deliver NK-cell activating signals as miR-186 and promote NK cell dysfunction by facilitating TGF-β-mediated immunosuppression.

It has recently been demonstrated that NK cells derived from NB patients display a defective uptake of glucose because of a lower expression of GLUT-1 transporter (89). Glycolysis is essential for the energy supply required for metabolic reprogramming, which triggers NK cell degranulation and cytotoxicity. Thus, the reported defect in glucose uptake hindered the proper activation of NK cells in NB. The lncRNA EPB41L4A-AS1 found overexpressed in NB patients' NK cells, was responsible for the inhibition of NK cells glycolysis. Importantly, lncRNA EPB41L4A-AS1 can be transferred via exosomes through different NK subsets, negatively affecting the glycolysis of recipient cells and, ultimately, leading to an immunosuppressive TME (89). The main features of NB cell-derived and NK cell-derived exosomes in NB contributing to the establishment of an immunosuppressive TME are summarized in Figure 3.

NK cell-based cancer therapy is potentiated using efficient activation methods. Shoae-Hassani A. et al. demonstrated that

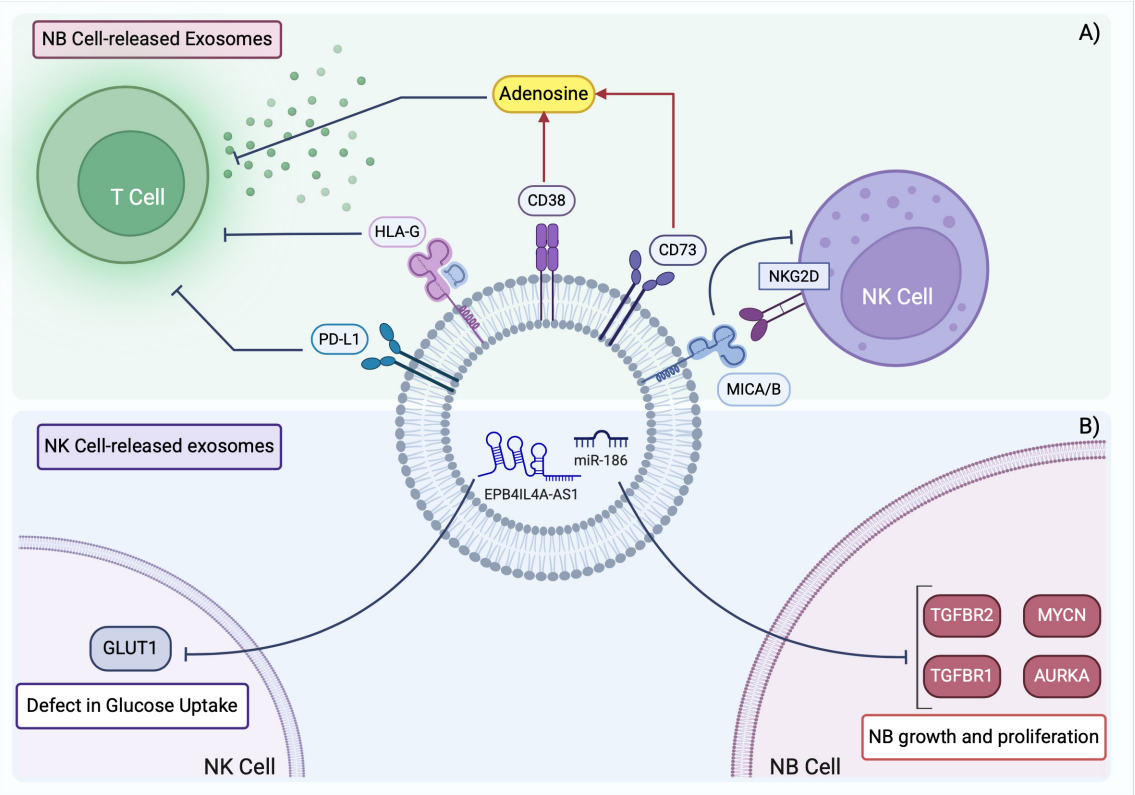


FIGURE 3
Exosomes derived from NB cells and NK cells shape the immunosuppressive TME in NB. **(A)** NB TME is characterized by the presence of TDEs expressing surface markers that prevent T cell activation and inhibit NK cell antitumor function. **(B)** NK cells in NB contain aberrant levels of non-coding RNAs that promote tumor development and further suppress the activation of neighboring NK cells. TME, tumor microenvironment; TDEs, tumor derived exosomes. Created with BioRender.com.

exposing naïve NK cells to exosomes derived from NK cells previously co-cultured with NB cells enhanced their anti-tumor cytotoxicity (90). Both *in vitro* and *in vivo* data confirmed that NK cell-derived exosomes can stimulate NK cell function by increasing the expression of receptors such as NKp44 and NKp30, and the production of cytokines with strong antitumor activity as IFN-γ and TNF-α. On the opposite side, NB-derived exosomes could inhibit NK cell function by altering cytokine secretion and downregulating the expression of activating receptors (90). These results suggest that exosomes derived from NK cells exposed to NB cells can educate naïve NK cells to exert cytotoxic effects on target cells. The formation of such NK cell memory could be beneficial for targeted therapy, making NK-derived exosomes a tool for improving NK-based cancer immunotherapy.

It has been reported that chemotherapy-induced senescence of tumor cells can represent a mechanism for escaping immune surveillance (91). In NB, senescent tumor cells overexpress the lncRNA MALAT1, which regulates the expression of ADAM10 metalloproteinase and the consequent release from tumor cells of the NKG2D ligand MICA/B. The release of MICA/B, occurring either in soluble form or within exosomes, can inhibit the NKG2D receptor and, thus, NK cell recognition and activation, shaping an immunosuppressive TME. MICA/B shedding is mainly regulated by metalloproteinases, and ADAM10 is a key enzyme in this process (92). MICA/B shedding involves lncRNA MALAT1, miR-92a, and ADAM10. Authors showed that silencing MALAT1 or ADAM10 could prevent MICA/B release, enhancing NK cell-mediated clearance of senescent tumor cells (93). These results show that NB-derived exosomes are directly involved in the suppression of NK cell activity through both surface ligands and regulatory RNAs. Importantly, the immunosuppressive effects exerted by NB-derived exosomes can be amplified by the developmental immaturity of the pediatric immune system. Indeed, in early life, the innate and adaptive immune responses are still immature, with NK cells characterized by reduced cytotoxicity and less active antigen presenting cells (94). These features may determine a higher susceptibility of pediatric patients to exosome-mediated immune modulation, facilitating the establishment of a pro-tumoral microenvironment.

## 5 Therapeutic targeting of TDEs

Targeting TDEs represents a compelling therapeutic opportunity, considering their key role in shaping the TME and impact on immunotherapy-based treatments. Strategies aimed at inhibiting the biogenesis, release, or uptake of TDEs are currently

being investigated, as well as the possibility of engineering exosomes released by immune cells to boost antitumor response.

## 5.1 Inhibition of TDEs biogenesis and uptake

As TDEs promote the transfer of key molecules aimed at facilitating tumor development and reducing the efficacy of immunotherapeutic treatment, inhibiting their synthesis and release may provide a useful therapeutic solution. In a recent study, Kim J. et al. showed the development of nanoparticles containing both an inhibitor of exosome release, GW4869, and a short interfering RNA for IRF3, a regulator of the activation of M2-like macrophages (95). *In vitro* experiments demonstrated the effectiveness of the synergistic action of TDEs release inhibition and IRF3 silencing, resulting in increased antitumor immune response. The data were confirmed *in vivo* in allograft murine breast cancer models: the treatment reduced tumor growth and metastases formation inhibited the release of TDEs expressing PD-L1, responsible for CD8+ T cell exhaustion, and reduced the number of immunosuppressive M2-type macrophages. Overall, these results highlight the potential of this combining therapeutic strategy in enhancing the anti-tumor immune response (95).

The GW4869 compound was also coupled with amlodipine (AM) and tested for inhibiting TDE secretion in hepatocellular carcinoma. AM was responsible for the autophagic degradation of PD-L1. This synergistic approach was effective in remodeling the TME by increasing the proliferation of functional CD8+ cytotoxic T cells, and innate lymphoid cells including NK cells, while reducing the populations of Treg and myeloid-derived suppressor cells (96).

Regarding the TDE uptake, it has been reported that heparan sulfate proteoglycans (HSPGs) are essential for the internalization of TDEs in recipient target cells. Although HSPG-independent mechanisms of TDE uptake exist, the entry pathway through HSPG interaction ensures the biological function of TDEs. Thus, the administration of small molecules targeting HSPG and preventing their interaction with exosomes may significantly reduce the internalization of functional TDEs, preventing tumor growth (97). To this purpose, heparin is a valuable candidate as it competes with HSPGs for binding exosomes-associated proteins. The persistent heparin treatment in oral squamous carcinoma cells (OSCCs) inhibited the uptake of exosomes that are responsible for the activation of tumor-promoting pathways as PI3K/Akt and MAPK/ERK axes. Although a continuous administration of heparin was needed to maintain the beneficial effects, the treatment significantly reduced the malignant potential of OSCCs (98).

## 5.2 NK cell-derived exosomes as a therapeutic tool

Exosomes derived by immune cell subpopulations can enhance anti-tumor immunity by targeting and inducing apoptosis in tumor cells and modulating the TME. The human origin of these vesicles confers higher stability, biocompatibility, and reduced immunogenicity compared to synthetic nanoparticles (99). NK-derived exosomes lack cellular components that can trigger adverse immune reactions including cytokine release syndrome (CRS) making them safer for administration in immunotherapy regimens. Moreover, exosomes can easily infiltrate the ECM of tumor tissue, exerting wider therapeutic effects (100). The ability of NK-derived exosomes to persist in an immunosuppressive TME is advantageous in solid tumors where functional exhaustion of immune effectors including NK cells occurs.

Importantly, exosomes can be engineered to be used as vehicles for the delivery of therapeutic molecules such as checkpoint inhibitors, chimeric antigen receptors (CARs), or siRNAs, further increasing their therapeutic potential. CAR-NK exosomes have been developed to target specific tumor antigens like HER2, showing efficacy in treating brain metastasis in breast cancer (101). Beyond HER2, other tumor-associated antigens such as EGFR, GD2, and EpCAM are being explored as potential targets for engineered NK exosomes in different cancer types. Additionally, exosomes can target novel tumor-associated antigens such as B7-H3, highly expressed in different tumors, or loaded with immune-stimulatory molecules, such as IL-5 or perforin, to enhance NK cytotoxicity and promote a more robust immune response within the TME.

Recent studies have also demonstrated the feasibility of combining NK-derived exosomes with existing immunotherapy approaches. Indeed, NK-derived exosomes can synergistically act with immunotherapies based on immune checkpoint inhibitors like PD-1/PD-L1 blockade or monoclonal antibodies by enhancing antigen presentation and boosting the cytotoxic activity of T cells and DCs.

The optimization of NK exosome production, purification, and delivery methods will be crucial for their successful clinical translation in cancer immunotherapy.

## 5.3 Challenges of exosome-based therapies and current clinical trials

The translation of exosome-based therapies into a clinical setting is hindered by technical challenges. There is no standardization in the isolation of exosomes, which can be carried out by numerous methodologies (e.g. ultracentrifugation, size-exclusion chromatography) differing in terms of yield, purify and scalability. Another limitation is due to targeting specificity, as the bioavailability of exosomes at the tumor site can be reduced by the mononuclear phagocyte system. To overcome this issue, different methods of surface protein modification or ligand engineering have been developed that, however, require further optimization. Moreover, the efficient and safe loading of therapeutic molecules, currently based on electroporation or transfection, is difficult, often resulting in low encapsulation efficiency (102). Despite these limitations, the interest in exosome-based therapies is rapidly growing. Compared to other standard drug delivery platforms, such as liposomes and polymeric nanoparticles, exosomes take

advantage of their endogenous origin, entailing lower toxicity and immunogenicity and higher stability (103).

Despite the encouraging preclinical results, there are no clinical trials currently investigating exosome-based therapies in pediatric oncology. Early-phase clinical trials have been activated only in adult cancers. Among the designed clinical trials involving exosomes, their application as cancer biomarkers represents the most prevalent focus, with therapeutic use being the second most explored area (104). Stem cell derived exosomes have been included in clinical trials for pancreatic cancer (NCT03608631) and acute myeloid leukemia (NCT06245746), DC-derives exosomes for lung cancer (NCT01159288) and plant-derived exosomes for colon (NCT01294072) and head and neck cancer (NCT01668849). The specific investigation of exosomes application in clinical solid cancer immunity is included in the Phase I clinical trial NCT05375604, exploring the delivery of the STAT6 anti-sense oligonucleotide, and in Phase I/II clinical trial NCT04592484 for the administration of the CDK-002 drug (105). These studies provide evidence of the feasibility of exosome administration and of their pharmacokinetics and safety profiles, paving the way to a broader clinical application. Considering the exosome characterization in NB, reported in section 4, actionable strategies tailored on NB biology could be developed. Indeed, exosomes could be engineered to deliver miR-186, which is able to restore NK cell cytotoxicity by avoiding TGF-β-mediated suppression. Furthermore, targeting exosomal PD-L1 or HLA-G with neutralizing antibodies or receptor-blocking strategies may hinder the establishment of the immunosuppressive TME within the BM metastatic niche. Finally, the exosomal transfer of regulatory RNAs, such as lncRNA EPB41L4A-AS1, which has been shown to impair NK cell metabolism, could be inhibited through RNA-targeting approaches, aiming at restoring NK cell function.

## 6 Conclusions

The scientific results here reported clearly show how TDEs play a pivotal role in creating an immunosuppressive TME that supports cancer progression and resistance to therapy. By modulating T cell immunity, macrophage function, and NK cell activity through PD-L1, TGF-β, and immunosuppressive microRNAs, TDEs effectively impair antitumor immune responses. Additionally, cancer stem cell (CSC)-derived exosomes contribute to tumor growth, metastasis, and angiogenesis while further modulating immune cell interactions to sustain an immune-evasive niche.

In NB and in other tumors, exosomes present a significant challenge to immunotherapy, hindering the efficacy of immune checkpoint inhibitors, adoptive cell therapies, and other targeted strategies. Given their central role in immune evasion, therapeutic strategies aimed at blocking TDE biogenesis, release, or uptake have emerged as promising solutions. Moreover, engineering NK cell-derived exosomes may provide an efficient immunotherapeutic tool to counteract the immunosuppressive effects of TDEs.

A deeper understanding of TDEs and CSC-derived exosomes in tumor-immune crosstalk will be essential for the development of novel therapeutic approaches. Targeting these vesicles could not only enhance current immunotherapies but also pave the way for more effective and stable cancer treatments.

## Author contributions

MM: Writing – review & editing, Writing – original draft, Conceptualization. CV: Writing – original draft, Writing – review & editing. MA: Writing – review & editing, Writing – original draft. AD: Writing – original draft, Writing – review & editing. KC: Writing – review & editing, Writing – original draft. CB: Conceptualization, Writing – review & editing, Funding acquisition, Writing – original draft. RC: Funding acquisition, Writing – review & editing, Conceptualization, Writing – original draft.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

## Publisher's note

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EDITED BY
Sheila Spada,
Hospital Physiotherapy Institutes (IRCCS), Italy

REVIEWED BY
Katia Cortese,
University of Genoa, Italy
Noemi Aloi,
CNR Area della Ricerca di Palermo, Italy
Anna Piro,
University of Studies G. d'Annunzio Chieti and Pescara, Italy

*CORRESPONDENCE
Yohan Han
dygks1876@wku.ac.kr
Jae Ho Seo
bionian9@wku.ac.kr

†These authors have contributed equally to this work

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# Cancer cell-derived extracellular vesicles: a potential target for overcoming tumor immunotherapy resistance and immune evasion strategies

Minseo Ahn[1,2†], Jeong-Geon Mun[3†], Yohan Han[2,4,5*] and Jae Ho Seo[1,2,5*]

[1]Department of Biochemistry, Wonkwang University School of Medicine, Iksan, Republic of Korea, [2]Sarcopenia Total Solution Center, Wonkwang University School of Medicine, Iksan, Republic of Korea, [3]Department of Oriental Pharmacy, College of Pharmacy, Wonkwang-Oriental Medicines Research Institute, Wonkwang University, Iksan, Republic of Korea, [4]Department of Microbiology, Wonkwang University School of Medicine, Iksan, Republic of Korea, [5]Institute of Wonkwang Medical Science, Wonkwang University School of Medicine, Iksan, Republic of Korea

Extracellular vesicles (EVs), including exosomes and microvesicles, play crucial roles in cancer progression by mediating the communication between cancer cells and their microenvironment. Cancer cell-derived EVs promote tumor growth, metastasis, and immune evasion by carrying bioactive materials, such as proteins, RNAs, DNA fragments, and lipids but, immunotherapy aims to enhance the immune response against cancer; however, resistance remains a major challenge. Cancer cell-derived EVs contribute to this resistance by delivering immunosuppressive molecules that impair T cell activation, promote the expansion of regulatory T cells (Tregs), and reduce natural killer (NK) cell cytotoxicity, thereby allowing cancer cells to evade immune surveillance. Additionally, cancer cell-derived EVs can carry immune checkpoint proteins, such as Programmed Death-Ligand 1 (PD-L1), which bind to the Programmed Death-1 (PD-1) receptor on T cells, leading to T cell exhaustion and reduced anti-tumor activity. This mechanism reflects how cancer cells directly evade immune detection and contributes to the overall resistance to immune checkpoint blockade therapies, such as anti-PD-1 or anti-PD-L1 antibodies. By delivering these immunomodulatory molecules, EVs not only contribute to local immune suppression but also create a systemic environment that is less favorable for effective anticancer immunity. Therefore, understanding the role of EVs in the immunotherapy resistance is crucial for developing targeted strategies to counteract their effects and ultimately improve therapeutic outcomes. Here we encourage researchers to pay more attention to the role of cancer cell-derived EVs in overcoming immunotherapeutic resistance, because such efforts may be one of the most promising approaches to address immunotherapy resistance in the future.

KEYWORDS

**extracellular vesicles, tumor microenvironment, cancer therapy, immunotherapeutic resistance, immune checkpoint inhibitors, immune cells**

# 1 Introduction

Extracellular vesicles (EVs) are nanosized, membrane-enclosed vesicles released by nearly all cell types, including cancer cells. These vesicles, mainly classified as exosomes (30–150 nm) and microvesicles (100–1,000 nm), function as intercellular messengers that transport bioactive molecules such as proteins, lipids, RNAs, and DNA fragments. By facilitating the exchange of these components, EVs influence a wide range of physiological and pathological processes, including immune modulation, tissue repair, and disease progression. In cancer, EVs play a pivotal role in shaping the tumor microenvironment (TME) and driving tumor growth, metastasis, and immune evasion through cell-to-cell communication (1). Cancer cell-derived EVs perform a range of functions and their influence on immune modulation has gained increasing attention. To evade immune detection, cancer cells utilize multiple strategies, and EVs play a crucial role in suppressing immune responses and facilitating immune evasion. EVs promote cancer progression while limiting the efficacy of immune-based therapies by transporting immunosuppressive factors, modulating immune cell activity, and reshaping the TME into an immune-resistant niche. A major concern regarding this phenomenon is the involvement of EVs in inducing resistance to immunotherapy, particularly to immune checkpoint inhibitors (ICIs), such as anti-Programmed Death-1 (PD-1) and anti-Programmed Death-Ligand 1 (PD-L1) antibodies. These therapies aim to restore anti-tumor immunity by reactivating exhausted T cells. However, growing evidence indicates that cancer cell-derived EVs can negatively regulate these responses by carrying immune checkpoint proteins, such as PD-L1, thus contributing to systemic immune suppression (2).

Although immunotherapy has the potential to transform cancer treatment, its overall success is hindered by primary or acquired resistance, which affects a substantial proportion of patients. A deeper understanding of the mechanisms underlying immunotherapy resistance is essential for developing novel therapeutic strategies to improve treatment outcomes. This review explores the role of EVs in cancer biology, emphasizing their involvement in immune evasion and resistance to immunotherapy. Additionally, we discuss potential strategies for counteracting EV-mediated immunosuppression and highlight future directions for enhancing cancer immunotherapy.

# 2 EVs in cancer biology

EVs are increasingly recognized as key mediators of intercellular communication in cancer, influencing tumor progression, immune modulation, metastasis, and therapeutic resistance. These vesicles, secreted by tumor cells, carry a diverse range of bioactive molecules, including proteins, lipids, nucleic acids, and metabolites, which contribute to shaping the TME. EVs contribute to fundamental cancer hallmarks by transferring oncogenic signals between cells, including uncontrolled proliferation, angiogenesis, immune evasion, and metastatic dissemination (1). Elucidation of these molecular mechanisms may enable the development of EV-targeted therapeutics and biomarker-driven strategies for precision oncology.

Cancer cells exploit EVs to enhance their survival and proliferation by transmitting oncogenic factors that support tumor growth and metabolic adaptation. These vesicles carry miRNAs, including *miR-21* and *miR-155*, which regulate pathways involved in cancer cell proliferation, apoptosis, and chemoresistance (3, 4). Additionally, EVs contain metabolic regulators including hexokinase 2 and lactate dehydrogenase, which promote the Warburg effect, a metabolic reprogramming process that enhances glucose uptake and lactate production to sustain rapid tumor growth. This metabolic shift provides cancer cells with a continuous energy supply, while modifying the surrounding environment to favor tumor expansion (5).

As tumors grow, they require an adequate blood supply, and EVs contribute to this process by promoting angiogenesis. Cancer cell-derived EVs carry key angiogenic factors, such as vascular endothelial growth factor, fibroblast growth factor, and hypoxia-inducible factor-1α (HIF-1α), which enhance endothelial cell proliferation and migration, leading to the formation of new blood vessels (6). Additionally, EVs containing *miR-210* suppress antiangiogenic regulators, thereby promoting the angiogenic switch (7). These molecular interactions not only ensure sufficient oxygen and nutrient supply to tumor cells, but also facilitate their infiltration into surrounding tissues (8).

Beyond local tumor progression, EVs play a pivotal role in metastatic dissemination by priming the colonization of distant organs. A key mechanism involves EV-associated integrins α6β4 and α6β1, which guide metastatic cancer cells to specific organs, such as the lungs, liver, and brain (9). In addition, EVs facilitate extracellular matrix remodeling by delivering matrix metalloproteinases that degrade structural barriers, allowing tumor cells to invade and establish secondary tumors. These interactions create a conducive environment for circulating tumor cells to initiate metastasis, ultimately inducing cancer progression to an advanced stage (10).

In addition to inducing tumor growth and metastasis, EVs contribute to evasion of the immune system, allowing cancer cells to escape immune surveillance. This is achieved by impairing T cell function through the induction of exhaustion and reduced cytotoxic activity, ultimately leading to diminished responsiveness to ICIs. Furthermore, EVs promote the expansion of regulatory T-cells (Tregs), contributing to the impairment of anti-tumor immunity by dampening cytotoxic T cell function. Another key immunosuppressive mechanism is the induction of myeloid-derived suppressor cells (MDSCs), which are expanded and activated through EV-mediated signaling, thereby suppressing T cell proliferation and anti-tumor immune responses (11–13). Additionally, EVs impair natural killer (NK) cell activity by downregulating activating receptors required for NK cell-mediated tumor elimination (14). By modulating immune regulatory pathways, EVs facilitate cancer immune evasion and ultimately reduce the efficacy of immune-based therapies.

Given their extensive role in shaping the TME, EVs have emerged as promising therapeutic targets. Efforts to block EV production,

neutralize their cargo, or harness them for drug delivery are being actively investigated to counteract their tumor-promoting effects. Moreover, the presence of cancer cell-derived EVs in bodily fluids highlights their potential as biomarkers for cancer diagnosis and treatment response monitoring. Despite advances in our understanding of EV functions, further research is essential to develop effective strategies that can selectively target EV-mediated signaling pathways without disrupting essential physiological processes.

## 3 Mechanisms of EV-mediated immune evasion and immunotherapy resistance

EVs mediate immune suppression, enabling cancer cells to evade immune destruction and resist immunotherapy. By delivering immunomodulatory molecules, EVs impair T-cell function, promote Treg expansion, inhibit NK cell cytotoxicity, and foster an immunosuppressive TME. Moreover, cancer cell-derived EVs carry immune checkpoint proteins that contribute to ICI resistance, thereby diminishing the efficacy of immunotherapy (15). However, one of the most critical mechanisms of EV-mediated immune suppression is the direct inhibition of T cell function. EVs suppress T cell function by delivering PD-L1, FasL, TGF-β, and IL-10, leading to T cell exhaustion and reduced anti-tumor immunity. Although this mechanism resembles PD-L1 expression in tumor cells, PD-L1-containing EVs have a broader impact by systemically circulating and suppressing immune responses at distant sites. Furthermore, EVs deliver FasL, which triggers apoptosis in activated T cells and further reduces anti-tumor immunity (16). By modulating these pathways, EVs establish an immunosuppressive environment that supports tumor survival and progression.

In addition, cancer-derived EVs enhance glycolysis in tumor-associated macrophages and myeloid-derived suppressor cells, further acidifying the TME and impairing effector T cell infiltration (17–19). Hypoxic EVs enriched in HIF-1α promote immune suppression by recruiting immunosuppressive myeloid cells and inhibiting cytotoxic T cell function (20–22). Notably, EVs also promote Treg expansion via TGF-β and IL-10, intensifying immune suppression within the TME. Consequently, EV-mediated Treg expansion contributes to immunotherapy resistance, because excessive Treg activity diminishes the efficacy of immune checkpoint blockade therapy (23). Similarly, cancer cell-derived EVs impair NK cell cytotoxicity via TGF-β, CD73, and FasL, downregulating NKG2D and facilitating immune evasion (24). Moreover, EVs contribute to MDSC expansion via HSP72- and *miR-21*-induced STAT3 activation, further suppressing T-cell responses (25, 26). Additionally, EVs facilitate metabolic reprogramming within the TME by transferring enzymes and metabolites that promote local hypoxia and acidity, creating an environment that is hostile to immune cells, but conducive to tumor progression (27).

A major concern in EV-mediated immune evasion is its contribution to resistance against ICIs, such as anti-PD-1 and anti-PD-L1 antibodies. Although ICIs are designed to restore T-cell function by blocking inhibitory signals, cancer cell-derived EVs act as decoys by carrying PD-L1, thereby neutralizing the therapeutic efficacy of these drugs. As a result, circulating PD-L1$^+$ EVs not only suppress T cell activation, but also sequester checkpoint inhibitors, reducing their ability to block tumor-associated PD-L1 (28). Importantly, the role of EVs in immune evasion and therapy resistance may differ depending on tumor type. Notably, their immunosuppressive functions have been more extensively studied in solid tumors, such as melanoma, triple-negative breast cancer (TNBC), and hepatocellular carcinoma, where EVs carry immune checkpoint ligands or immunomodulatory RNAs that suppress anti-tumor immunity (29, 30). In contrast, EVs in hematological malignancies often participate more prominently in altering the bone marrow microenvironment, promoting niche remodeling and facilitating chemoresistance (31, 32). Such tumor-specific variations necessitate the development of more sophisticated and individualized EV-based therapeutic approaches tailored to the distinct biological contexts of each cancer type. Thus, targeting EVs production or their immunosuppressive cargo may enhance the efficacy of immunotherapy.

Emerging approaches include pharmacological inhibition of EVs release, with agents such as GW4869, a neutral sphingomyelinase inhibitor that effectively reduces exosome secretion and tumor-promoting signals (33). In addition, neutralization of immunosuppressive EVs-associated cargo, such as PD-L1, has been shown to restore T-cell activity and enhance responses to ICIs (34). The development of engineered EVs capable of delivering therapeutic agents to specific immune or tumor targets represents another innovative approach (35). Moreover, the identification of EV-derived biomarkers predictive of immunotherapy response holds significant promise for advancing precision oncology, although clinical validation remains an ongoing challenge (36, 37). Collectively, these strategies highlight the therapeutic potential of EV-targeting interventions, but systematic preclinical and clinical validation is needed to translate these findings into clinical practice.

## 4 Cancer cell-derived EVs and immunotherapy resistance

Immunotherapy has emerged as an innovative approach that uses the immune system to treat cancer. However, resistance to immunotherapy remains a significant challenge, and increasing evidence suggests that cancer cell-derived EVs play a pivotal role in this process. In this section, we describe the role of cancer cell-derived EVs in immune suppression and resistance to cancer therapy based on previous studies. These data are summarized in Table 1.

Cancer cell-derived EVs directly contribute to immunotherapy resistance by interfering with ICIs and chimeric antigen receptor (CAR)-T cells. ICIs, such as anti-PD-1/PD-L1 antibodies, are powerful treatments that enhance the ability of the immune system to recognize and attack cancer cells by blocking inhibitory

TABLE 1 Role of cancer-derived extracellular vesicles in immune suppression and resistance to cancer therapy.

| Cancer Type | Cargo loaded in EVs | Description | Reference |
|---|---|---|---|
| Prostate and colon cancer, adenocarcinoma | PD-L1 | Act as decoys, accelerating anti-PD-L1 clearance by macrophages | (23) |
| Breast, colon, and lung cancer | PD-L1 | Lower the response to immune checkpoint blockade drugs | (24) |
| Metastatic melanoma | PD-L1 | Suppress the function of $CD8^+$ T cells and facilitate tumor growth | (25) |
| Leukemia | CD19 | Induce CAR-T cell exhaustion and reducing cytotoxicity | (26) |
| Breast cancer | CXCL1 | Increase PD-L1 expression levels in tumor-associated macrophages | (27) |
| Breast cancer | Efflux transporters, miRNAs | Enhance survival, inhibit apoptosis, and suppress immunity. | (28) |
| Colorectal cancer | – | Induce $CD8^+$ T-cell apoptosis and alter cytokine expression | (29) |
| Liver cancer | HSP90α | Promote IL-6/IL-8 secretion, suppress $CD8^+$ T cells, and reduce anti-PD-1/PD-L1 efficacy | (30) |
| Breast, prostate, renal, ovarian, renal, and bladder cancer, hepatocellular carcinoma | EBAG9 | Suppressing T-cell cytotoxicity and modulate immune gene expression | (31) |
| Breast and ovarian cancer, acute myelogenous leukemia, head and neck squamous cell carcinoma, melanoma | FasL, MHC class I molecules | Induce $CD8^+$ T-cell apoptosis | (32) |
| Pancreatic cancer | *miR-203* | Downregulate TLR4 in dendritic cells, reduce TNF-α and IL-12 production, and suppress DC-mediated immunity. | (33) |
| Breast and ovarian cancer, acute myelogenous leukemia, head and neck squamous cell carcinoma, melanoma | NKG2D ligands | Suppress NK cell function by downregulating NKG2D expression levels and reducing cytotoxicity | (32) |
| Pancreatic ductal adenocarcinoma | T and B cell epitopes | Reduce B cell function by triggering autoantibody production and absorbing complement attacks | (34) |
| Esophageal | LAMP1, MMP9 | Suppress $CD8^+$ T-cell proliferation by differentiating naive B cells into regulatory B cells | (35) |
| Hepatocellular carcinoma | HMGB1 | Induce $CD8^+$ T-cell suppression by expanding Bregs via TLR2/4-MAPK signaling | (36) |
| Ovarian | *miR-222-3p* | Induce macrophage polarization into the tumor-promoting M2 phenotype via the SOCS3/STAT3 pathway | (37) |
| Nasopharyngeal carcinoma | *hsa-miR-24-3p*, hsa-miR-891a, *hsa-miR-106a-5p*, *hsa-miR-20a-5p*, and *hsa-miR-1908* | Promote Treg induction, inhibit Th1/Th17 differentiation, and alter T-cell signaling pathways | (38) |
| Gastric cancer | PD-L1 | Enhance MDSC expansion via IL-6/STAT3 signaling | (39) |
| Glioblastoma | EGFRvIII | Promote tumor growth and resistance to targeted therapies by activating MAPK and Akt pathways | (1) |
| Gastric and lung cancer, melanoma | TGF-β, Src, Wnt3, HIF1 α | Induce stromal transformation and create a pro-tumor niche | (41) |
| Pancreatic cancer | CD73 | Activate mast cells via adenosine signaling, upregulate pro-angiogenic and tissue-remodeling factors | (42) |

The table presents an overview of the roles of cancer-derived EVs in immune suppression and resistance to cancer therapy across various cancer types. It categorizes different cancer types, the specific cargo loaded in EVs, their functional effects on immune cells, and their contribution to tumor progression and therapy resistance. Key molecules such as PD-L1, CXCL1, HSP90α, miRNAs, and FasL are highlighted, demonstrating their impact on T-cell apoptosis, immune evasion, and suppression of NK cell function.

signals that suppress T cell activity. PD-L1 present in cancer cell-derived EVs can contribute to immunotherapy resistance by acting as a decoy that binds to anti-PD-L1 antibodies (38). Tumor-cell-derived EVs carrying PD-L1 also interact with PD-1 on T cells, leading to immune suppression and reduced efficacy of immune checkpoint blockade therapies (39). PD-L1-containing EVs have been shown to correlate with the patient response to anti-PD-1 therapy in metastatic melanoma (37). In addition, cancer cell-derived EVs impair the efficacy of CAR-T cell therapy. EVs containing CD19 interact with CAR-T cells, inducing proinflammatory cytokine secretion and promoting T cell exhaustion. This interaction weakens the therapeutic efficacy and reduces CAR-T cell cytotoxicity (40).

Chemotherapy is widely used for cancer treatment; however, several studies have suggested that cancer cell-derived EVs formed after chemotherapy reduce the efficacy of immunotherapy. Chemotherapy-induced cancer cell-derived EVs increase PD-L1 expression levels in tumor-associated macrophages (TAMs) (41). Similarly, EVs from drug-resistant breast cancer cells deliver efflux transporters and miRNAs that enhance cell survival, inhibit apoptosis, and inhibit immune responses (42). In addition, after chemotherapy, cancer cell-derived EVs induce $CD8^+$ T-cell apoptosis and alter cytokine-related gene expression, leading to an immunosuppressive TME that hinders effective anti-tumor immunity (43).

Cancer cell-derived EVs contain various molecules that significantly contribute to immunotherapy resistance by affecting both immune and immunosuppressive cells. For example, cancer cell-derived EVs carrying HSP90-alpha promote the secretion of IL-6 and IL-8 by monocytes and neutrophils, resulting in $CD8^+$ T cell suppression and reduced efficacy of anti-PD-1/PD-L1 treatment (44). Cancer cell-derived EVs transfer estrogen receptor-binding fragment-associated antigen 9 to T cells, suppressing cytotoxicity and modulating immune-related gene expression (45). Cancer cell-derived EVs can also induce $CD8^+$ T-cell apoptosis via the membrane-associated form of the FasL and MHC class I molecules (46).

Cancer cell-derived EVs suppress the function of other immune cells, including dendritic, NK, and B cells, contributing to immune evasion and tumor progression. Pancreatic-cancer cell-derived EVs transfer *miR-203* to dendritic cells (DCs), leading to TLR4 downregulation and reduced TNF-α and IL-12 production, thereby suppressing DC-mediated immune responses (47). Cancer cell-derived EVs suppress NK cell function by down-regulating NKG2D receptor expression and decreasing cytotoxicity (46). They present tumor antigens to B cells to trigger autoantibody production, while simultaneously acting as decoys that absorb complement attacks, thereby reducing complement-mediated cytotoxicity against cancer cells (48). Cancer cell-derived EVs not only impair B cell function, but also induce their differentiation into regulatory B cells (Bregs), a subset of B cells with immunosuppressive functions that inhibit excessive immune responses and promote immune tolerance. Esophageal cancer cell–derived EVs induce naive B cells to differentiate into Bregs, which suppress $CD8^+$ T cell proliferation (49). In hepatocellular carcinoma, Cancer cell-derived exosomes induce the expansion of Bregs via HMGB1-TLR2/4-MAPK signaling, enhancing their ability to suppress $CD8^+$ T cell activity (50).

Cancer cell-derived EVs promote the functions of other immunosuppressive cells. Epithelial ovarian cancer cell–derived EVs contain *miR-222-3p*, which regulates the SOCS3/STAT3 pathway and induces macrophage polarization to the tumor-promoting M2 phenotype (51). Cancer cell-derived EVs from nasopharyngeal carcinoma promote Treg induction by inhibiting Th1 and Th17 differentiation and altering T-cell signaling pathways, contributing to immune suppression (52). MDSC expansion can be promoted by cancer cell-derived EVs carrying PD-L1 via IL-6/STAT3 signaling, enhancing immune suppression and tumor progression in gastric cancer (53).

Cancer cell-derived EVs can also act as messengers that facilitate communication between cancer cells, potentially contributing to resistance to immunotherapy. Cancer cell-derived EVs transfer EGFRvIII between tumor cells, activating the MAPK and Akt signaling pathways, which promote tumor growth and metastasis, leading to enhanced resistance to targeted therapies (1). Additionally, EVs facilitate intercellular communication and tumor progression by transferring nucleic acids and proteins between tumor cells and modulating the TME to form a pre-metastatic niche (54).

The TME, which regulates various signals and immunosuppressive cells, significantly influences the efficacy of immunotherapy. For instance, cancer cell-derived EVs containing TGF-β, Src, Wnt3, and HIF1α are taken up by TAMs, which subsequently release membrane blebs, transferring these components to stromal cells (55). Blebs secreted by TAMs carrying cancer cell-derived components induce myofibroblastic changes in the recipient stromal cells, creating a favorable niche for cancer cells (55). Cancer cell-derived EVs activate mast cells through CD73-mediated adenosine signaling, leading to the upregulation of pro-angiogenic and tissue remodeling factors, which contribute to an immunosuppressive TME (56).

Despite significant advances in elucidating the immunosuppressive roles of tumor-derived EVs, emerging evidence points to their dual and context-dependent functions. As demonstrated in preclinical vaccination models, some EVs can stimulate antitumor immunity by delivering tumor-associated antigens and MHC molecules to dendritic cells (57). These contrasting findings highlight the complexity of EVs-mediated immune modulation and indicate the need to identify the molecular and contextual factors determining whether EVs suppress or activate immunity. Moreover, EVs heterogeneity within the tumor microenvironment remains a critical yet underexplored factor. Addressing these challenges through standardization efforts, such as the MISEV guidelines (58), and conducting research towards greater standardization in future studies. While certain EVs-derived signatures show promise as predictive immunotherapy response biomarkers, clinical validation remains incomplete. Addressing these challenges will be crucial for fully harnessing the translational potential of EVs in cancer immunotherapy.

## 5 Conclusions

Cancer cell-derived EVs play a crucial role in immunotherapy resistance by modulating the immune system and promoting an immunosuppressive TME (Table 2). These vesicles carry various immunosuppressive molecules, including PD-L1, TGF-β, and FasL, which impair the function of immune cells such as T cells, NK cells, and DCs. By influencing immune regulation and fostering immune evasion, EVs can contribute to the failure of ICIs and CAR-T cell therapies. Furthermore, EVs facilitate communication between tumor cells, thereby enhancing metastasis and resistance to targeted therapies. Understanding the mechanisms by which EVs contribute to immunotherapy resistance is essential for developing novel therapeutic strategies that can counteract their effects and improve the efficacy of cancer immunotherapies. Targeting EVs is a promising approach to overcome resistance and enhance treatment outcomes in cancer therapy. In this review, we highlighted the critical role of cancer cell-derived EVs in promoting immunotherapy resistance, underscoring the need for targeted strategies to counteract EV-mediated immune evasion and improve therapeutic outcomes (Figure 1).

Although EV-targeted therapies show promise, several limitations must be considered. Systemic inhibition of EVs may lead to unintended toxicity by interfering with physiological intercellular communication. Moreover, off-target effects remain a concern due to the challenge of distinguishing tumor-derived EVs from those released by normal cells (58). Strategies for selective targeting, such as ligand-directed delivery or nanoparticle engineering, are under development but require further optimization (59, 60).

## 6 Discussion

Concrete bioengineering strategies involve genetic modification of donor cells to enrich EVs with therapeutic cargos (e.g., siRNAs, ICIs), electroporation-based loading methods, and surface functionalization with antibodies or targeting ligands (e.g., anti-EGFR antibodies) (60–63). Preclinical models utilizing engineered EVs have shown promising results in overcoming immune resistance and enhancing the delivery of checkpoint inhibitors (64).

Recent advances in detection technologies such as next-generation sequencing (NGS), nanoplasmonic platforms, and microfluidic enrichment have improved EV analysis by enhancing the sensitivity and specificity of EV-based diagnosis (65–68). For instance, plasma-derived EV RNA signatures have been employed to predict therapy response in non-small cell lung cancer (NSCLC) (69). Several clinical studies have demonstrated the prognostic and predictive value of EV biomarkers, such as PD-L1+ EVs, in lung cancer, melanoma, and colorectal cancer (70–73). Ongoing clinical trials further support these findings, evaluating EV-based biomarkers for treatment response monitoring, minimal residual disease detection, and early cancer diagnosis across various tumor types such as lung cancer, breast cancer, pancreatic cancer, and colorectal cancer (74).

Despite the growing interest in EVs as key players in cancer progression, several controversies and unresolved challenges remain. A major issue is their heterogeneity and the classification of EVs to distinguish them from exosomes, microvesicles, and other subtypes remains unclear, because of overlapping size ranges and the absence of universally accepted markers (75). This complicates the standardization of EV isolation and characterization, leading to

TABLE 2 Overview of immunomodulatory roles and therapeutic implications of cancer-derived EVs.

| Category | EV-mediated Mechanisms | Mechanistic Detail |
|---|---|---|
| ❖ Scope of EVs Action | ◆ Local (Tumor Microenvironment) | - Suppression of cytotoxic T cells<br>- Induction of Tregs and MDSCs<br>- NK cell dysfunction<br>- Promotion of angiogenesis and stromal remodeling |
| | ◆ Systemic (Distal Sites) | - Circulating PD-L1$^+$ EVs mediating systemic immunosuppression<br>- Preparation of pre-metastatic niches |
| ❖ Mechanisms of Immune Modulation | ◆ Direct Effects | - Delivery of immunosuppressive molecules<br>- Induction of T cell apoptosis and exhaustion |
| | ◆ Indirect Effects | - Expansion of Tregs and MDSCs<br>- Reprogramming of myeloid and lymphoid compartments |
| ❖ Targeted Immune Components | ◆ Innate Immunity | - Inhibition of NK cell cytotoxicity<br>- Impairment of dendritic cell function |
| | ◆ Adaptive Immunity | - Suppression of effector T cell responses<br>- Expansion of immunoregulatory populations |
| ❖ Therapeutic Implications | ◆ Targetable Processes | - Inhibition of EVs production and release<br>- Neutralizing immunosuppressive cargo<br>- Engineering EVs for therapeutic delivery |
| | ◆ Biomarker Applications | - Development of EVs-derived molecular signatures for predicting immunotherapy outcomes |

This table summarizes the immunomodulatory roles of cancer-derived EVs, including their local and systemic effects, mechanisms of immune suppression, targeted immune components, and potential therapeutic and biomarker applications.

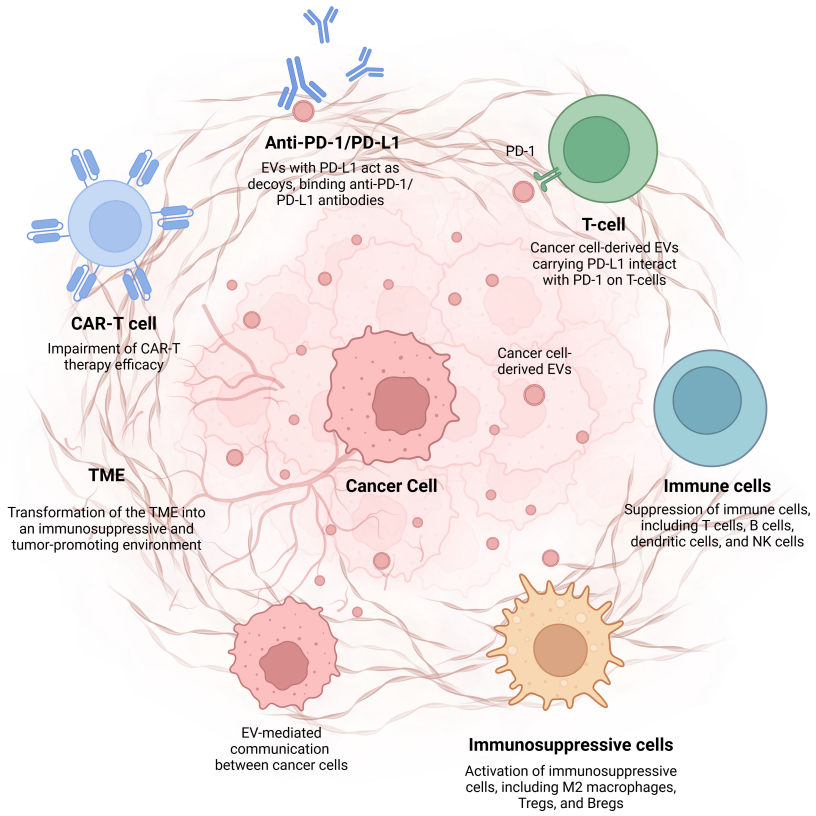


FIGURE 1
Cancer cell-derived EVs and their role in immunotherapy resistance. This figure illustrates the mechanisms by which cancer cell-derived extracellular vehicles (EVs) contribute to immunotherapy resistance by modulating the tumor microenvironment and suppressing the immune response. EVs, Extracellular Vesicles; PD-LI, Programmed Death-Ligand 1; PD-1, Programmed Death-1; NK, Natural Killer; Tregs, Regulatory T cells; Bregs, Regulatory B cells; TME, Tumor Microenvironment; CAR-T cell, Chimeric Antigen Receptor-T cell. Image was created with BioRender (https://biorender.com/)

inconsistencies across studies (76). Additionally, although cancer cell-derived EVs carry oncogenic cargo, distinguishing them from normal-cell-derived EVs remains difficult, limiting their diagnostic potential (77–79).

Beyond tumor progression, cancer cell-derived EVs critically contribute to immune evasion and resistance to immunotherapy. ICI therapies, such as anti-PD-1/PD-L1 therapies, can induce the release of PD-L1$^+$ EVs, which act as decoys to neutralize therapeutic antibodies and suppress T-cell responses (80). Similarly, tumors treated with CAR-T cells secrete EVs carrying inhibitory molecules that induce T cell exhaustion (81). Furthermore, chemotherapy-induced EVs can enhance PD-L1 expression levels in TAMs, creating an immunosuppressive TME (82). These EVs also carry immunosuppressive molecules, including PD-L1, TGF-β, and IL-10, which inhibit T cell activation or promote components of immune suppressive TME, such as Tregs and MDSCs, further dampening anti-tumor immunity (83, 84). The ability of EVs to establish an immunosuppressive microenvironment directly contributes to tumor immune escape and reduces the efficacy of ICIs.

To fully exploit EVs in cancer diagnosis and therapy, future research should focus on standardizing high-throughput EV isolation techniques (85) and utilizing advanced analytical tools, such as super-resolution microscopy and microfluidics-based platforms, to better understand EV heterogeneity (86). Additionally, engineered EVs are being explored as therapeutic vesicles using approaches such as selectively loading EVs with ICIs or small interfering RNAs to counteract tumor-induced immune suppression (87).

Bridging the gap between EV research and clinical applications requires collaborations among researchers, clinicians, and engineers. Large-scale clinical trials are needed to validate EV-based biomarkers and therapies for regulatory approval and widespread adoption (88). PD-L1-expressing EVs are emerging predictive biomarkers of ICI efficacy, enabling patient stratification for personalized treatment (89). Engineered EVs carrying immunomodulatory agents may offer novel strategies to overcome immune resistance (90). These emerging applications emphasize the need for further research on EVs as mediators and therapeutic targets in immunotherapeutic resistance.

## Author contributions

MA: Investigation, Writing – original draft. J-GM: Investigation, Writing – original draft. YH: Conceptualization, Supervision, Investigation, Writing – review & editing, Writing – original draft. JS: Writing – review & editing, Investigation, Conceptualization, Writing – original draft, Supervision.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

## Publisher's note



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EDITED BY
Anirban Ganguly,
All India Institute of Medical Sciences Deoghar, India

REVIEWED BY
Dwijendra K. Gupta,
Allahabad University, India
Zhijin Fan,
Sun Yat-sen University, China

*CORRESPONDENCE
Qi Wang
qwangk@naver.com

†These authors have contributed equally to this work and share first authorship

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# Advances in the mechanism of small extracellular vesicles promoting the development of hepatocellular carcinoma through multi-network fusion

Xiaoying Yuan[1†], Defa Huang[2†], Liang Peng[3†], Yilong Lin[4], Lijuan Wang[4], Jiawei Yan[4], Youming Qiu[1], Chenggui Song[1] and Qi Wang[1*]

[1]The First School of Clinical Medicine, Gannan Medical University, Ganzhou, China, [2]Department of Laboratory Medicine, The First Affiliated Hospital of Gannan Medical University, Ganzhou, China, [3]Department of Research and Education, The Second People's Hospital of Jingdezhen, Jingdezhen, China, [4]School of Medical Technology, Gannan Medical University, Ganzhou, China

Hepatocellular carcinoma (HCC) is a highly malignant epithelial tumor characterized by global high incidence and poor clinical prognosis. Radical surgical resection, as the standard treatment for early-stage HCC patients, has been extensively validated for its therapeutic efficacy. However, epidemiological studies indicate that most patients are already in advanced stages at initial diagnosis, losing eligibility for radical treatment. Notably, HCC pathogenesis exhibits marked etiological heterogeneity, posing significant challenges for clinical management. Although significant breakthroughs have been made in understanding HCC drivers at pathophysiological levels, translational applications of these findings remain hindered by multiple barriers. Currently, elucidating the molecular mechanisms of HCC pathogenesis and identifying effective therapeutic targets constitute major research priorities in this field.Small extracellular vesicles (sEVs) are phospholipid bilayer vesicles (30-150 nm in diameter) carrying functional proteomes and nucleic acids (e.g., miRNAs, lncRNAs) with substantial biological activity. Studies demonstrate that sEVs contribute to malignant phenotype acquisition by modulating key signaling pathways such as PI3K/AKT and Wnt/β-catenin. These molecular cascades ultimately confer hallmark pathological features including aberrant proliferation, apoptosis resistance, and immune evasion to tumor cells. Within multi-network regulatory systems, sEVs serve as crucial intercellular messengers mediating tumor cell interactions with other tumor microenvironment (TME) components (e.g., cancer-associated fibroblasts, immune cells). Such communication facilitates TME reprogramming, pro-angiogenic phenotypic shifts, and therapy resistance development. Nevertheless, the precise molecular mechanisms of sEVs in HCC pathogenesis remain incompletely understood, warranting further exploration of their translational potential in clinical practice.

KEYWORDS

**hepatocellular carcinoma, small extracellular vesicles, multi-network fusion, mechanism, therapy**

# 1 Introduction

Hepatocellular carcinoma (HCC) ranks among the most prevalent and lethal malignancies worldwide, with escalating incidence and mortality rates (1). HCC exhibits multifactorial etiology, with primary risk factors encompassing chronic HBV/HCV infections, alcoholic liver disease, and non-alcoholic fatty liver disease (2). Current therapeutic strategies for HCC merely extend nominal survival curves while inducing broad-spectrum toxicities. This ultimately leads to treatment resistance development in patients (3). Consequently, developing novel therapeutic approaches is imperative. Recent advances in fundamental medical research have progressively unraveled HCC pathogenesis mechanisms. Small extracellular vesicles (sEVs), as critical tumor microenvironment components, have garnered substantial research attention.

sEVs are nanoscale membranous vesicles secreted by diverse cell types, transporting bioactive cargo (proteins, lipids, mRNAs, miRNAs) to mediate intercellular communication and signaling (4). Studies demonstrate HCC-derived sEVs interact not only with tumor cells but also with TME components (fibroblasts, endothelial cells, immune cells), promoting hepatocarcinogenesis and progression via multinetwork fusion mechanisms (5). Although preliminary understanding of sEVs' mechanistic roles in HCC exists, their complex signaling networks and clinical potential require further exploration.

This review systematically elucidates the multinetwork regulatory mechanisms of sEVs in HCC pathogenesis. Integrating current evidence, we analyze how sEVs drive HCC progression by: (a) modulating pivotal pathways (PI3K/AKT, Wnt/β-catenin); (b) reprogramming TME cellular composition/functionality; (c) enhancing malignant behaviors (proliferation, metastasis). Building upon these mechanisms, we evaluate sEVs' translational value as precision medicine targets. This review addresses three key questions: (a) sEVs biogenesis/molecular signatures; (b) pathological mechanisms of sEVs-mediated network crosstalk; (c) clinical applications and translational prospects.

# 2 sEVs

## 2.1 Definition and classification of sEVs

The International Society for Extracellular Vesicles (ISEVS) defines extracellular vesicles as phospholipid bilayer-enclosed membranous structures ranging from 30–5000 nm in diameter. Their fundamental biological characteristics include cellular origin, lack of replicative capacity, and intercellular communication functions (6). Current classification criteria are based on physical properties and biogenesis pathways: by size as small EVs (sEVs, <200 nm) and large EVs (lEVs, >200 nm); by origin as exosomes (endosomal pathway), microvesicles (plasma membrane budding), and apoptotic bodies (programmed cell death products). Notably, ISEVS recommends using the operational term "small extracellular vesicles" (sEVs) rather than the mechanistically suggestive "exosomes". This recommendation stems from: technical limitations in distinguishing biogenesis pathways; absence of specific molecular markers; and substantial heterogeneity in clinical samples (6, 7).

## 2.2 Molecular characteristics and characterization techniques of sEVs

sEVs exhibit characteristic nanoscale size distribution (30–200 nm) and marked morphological heterogeneity. Their bilayer membranes are enriched with tetraspanins (CD63/CD81/CD9) and tissue-specific markers (8, 9). Modern characterization techniques include: (a) Nanoparticle tracking analysis (NTA) for size quantification; (b) Transmission electron microscopy (TEM) for ultrastructure; (c) Super-resolution microscopy overcoming optical limits; (d) Mass spectrometry for molecular profiling (10, 11). Key technical challenges persist: *in vitro* models are culture-condition dependent (e.g., FBS starvation alters proteomes) (12); xenografts fail to recapitulate full TME interactions (13); clinical samples suffer lipoprotein co-isolation (plasma concentration ~$10^{16}$/ml) (14). Optimization strategies combine separation techniques (e.g., SEC-density gradients) and surface marker capture, requiring purity-yield tradeoffs (15).

## 2.3 Biogenesis and uptake of sEVs

Rab GTPases are small GTPases belonging to the Ras superfamily that primarily regulate intracellular membrane trafficking and vesicular transport (16). They cycle between GTP-bound (active) and GDP-bound (inactive) states to modulate functional status, recruiting effector proteins to specific membrane compartments to control vesicle formation, trafficking, and fusion (17). This mechanism is crucial for the biogenesis of small extracellular vesicles (sEVs).

sEVs formation initiates with membrane invagination of early endosomes to generate intraluminal vesicles (ILVs), which subsequently develop into multivesicular bodies (MVBs) (18). Rab GTPases influence sEVs production and release by regulating multiple steps of this process. For instance, Rab27a and Rab27b promote MVB docking with the plasma membrane to enhance sEVs secretion (19), while Rab7 determines whether MVBs undergo degradation or sEVs release (20). Furthermore, Rab11- and Rab35-regulated sEVs secretion appears ESCRT-independent but Rab27-dependent for ILV formation (21), demonstrating the diverse functions of Rab proteins in sEVs biogenesis. Distinct Rab proteins precisely control sEVs generation through specific effector protein networks. Rab5 initiates ILV formation at the early endosome stage (22), while Rab11 affects the recycling endosome pathway (23), collectively ensuring proper sEVs assembly and function.

Selective uptake of sEVs represents a core aspect of intercellular communication, being tightly regulated rather than stochastic. The membrane protein composition of sEVs serves as a key determinant

for selective uptake. Integrin family proteins direct sEVs homing to specific tissues (24), explaining why tumor-derived sEVs preferentially target particular organs. Tetraspanins (CD9, CD63, CD81) mediate cell-specific recognition through interactions with receptor cell surface ligands (25). Multiple mechanisms exist for sEVs entry into recipient cells, including clathrin-dependent endocytosis, caveolin-mediated endocytosis, macropinocytosis, and direct membrane fusion (26) (Figure 1).

## 2.4 Research limitations of sEVs

Despite established definitions, high-purity sEVs isolation remains challenging due to incomplete biological understanding. *In vitro* models: Cell line-derived sEVs are experimentally controllable but their biogenesis is altered by artificial conditions (e.g., serum-free media), modifying molecular composition. FBS starvation alters sEVs yield, proteome, protein metabolic regulation, and membrane raft assembly functions (12). Given these effects, sEVs-depleted serum is recommended for *in vitro* studies. Xenograft models preserve tumor characteristics but fail to replicate dynamic TME interactions during tumor growth. Immunodeficient mice lack complete immune environments and human-mouse cellular interactions, limiting translational studies of sEVs-mediated immunomodulation (13). Clinical samples: Plasma-derived sEVs are clinically relevant but show inter-individual variability and lipoprotein contamination, requiring stringent characterization. Current methods (dUC, ExoQuick) co-isolate plasma proteins/lipoproteins and may induce vesicle aggregation. Notably, plasma lipoproteins ($\sim 10^{16}$/ml) share size/density characteristics with sEVs (chylomicrons/VLDL/HDL) (14).

## 2.5 Challenges and optimization in sEVs isolation technology

### 2.5.1 Inefficient separation of non-vesicular contaminants

Although traditional methods such as ultracentrifugation effectively enrich sEVs, they also co-precipitate contaminants including lipoproteins and protein aggregates (27). The MISEVS2023 guidelines recommend a multi-parametric evaluation strategy, incorporating immunoblotting or mass spectrometry to detect negative markers such as apolipoproteins.

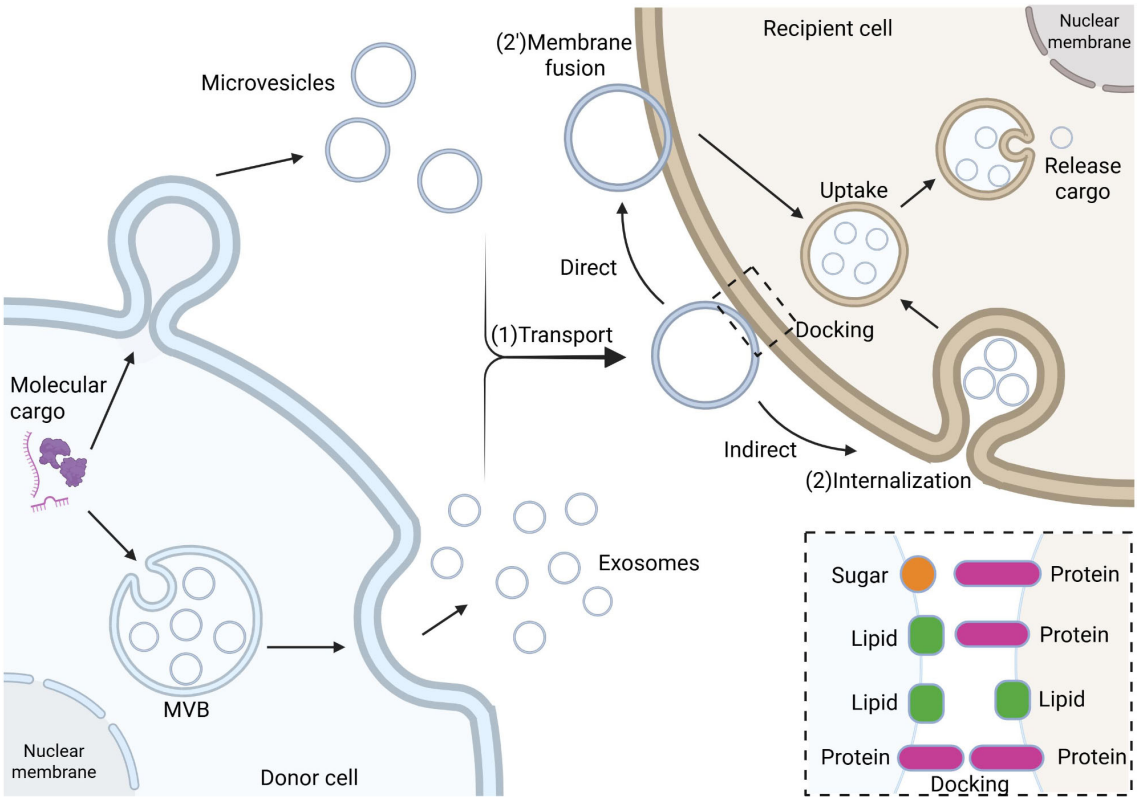


FIGURE 1
The biogenesis, transport, and internalization mechanisms of sEVs. Biogenesis of sEVs initiates from early endosomes, where the endosomal membrane invaginates to form intraluminal vesicles (ILVs) through membrane budding and sorting mechanisms, which subsequently mature into late endosomes, also known as multivesicular bodies (MVBs). MVBs fuse with the plasma membrane to release their intraluminal sEVs into the extracellular matrix (ECM). During intercellular communication, sEVs bearing surface ligands (e.g., transmembrane proteins or lipids) bind to specific receptors on target cell membranes, followed by internalization via endocytosis (including clathrin-dependent or -independent pathways) or membrane fusion. The newly formed early endosomes undergo maturation in the cytoplasm, ultimately releasing their bioactive cargo (e.g., nucleic acids, proteins) into the target cell cytoplasm, thereby modulating cellular physiological or pathological processes.

Optimization strategies include combining ultracentrifugation with size-exclusion chromatography, employing density gradient centrifugation for enhanced resolution, and exploring emerging technologies such as microfluidics (6). Standardized documentation of isolation methods and contaminant profiles is crucial to ensure reproducibility.

### 2.5.2 Significant variability in protein marker expression

Commonly used markers such as CD63 and CD9 exhibit heterogeneous distribution across sEVs subpopulations, with expression dynamically influenced by cellular origin and disease state (28). Researchers should employ a combination of universal and cell-specific markers for validation and enhance detection accuracy using advanced techniques such as high-resolution flow cytometry.

### 2.5.3 Technical bottlenecks in clinical-scale applications

Size-exclusion chromatography suffers from low recovery rates (30-60%) and limited throughput (29), whereas microfluidic technology demonstrates significant advantages, achieving >80% recovery, reducing processing time to minutes, and enabling specific capture of disease-associated sEVs subpopulations (30). Future efforts should focus on standardization through multicenter validation and the development of integrated automated workstations to address scalability challenges.

## 2.6 Single-vesicle analysis technologies

Conventional bulk analysis methods fail to resolve the high heterogeneity of sEVs, driving the need for single-vesicle detection technologies. Next-generation single-vesicle analysis enables precise characterization of individual vesicles' physical properties and molecular composition, offering novel insights into sEVs biological functions (31).

Advanced microscopy techniques are revolutionizing sEVs observation. Super-resolution microscopy (STORM/PALM) overcomes the optical diffraction limit, revealing nanoscale structural features of sEVs (32). Cryo-EM preserves native sample states, providing authentic 3D morphological information of sEVs (33).

Single-molecule detection significantly enhances sEVs analysis precision. Single-molecule fluorescence tracks dynamic surface interactions, while nanopore sequencing enables direct RNA detection without amplification (34–36). These approaches offer unique advantages for low-abundance biomarker discovery.

Microfluidic platforms provide high-throughput solutions for sEVs analysis. Integrated with fluorescent labeling or Raman spectroscopy, these chip systems enable rapid sorting and characterization of individual sEVs (37). Digital microfluidics advances further by permitting multiplexed analysis of captured single vesicles.

Machine learning algorithms are transforming sEVs data processing. Deep learning models automatically identify characteristic patterns of sEVs subpopulations, while clustering analysis aids in discovering novel functional classifications (38). These methods are particularly suited for handling massive single-vesicle datasets.

Despite promising prospects, single-vesicle analysis faces sEVseral technical challenges. Key issues requiring resolution include balancing sensitivity with throughput, standardizing detection methods, and ensuring clinical translation feasibility. Overcoming these challenges will determine the technology's practical utility.

Next-generation technologies will focus on multidimensional integrated analysis. Integrating nanotechnology, biosensing, and advanced computational methods, future single-vesicle analysis may achieve higher-precision multi-omics detection. This will open new possibilities for precision medicine and fundamental research.

# 3 Network regulation of tumor microenvironment by sEVs in hepatocellular carcinoma

The tumor microenvironment (TME) constitutes a complex ecosystem comprising diverse cell types and their secretory factors (39). This system primarily consists of: (a) Tumor cells - the central component exhibiting uncontrolled proliferative potential and invasiveness (40); (b) Cancer-associated fibroblasts (CAFs) - secreting bioactive factors (growth factors, cytokines, ECM components) to critically regulate tumor progression and metastasis (41); (c) Endothelial and pericytes - forming structural/functional units of tumor vasculature that enhance hematogenous metastasis via angiogenesis (42); (d) Immune cells (T/B cells, TAMs, DCs, MDSCs) - collectively participating in immunesurveillance, immunosuppression and immune evasion (43) (Figure 2).

sEVs serve as crucial signaling mediators in TME, orchestrating intercellular communication, metabolic reprogramming and immunomodulation (44, 45). By transporting diverse bioactive molecules, sEVs mediate complex crosstalk among tumor, stromal and immune cells to drive malignant progression (46, 47). Metabolically, sEVs remodel TME metabolism by transferring metabolites and regulators to fuel tumor proliferation (48). Notably, sEVs exhibit dual immunoregulatory roles: suppressing effector immune cells while activating immunosuppressive populations to establish an immune-tolerant niche (49). These findings establish sEVs as both essential TME components and promising therapeutic targets, offering novel avenues for treatment optimization and prognostic evaluation.

## 3.1 sEVs in communication network regulation

### 3.1.1 sEVs and common oncogenic mechanisms in HCC

sEVs participate in the common pathological processes of HCC by mediating intercellular communication. At the molecular regulatory level, the long non-coding RNA HULC competitively

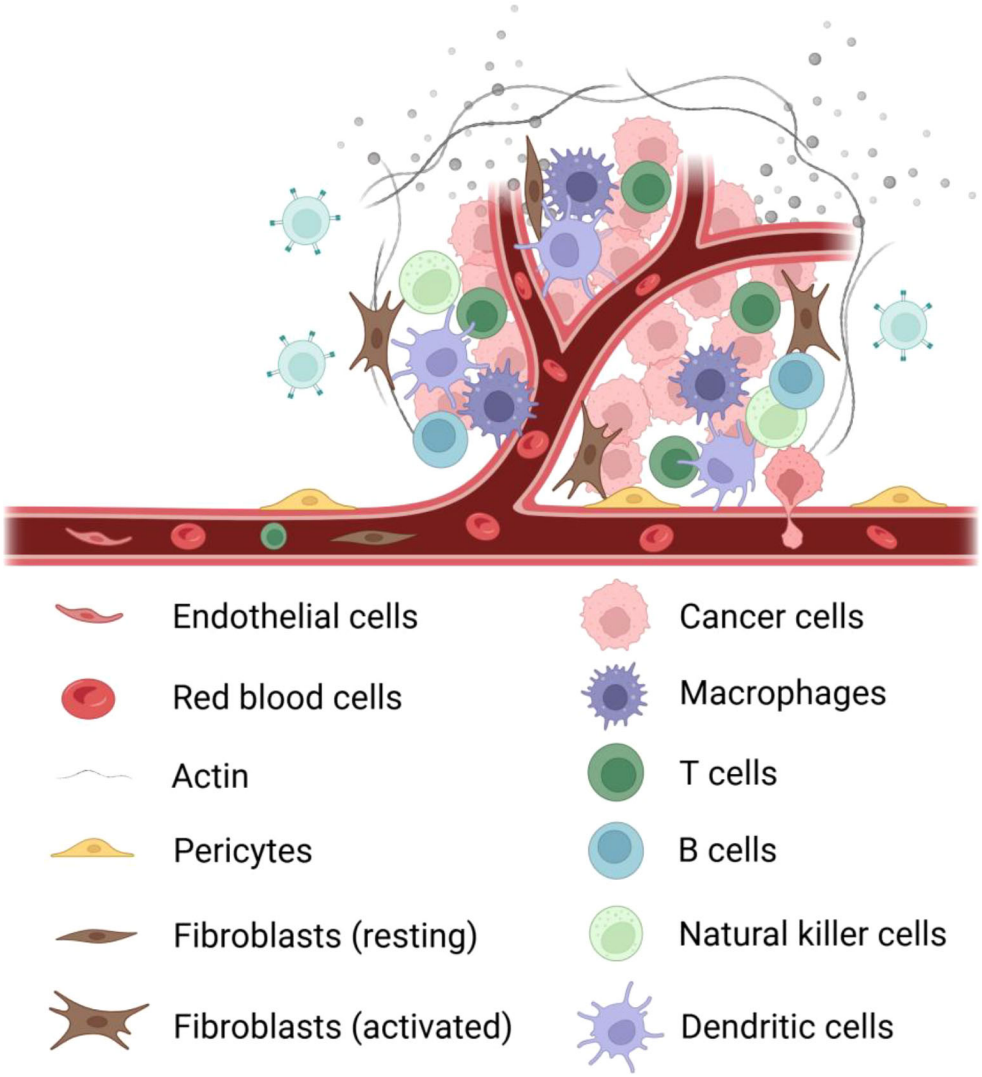


FIGURE 2
Components of the tumor microenvironment. It mainly contains the following cells: ① tumor cells; ② immune cells: tumor-associated macrophages, T cells, B cells, natural killer cells, dendritic cells; ③ fibroblasts: activated fibroblasts, resting fibroblasts; ④ blood vessels: erythrocytes, pericytes, endothelial cells ; ⑤ extracellular matrix; ⑥ signaling molecules.

inhibits miR-372-3p expression, leading to upregulation of Rab11a protein, thereby promoting sEVs secretion and accelerating HCC progression. Notably, the expression level of HULC in serum sEVs of HCC patients is significantly higher than in healthy controls, suggesting its potential as a diagnostic biomarker (50). In drug resistance regulation, upregulated Rab27B expression in drug-resistant HCC cells enhances sEVs secretion, promoting the efflux of chemotherapeutic agents (e.g., 5-fluorouracil) and reducing intracellular drug concentration; genetic knockout of Rab27B reverses this resistant phenotype (51). Furthermore, sEVs-mediated transfer of circPAK1 is a key mechanism of acquired resistance in HCC, as resistant cells transmit circPAK1 to sensitive cells via sEVs, conferring drug resistance (52).

sEVs facilitate malignant behaviors in HCC by transferring specific RNAs and proteins (53). Upregulation of NEAT1 reduces tumor-suppressive miRNAs (e.g., miR-634, miR-638) in sEVs, enhancing the proliferation and invasion of HCC cells (54). Moreover, sEVs secreted by highly metastatic HCC cells carry carboxypeptidase E (CPE), which can be taken up by low-metastatic cells, promoting their malignant transformation, whereas CPE inhibition reverses this effect (55). Overexpression of p62 protein increases sEVs secretion, enhancing the migration and invasion of recipient cells (56). Ribosomal protein L9 (RPL9) transmits miR-24-3p and miR-185-5p via sEVs, further promoting HCC progression (57). DEAD-box helicase 55 (DDX55) is enriched in HCC-derived sEVs and promotes tumor invasion and angiogenesis through intercellular transfer (58). These findings indicate that sEVs drive the malignant phenotype of HCC by regulating key molecular networks, highlighting their importance in targeted therapy.

### 3.1.2 sEVs and unique oncogenic mechanisms in HCC

In virus-associated HCC, sEVs exhibit distinct regulatory features. CD81-positive sEVs mediate viral immune evasion and promote tumor progression in HCV-associated HCC; HCV viral particles exploit CD81-positive sEVs for transmission, and this sEVs subpopulation is significantly enriched in HCV-positive HCC patients, suggesting its potential as a therapeutic target (59). In HBV-associated HCC, hepatitis B virus core antigen (HBcAg) delivers miR-135a-5p via sEVs, suppressing VAMP2 expression, thereby enhancing anti-apoptotic capacity and fostering drug resistance in HCC cells (60). Additionally, dysregulated autophagy in HCC patients leads to aberrant release of Glypican-3 (GPC3) in sEVs, and its high expression profile makes it a candidate molecular marker for early diagnosis (61).

### 3.1.3 sEVs and universal tumor-suppressive mechanisms in HCC

sEVs suppress HCC malignant progression by delivering tumor-suppressive molecules or regulating key signaling pathways. Studies demonstrate that tumor-suppressive long non-coding RNAs (e.g., SENP3-EIF4A1) delivered by sEVs significantly inhibit HCC cell proliferation and induce apoptosis (62). Hesperidin modulates sEVs molecular composition by reducing oncogenic RNA cargo (e.g., RAB11A mRNA and lncRNA-RP11-583F2.2) while upregulating tumor-suppressive miR-1298 expression, thereby effectively inhibiting hepatic precancerous lesion development (63). Resveratrol downregulates Rab27a to reduce sEVs secretion and alters lncRNA SNHG29 expression in sEVs, consequently inhibiting Wnt/β-catenin signaling and autophagy processes (64). The transcription factor KLF4 suppresses HCC progression by upregulating sEVs surface markers CD9 and CD81, whereas low expression of RNA helicase DDX3 promotes sEVs secretion and enhances stemness features and drug resistance in HCC cells (65, 66).

In therapeutic applications, engineered sEVs demonstrate remarkable targeted delivery potential. For instance, anti-GPC3 antibody-modified sEVs efficiently deliver miR-26a, significantly suppressing HCC growth (67). GalNAc-modified sEVs co-deliver paclitaxel (PTX) and miR122, synergistically enhancing antitumor effects (68). Furthermore, sEVs-based gene editing systems show promising applications; AAV6 vectors effectively deliver suicide genes (e.g., inducible caspase 9), markedly enhancing HCC cell killing (69). The CRISPR-Cas9 ribonucleoprotein system delivered by sEVs also exhibits high-efficiency gene editing capability (70). Advanced studies reveal that multiplex siRNA delivery systems (targeting GPX4 and DHODH) enhance sorafenib-induced ferroptosis to overcome HCC drug resistance (71).

### 3.1.4 sEVs and HCC-specific tumor-suppressive mechanisms

Certain tumor-suppressive mechanisms exhibit HCC-specific regulatory characteristics. Serum cathelicidin antimicrobial peptide (CAMP) levels are significantly reduced in HCC patients, and CAMP supplementation effectively inhibits HCC cell proliferation, suggesting its potential as a diagnostic biomarker (72). Natural killer (NK) cell-derived sEVs selectively target HCC cells and induce apoptosis (73). Additionally, HEK293 cell-derived sEVs delivering miR-365a-3p significantly suppress HCC proliferation and promote apoptosis (74). Notably, Parkinson's disease cell-derived sEVs enriched with α-synuclein inhibit HCC growth and migration (75).

In summary, sEVs play pivotal roles in HCC pathogenesis, drug resistance development, and metastasis by participating in complex molecular network regulation. These mechanisms encompass both universal HCC regulatory pathways and virus-specific modes of action, highlighting their translational value as diagnostic biomarkers and targeted therapeutic vehicles (Table 1).

## 3.2 sEVs in metabolic network regulation

### 3.2.1 sEVs and hypoxia

The mechanistic role of hypoxic microenvironment in HCC progression has been extensively elucidated. Hypoxia regulates HCC malignancy through sEVs-mediated mechanisms. Hypoxic conditions modulate sEVs secretion via HIF-1α, influencing HCC proliferation, metastasis, and immune evasion. HIF-1α facilitates GPC3 loading into sEVs, reducing intracellular GPC3 to suppress Wnt/β-catenin signaling and tumor growth (76). Hypoxia-regulated sEVs biogenesis promotes angiogenesis through miRNA transfer. HIF-1α upregulates miR-3174 under hypoxia and enhances its packaging into sEVs. These sEVs are delivered to endothelial cells, inhibiting HIPK3 signaling to enhance angiogenesis/vascular permeability and accelerate HCC metastasis (77).

Regarding sEVs-mediated malignant transformation, miR-1273f activates Wnt signaling to promote HCC invasion. Hypoxic HCC-derived sEVs enriched with miR-1273f activate Wnt/β-catenin signaling to enhance proliferation, migration, and EMT (78). Further studies reveal hypoxic sEVs alter hepatocyte mechanical properties. Hypoxic sEVs (H-exos) promote proliferation/migration at lower concentrations than normoxic sEVs, inducing cytoskeletal reorganization and reduced elastic modulus (79). Crucially, hypoxic sEVs induce malignant transformation of normal hepatocytes. Chronic hypoxia enables HCC-sEVs to transform HL-7702 cells, enhancing proliferation/migration, tumor marker expression, and mechanical changes, while promoting tumor growth and liver damage *in vivo* (80).

Regarding metastasis, sEVs facilitate pre-metastatic niche formation. Hypoxic HCC-sEVs activate fibroblast ERK1/2-NFκB signaling to establish pulmonary PMN. Oleanolic acid (OA) inhibits this pathway to block PMN formation, showing anti-metastatic potential (81). Recent studies show CAF-derived sEVs containing circHIF1A promote immune evasion. Hypoxic CAF-sEVs deliver circHIF1A to stabilize PD-L1, enhance malignancy, and suppress CD8+ T cells, suggesting immunotherapeutic targets (82).

TABLE 1 Main mechanisms involved in the regulation of communication networks by sEVs.

| Effect | Key signals | Main mechanisms | Ref. |
|---|---|---|---|
| Pro-cancer | lncRNA HULC | Inhibition of miR-372-3p expression, up-regulation of Rab11a protein expression, and enhancement of sEVs secretion | (50) |
| | HCV | Evades immune surveillance by binding to CD81+ sEVs | (59) |
| | GPC3 | Secretion through sEVs as an early diagnostic marker for HCC | (61) |
| | Rab27B | Exclusion of chemotherapeutic drugs from cells via sEVs reduces intracellular drug concentrations and enhances drug resistance | (51) |
| | HBc | Up-regulation of miR-135a-5p expression in sEVs, inhibition of its target gene VAMP2, and enhancement of anti-apoptotic and chemotherapy resistance | (60) |
| | CircPAK1 | Delivery to sensitive cells via sEVs, conferring cellular drug resistance | (52) |
| | NEAT1 | Promotes secretion of sEVs and regulates significant down-regulation of oncogenic miRNA expression in sEVs | (54) |
| | CPE | Low-metastatic cells significantly promote their malignant behavior after uptake of carboxypeptidase E released by high-metastatic tumor cells via sEVs | (55) |
| | P62 | Increased secretion of sEVs, enhanced malignant behavior of receptor cells | (56) |
| | RPL9 | Delivery of specific miRNAs via sEVs | (57) |
| | DDX55 | Enhancement of tumor cell invasiveness and angiogenesis through sEVs delivery between tumor cells and endothelial cells | (58) |
| | Gremlin-1 | Enhancement of invasiveness and metastasis of HCC cells by sEVs, activation of Wnt/β-catenin and BMP signaling pathways, and enhancement of drug resistance | (135) |
| Anti-cancer | Hesperidin | Significantly decreased the expression of RAB11A mRNA and lncRNA-RP11-583F2.2 in sEVs and increased the expression of miR-1298 in sEVs | (63) |
| | AAV6 | Delivery of an inducible caspase 9 suicide gene and significant enhancement of tumor cell killing | (69) |
| | lncRNA SENP3-EIF4A1 | Delivery of SENP3-EIF4A1 to HCC cells via sEVs. Inhibits their proliferation and migration and promotes their apoptosis | (62) |
| | KLF4 | Inhibition of HCC progression by altering sEVs subtypes through upregulation of sEVs surface proteins CD9 and CD81 | (65) |
| | DDX3 | Promoting the secretion of sEVs and enhancing the expression of sEVs-related proteins, thereby promoting stem cell properties and drug resistance in HCC cells | (66) |
| | miR-26a | Delivery of miR-26a to HCC cells via sEVs significantly inhibits tumor growth | (67) |
| | siRNA | Delivery of siRNAs targeting GPX4 and DHODH via sEVs significantly enhances the iron death effect of sorafenib in therapy | (71) |
| | CAMP | CAMP supplementation inhibits the proliferation of HCC cells | (72) |
| | α-synuclein | Significant inhibition of HCC growth and migration through sEVs uptake by HCC cells | (75) |
| | Resveratrol | Downregulation of Rab27a expression, inhibition of sEVs secretion, alteration of lncRNA expression in sEVs, inhibition of Wnt/β-catenin signaling pathway and autophagy | (64) |
| | Hsa-mir-365a-3p | Delivery of hsa-miR-365a-3p to HCC cells via sEVs significantly inhibited cell proliferation, increased oxidative stress, and induced apoptosis | (74) |

### 3.2.2 sEVs and glycolysis

HCC-derived sEVs regulate glycolysis via lncRNA transfer to promote progression. sEVs-carried ZFPM2-AS1 suppresses miR-18b-5p to upregulate PKM, activating HIF-1α-dependent glycolysis and enhancing HCC malignancy. ZFPM2-AS1 also promotes M2 macrophage polarization to accelerate progression (83). sEVs-delivered miR4458HG binds IGF2BP2 to stabilize HK2/SLC2A1 mRNAs, enhancing glycolysis and HCC growth (84).

circRNAs modulate HCC glycolysis via miRNA sponging. circFBLIM1 (enriched in HCC-sEVs) sequesters miR-338 to derepress LRP6, promoting glycolysis (85). Similarly, circ-ZNF652 inhibits miR-29a-3p to upregulate GUCD1, enhancing glycolytic flux - its knockout suppresses HCC glycolysis (86).

Highly metastatic HCC cells (e.g., 97H/LM3) secrete sEVs enriched with glycolytic/gluconeogenic/PPP proteins to enhance invasiveness (87). Conversely, senescent HCC cells deliver miR-

146a-5p via sEVs to suppress glycolysis. This miRNA targets IRF7 to downregulate PFKL, reducing glucose metabolism and tumor growth (88).

### 3.2.3 sEVs and other metabolic pathways

FTO demethylates GPNMB mRNA to stabilize its expression and promote sEVs loading. sEVs-delivered GPNMB binds SDC4 on CD8+ T cells to suppress activation, enabling immune evasion. This FTO/m6A/GPNMB axis reveals key HCC mechanisms and therapeutic targets (89).

HCC cells enhance sEVs biogenesis/secretion via ferroptosis to clear misfolded proteins and alleviate ERS. Unsaturated fatty acids (e.g., arachidonic acid) augment this process. Ferroptosis inhibition reduces sEVs release and increases ERS sensitivity, revealing its cytoprotective role (90).

HMGB1/RICTOR upregulate PD-L1 expression and PD-L1+ sEVs release to impair immune function and anti-PD-L1 efficacy. They also enhance glutaminolysis via mTORC2-AKT-c-MYC (upregulating GS) and mTORC1-mediated GDH derepression (91) (Table 2).

sEVs bilayers contain phosphatidylserine, sphingomyelin, and cholesterol, with LPC modulating membrane stability/function. Exogenous LPC/PGD2 activate TGF-β via TLR2/DP1 to promote fibrosis/immunomodulation. Cholesterol-conjugated siRNAs enhance sEVs delivery efficiency, while vitamin E modifications improve cargo loading (92–94). miR-23b-3p is enriched in sEVs from aged mice/FH patients, accelerating senescence/metabolic dysfunction via Tnfaip3 suppression. Targeting miR-23b-3p may treat age-related liver/metabolic disorders (95). While sEVs roles in HCC lipid metabolism require further study, their regulatory functions show significant research value.

## 3.3 sEVs in immune network regulation

### 3.3.1 sEVs and macrophages

Macrophage-derived sEVs play crucial regulatory roles in HCC invasive phenotypes. Studies demonstrate macrophages enhance HCC invasiveness by secreting miR-92a-2-5p-enriched sEVs. These sEVs are internalized by HCC cells to downregulate androgen receptor (AR) expression, activating the AR/PHLPP/p-AKT/β-catenin signaling axis and promoting tumor progression. Experimental evidence shows inhibiting sEVs secretion or miR-92a-2-5p knockdown significantly attenuates macrophage-mediated HCC invasion (96).

sEVs-mediated immune evasion and immunosuppression in HCC microenvironment have been extensively investigated. HCC-derived sEVs deliver PCED1B-AS1 to T cells/macrophages, reducing hsa-miR-194-5p to upregulate PD-L1/PD-L2, inducing immune cell apoptosis/dysfunction (97). GOLM1 facilitates PD-L1 transfer via HCC-sEVs to tumor-associated macrophages (TAMs), enhancing immune evasion and CD8+ T cell suppression. Zoledronic acid combined with anti-PD-L1 effectively reverses this immunosuppression (98).

Recent breakthroughs reveal therapeutic potential of macrophage-derived sEVs in HCC. RBPJ-overexpressing macrophage sEVs (RBPJ+/+ Mφ-Exo) deliver hsa_circ_0004658 to suppress HCC proliferation and induce apoptosis. This circRNA sponges miR-499b-5p to derepress JAM3, exerting antitumor effects (99). Conversely, HCC-derived circTMEM181-enriched sEVs upregulate macrophage CD39, activating ATP-adenosine pathway to create immunosuppressive microenvironment and impair anti-PD1 efficacy (100). These findings provide novel directions for sEVs-targeted HCC therapies.

### 3.3.2 sEVs and M1 macrophages

HBV-associated HCC sEVs exhibit significant miR-142-3p upregulation. These sEVs deliver miR-142-3p to induce M1 macrophage ferroptosis, promoting tumor progression. Mechanistically, miR-142-3p targets SLC3A2 to regulate macrophage ferroptosis. This reveals how sEVs promote HBV+ HCC by modulating macrophage function (101). Additionally, HCC-derived sEVs can drive M1 macrophage polarization. FTCD-mediated sEVs signaling promotes M1 polarization to suppress HCC proliferation (102).

sEVs combined with superparamagnetic iron oxide nanoparticles (PIONs@E6) enhance M1 polarization. This combination increases proinflammatory cytokines (IL-12, TNF-α) and ROS production, effectively suppressing HCC growth in mice. sEVs-nanoparticle conjugates enhance macrophage antitumor immunity (103). A novel sEVs-mimetic nanosystem reprograms immunosuppressive M2 TAMs to antitumor M1 phenotype. Near-infrared irradiation increases M1 macrophages, inhibits tumor growth, and enhances immune activity in TME (104).

### 3.3.3 sEVs and M2 macrophages

HCC cells regulate macrophage polarization via sEVs secretion to promote tumor development. HCC-sEVs deliver hsa_circ_0074854 and other ncRNAs to induce M2 polarization, enhancing migration/invasion. hsa_circ_0074854 inhibition reverses this effect, confirming its key role in tumor-immune crosstalk (105).

sEVs-delivered miRNAs promote M2 polarization by targeting specific genes. miR-452-5p and miR-21-5p downregulate TIMP3 and RhoB respectively, enhancing HCC malignancy (106, 107). miR-200b-3p reinforces M2 polarization via ZEB1/JAK/STAT pathway (108). IL-6-stimulated HCC cells secrete miR-143-3p-enriched sEVs that promote M2 polarization via MARCKS regulation (109).

M2 macrophage-derived sEVs reciprocally promote tumor progression. Their miR-27a-3p and miR-660-5p suppress TXNIP and KLF3 respectively, enhancing HCC stemness/invasiveness (110, 111). lncRNAs (PSMA5, HEIH) in HCC-sEVs activate JAK2/STAT3 to induce M2 polarization (112, 113).

M2 macrophage sEVs mediate HCC drug resistance and vascular remodeling via specific miRNAs. miR-200c-3p activates PI3K/AKT pathway to induce sorafenib resistance (114). miR-23a-3p targets PTEN/TJP1 to disrupt vascular barriers and promote metastasis (115). These findings reveal multifaceted regulatory roles of sEVs in HCC microenvironment.

TABLE 2 Main mechanisms involved in the regulation of metabolic networks by sEVs.

| Metabolic type | Key signals | Main mechanisms | Ref. |
|---|---|---|---|
| Lacking oxygen | lncRNA HMMR-AS1 | Activates HIF-1α and significantly increases lncRNA HMMR-AS1 expression. Delivery to macrophages via sEVs induces M2-type polarization | (138) |
| | Glycosaminoglycan-3 | Activation of HIF-1α, reduction of GPC3 expression, inhibition of HCC cell proliferation, migration and epithelial-mesenchymal transition, inhibition of Wnt/β-catenin signaling pathway, inhibition of tumor growth and angiogenesis | (76) |
| | miR-3174 | Activation of HIF-1α, up-regulation of miR-3174 expression, delivery to human umbilical vein endothelial cells via sEVs, inhibition of HIPK3 signaling pathway, enhancement of angiogenesis and vascular permeability, and promotion of HCC growth and metastasis | (77) |
| | miR-1273f | Activation of Wnt/β-catenin signaling pathway enhances proliferation, migration, invasion, and epithelial-mesenchymal transition of HCC cells | (78) |
| | oleanolic acid | Inhibition of ERK1/2-NFκB signaling pathway and effective prevention of hypoxia-induced formation of distal pre-metastatic microenvironment | (81) |
| | CircHIF1A | Binds to HuR, stabilizes PD-L1 expression, and enhances proliferation, migration, invasion and epithelial-mesenchymal transition of HCC cells. Inhibits cytotoxicity of CD8+ T cells, leading to immune escape | (82) |
| Glycolysis | lncMMPA | Competes with miR-548s for binding, increases ALDH1A3 expression, and promotes glycolytic activity and proliferation of HCC cells | (139) |
| | lncRNA TUG1 | Inhibition of miR-524-5p, up-regulation of SIX1 expression, and promotion of glycolysis-related gene activity | (140) |
| | ZFPM2-AS1 | Inhibition of miR-18b-5p, enhancement of PKM expression, activation of the HIF-1α-dependent glycolytic pathway, promotion of proliferation, migration and invasion of HCC cells, and promotion of M2-type polarization in tumor-associated macrophages | (83) |
| | miR4458HG | Binds to the m6A reader IGF2BP2, stabilizes mRNAs of glycolysis-related genes, and enhances the glycolytic process in HCC cells | (84) |
| | CircFBLIM1 | Reduction of LRP6 inhibition by miR-338, promotion of LRP6 expression, enhancement of glycolysis and tumor progression in HCC cells | (85) |
| | CircZNF652 | Reduction of GUCD1 inhibition by miR-29a-3p, enhancement of glycolysis-related metabolic activities such as glucose uptake, pyruvate levels, lactate production and ATP production | (86) |
| | miRNA-146a-5p | IRF7 upregulates the expression of PFKL, a key enzyme in glycolysis. Targeted inhibition of IRF7 reduces glucose uptake, lactate production and ATP yield. Accelerates cellular senescence | (88) |
| M6a modification | HBeAg | Enhancement of m6A methylation modification and stabilization of MAAS in M2-type macrophages. delivery of MAAS to HBV-associated HCC cells via sEVs and promotion of tumor cell proliferation | (141) |
| | miR-628-5p | Delivery of miR-628-5p into HCC cells via sEVs, inhibition of METTL14 expression, reduction of m6A modification of circFUT8, and blocking of circFUT8 translocation from nucleus to cytoplasm | (142) |
| | FTO | Removal of m6A modification on GPNMB mRNA, stabilization of GPNMB expression, binding of GPNMB to SDC4 receptor on CD8+ T cells via sEVs, inhibition of T cell activation, promotion of immune escape | (89) |
| Other metabolism | HMGB1和RICTOR | Modulation of PD-L1 expression, promotion of PD-L1+ sEVs generation, inhibition of cytotoxicity in immune cells, and attenuation of the effects of anti-PD-L1 immunotherapy | (91) |
| | HMGB1和RICTOR | Activation of the mTORC2-AKT-C-MYC pathway, upregulation of glutamine synthetase expression, deregulation of glutamate dehydrogenase inhibition, and enhancement of glutamine metabolism | (91) |

### 3.3.4 sEVs and T lymphocytes

HCC-derived sEVs modulate immune cell functions within the tumor microenvironment through multiple mechanisms, thereby influencing tumor progression. HCC-derived sEVs deliver 14-3-3ζ protein to tumor-infiltrating lymphocytes (TILs), impairing their activation, proliferation, and antitumor functions while accelerating T cell exhaustion. This mechanism demonstrates how sEVs suppress TIL immunocompetence to attenuate antitumor

responses and promote HCC progression (116). Furthermore, sEVs play pivotal roles in regulatory T cell (Treg) expansion. HCC-sEVs carrying circGSE1 activate the TGFBR1/Smad3 pathway by sponging miR-324-5p, thereby enhancing Treg-mediated immunosuppression. This process inhibits CD8+ T cell antitumor activity and facilitates HCC immune evasion (117).

Multiple studies have investigated HCC-sEVs regulation of dendritic cells (DCs) and DC-mediated T cell responses. For instance, HCC-sEVs are internalized by DCs to present tumor antigens and activate CD8+ T cells, inducing antitumor immunity. However, sEVs concurrently suppress DC IL-12 secretion, which can be restored by IL-12 supplementation to enhance CTL-mediated tumor killing (118, 119).

The immunomodulatory properties of sEVs confer potential as antitumor vaccines. DC-derived sEVs (Dex) combined with microwave ablation (MWA) enhance CD8+ T cell infiltration while reducing Tregs, remodeling the immunosuppressive microenvironment comparably to DC vaccines (120). Moreover, tumor antigen-loaded sEVs potently enhance T cell function, demonstrating robust antitumor activity both *in vitro* and in murine models (121, 122).

The synergy between sEVs and immune checkpoint inhibitors has garnered significant attention. Antigen-loaded DC-derived sEVs (DC-TEX) increase intratumoral CD8+ T cells and elevate IFN-γ/IL-2 cytokine levels. Combined with anti-PD-1, they reverse T cell exhaustion and significantly enhance antitumor immunity (123). Beyond antitumor immunity, sEVs exhibit potential in antiviral immunity. HDV antigen-loaded DC-sEVs activate CD8+ T cells and promote Th1 responses via JAK/STAT signaling to suppress HDV replication (124).

### 3.3.5 sEVs and natural killer cells

HCC-derived sEVs significantly regulate natural killer (NK) cell immune functions. HCC-sEVs deliver miR-92b to NK cells, downregulating CD69 expression and impairing cytotoxicity to facilitate immune evasion (125). Additionally, HCC-sEVs transfer circUHRF1 to downregulate miR-449c-5p and upregulate TIM-3, inducing NK cell exhaustion and impairing anti-PD1 efficacy (126). Another mechanism involves miR-17-5p transfer, which suppresses the RUNX1-NKG2D axis to further compromise NK cell tumoricidal activity (127).

In contrast, NK cell-derived sEVs (NK-exo) enriched with cytotoxic proteins induce HCC apoptosis by inhibiting AKT/ERK1/2 signaling (73). IL-15/IL-21-stimulated NK-exos exhibit enhanced antitumor activity due to elevated cytotoxic protein content (128).

HCC-mediated immunosuppression via sEVs reveals novel immune escape mechanisms, while NK-exos demonstrate therapeutic potential. Future studies should explore blocking protumor sEVs or leveraging NK-exos to enhance antitumor immunity.

### 3.3.6 sEVs and fibroblasts

Cancer-associated fibroblast (CAF)-derived sEVs regulate HCC migration/invasion via noncoding RNAs. Reduced miR-150-3p in CAF-sEVs enhances HCC migratory/invasive capacities. Low miR-150-3p correlates with poor HCC prognosis, suggesting its regulatory role (129). CAF-sEVs deliver miR-92a-3p to activate Wnt/β-catenin signaling, promoting HCC proliferation/stemness (130). CAFs also modulate tumor suppressors to influence HCC progression. CAF-sEVs transfer miR-20a-5p to suppress LIMA1 and enhance HCC malignancy (131).

During metastasis, B[a]P-treated HCC cells transfer circ_0011496 via sEVs to activate lung fibroblasts into CAFs. This circRNA enhances profibrotic/proinflammatory functions via miR-486-5p/TWF1/MMP9 to drive pulmonary metastasis (132). Conversely, CAF-sEVs-delivered miR-29b suppresses metastasis by downregulating DNMT3b and upregulating MTSS1 (133).

Regarding chemoresistance, CAF-sEVs-circZFR enhances cisplatin resistance by inhibiting STAT3/NF-κB signaling (134). sEVs-transferred Gremlin-1 reduces sorafenib sensitivity via EMT and Wnt/β-catenin/BMP pathway modulation (135). These findings highlight CAF roles in HCC TME and suggest therapeutic strategies (Table 3).

### 3.3.7 sEVs and complement system

sEVs employ complement regulators for self-protection. Surface CD55/CD59 inhibit membrane attack complex (MAC) formation to prevent complement-mediated lysis. This enhances sEVs stability in bodily fluids for prolonged immunomodulation. Antigen-presenting cell-derived sEVs maintain structural integrity via this mechanism (136).

sEVs modulate complement via C3 fragments. B cell/macrophage-derived sEVs containing C3 fragments promote complement activation. This enhances antigen presentation and T cell responses. C3 fragments may also confer additional complement resistance (137).

## 3.4 sEVs in multi-network regulation

sEVs participate in complex intercellular communication networks by transporting bioactive molecules including proteins, nucleic acids, and lipids. They play pivotal roles in metabolic regulation and immunomodulation: modulating insulin sensitivity, glycolipid metabolic enzyme activity and mitochondrial function to maintain energy homeostasis, while precisely controlling immune responses through antigen presentation, immune receptor interactions and cytokine regulation. This regulation exhibits high specificity depending on sEVs cargo composition and microenvironmental conditions (Figure 3).

In HCC, sEVs-mediated intercellular communication significantly influences tumor progression. Hypoxic conditions activate HIF-1α, promoting HCC cells to secrete HMMR-AS1 lncRNA-enriched sEVs. Upon macrophage uptake, these vesicles competitively bind miR-147a to upregulate ARID3A, inducing M2 polarization that enhances immunosuppression and accelerates tumor progression (138).

Tumor-associated macrophage (TAM)- and cancer-associated fibroblast (CAF)-derived sEVs regulate HCC metabolism through noncoding RNA delivery. TAM-secreted lncMMPA suppresses

TABLE 3 Main mechanisms involved in the regulation of immune networks by sEVs.

| Immune cell | Key signals | Main mechanisms | Ref. |
|---|---|---|---|
| Macrophage | miR-92a-2-5p | Reduction of androgen receptor expression, enhancement of hepatocellular carcinoma cell invasiveness, and modulation of AR/PHLPP/p-AKT/β-catenin signaling pathway | (96) |
| | PCED1B-AS1 | Release of PCED1B-AS1 via sEVs, resulting in decreased levels of hsa-miR-194-5p in immune cells. Increases PD-L1 and PD-L2 expression, triggering apoptosis and decreased viability of immune cells. | (97) |
| | GOLM1 | Promotion of PD-L1 stability, delivery of PD-L1 to tumor-associated macrophages via sEVs, increase of PD-L1 expression on macrophages, enhancement of immune escape, inhibition of CD8+ T cell activity | (98) |
| | RBPJ | Carrying up-regulated hsa_circ_0004658 by sEVs, competitive adsorption of miR-499b-5p, deregulation of JAM3, and up-regulation of JAM3 expression | (99) |
| | CircTMEM181 | Delivery of circTMEM181 to macrophages via sEVs, promotion of CD39 expression in macrophages, activation of the ATP-adenosine pathway, formation of an immunosuppressive microenvironment, and weakening of the antitumor effect of CD8+ T cells | (100) |
| | lncMMPA | Interacts with miR-548s, enhances ALDH1A3 expression | (139) |
| M1 macrophage | miR-142-3p | Delivery of miR-142-3p via sEVs, targeted down-regulation of SLC3A2 expression, and induction of iron death in M1-type macrophages | (101) |
| | FTCD | Promotion of macrophage polarization to M1 type by sEVs | (102) |
| | miR-628-5p | Delivery of miR-628-5p into HCC cells via M1-Exo, inhibition of METTL14 expression, reduction of m6A modification of circFUT8, and blocking of circFUT8 translocation from nucleus to cytoplasm | (142) |
| | PIONs@E6 | Significantly promotes polarization of M1 macrophages | (103) |
| | MPDA/ICG@M1NVs | Repolarization of immunosuppressive M2 tumor-associated macrophages into anti-tumor M1 macrophages | (104) |
| M2 macrophage | Hsa_circ_0074854 | Delivery of hsa_circ_0074854 to macrophages and promotion of macrophage M2 polarization via sEVs | (105) |
| | miR-452-5p | Targeting TIMP3 and promoting M2 polarization in macrophages | (106) |
| | miR-21-5p | Targeting RhoB and promoting M2 polarization in macrophages | (107) |
| | miR-200b-3p | Inhibition of ZEB1, activation of the JAK/STAT signaling pathway, and promotion of M2 polarization in macrophages | (108) |
| | miR-143-3p | Regulation of the MARCKS gene in TAMs, promotion of M2 polarization in macrophages | (109) |
| | miR-27a-3p | Down-regulation of TXNIP gene, enhancement of stemness characteristics and malignant behavior of HCC cells | (110) |
| | miR-660-5p | Down-regulation of KLF3 gene, enhancement of stemness characteristics and malignant behavior in HCC cells | (111) |
| | PSMA5 | Activation of JAK2/STAT3 pathway, induction of M2 polarization in macrophages | (112) |
| | HEIH | Activation of miR-98-5p/STAT3 pathway, induction of M2 polarization in macrophages | (113) |
| | HMMR-AS1 | Delivery to macrophages via sEVs, competitive adsorption of miR-147a, prevention of its degradation by ARID3A, promotion of M2 polarization | (138) |
| | LncRNA MAPKAPK5_AS1 | Delivery of MAAS via sEVs and promotion of HCC cell proliferation. HBeAg stabilizes MAAS expression in M2 macrophages by enhancing m6A methylation modification | (141) |
| | miR-200c-3p | Activation of PI3K/AKT signaling pathway and enhancement of sorafenib resistance in HCC cells | (114) |
| | miR-23a-3p | Targeting PTEN and TJP1, increasing vascular permeability, and weakening intercellular tight junctions | (115) |
| T lymphocyte | 14-3-3ζ protein | Delivery of 14-3-3ζ protein to tumor-infiltrating T lymphocytes via sEVs results in decreased T cell activation and proliferation, reduced anti-tumor activity, and a greater tendency to depletion | (116) |
| | CircGSE1 | Acts as a sponge for miR-324-5p, activates the TGFBR1/Smad3 signaling pathway, enhances the function of Tregs, and inhibits the anti-tumor activity of effector T cells | (117) |
| | Rab27a | Increased secretion of sEVs, significant promotion of dendritic cell maturation, expression of higher levels of MHC class II molecules and co-stimulatory molecules CD80 and CD86, inhibition of IL-12 secretion by DCs | (119) |
| Natural killer cell | miR-92b | Delivery of miR-92b to natural killer cells via sEVs, inhibition of CD69 expression, and impaired cytotoxicity of NK cells | (125) |
| | CircUHRF1 | Degradation of miR-449c-5p and up-regulation of TIM-3 expression, inhibition of anti-tumor activity of NK cells | (126) |

*(Continued)*

TABLE 3 Continued

| Immune cell | Key signals | Main mechanisms | Ref. |
|---|---|---|---|
| | miR-17-5p | Delivery of miR-17–5 to natural killer cells via sEVs, inhibition of RUNX1-NKG2D axis expression, and attenuation of cytotoxicity and killing capacity of natural killer cells | (127) |
| Fibroblast | miR-150-3p | Reduction of miR-150-3p significantly enhances migration and invasion of HCC cells | (129) |
| | miR-29b | Down-regulation of DNA methyltransferase 3b and up-regulation of tumor suppressor MTSS1 | (133) |
| | Circ_0011496 | Activation of lung fibroblasts, promotion of their transformation to CAFs, regulation of the miR-486-5p/TWF1/MMP9 axis, and enhancement of pro-fibrotic and pro-inflammatory functions of fibroblasts | (132) |
| | miR-92a-3p | Inhibition of AXIN1 gene expression, activation of the Wnt/β-catenin signaling pathway, significant promotion of proliferation and stemness characteristics of HCC cells, and enhancement of tumor invasiveness and metastatic potential | (130) |
| | CircZFR | Enhancement of cisplatin resistance and inhibition of STAT3/NF-κB signaling pathway in HCC cells | (134) |
| | lncRNA TUG1 | Inhibition of miR-524-5p and up-regulation of SIX1 gene expression | (140) |
| | Gremlin-1 protein | Enhancement of epithelial-mesenchymal transition, modulation of Wnt/β-catenin and BMP signaling pathways, and reduction of sensitivity to sorafenib in HCC cells | (135) |
| | miR-20a-5p | Inhibition of the expression of the oncogene LIMA1 | (131) |

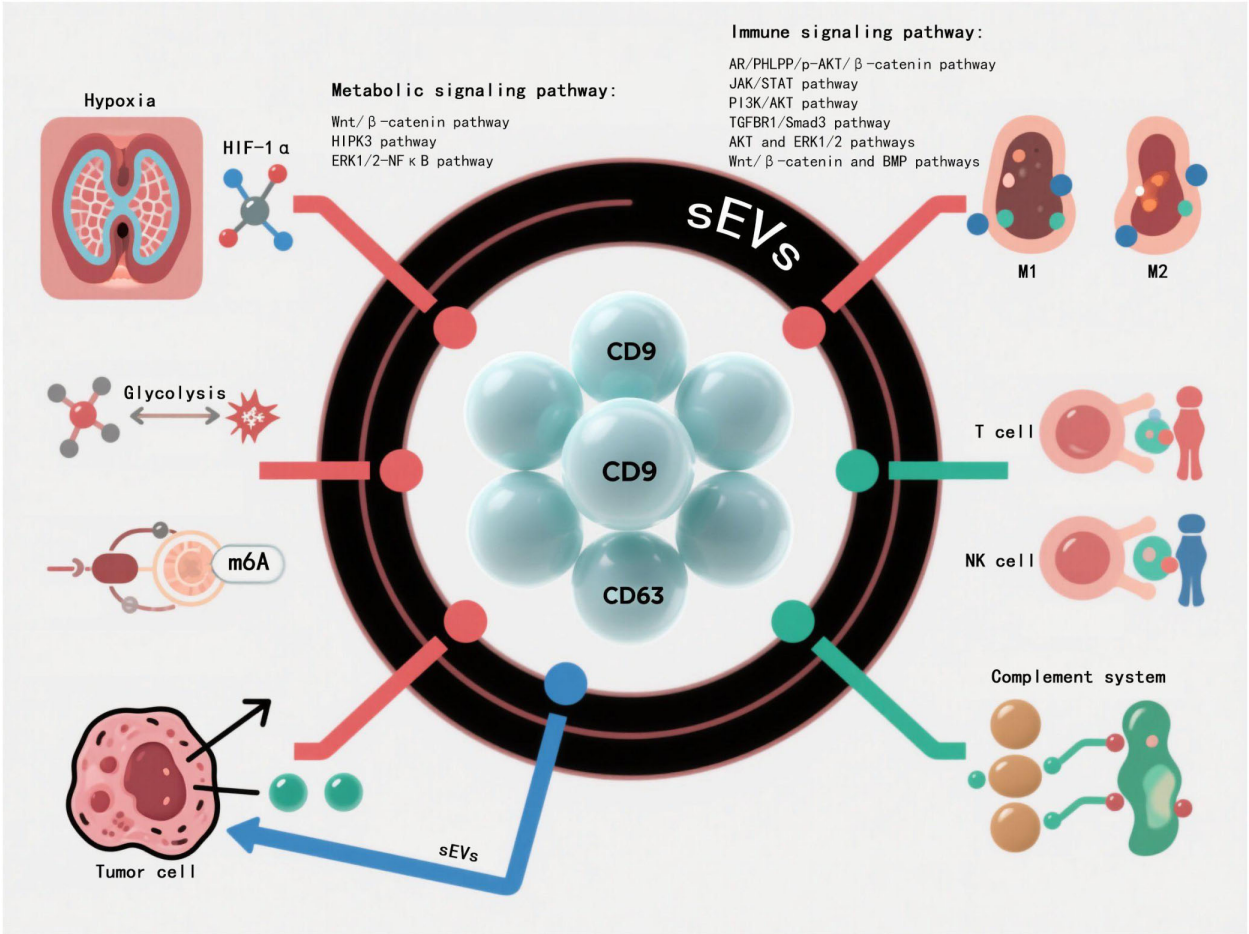


FIGURE 3
sEVs play a core role in multi-network regulation. sEVs systematically integrate into the highly complex intercellular communication network system through the bioactive molecular libraries such as proteomics,nucleic acid components and lipid groups they carry. The metabolic-immune cross-regulatory network mediated by them shows multi-dimensional regulatory characteristics, among which multiple signal transduction pathways constitute the key molecular hubs of cascade regulation.

miR-548s to upregulate ALDH1A3, enhancing glycolysis and tumor proliferation (139). Similarly, CAF-derived TUG1 inhibits miR-524-5p to activate SIX1, promoting glycolysis and invasive capacity (140). These findings demonstrate the central role of sEVs in HCC metabolic reprogramming.

In HBV-associated HCC, HBeAg stabilizes lncRNA MAAS in macrophages via m6A modification, promoting its enrichment in sEVs. MAAS delivery to HCC cells significantly enhances proliferation (141). Conversely, M1 macrophage-derived sEVs deliver miR-628-5p to suppress METTL14-mediated m6A modification of circFUT8, thereby inhibiting tumor growth (142). This contrast highlights the bidirectional regulation of HCC by sEVs through epigenetic mechanisms.

## 4 Therapeutic applications of sEVs in hepatocellular carcinoma microenvironment

sEVs play a central role in regulating the HCC microenvironment. By mediating the transfer of various oncogenic molecules and signaling pathways, sEVs critically regulate the formation and evolution of the HCC tumor microenvironment. In HCV-associated HCC, CD81+ sEVs significantly impair host immune surveillance through immune evasion mechanisms, establishing them as promising therapeutic targets (59). The Rab27B-dependent sEVs-mediated drug efflux mechanism has been shown to substantially enhance chemoresistance in HCC cells (51). HBV core antigen (HBc) upregulates miR-135a-5p in sEVs to inhibit VAMP2 function, promoting anti-apoptotic properties and drug resistance in HCC cells (60). These studies elucidate the molecular mechanisms of sEVs-mediated therapy resistance in HCC and identify multiple potential targets for therapeutic intervention.

sEVs exhibit multifaceted regulatory functions in HCC immune evasion. sEVs surface-associated immune checkpoint molecules like PD-L1 effectively suppress T cell antitumor activity, reducing clinical response to immunotherapy (82, 91, 97, 98). Under hypoxic conditions, sEVs selectively enrich and deliver specific miRNAs/lncRNAs to enhance immunosuppression and accelerate HCC progression (76–78, 81, 82, 138).

With inherent biocompatibility and targeting capabilities, sEVs offer distinct advantages for drug delivery systems. Nanoengineered sEVs significantly improve targeting precision and bioavailability of therapeutic agents (68, 103, 104, 143). For gene/immunotherapies, sEVs demonstrate remarkable clinical potential by efficiently delivering functional nucleic acids or immunomodulators to enhance T cell activation and antitumor immunity (118, 122).

Artificial intelligence is transforming methodological approaches in sEVs research. Machine learning algorithms significantly enhance TEM and cryo-EM capabilities for sEVs ultrastructural analysis, enabling automated classification and quantification. AI-driven multi-omics integration efficiently identifies sEVs-associated diagnostic biomarkers, with random forest models demonstrating reliability for liquid biopsy applications. Computational biology frameworks integrate sEVs-mediated intercellular networks with tumor ecosystem dynamics, providing novel paradigms for studying oncogenesis.

## 5 Conclusion

HCC ranks among the most prevalent and lethal malignancies worldwide. Emerging fundamental research demonstrates that sEVs play pivotal regulatory roles in HCC pathogenesis and progression. As crucial intercellular communication vehicles, sEVs orchestrate HCC initiation, progression and malignant transformation through complex molecular networks encompassing cellular communication, metabolic regulation and immunomodulation.

By transporting diverse bioactive molecules (miRNAs, proteins, lipids), sEVs establish sophisticated signaling networks between cancer cells and microenvironmental components. These molecules enhance cancer cell proliferation, invasion and metastatic potential. Specifically, sEVs-enclosed miRNAs can selectively suppress tumor suppressor genes to accelerate HCC malignancy. Furthermore, sEVs reinforce malignant phenotypes by reprogramming cancer cell gene expression profiles.

Regarding metabolic regulation, sEVs modulate HCC metabolic characteristics through multiple mechanisms. They transfer critical metabolic enzymes/regulators and substantially alter glucose, lipid and energy metabolism pathways in HCC cells. Studies show sEVs enhance aerobic glycolysis via specific metabolic enzymes, sustaining proliferative capacity even under hypoxic conditions.

In immunomodulation, sEVs critically contribute to HCC immune evasion. They deliver immune checkpoint molecules (e.g., PD-L1) to impair immune surveillance and tumor clearance. Concurrently, sEVs modulate tumor-associated macrophage polarization while suppressing T/NK cell antitumor activity, establishing an immunosuppressive niche favorable for tumor growth.

In summary, sEVs comprehensively participate in shaping the HCC microenvironment by integrating cellular communication, metabolic reprogramming and immune evasion networks. These findings not only deepen our understanding of HCC pathogenesis but also provide theoretical foundations for novel diagnostic/therapeutic strategies. Future studies should further elucidate sEVs molecular mechanisms in HCC and explore clinical applications of sEVs-based targeted therapies and drug delivery systems. With advancing research, sEVs may emerge as crucial breakthroughs in HCC diagnosis and treatment.

## Author contributions

XY: Writing – original draft. DH: Writing – original draft. LP: Writing – original draft. LW: Writing – review & editing. YL: Writing – review & editing. JY: Writing – review & editing. YQ: Writing – review & editing. CS: Writing – review & editing. QW: Writing – review & editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Generative AI statement

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EDITED BY
Qiang Fu,
Binzhou Medical University, China

REVIEWED BY
Etsuro Ito,
Waseda University, Japan
Tao Wu,
Tsinghua University, China

*CORRESPONDENCE
Jianmei Yin
yinjianmei1985@jlu.edu.cn

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# Recent progress in the study of exosomes in the gastric cancer immune microenvironment

Haibo Liu[1], Lei Zhu[1] and Jianmei Yin[2*]

[1]Emergency Department, The First Hospital of Jilin University, Changchun, Jilin, China, [2]Department of Otolaryngology-Head and Neck Surgery, The First Hospital of Jilin University, Changchun, Jilin, China

Gastric cancer (GC) ranks among the most prevalent forms of cancer and contributes significantly to cancer-related mortality. There exists a pressing need to investigate novel approaches for GC management to improve diagnostic methods, therapeutic interventions, and patient outcomes. Exosomes are nanoscale extracellular vesicles (EVs) derived from various cell types that carry a diverse range of biomolecular cargo, including DNA, RNA, proteins, lipids, and other bioactive constituents. They play significant roles in GC pathogenesis and tumor microenvironment (TME) modulation. Exosomes derived from cancer cells can enhance tumor progression, transform the TME, and modulate immune responses. Immune cell-derived exosomes can similarly modulate immune functions and the TME. Immunotherapy represents a GC treatment breakthrough and is expected to show efficacy when combined with exosome-targeted therapy. Abundant research has demonstrated that exosomes are crucial for tumor growth, immune evasion, immune microenvironment reconfiguration, and immunotherapy efficacy in GC. This review describes the role of exosomes in the GC microenvironment, focusing on the mechanisms by which exosomes regulate immune responses to GC, and summarizes the current status of and challenges in the development of exosome-based diagnostics and immunotherapy for GC.

KEYWORDS

**gastric cancer, exosomes, immunity, tumor microenvironment, tumor immunotherapy**

## 1 Introduction

Gastric cancer (GC) is the fourth-most common cause of cancer-related deaths and the fifth-most common cancer type globally (1). Factors affecting the development of GC include genetic polymorphisms, environmental exposures, age, sex, and infection with *Helicobacter pylori* (2). Due to the low proportion of early-stage diagnoses and lack of definite clinical symptoms, GC is commonly detected in the advanced metastatic phase, where the 5-year survival rate is only approximately 32% (3). Effective treatment options for GC include surgery, radiotherapy, chemotherapy, targeted therapy, and immunotherapy, but even after surgery, approximately 60% of patients experience local

recurrence or distant metastasis (4). As a result, the exploration of new, robust biomarkers and therapeutic strategies is crucial for improving the prognosis and quality of life of GC patients.

The prognosis of GC patients and their responses to immunotherapy are impacted considerably by morphological and molecular heterogeneity in the tumor microenvironment (TME). Immunomodulatory cells in the GC TME include regulatory T cells, tumor-infiltrating myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), and natural killer (NK) cells (5). Immune cells can also interact with cancer cells to influence the onset and progression of cancer.

Exosomes are a type of extracellular vesicle (EV) with diameters of 30–150 nm. They are secreted by virtually all types of cells and can be stably present in a variety of biological fluids (6). The biogenesis of exosomes commences with the invagination of the plasma membrane, a process that gives rise to early endosomes. These early endosomes subsequently mature into multivesicular bodies (MVBs), which fuse with the plasma membrane and are released into the extracellular milieu as exosomes (7) (Figure 1A). Under strict cellular regulation, released exosomes encapsulate a diverse array of biomolecules, including proteins, RNA, DNA, lipids, and cytokines (8). Exosomes are composed of a lipid bilayer that contains transmembrane proteins; delivery of this cargo can mediate local and distant cell communication under physiological and pathological conditions (9). Several studies have shown that exosome-mediated transport of bioactive signaling molecules in the TME has diagnostic and therapeutic functions (10). Indeed, numerous lines of investigation have demonstrated that the delivery of exosome cargo is vital for GC proliferation, metastasis, drug resistance, immune response, and treatment (11). Exosomes have also attracted attention in the field because they are more stable than circulating proteins and hormones and can serve as early biomarkers for cancer detection and disease progression (12). In addition, improvements in exosome engineering technology have led to the development of targeted exosomes, a promising way to deliver anti-neoplastic therapies (13). Indeed, due

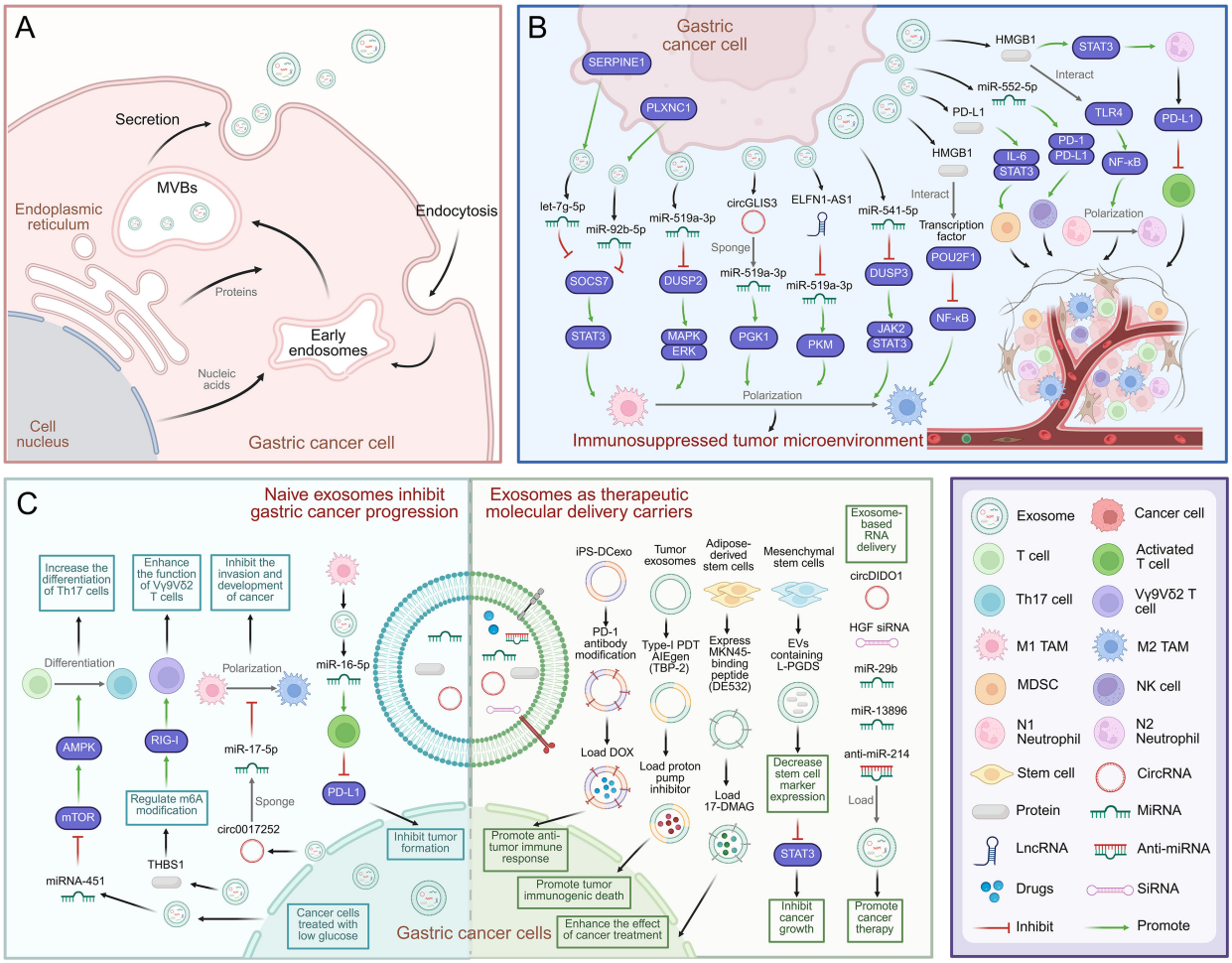


FIGURE 1
Mechanisms of exosome-mediated immune escape and immunotherapy in GC. **(A)** Schematic diagram of exosome biogenesis in GC cells. **(B)** Exosomes from GC mediate communication between GC cells and immune cells, and promote GC cell development and immunosuppression in immune TME. **(C)** Mechanism of natural exosomes promoting immunotherapy in gastric cancer. Exosomes can be used as carriers to deliver therapeutic molecules to promote the treatment of gastric cancer, including chemotherapy drugs, proteins and RNA. Created with BioRender.com.

to their biocompatibility, low immunogenicity, and ability to transport biomolecules between cells and cross biological barriers, they are suitable for the targeted delivery of various therapeutic modalities such as small molecules, siRNA, and miRNA (14).

The tumorigenesis and progression of GC are intimately associated with immune cells and other types of mesenchymal stromal cells (MSCs), cytokines, and exosomes in the TME (15). Exosomes derived from cancer cells or tumor-associated immune cells can carry factors that suppress immune cell activity and help tumors evade immune surveillance (8). Therefore, it is important to investigate the mechanisms by which exosomes regulate the immune microenvironment of GC. To this end, this review aims to summarize the mechanisms of exosome-mediated GC development, focusing on recent studies of exosome-mediated immune escape. In addition, tumor immunotherapies, which consist of immune checkpoint inhibitors, cellular immunotherapy, and therapeutic cancer vaccines, have drawn substantial attention within the field. Identifying key targets and elucidating the molecular mechanisms associated with GC immunity will improve our understanding of GC pathogenesis and augment immunotherapy efficacy (16). Thus, we have reviewed the role of exosomes in GC immune responses, as well as recent advances in exosome-targeted treatments. Together, these studies provide a basis for potential GC treatment strategies.

## 2 Exosomes in GC diagnosis and prognosis

Abundant evidence indicates that exosomes are intricately linked to GC tumorigenesis, progression, metastasis, immune escape, and drug resistance through the delivery of functional biomolecules (17). Cancer initiation and development are generally influenced by the function of cancer cells or cells in the microenvironment. The bioactive substances carried by exosomes, including exosomal proteins, miRNAs, long non-coding RNAs (lncRNAs), and circular RNAs (circRNAs), are involved in many important processes (18) as described below, including the diagnosis, prognosis, and clinical treatment of GC (Table 1).

Early detection and diagnosis of GC are crucial for improving patient prognosis. Liquid biopsy is a non-invasive method for detecting circulating tumor cells, circulating tumor DNA, and EVs in serum and other bodily fluids (19). Research has employed AI to analyze surface-enhanced Raman spectra (SERS) of exosomes, leveraging classification models that recognize signal patterns in plasma exosomes to enable simultaneous identification of multiple early-stage cancers—including GC (20). Exosomal ncRNAs and proteins have shown great promise as molecular cancer diagnostic biomarkers (21). Indeed, 182 candidate GC

TABLE 1 Exosomes in GC diagnosis and prognosis.

| Origin | Target | Contents | Mechanism | Effect | Ref. |
|---|---|---|---|---|---|
| GC cells | Circulating EVs | LncGC1 | As an early marker of therapeutic efficacy of neoCT | | (25) |
| GC cells | Serum | PD-L1 | Reflect the immunosuppressive state of advanced GC patients | | (26) |
| GC cells | Plasma | PKM2 | Plasma exosome PKM2 is a novel biomarker and can be used for the clinical diagnosis of GC | | (27) |
| GC cells | Plasma | Circ_0079439 | Exosomal circ_0079439 may be a potential biomarker for the early and late diagnosis of GC | | (28) |
| GC cells | Serum | LncHOTTIP | As a potential diagnostic and prognostic biomarker for gastric cancer | | (29) |
| GC cells | Peritoneal lavage samples | Let-7g-3p and miR-10395-3p | Can be used as a biomarker to predict the effect of peritoneal metastasis and systemic chemotherapy | | (30) |
| GC cells | GC cells | GRP78 | Promote cancer stemness | | (31) |
| GC cells | Lymphatic endothelial cells | LncAKR1C2 | Exosomal lncAKR1C2 promotes CPT1A expression by regulating YAP phosphorylation | Enhance tube formation and migration of lymphatic endothelial cells, and promote lymphangiogenesis and lymphatic metastasis *in vivo* | (33) |
| GC cells | Human umbilical vein endothelial cells | NOS3 | MiR-605-3p mediates the production of exosomal NOS3 | Induce angiogenesis, establishe liver PMN, and promote liver metastasis | (35) |
| GC cells | Serum | CircHIPK3 | CircHIPK3 blocks autophagy dependent ferroptosis | Can be used as a noninvasive index to evaluate cisplatin resistance in GC | (38) |
| Cisplatin-resistant GC cells | GC cells | MiR-769-5p | Exosomal miR-769-5p targets caspase-9 and promote ubiquitination degradation of p53 | Lead to cisplatin resistance in GC and promote cancer progression | (39) |
| Cisplatin-resistant GC cells | GC cells | Lnc00852 | Exosomal lnc00852 regulates COMMD7 through miR-514a-5p | Lead to cisplatin resistance in GC | (40) |
| CAFs | GC cells | MiR-522 | Exosomal miR-522 targets ALOX15 and inhibits ferroptosis | Mediate acquired chemotherapy resistance in GC | (42) |

biomarkers in serum exosomes have been identified via RNA sequencing, facilitating the machine learning-assisted identification of exosomal ncRNA characteristics for non-invasive, early detection of GC (22). The characteristics of miRNA in circulating exosomes can also predict peritoneal metastasis in patients with advanced gastric cancer (23). In addition, the characteristics of circulating exosome-derived mRNA, miRNA, and lncRNA in liquid biopsies have the potential to predict the therapeutic outcomes of neoadjuvant chemotherapy (neoCT) in advanced GC patients (24). Still other research has demonstrated that lncRNA-GC1 derived from circulating EVs serves as an early indicator of neoCT efficacy and can predict the survival rates of GC patients undergoing this therapy (25). Additionally, exosomal programmed death ligand 1 (PD-L1) is linked to systemic inflammatory markers, immunomodulatory cytokines, and T cells, and exosomal PD-L1 in serum may reflect an immunosuppressive state in advanced GC patients (26). Plasma-derived exosomal PKM2 modulates the TME by activating the SREBP1-associated lipid synthesis pathway in macrophages. Elevated expression of exosomal PKM2 correlates with unfavorable prognosis in GC patients and represents a promising novel biomarker (27). Another study result indicates that the upregulated plasma exosomal hsa_circ_0079439 in patients with GC may be a potential biomarker for the early and late diagnosis of GC (28). Besides, it has been demonstrated that exosomal-derived lncRNA HOTTIP may be a novel biomarker for GC diagnosis and outcome prediction (29). Other studies have shown that exosomes from GC peritoneal lavage fluid are rich in numerous miRNAs associated with peritoneal metastasis, such as let-7g-3p and miR-10395-3p, which can be used as biomarkers of peritoneal metastasis and chemotherapy efficacy (30). Moreover, studies have employed ultrasensitive ELISA combined with thio-NAD cycling to quantify trace amounts of 78-kDa glucose-regulated protein (GRP78) in exosomes released by GC cells. It was found that GRP78-enriched exosomes can promote cancer stemness (31).

Exosomes also have the capacity to facilitate the migration/metastasis of GC cells to local or distant tissues and organs. At initial diagnosis, over half of GC patients have detectable lymph node metastases, which frequently results in the development of distant metastasis and a poor prognosis (32). GC cell-derived exosomal lncAKR1C2 encodes the micro-protein pep-AKR1C2, which enhances CPT1A expression through the regulation of YES-associated protein 1 (YAP1) phosphorylation. It also improves the tube-forming ability and migratory capacity of lymphatic endothelial cells, thereby facilitating lymphangiogenesis and promoting tumor lymphatic metastasis *in vivo* (33). The establishment of pre-metastatic niches (PMN) in distant organs is critical for tumor metastasis (34). Studies have shown that nitric oxide synthase 3 (NOS3) originating from GC cell-derived exosomes can increase the concentration of nitric oxide within human umbilical vein endothelial cells, triggering angiogenesis, facilitating the formation of liver PMNs, and augmenting GC hepatic metastasis (35). Additionally, studies have isolated plasma exosomes from metastatic GC patients undergoing systemic chemotherapy and developed a liquid biopsy assay based on exo-miRNAs, and differentially expressed exo-miRNAs were identified as potential biomarkers for predicting chemotherapy resistance in GC patients (36).

Exosomes also mediate intercellular crosstalk during cancer progression and enhance therapeutic resistance. Currently, cisplatin-based chemotherapy is the primary treatment choice for advanced GC patients; however, a significant number of patients exhibit cisplatin resistance due to epigenetic alterations, signaling pathway aberrations, and disruptions in cell metabolism (37). CircHIPK3 has been shown to promote GC cisplatin resistance by obstructing ferroptosis and, when present in serum exosomes, may be a non-invasive marker of cisplatin resistance (38). In addition, exosomal miR-769-5p from cisplatin-resistant GC cell lines imparts cisplatin resistance to recipient GC cells. It further promotes cancer progression by targeting caspase-9 and enhancing the ubiquitination and degradation of p53 (39). Furthermore, exosomal lnc00852 originating from cisplatin-resistant GC cells also modulates COMM domain containing 7 (COMMD7) via miR-514a-5p to promote cisplatin resistance in recipient cells (40). Finally, cancer-associated fibroblasts (CAFs) are the primary stromal cell type in the TME (41). These cells secrete exosomal miR-522, which inhibits cancer cell ferroptosis by targeting arachidonate 15-lipoxygenase (ALOX15) and blocking lipid peroxidation, mediating acquired chemotherapy resistance in GC (42).

# 3 Exosomes can mediate GC immune escape

## 3.1 Exosomes derived from GC cells mediate immune escape

Cancer cells can regulate the immune milieu by releasing exosomes. Many studies have revealed that exosomes make critical contributions to the reconfiguration of the TME, thereby facilitating cancer cells' escape from the immune system (43) (Figure 1B). Research has demonstrated that exosomes extracted from GC cell lines can alter the function and gene expression of CD8+ T cells, increase the frequency of effector memory CD4+ T cells and MDSCs, and reduce the frequency of CD8+ T cells and NK cells. Consistent with this observation, mice injected with GC cell-derived exosomes develop an immunosuppressive pulmonary TME. Studies have shown that exosomes originating from GC cells regulate the TME by suppressing immune function (44). Therefore, exploring the molecular characteristics of exosomes is critical for improving our understanding of immune escape mechanisms in GC (45).

T cell activation is central to the anti-tumor immune response (46). Some studies have found that exosomal circMAN1A2 can promote the development of GC and inhibit T cell anti-tumor activity. Specifically, circMAN1A2 competes with F-Box and WD repeat domain containing 11 (FBXW11) to bind and stabilize splicing factor proline and glutamine rich (SFPQ) expression,

inhibiting T cell receptor stimulation and reducing T cell anti-tumor activity (47). Moreover, other studies have shown that lysine-specific demethylase 1 (LSD1) restricts T cell responses in the GC TME by triggering the aggregation of PD-L1 in GC exosomes, providing a novel target for GC immunotherapy (48). Finally, Vγ9Vδ2 T cells can effectively internalize exosomes derived from GC cells that carry miR-135b-5p. The miR-135b-5p impairs the function of Vγ9Vδ2 T cells by targeting specific protein 1 (SP1), inducing apoptosis, and reducing the production of the cytotoxic cytokines IFN-γ and TNF-α. Thus, targeting the exosomal miR-135b-5p/SP1 axis may improve the efficiency of Vγ9Vδ2 T cell-based GC immunotherapy (49).

TAMs, particularly M2-polarized TAMs, can be recruited and regulated by tumor-derived inflammatory cytokines and immunosuppressive metabolites, rendering them important mediators of GC tumor progression, immune escape, and therapeutic resistance (50). Studies have demonstrated that in GC cells, increased serpin family E member 1 (SERPINE1) expression leads to higher levels of let-7g-5p in exosomes. In turn, exosomal let-7g-5p is transferred to and taken up by macrophages, reducing the levels of suppressor of cytokine signaling 7 (SOCS7). By disrupting the interaction between SOCS7 and signal transducer and activator of transcription 3 (STAT3), it removes the inhibitory effect on STAT3 phosphorylation, leading to STAT3 over-activation and driving M2 polarization (51). Additionally, Biglycan belongs to the family of small proteoglycans. GC cell-derived exosomes containing Biglycan are delivered to macrophages, which triggers M2 polarization and upregulates CXCL10 expression. Consequently, this mechanism activates the JAK/STAT1 signaling pathway and enhances the proliferative, invasive, and metastatic capabilities of GC cells (52). Liver metastasis (LM) confers a poor prognosis to individuals suffering from GC. Notably, miR-519a-3p expression in exosomes from GC patients with LM is strikingly elevated compared to exosomes from GC patients without LM. Exosomal miR-519a-3p triggers the activation of the MAPK/ERK pathway by targeting dual specificity phosphatase 2 (DUSP2), resulting in the M2-like polarization of macrophages, facilitating the establishment of a pre-metastatic intrahepatic niche, and promoting GC-LM progression (53). In addition, plexin C1 (PLXNC1) inhibits SOCS7-STAT3 interactions by transferring GC cell-derived exosomal miR-92b-5p to macrophages, activating STAT3, and promoting GC cell proliferation and M2 TAM polarization (54). In addition, exosomal circGLIS3 promotes GC metastasis and the M2-like polarization of macrophages. Mechanistically, circGLIS3 sequesters miR-1343-3p, up-regulating PGK1 expression and modulating vimentin phosphorylation to drive GC tumorigenesis (55). Additionally, ELNF1-AS1 is highly enriched in GC-derived exosomes and targets miR-4644 to trigger pyruvate kinase M1/2 (PKM) expression. Exosomal ELNF1-AS1 in GC exosomes can also regulate glycolysis through PKM in a hypoxia inducible factor 1 subunit α (HIF-1α)-dependent manner, where it contributes to M2 TAM polarization and macrophage recruitment, thereby enhancing the growth and metastatic capacity of GC cells (56). Another study demonstrated that GC cells can also induce macrophage M2 polarization through the DUSP3/JAK2/STAT3 pathway, which is mediated by exosomal miR-541-5p (57). Moreover, the polarization of macrophages towards the M2-like phenotype is regulated by the deactivation of the NF-κB signaling pathway. This process results from the inhibition of p50 transcriptional activity via the engagement of high mobility group box-1 protein (HMGB1), present in exosomes derived from GC cells, with the transcription factor POU class 2 homeobox 1 (POU2F1) (58).

MDSCs are the principal immunosuppressive cells in the TME, and up-regulation of PD-L1 expression in the gastric epithelium can increase the number of tumor-infiltrating MDSCs (59). Studies have revealed that exosomal PD-L1 from GC cells may promote immunosuppression by promoting MDSC clustering and proliferation by activating the IL-6/STAT3 signaling pathway (60). Neutrophils are also important players in cancer development and progression and can promote cancer growth, metastasis, angiogenesis, and immunosuppression (61). Some studies have shown that neutrophils can promote tumor phenotypes through polarization. Specifically, exosomes derived from GC cells induce neutrophil autophagy and promote tumor activation through HMGB1/TLR4/NF-κB signaling (62). In addition, EVs in the GC microenvironment convey HMGB1 to trigger STAT3 activation, which up-regulates PD-L1 gene expression in neutrophils, thus inhibiting T cell-mediated immunity and highlighting the multidimensional role of EVs in regulating the immunosuppressive microenvironment (63). Furthermore, NK cells are crucial for immune homeostasis and preventing tumorigenesis; however, reductions in their efficiency have been noted in both GC tissue and peripheral blood (64). Indeed, studies have revealed that miR-552-5p derived from GC cell exosomes can drive the progression of GC by modulating the PD-1/PD-L1 axis, which affects NK cell function and impacts GC EMT (65).

## 3.2 Exosomes from immune cells mediate GC immune escape

Considerable evidence has shown that exosomes produced by immune cells can also impact the TME and are important regulators of tumor progression (Supplementary Figure 1). In particular, the multifunctional role of M2 TAM-derived exosomes in cancer progression has been extensively studied. Studies have shown that MALAT1 from M2 TAM-derived exosomes engages with the δ-catenin protein and impedes its ubiquitination and degradation via β-transducin repeats-containing proteins (β-TRCP). Moreover, MALAT1 sequesters miR-217-5p to up-regulate HIF-1α expression, thereby enhancing aerobic glycolysis in GC cells. These findings imply that M2 TAM-derived exosomes facilitate GC progression through MALAT1-mediated glycolytic regulation, presenting a potential target for GC treatment (66). Moreover, TAMs are a unique group of immune cells that express apolipoprotein E (ApoE) in the GC microenvironment. Indeed, M2 macrophage-derived exosomes trigger the activation of the PI3K/AKT signaling pathway in recipient GC cells via ApoE, thus enhancing the migration of GC cells (67).

TAMs are abundant in the TME and can regulate chemotherapy resistance (68). It has been shown that M2 macrophage-derived exosomes containing circTEX2 regulate the miR-145/ABCC1 axis, thereby increasing GC cell cisplatin resistance. These data suggest that exosome transfer between macrophages and cancer cells may be a robust target for reducing cisplatin resistance in GC (69). Furthermore, circ0008253 from M2-polarized TAM-derived exosomes can be transferred from TAMs to GC cells, ultimately enhancing GC cell resistance to oxaliplatin (70). Furthermore, the lncRNA CRNDE is enriched in exosomes derived from M2-polarized TAMs and can be transferred to GC cells. Mechanistically, CRNDE promotes NEDD4-1-mediated PTEN ubiquitination and reduces cisplatin resistance in GC (71). Additional work demonstrated that exosomal miR-588 secreted by M2 macrophages encourages cisplatin resistance in GC cells by partially targeting CYLD (72).

Tumor-associated neutrophils (TANs) play dual roles in tumors, where N1 TANs have anti-tumor functions and N2 TANs exhibit pro-tumor activities (73). Neutrophil-derived exosomes regulate the initiation and progression of tumors by delivering mRNA, miRNA, and piRNA molecules. Studies have shown that exosomes from N2 TANs transfer miR-47445-5p/miR-3911 to GC cells, down-regulating the expression of slit guidance ligand 2 (SLIT2) and promoting GC metastasis (74).

# 4 The role of exosomes in GC immunotherapy

## 4.1 Exosomes can augment GC immunotherapy

Many recent studies have revealed that exosome-mediated crosstalk between cancer cells and immune cells in the TME can impact the outcome of immunotherapy (16) (Figure 1C). For example, γδ T cells play crucial roles in innate and adaptive immune surveillance and are receiving increasing attention in the context of cancer immunotherapy. Immunotherapies based on γδ T cells have shown favorable safety profiles and clinical responses in patients with a variety of cancers (75). Indeed, studies have demonstrated that thrombospondin 1 (THBS1) in exosomes derived from GC cells regulates m6A modification in Vγ9Vδ2T cells and activates the retinoic acid-inducible gene-I (RIG-I) receptor signaling pathway, leading to increased Vγ9Vδ2T cell cytotoxicity toward GC cells. Thus, targeting the exosomal THBS1/m6A/RIG-I axis could be of great significance for Vγ9Vδ2T cell-based GC immunotherapy (16). Furthermore, exosomes facilitate the transfer of miR-451 from GC cells to infiltrating T cells, leading to an increase in T cell Th17 polarization via reduced AMP-activated protein kinase (AMPK) and enhanced mammalian target of rapamycin (mTOR) activity (76).

Macrophage-derived exosomes carrying ncRNAs and immune factors can promote immune activation by regulating B cells, T cells, and NK cells (77). Studies have demonstrated that exosomes derived from M1 macrophages, which contain miR-16-5p, can initiate T cell immune responses and impede the formation of GC tumors by decreasing the expression of PD-L1 (78). Moreover, exosomal circ0017252 released from GC cells can efficiently suppress M2-like polarized macrophages and inhibit the invasion and malignant progression of GC cells by sequestering miR-17-5p (79).

## 4.2 Exosomes can be used as delivery vehicles for GC treatment

Although numerous cytotoxic chemotherapeutic agents, targeted therapies, and immunomodulators have demonstrated remarkable cancer treatment efficacy, challenges such as drug resistance and side effects remain, and the development of new approaches is crucial. Recent studies indicate that the loading of therapeutic agents into nanoparticles designed specifically to target GC may improve treatment outcomes and greatly reduce adverse effects (80) (Figure 1C). For example, some studies have investigated the effects of a tumor-targeting nanosystem, in combination with chemotherapy and immunotherapy, on GC treatment and prognosis. Specifically, a tumor-targeting system based on a fusion vector of modified iPSC and DC exosomes, DOX@aiPS-DCexo, was developed and modified with an anti-PD-1 antibody. Additionally, when the chemotherapy drug doxorubicin (DOX) was loaded into the DOX@aiPS-DCexo fusion system, it was capable of specifically targeting and eliminating tumor tissues. The system also had the ability to activate and enhance a range of local immune responses and mitigate tumor-associated immunosuppression, highlighting the efficacy of combined chemotherapy and immunotherapy treatment (81). In addition, the efficacy of aggregation-induced emission luminogen (AIEgen)-based photodynamic therapy (PDT) is constrained by cellular glutathione (GSH), the latter of which must be reduced to effectively induce oxidation within tumor cells. Consistent with this observation, studies have leveraged tumor-derived exosomes for the co-delivery of AIEgens and proton pump inhibitors in the context of tumor combination treatment. This system can restrain cell glutamine metabolism, inhibit the generation of GSH and ATP in tumor cells, improve the effect of AIEgen type I PDT, and promote immunogenic tumor death (82). Other studies have described the development of engineered exosomes, consisting of genetically engineered adipose-derived stem cells that express the MKN45-binding peptide DE532 on their surfaces and in which 17-(dimethylaminoethyl amino)-17-demethoxygeldanamycin (17-DMAG) has been encapsulated. These targeted, 17-DMAG-loaded DE532 exosomes effectively delivered anti-cancer agents, enhancing the therapeutic responses of GC (13). Moreover, the lipid carrier protein prostaglandin D2 synthase (L-PGDS) has been shown to inhibit GC growth. L-PGDS-loaded EVs (EVs-L-PGDS) were produced by transducing MSCs with adenovirus encoding L-PGDS. These EVs-L-PGDS decreased the expression of stem cell markers such as Oct4, Nanog, and Sox2, and blocked STAT3 phosphorylation, suppressing GC tumor development and suggesting that MSC-derived EVs can act as efficient nano-capsules (83).

In addition, exosome-mediated delivery of RNA has demonstrated remarkable cancer treatment potential. Indeed, exosome-mediated siRNA, miRNA, and anti-miRNA oligonucleotide delivery has been extensively studied in the treatment of diverse cancers; modification of these exosomes through engineering can further improve their targeting capacity and therapeutic efficacy (84). Studies have demonstrated that circDIDO1 suppresses GC progression via the regulation of the miR-1307-3p/SOSC2 axis and that the use of RGD-modified exosomes with circDIDO1 (RGD-Exo-circDIDO1) can reduce GC occurrence and invasion. These results suggest that engineered RGD-Exo-circDIDO1 could represent a feasible nanomedicine for GC treatment (85). Additionally, exosomes acting as nanoparticles impede tumor advancement and angiogenesis in GC by transporting hepatocyte growth factor (HGF) siRNA (86). In another study, the quantity of miR-29b within the peritoneal exosomes of patients with pre-metastases (PMs) was markedly reduced. Transduction of human bone marrow-derived MSCs with an integrated recombinant lentiviral vector encoding miR-29b confirmed that sEVs from bone marrow MSCs were effective carriers of miR-29 that could inhibit the development of PM in GC (87). Still another study used electroporation to insert miR-13896 into human umbilical cord MSC-EVs. These engineered EVs were effectively transported to tumor sites where miR-13896 specifically targets and down-regulates the ATG2A-mediated autophagy pathway, thereby significantly suppressing the growth and metastasis of GC cells (88). Furthermore, exosomes were utilized to deliver anti-miR-214, with the aim of reversing cisplatin-based chemotherapy resistance in GC, and successfully suppressed tumor growth (89).

## 5 Discussion

GC poses a global healthcare challenge. By 2040, it is projected that the incidence of GC will rise by 62%, resulting in a substantial burden on public health services, costs, and patient quality of life (90). GC patients treated with chemotherapy and surgery have a poor prognosis, and many current clinical trials of late-stage tumors are evaluating targeted agents and immunotherapies (91). The GC TME is a highly structured ecological system containing cancer cells, immune cells, CAFs, endothelial cells, pericytes, and various other cell types. These elements work together to sustain proliferation signals, initiate invasion and metastasis, and suppress immune reactions (92). Exosomes are important messengers between cancer cells and TME cells. Indeed, preliminary studies have shown that exosomes generated by cancer cells control the phenotypes and functions of TME cells, driving tumor growth, metastasis, and the emergence of treatment resistance. Exosomes derived from TME cells also contain a broad spectrum of bioactive molecules and have been implicated in the regulation of tumor malignancy (74).

Exosomes play crucial roles in GC-associated immune responses. Specifically, numerous studies have shown that tumor-derived exosomes affect the differentiation, proliferation, and functional regulation of various immune cell populations in the TME, including T cells, macrophages, neutrophils, and MDSCs (93). This review focuses on the complex mechanisms by which tumor-derived exosomes regulate GC progression, the immune microenvironment, and immune escape. In addition, exosomes derived from immune cells can also regulate immune responses to GC cells and reshape the immune microenvironment through the delivery of biological molecules such as ncRNAs. Therefore, targeting exosome-secreting immune cells may represent a promising approach to improve the efficacy of GC immunotherapy (94). Furthermore, exosome biogenesis is intricately regulated. GC cells regulate exosome secretion to facilitate pre-metastatic niche formation (95). Meanwhile, crosstalk between cancer cells and other cells in TME via signal transduction enhances exosome biogenesis, which in turn modulates the TME (96). Hypoxia, for instance, also promotes GC exosome release through a HIF-1α-dependent pathway, impacting tumor development and metastasis (97). Therefore, the study of exosomes in the immune microenvironment of GC tumors can facilitate the development of more personalized targeted therapy and immunotherapy regimens for GC patients; improve our understanding of the molecular mechanisms underlying GC proliferation, progression, metastasis, and treatment resistance; and reveal new diagnostic and prognostic biomarkers and potential immunotherapeutic targets.

The success of immunotherapy, which aims to reinstate normal anti-tumor immune responses, reinitiate anti-tumor immunity, and further eliminate tumor cells, demonstrates that immune escape is crucial for tumor development and growth (98). Immunotherapy for GC has greatly improved in recent years, but there is still a paucity of targets that reliably evoke anti-tumor immunity, and challenges persist with respect to achieving precise and personalized GC immunotherapy results (99). Due to their innate capacity for long-distance communication, outstanding biocompatibility, and ability to traverse barriers, exosomes are ideal carriers for the delivery of various molecules, including proteins, nucleic acids, chemotherapy drugs, and gene therapy molecules (80), with great potential for GC immunotherapy applications. Moreover, there has been a lot of evidence indicating that cancer immunotherapy can also target the production of exosomes. For example, tumor cells directly inhibit T cell function by releasing PD-L1 through exosomes, while anti-PD-1 therapy promotes cancer treatment by blocking the PD-1/PD-L1 signaling pathway and reducing the secretion of PD-L1 in exosomes (100).

In the present work, we have explored the possibility of augmenting GC immunotherapy with exosome-loaded molecules and proposed the potential of exosomes as therapeutic delivery carriers. Nevertheless, the utilization of exosomes in this context is still impeded by numerous risks and challenges, including the precision and standardization of exosome extraction procedures, the need to enhance the specificity and detection efficacy of

techniques such as liquid biopsy, and the absence of clinical sample verification (101). In addition, given their immunomodulatory effects, the use of exosomes as carriers to construct targeted chemotherapy drugs may become a new approach for personalized GC treatment. Accordingly, there is an urgent need to increase the efficiency of loading drugs or antigens into exosomes and develop more convenient methods to evaluate this process.

In summary, the molecular mechanisms underlying exosome-mediated GC occurrence, development, and immune escape or immune activation should be clarified. Moreover, targeted exosomes that amplify anti-tumor immune responses should be explored so as to overcome challenges in the standardization and clinical application of exosomes. Promoting the development of novel exosome-dependent or exosome-targeted drugs will help achieve precise delivery and synergistic therapy in the context of GC immunotherapy.

## 6 Conclusion

In conclusion, exosomes mediate communication between GC cells and other cell types within the TME. Exosomes regulate cancer initiation, progression, metastasis, and immune responses by delivering different biomolecules. Herein, we summarized recent studies on the molecular mechanisms underlying exosome-mediated GC development. We also described the role of exosomes as biomarkers for GC diagnosis and treatment, focusing on how exosomes derived from GC or immune cells modulate GC immune escape. Moreover, exosomes are promising vectors for targeted drug delivery and have great potential in GC immunotherapy applications. More extensive studies are needed to thoroughly understand the regulatory mechanisms by which exosomes are released by cells in the immune GC microenvironment and further explore the utility of exosomes in the augmentation of immunotherapy. Together, this work will facilitate the development of novel diagnostic, prognostic, and therapeutic strategies and targets.

## Author contributions

HL: Writing – review & editing, Writing – original draft. LZ: Writing – review & editing, Writing – original draft. JY: Writing – review & editing, Writing – original draft.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2025.1595124/full#supplementary-material

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