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Extracellular vesicles as modulators of cancer cell adaptive responses linked to therapy resistance

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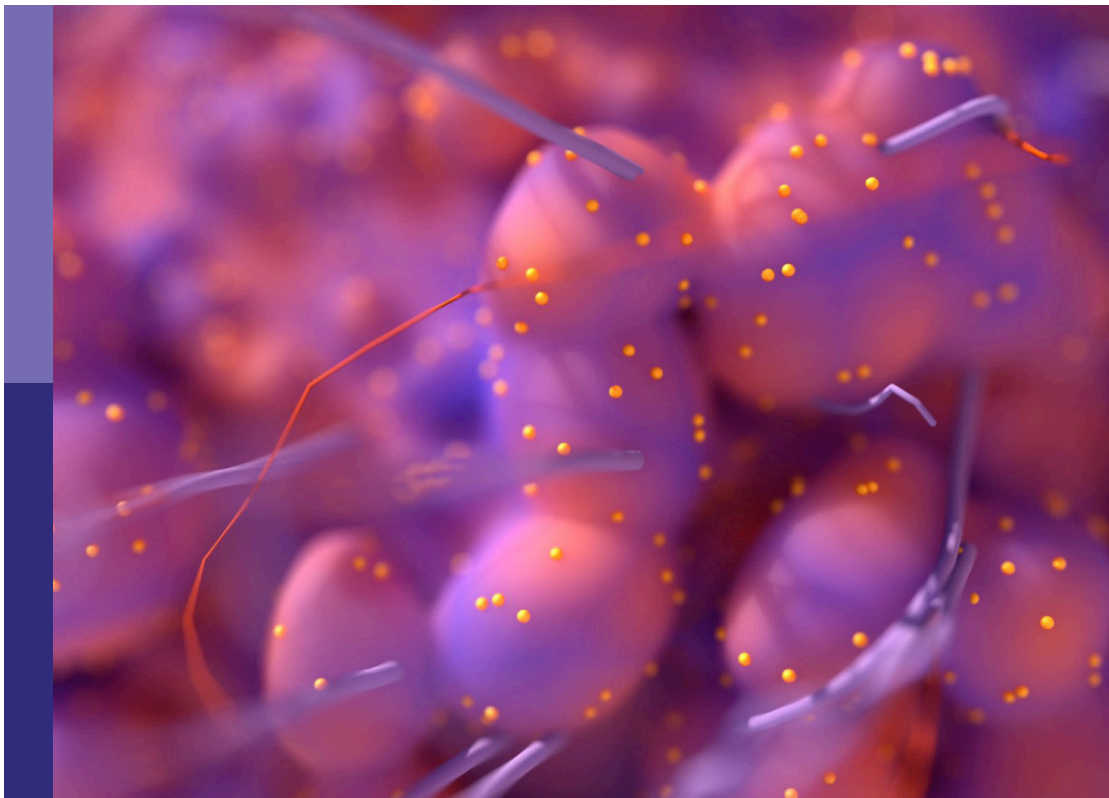

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Extracellular vesicles as modulators of cancer cell adaptive responses linked to therapy resistance

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Editorial: Extracellular vesicles as modulators of cancer cell adaptive responses linked to therapy resistance

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Editorial on the Research Topic

Extracellular vesicles as modulators of cancer cell adaptive responses linked to therapy resistance

Cancer still ranks as the leading cause of death, with approximately 20 million new cases per year worldwide (1). The main obstacle to cancer eradication is that anticancer approaches are often hindered by innate or acquired resistance to treatments (2). Cancer cells escape toxicity of therapeutics *via* genetic heterogeneity, enhanced pro-survival signals, metabolic reprogramming and improved detoxification and antioxidant scavenging, among other mechanisms (3–5). The crosstalk between tumor cells and the surrounding tumor microenvironment (TME) through the extracellular vesicle (EV)-based communication system plays a major role in influencing the behavior and phenotype of cancer cells through a wide array of molecular cargoes, such as proteins, nucleic acids, lipids, and metabolites (6, 7).

Unfortunately, a comprehensive view of the molecular mechanisms through which EVs affect resistance to anticancer treatments is yet to be depicted. Some evidence suggests that key roles might be played by regulatory RNAs (namely, lncRNAs and miRNAs) and drug efflux pumps (8, 9), metabolism reprogramming in cancer cells and in the TME (10), changes in mitochondrial function, bioenergetics, reactive oxygen species production and disposal, as well as in genomic stability and epigenetic control of gene expression (11–13).

The aim of this Research Topic was to collect contributions focused on how EVs affect molecular phenotype and behavior of cancer cells, in terms of their response to anticancer interventions.

Pompili et al. summarized the current state of knowledge on the most important cellular pathways involved in the cytoprotective effects of EVs in cancer cells, which can gain resistance to chemotherapy *via* EV-dependent extrusion of therapeutics, or even through the uptake of diverse molecular cargoes, including ABC transporter proteins, inhibitors of apoptosis, phase II detoxification enzymes, proliferation enhancers and non-coding RNAs. Similarly, Palazzolo et al. reviewed how EV molecular cargoes can change the response profile of cancer cells to chemotherapeutics, for example by inducing epithelial-mesenchymal transition (EMT) and cancer stem cell (CSC) phenotypes, by stimulating the expression of ATP-dependent efflux pumps, such as P-gp, or even impairing caspase 3-dependent apoptosis. Pompili et al. and Palazzolo et al. also discussed how natural or modified EVs may serve as drug delivery systems, and how the EV-dependent cell-to-cell communication may be targeted to reduce chemoresistance in cancer.

Studies provided evidence for an EV-mediated cell-to-cell transmission of drug-resistance traits in malignancies (14–16). The ability of EVs to transfer resistance to recipient cells was investigated by Lombardi et al., who observed that (TMZ)-sensitive glioblastoma multiforme cells became less responsive to TMZ after internalization of cyclooxygenase-2-containing EVs derived from TMZ-resistant cancer cells.

The involvement of the redox milieu in the EV-dependent modification of cancer cell behavior was investigated by some of us. Ponzetti et al. showed that osteoblast-derived EVs (OB-EVs) reduced osteosarcoma cells' aggressiveness and viability by impairing the redox balance of glutathione, a critical endogenous antioxidant molecule with key functions in detoxification and reactive oxygen species (ROS) scavenging within cells (17). Interestingly, OB-EVs did not alter the energy-related metabolic balance or mitochondrial dynamics.

NF-κB plays a major role in the execution of redox cellular responses. As reviewed by Di Vito Nolfi et al., NF-κB, whose expression governs key pro-survival pathways, is positively regulated by the EV-dependent release of specific tumor-promoting factors in the TME. A reciprocal regulation exists between EVs and NF-κB signaling, with NF-κB being directly involved in EVs trafficking and EVs-mediated chemoresistance, along with EVs playing a role in the activation of NF-κB. The authors discussed also how other proteins, molecules, molecular mechanisms and pathways possibly play a role in chemoresistance. The EV-mediated intercellular communication contributes to pathway activation, immune escape, and drug resistance Di Vito Nolfi et al. Beyond its important role in the redox response, NF-κB regulates an array of genes involved in immune and inflammatory responses (18). Mezzasoma et al. summarized how EVs and the pro-inflammatory TME could lead to cancer drug resistance, for example by modulating the activity of the NLRP3-dependent cascade, thus altering the inflammasome activation in cancerous recipient cells, as well as stimulating immune-escape or immune-stimulation, depending on the nature of the EV-releasing and -receiving cells. The authors also discussed how potential inhibitors of the inflammasome machinery could be effectively exploited to develop new anti-cancer strategies. Simón et al. also discussed how the proinflammatory TME elicits pro-survival effects through EV release. These authors summarized the role of hypoxia and chemotherapy in promoting release of EVs. Moreover, they described how macrophages and adipocytes, main contributors to pro-inflammatory disorders, can also induce release of EVs eventually leading to increased chemoresistance. Finally, the authors also provided an interesting picture of the pro-survival molecular pathways activated by CSC- and cancer associated fibroblast (CAF)-derived EVs. CAFs promote cancer progression by facilitating metastasization, angiogenesis, immunosuppression and drug resistance (19). In this context, Giusti et al. clearly demonstrated that tumor-derived EVs activate fibroblasts into a CAF-like phenotype, supporting their proliferation, motility, invasiveness and enzyme expression.

Increasing evidence underlines a crucial role for EVs within the TME as one of the main determinant for the immune function of neutrophils in malignancies (20). Zippoli et al. reviewed how tumor-derived EVs promote the differentiation of a pro-tumoral immune-suppressive sub-population of tumor associated neutrophils (TANs) and suppress T cell-mediated immunity by increasing the expression of programmed death-ligand 1 (PD-L1) in neutrophils. Interestingly, the authors reviewed also literature that suggests that neutrophil-derived EVs may serve as predictors of cancer outcome.

Lu et al. experimentally demonstrated that exosomes (EXOs) from dendritic cells infected with *Toxoplasma gondii* inhibited tumor growth in a mouse model of colorectal cancer (CRC), thus providing insights of how parasite-based anticancer strategies may achieve interesting results. Further research should identify the specific components of the exosomes involved in this effect.

The regulatory RNAs shuttled by EXOs may be involved in modulating the response to anticancer drugs (21). Wu et al. described the role of circRNAs shuttled by EVs as either suppressors or promoters of resistance to radiation in various cancer models. Accordingly, circRNAs could serve as novel clinical radiosensitizers, and as biomarkers to predict the effect of radiotherapy on tumors, thus providing a basis for targeted precision treatment in the future. In addition to the direct effect of radiation on irradiated cells, the authors also observed a process known as the radiation-induced bystander effect (RIBE), in which non-irradiated cells are also indirectly affected by radiation. RIBE appears to play a major role in determining the success of cancer radiotherapy. Further research is needed to

identify if circRNAs can also induce RIBE through EXOs. As discussed in the mini-review from Zelli et al., exosomal miRNAs are highly biocompatible, scarcely immunogenic, and have the ability to cross the blood-brain barrier, thus representing potential therapeutic delivery agents to suppress or prevent further tumor progression.

EVs cargo also include lipids, such as cholesterol, ceramide, sphingomyelin, and phosphatidylserine. Interestingly, in the original article from Chen et al. atorvastatin was found to reduce the release of EVs and their lipid content in ovarian adenocarcinoma cells, while promoting the release of cholesterol-enriched EVs. These effects were linked to reduced cell proliferation, migration, invasion, and to an increase in chemosensitivity to paclitaxel.

Acquired resistance to drugs is a major cause for hepatocellular carcinoma (HCC) being a highly relapsing disease and a leading cause of cancer mortality (22). Wang et al. reviewed how specific HCC-derived cargoes promote the conversion of hepatic stellate cells to CAFs, induce a pro-angiogenic effect and reduce endothelial integrity, eventually promoting tumor invasion. In addition, the authors discussed how specific EVs-associated miRNAs could be used as valuable biomarkers for HCC diagnosis.

This guest editorial board hopes that the contributions here collected offer innovative and interesting mechanistic insights on the decisive role of EVs as key regulators of critical aspects of cancer cell phenotype and behavior, in terms of their capacity to stimulate the cellular stress response upon treatment, as well as in terms of their ability to enable cancer cells to escape death upon exposure to antitumor agents.

We wish to thank all the Authors for sharing novel findings and interesting views of the current state of understanding on this Research Topic. We also greatly appreciated the valuable support given by the independent experts during the peer-review of all the submitted manuscripts.

Author contributions

AG: Conceptualization, Writing - Original Draft, Writing - Review & Editing; NR: Conceptualization, Writing - Original Draft, Writing - Review & Editing; SF: Conceptualization, Writing - Original Draft, Writing - Review & Editing. All authors contributed to the article and approved the submitted version.

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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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ORIGINAL RESEARCH
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Tumor-Derived Extracellular Vesicles Activate Normal Human Fibroblasts to a Cancer-Associated Fibroblast-Like Phenotype, Sustaining a Pro-Tumorigenic Microenvironment

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Fibroblasts in the tumor microenvironment have been proven to actively participate in tumor progression; they can be "educated" by cancer cells acquiring an activated state and, as such, are identified as cancer-associated fibroblasts (CAFs); CAFs, in turn, remodel tumor stroma to be more advantageous for cancer progression by modulating several processes, including angiogenesis, immunosuppression, and drug access, presumably driving the chemoresistance. That is why they are believed to hamper the response to clinical therapeutic options. The communication between cancer cells and fibroblasts can be mediated by extracellular vesicles (EVs), composed of both exosomes (EXOs) and microvesicles (MVs). To verify the role of different subpopulations of EVs in this cross-talk, a nearly pure subpopulation of EXO-like EVs and the second one of mixed EXO- and MV-like EVs were isolated from ovarian cancer cells and administered to fibroblasts. It turned out that EVs can activate fibroblasts to a CAF-like state, supporting their proliferation, motility, invasiveness, and enzyme expression; EXO-like EV subpopulation seems to be more efficient in some of those processes, suggesting different roles for different EV subpopulations. Moreover, the secretome of these "activated" fibroblasts, composed of both soluble and EV-associated molecules, was, in turn, able to modulate the response of bystander cells (fibroblasts, tumor, and endothelial cells), supporting the idea that EVs sustain the mutual cross-talk between tumor cells and CAFs.

Keywords: extracellular vesicles, cancer-associated fibroblasts, CAFs, ovarian cancer, tumor microenvironment, vesicles subpopulations

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Abbreviations: α-SMA, α-smooth muscle actin; ABs, apoptotic bodies; CAFs, cancer-associated fibroblasts; CM, conditioned medium; ECGF, endothelial cell growth factor; EGFs, epidermal growth factors; EVs, extracellular vesicles; EXOs, exosomes; FAP, fibroblast activation protein; FBS, fetal bovine serum; FGFs, fibroblast growth factors; HGF, hepatocyte growth factor; HUVECs, human umbilical vein endothelial cells; lEVs, large extracellular vesicles; MMPs, matrix metalloproteinases; MVs, microvesicles; NCS, newborn calf serum; NHDF, normal human dermal fibroblasts; PDGFs, platelet-derived growth factors; SEM, scanning electron microscopy; sEVs, small extracellular vesicles; TGF-β, transforming growth factor β; VEGF, vascular endothelial growth factor.

INTRODUCTION

The term "extracellular vesicles" (EVs) is used to describe all spherical and membrane-enclosed vesicles released into the extracellular space by both normal and tumor cells (1). When their size and cellular origin are considered, it is possible to distinguish three subpopulations of EVs: exosomes (EXOs), microvesicles (MVs), and apoptotic bodies (ABs) (2).

ABs are released from the plasma membrane as blebs when cells undergo apoptosis and have a size ranging between 1 and 4 μm in diameter. EXOs and MVs are released, instead, from viable cells; EXOs are the smallest EVs, ranging from 40 to 150 nm in diameter, and originate from the formation of an early endosome at the plasma membrane and the subsequent maturation into multivesicular bodies, where intraluminal vesicles (ILVs) form in the lumen by inward budding of the membrane; their final fusion with the plasma membrane results in the release of the ILVs into the extracellular space originating EXOs. MVs are larger than EXOs, being around 100 nm to 1 μm in size, and originate directly from the outward budding of the plasma membrane (2).

To date, EVs are considered as an intercellular communication mechanism acting as molecular shuttles packaged with a bioactive cargo of proteins, lipids, and nucleic acids that are used by cells to interact with the neighboring ones to modulate their environment (3, 4); once released, indeed, they can interact with target cells, releasing their content into extracellular space following EV lysis, interacting with their receptors, by fusion, or other mechanisms yet to be identified (3–5).

As such, EVs are involved in many physiological and pathological processes (6–9); among the latter, cancer has been the focus in the past years given the cancer-derived EV involvement in many tumor-related processes such as angiogenesis induction, invasion, motility, evasion from immune surveillance, apoptosis escape, and drug resistance promotion (10–17).

Over the last few years, some evidence has emerged suggesting that, during cancer progression, EVs are also able to support the creation of a microenvironment encouraging cancer growth, progression, and metastasis by conveying messages to nearby stromal cells, including the so-called "cancer-associated fibroblasts" (CAFs) (18, 19).

CAFs, along with the extracellular matrix and several cell types (including endothelial cells, immune cells, and adipocytes), constitute the tumor stroma in many types of cancer, including ovarian cancer. In this kind of tumor, the stroma could account for a large percentage of tumor tissue (up to 83%), leading to hypothesize a relevant role for CAFs (20, 21). CAFs have been demonstrated to actively participate in cancer progression, being involved in cancer metastasis, angiogenesis stimulation, immunosuppression induction, and drug resistance (22–24).

Our previous study has demonstrated that the human ovarian cancer cell line CABA I releases different EV subpopulations in a time-dependent mode; starved CABA I cells, indeed, once stimulated with fetal bovine serum (FBS), released a first nearly pure population of EXO-like EVs (mean size ~100 nm) and a second one mix of EXO- and MV-like EVs (size > 100 nm) (25). These data highlighted that different time intervals lead to the release of different subpopulations of EVs, in terms of not only size but also amount and molecular composition, suggesting possible different cargoes and, consequently, different biological roles for the different subpopulations.

This work aimed to verify if specific EV subpopulations released from CABA I were able to activate normal human fibroblasts into CAF-like cells and to verify the effect of such activation on surrounding cells (cancer cells, endothelial cells, and not activated fibroblasts).

Our present findings support the idea that ovarian cancer cells can modulate fibroblast behavior through the release of EVs, activating them to a CAF-like state that is able, in turn, to stimulate the nearby cells. However, the different subpopulations of EVs show a different ability to stimulate these processes: the EXO-like EVs rather than the mixed population of EXO- and MV-like EVs seem to be more efficient in some activation processes. Overall, these findings suggest that EVs, particularly EXOs, can be considered pivotal targets of novel anticancer therapies to hamper fibroblast activation.

MATERIALS AND METHODS

Cell Cultures

CABA I cell line was established from the ascitic fluid of an ovarian carcinoma patient not undergoing drug treatment (26). Cells were grown as monolayers in Roswell Park Memorial Institute-1640 (RPMI-1640) supplemented with 5% (v/v) heat-inactivated FBS, 1× penicillin/streptomycin, and 2 mM of L-glutamine.

Normal human dermal fibroblasts (NHDF) cell line was purchased from Lonza (Walkersville, MD, USA) and grown as a monolayer in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) heat-inactivated FBS, 2 mM of L-glutamine, penicillin, and streptomycin. Cells were subcultured and used within the 15th doubling, as suggested by Lonza's protocols.

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord veins; the study was conducted in accordance with the Declaration of Helsinki, approved by the Internal Review Board of L'Aquila University (protocol code 07/2018, February 2018), and informed consent was obtained from all subjects involved. Endothelial cells were grown on 1% gelatin-coated flasks in DMEM supplemented with 10% (v/v) heat-inactivated FBS, 10% (v/v) heat-inactivated newborn calf serum (NCS), 20 mM of HEPES [*N*- (2-hydroxyethyl) piperazine-*N*′- (2-ethane sulfonic acid)], 6 U/ml of heparin, 2 mM of L-glutamine, 50 μg/ml of endothelial cell growth factor (ECGF), penicillin, and streptomycin. These cells were used within the fifth passage.

All cell lines were cultured at 37°C in a humidified atmosphere with 5% CO_2, and experiments were carried out on sub-confluent (except for wound-healing assays) and mycoplasma-negative cells.

FBS, RPMI, DMEM, glutamine, penicillin, and streptomycin were purchased from Euroclone (Euroclone SpA, Milan, Italy); Hepes and ECGF were from Sigma-Aldrich (St. Louis, MO, USA);

and NCS was from Gibco (Gibco, Thermo Fisher Scientific, Waltham, MA, USA).

Extracellular Vesicle Isolation From Culture Media

The protocol to isolate the two different EV subpopulations, used to stimulate NHDF, had been previously set (25). Briefly, CABA I cells were starved in serum-free medium for 24 h to avoid EV release and subsequently stimulated with 5% of 40-nm-filtered FBS HyClone (Thermo Scientific, Rockford, IL, USA) in RPMI-1640; conditioned media (CMs) containing EVs were collected in sterile working conditions after 30 min and 18 h from the HyClone supplement.

To isolate EVs, these CMs were firstly centrifuged at 4°C at 600×*g* for 15 min and then at 1,500×*g* for 30 min to remove cells and large debris, respectively. The resulting supernatants were centrifuged at 100,000×*g* (Rotor 70Ti, Quick-Seal Ultra-Clear tubes, k_{adj} 221, brake 9) for 2 h at 4°C in an Optima XPN-110 Ultracentrifuge (Beckman Coulter, Brea, CA, USA). For each preparation, the EVs were derived from a starting cell number of 4,500,000–5,000,000 cells for 30-min collection and 7,500,000–9,000,000 for the 18-h collection. Isolated vesicles were resuspended in Dulbecco's phosphate-buffered saline (PBS) (EuroClone, Milan, Italy), and the determination of vesicle quantification was carried out by measuring the vesicle-associated protein levels using the Bradford method (27) (Bio-Rad, Milan, Italy) with bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) as the standard.

The EV subpopulations obtained with this experimental protocol have already been previously characterized by markers, NanoSight assay, and transmission electron microscopy (25). Hereinafter, EVs from CMs collected after 30 min and 18 h from the HyClone supplement will be indicated, respectively, as $EVs_{30'}$ and EVs_{18h}.

Fibroblast Treatments With $EVs_{30'}$ and EVs_{18h}

NHDF were administered with $EVs_{30'}$ and EVs_{18h} by supplying 1 μg of EVs/ml every day for up to 5 days, in a cumulative way: EVs were added every 24 h without replacing the medium for the entire duration of the treatment, so as to mimic the continuous release of EVs by cancer cells within the tumor microenvironment and the persistent exposure of fibroblasts to EVs. Treatments were performed by adding the EVs to culture media supplemented with a reduced percentage of FBS (2%) to limit the serum stimulatory effect while ensuring fibroblast survival.

Hereinafter, NHDF treated with $EVs_{30'}$ and EVs_{18h} will be, respectively, indicated as $NHDF_{30'}$ and $NHDF_{18h}$. Untreated fibroblasts will be indicated as NHDF.

Western Blotting

To verify the NHDF activation into a CAF-like state, 48 h after the end of a 5-day treatment with EVs, NHDF, $NHDF_{30'}$, and $NHDF_{18h}$ were washed three times with PBS and lysed in radioimmunoprecipitation assay (RIPA) Lysis Buffer, containing 50 mM of Tris-HCl, pH 7.5, 150 mM of NaCl, 0.5% sodium deoxycholate, 1% Triton-X, 0.1% sodium dodecyl sulfate (SDS), 5 mM of EDTA, 100 mM of sodium fluoride (NaF), 2 mM of sodium orthovanadate (Na_3VO_4), 10 mM of sodium pyrophosphate (NaPPi), 1 mM of phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml of leupeptin, 1 μg/ml of aprotinin, and 100 μg/ml of trypsin inhibitor (Sigma, St. Louis, MO, USA). Fibroblasts' protein content was determined by the Bradford method, as described above. Fibroblast activation protein (FAP) and α-smooth muscle actin (α-SMA) expression were identified in samples containing 12 and 15 μg of protein (for FAP and α-SMA, respectively) resolved by 7.5% and 12.5% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (for FAP and α-SMA, respectively) under reducing conditions and with heating. Separated proteins were then blotted onto a nitrocellulose membrane (Whatman-GE Healthcare Life Sciences, London, UK), and non-specific binding sites were blocked for 2 h in 10% non-fat dry milk in TBS containing 0.5% Tween-20 (TBS-T) at room temperature.

Blots were then probed with the specific primary antibody at 4°C overnight: FAP (rabbit monoclonal, 1:1,000 dilution, ab207178, Abcam, Cambridge, UK) and α-SMA (rabbit monoclonal, 1:5,000 dilution, ab32575, Abcam, Cambridge, UK). GAPDH (mouse monoclonal, 1:5,000 dilution; MA5-11114; Thermo Scientific) was used as a normalizer. After several washes in TBS-T, the membranes were incubated in appropriate horseradish peroxidase (HRP)-conjugated secondary Abs: goat anti-mouse IgG-HRP, dilution 1:10,000 (sc-2005, Santa Cruz Biotechnology, Dallas, TX, USA) or goat anti-rabbit IgG-HRP, dilution 1:7,500 (sc-2204, Santa Cruz Biotechnology) for 1 h. All the antibodies were diluted in blocking buffer (TBS-T containing 1% non-fat dry milk). After being washed in TBS-T, the reactive bands were visualized with a chemiluminescence detection kit (SuperSignal West Femto Chemiluminescent Substrate, Thermo Scientific).

Images were recorded and analyzed with the gel documentation system Alliance LD2 (Uvitec, Cambridge, UK).

Collection of Normal Human Dermal Fibroblast Conditioned Media

To verify if treated NHDF modify their secretome, after the 5 days of cumulative treatment with 1 μg of EVs/ml, cells were washed with serum-free DMEM and then incubated for 24 h in a complete medium in which FBS was replaced with 0.2% Lactalbumin Enzymatic Hydrolysate (LEH; Sigma, St. Louis, MO, USA) to remove the contribution of enzymes/growth factors from the serum. Parallelly, CM was prepared in the same manner from untreated fibroblasts (controls). Cells and cell debris were removed by centrifugation at 600–1,550×*g* from all the CMs. Then, CMs were concentrated using Centricon Ultracel YM-10 filters (Amicon Bioseparations; Millipore Corporations, MA, USA; cutoff, 10 kDa) to be analyzed by casein–plasminogen zymography assays or were used unconcentrated for tests such as proliferation, migration, and invasion assays, in addition to gelatin zymography assays.

Hereinafter, CMs obtained from NHDF, $NHDF_{30'}$, and $NHDF_{18h}$ will be indicated, respectively, as CM NHDF, CM $NHDF_{30'}$, and CM $NHDF_{18h}$.

Proliferation Assay

NHDF (1,000 cells/well) were seeded onto a 96-well plate, incubated for 24 h in complete medium to enable cell adhesion and spreading, and then treated with $EVs_{30'}$ and EVs_{18h} as explained above (1 μg of EVs/ml every day for 5 days). The effects of EVs on NHDF proliferation were evaluated by the XTT assay on the 5th day, i.e., at 96 h from the beginning of the EV treatment, while treatment was still in progress. Untreated fibroblasts, grown in the same medium but without EVs, were used as control.

For experiments with CM, NHDF (1,000 cells/well), ovarian cancer cells CABA I (1,500 cells/well), and HUVECs (1,000 cells/well) were seeded into 96-well plates (gelatin-coated for HUVECs), allowed to adhere and spread for 24 h at 37°C and 5% CO_2, and then cultured for 96 h (NHDF) or 72 h (CABA I and HUVECs) with the CM $NHDF_{30'}$ and CM $NHDF_{18h}$. At the end of each specified interval, the proliferation was assessed with the XTT assay.

CMs for experiments on NHDF were supplemented with 1% FBS to ensure fibroblast survival, without stimulating their growth; for the same reason, CMs for HUVEC experiments were supplemented with 5% FBS, 5% NCS, HEPES, heparin, and ECGF; CABA I cells were incubated with unsupplemented CM. Cells incubated with CM NHDF were used as controls.

For the proliferation assay, 1 mg/ml of XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxamide] (Sigma, St. Louis, MO, USA) and 1.53 mg/ml of phenazine methosulfate (PMS; Sigma-Aldrich) were mixed, and 50 μl of this solution was added to each well. Plates were incubated for 4 h at 37°C, 5% CO_2; after this interval, the optical density (OD) of the colored, non-toxic, water-soluble formazan originated by the metabolic reduction of XTT mixed with PMS by mitochondria of living cells was measured by an ELISA reader at 450 nm. Values obtained in the absence of cells were considered as background and subtracted from the OD values of the samples. XTT tests were performed before the cells reached confluence to prevent any possible artifact decrease in the results due to contact inhibition.

Each experiment was performed in triplicate and repeated at least twice. The data are expressed as the means ± SDs.

In Vitro Scratch Wound-Healing Assay

The wound-healing assay is one of the earliest developed tests to study directional cell migration *in vitro*, and it is based on the observation of cell migration into a scratch "wound" created on a cell monolayer.

NHDF were cultured in 24-well microplates and treated as previously explained. The scratch was performed at 48 h after the end of 5 days' treatment with $EVs_{30'}$ and EVs_{18h} when the cells had reached the full confluency; a previously sterilized 200-μl plastic tip was drawn across the cellular stratum to produce a wound, floating cells were removed, and wells were washed 3 times with PBS to remove debris and to smooth the edge of the wound. During the migration into the wound, cells were maintained in an FBS reduced culture medium (2% FBS) that avoided scratch closure by means of cell growth.

The status of the scratch wounds was monitored up to 48 h using a contrast-phase microscope; representative images were collected at the beginning of the assay and at regular intervals. The surface of the wounded area in each image was quantified with the ImageJ software, and the data were reported as % of wound closure (compared to 100%, conventionally assigned to the original scratch area).

Invasion Assay

The study of cell invasiveness was accomplished using modified Boyden chambers, separating the upper and lower compartments with filters (8-μm pore size polycarbonate polyvinylpyrrolidone-free Nucleopore filters) coated with a thin layer of Matrigel® Growth Factor reduced (Beckton Dickinson, Franklin Lakes, NJ, USA) diluted in serum-free medium to a concentration of 0.5 mg/ml.

Briefly, NHDF, $NHDF_{30'}$, and $NHDF_{18h}$ (1,000 cells/well) were added to the upper chamber in 45 μl of serum-free medium, and their motility abilities were tested using as chemoattractant some DMEM containing 10% FBS, which was added into the lower chamber; NHDF were used as controls.

In experiments with ovarian cancer cells, CABA I cells (1,000 cells/well) were added to the upper chamber in 45 μl of serum-free medium, and in the lower chamber were added the serum-free CM $NHDF_{30'}$ and CM $NHDF_{18h}$ to test their effect as chemoattractant; cells invading in response to CM NHDF were used as controls.

The cells were allowed to invade the Matrigel® for 24 h at 37°C, 5% CO_2. The non-invading cells on the upper surface of the 8-μm pore filters were removed with a cotton swab. The invading cells on the filters' lower surface were fixed and stained in 1% crystal violet in methanol. Invading cells in five random microscope fields for each well were counted at 20× magnifications.

Zymography Assays

Serum-free CM NHDF, CM $NHDF_{30'}$, and CM $NHDF_{18h}$ were subjected to both gelatin and casein–plasminogen zymography assays. Gelatin zymography was performed using 7.5% SDS-PAGE copolymerized with 1 mg/ml of gelatin type B (Sigma, St. Louis, MO, USA); the CMs were diluted in SDS-PAGE sample buffer and analyzed under non-reducing conditions without heating. After electrophoresis, the gels were washed three times, 15 min each, at room temperature, in a washing buffer containing 50 mM of Tris-HCl (pH 7.4) and 2.5% Triton X-100 (Sigma-Aldrich); they were, then, incubated overnight in an activation Tris buffer (50 mM of Tris-HCl, pH 7.4, 5 mM of $CaCl_2$, and 120 mM of NaCl) at 37°C. To visualize the lytic bands, the gels were stained with Coomassie Blue R 250 (Bio-Rad, Hercules, CA, USA) dissolved in a mixture of methanol:acetic acid:water (4:1:5) for 30 min and then destained in the same solution without dye.

The plasminogen activators (PAs) in the concentrated culture CM were examined using the casein–plasminogen zymography under non-reducing conditions and without heating. Proteins were separated by electrophoresis in 10% SDS-PAGE copolymerized with 0.2% casein (Sigma-Aldrich, St. Louis, MO, USA) and 10 mg/ml of human plasminogen (Sigma-Aldrich, St. Louis, MO, USA). After electrophoresis, the gel was washed in the same buffer used for the gelatinase assay

and then incubated for 48 h at 37°C in 50 mM of Tris-HCl, pH 7.4 + 0.02% NaN_3. Staining and destaining were performed as previously described. Activities of gelatinases and PAs appeared as clear and distinct bands, which indicated proteolysis of the substrate, on a blue background: those digestion bands were quantified by ImageJ software.

Electron Microscopy

Scanning electron microscopy (SEM) analysis was performed on fibroblasts treated with $EVs_{30'}$ and EVs_{18h} for up to 5 days. Forty-eight hours after the end of this treatment, NHDF, $NHDF_{30'}$, and $NHDF_{18h}$ were detached, washed, and allowed to grow to subconfluence on coverslips for an additional 96 h; then cells were fixed in 2% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) in PBS for 3 min.

After being dehydrated with a graded scale of ethanol (30% to 100%) and critical point-dried, the samples were glued onto stubs, coated with gold in an SCD040 Balzer Sputterer, and detected with Philips 505 SEM at 20 kV.

Migration Assay

The migration of normal fibroblasts and CABA I cells was tested in response to CM $NHDF_{30'}$ and CM $NHDF_{18h}$ (added as a chemoattractant in the lower chambers, the same volume for each sample). Cells migrated in response to CM NHDF were used as controls. Briefly, cells were detached, washed three times in serum-free medium, and seeded on the upper wells (5,000 cells/wells in serum-free medium) of the modified Boyden chamber. Gelatin-coated polycarbonate membranes with 8-μm pores were used to separate the upper wells from the lower ones. Each condition to be tested was analyzed in triplicate. The Boyden chambers were incubated for 24 h at 37°C in a CO_2 incubator, and then migrated cells were visualized as described for the invasion assay. The number of cells, migrated to the lower surface of the polycarbonate membrane, was counted in five random 20× fields within each well, under a microscope. The mean number of cells per field was calculated as cell counts.

Tube Formation Assay

This *in vitro* test measures the ability of endothelial cells to invade, migrate, organize, and differentiate into capillary-like tubular structures within a three-dimensional matrix constituted by Matrigel® Growth Factor Reduced 10 mg/ml (BD56230, Franklin Lakes, NJ, USA). Briefly, Matrigel® was plated on the bottom of 96-well plates and allowed to gel at 37°C for 1 h. HUVECs were detached, counted, washed in serum-free medium, and resuspended in serum-free CM NHDF, CM $NHDF_{30'}$, and CM $NHDF_{18h}$. Then, 20,000 cells/well were seeded on Matrigel® and incubated at 37°C, 5% CO_2; the tube formation was observed at 7 h after cell seeding. Several images were acquired per well and processed using the Angiogenesis Analyzer plugin with ImageJ software (28) downloadable from the National Institutes of Health website. The total length of the capillary-like structures, the number of nodes, and the number of segments normalized per area were used for data analysis.

Statistical Analysis

Data are expressed as the mean ± standard error. Comparisons between the means of control groups and treated groups were performed using the one-way ANOVA followed by Tukey's post-test; results were considered statistically significant when $p < 0.05$ (*), $p < 0.01$ (**).

RESULTS

Normal Fibroblasts Treated With Extracellular Vesicles Acquire Cancer-Associated Fibroblast-Like Morphologyand Express Their Markers

Potential NHDF morphological changes, a typical signature of fibroblast activation, induced by $EVs_{30'}$ and EVs_{18h} were observed by an inverted optical microscope.

$EVs_{30'}$ and EVs_{18h} are EVs isolated from CMs collected after 30 min and 18 h, respectively. As mentioned above and discussed further below, in previous work (25), we highlighted that the human ovarian cancer cell line CABA I releases two specific subpopulations of sEVs "(EVs30') and lEVs+sEVs (EVs18h).

Such morphological changes were visible in NHDF treated with ovarian cancer $EVs_{30'}$ and EVs_{18h} starting after 72 h at the beginning of treatment (**Figure 1**), whilst untreated fibroblasts exhibit typical elongated and spindle-shaped morphology, some $NHDF_{30'}$ and $NHDF_{18h}$ underwent a morphological change, acquiring the typical morphology of activated fibroblasts ($NHDF_{30'}$ and $NHDF_{18h}$ are, respectively, NHDF treated with $EVs_{30'}$ and EVs_{18h}): they appeared very spread with many visible stress-contractile fibers inside the cytoplasm.

To confirm the cell activation, at the end of the EV treatment, NHDF, $NHDF_{30'}$, and $NHDF_{18h}$ were lysed as described, and protein extracts were analyzed to detect the expression of typical markers of CAFs: FAP and α-SMA (**Figure 2**).

The quantitative analysis detected a statistically significant increase in the expression of α-SMA (calculated molecular weight: ~44 kDa) in both $NHDF_{30'}$ and $NHDF_{18h}$ when compared to NHDF (1.44 and 1.35, respectively) (**Figure 2**). FAP was also increased in both $NHDF_{30'}$ and $NHDF_{18h}$ when compared to NHDF (3.3 and 4.5, respectively).

Extracellular Vesicle Subpopulations Differently Affect Fibroblast Proliferation, Motility, Invasiveness, Enzyme Expression, and Microvesicle Release

Proliferation rate alteration induced by EV treatments was evaluated. It was tested while the treatment was still ongoing on the 5th day of the EV treatment (96 h from the beginning of treatments) (**Figure 3**): EVs_{18h} did not induce any significative increase, while the treatment with $EVs_{30'}$ resulted in a significant increase when compared to the untreated cells NHDF (+15%).

The motility induced by $EVs_{30'}$ and EVs_{18h} treatments was tested with the scratch wound assay (**Figure 4**). Migration was observed at different time intervals (24, 32, and 48 h), and the

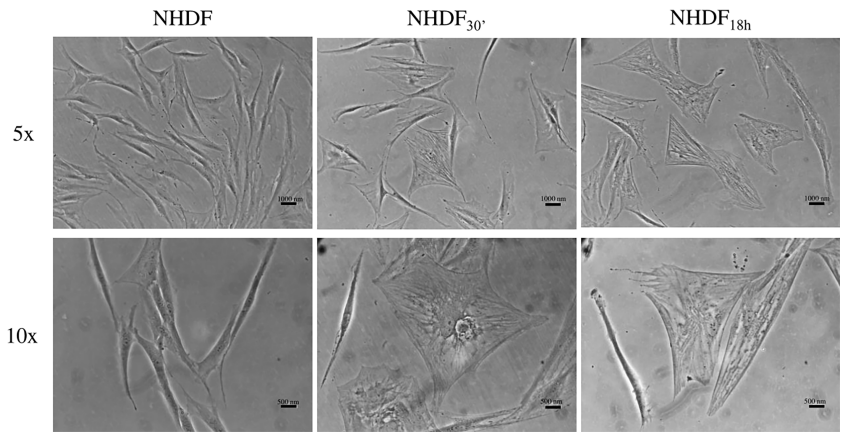

FIGURE 1 | Optical images of fibroblasts showing morphological changes induced by extracellular vesicle (EV) subpopulations $EV_{30'}$ and EV_{18h}. Representative images of untreated control fibroblasts (NHDF) and fibroblasts treated with the two EV-subpopulations ($NHDF_{30'}$ and $NHDF_{18h}$). The scale bar is 1,000 nm in the top row and 500 nm in the bottom row. Images were captured with 5× and 10× objectives of an inverted optical microscope.

most significant changes were captured after the beginning point (time zero); the observation of the wounded area showed that $NHDF_{30'}$ and $NHDF_{18h}$ have a greater tendency to close the wound by migrating inside it compared to NHDF (**Figure 4A**);

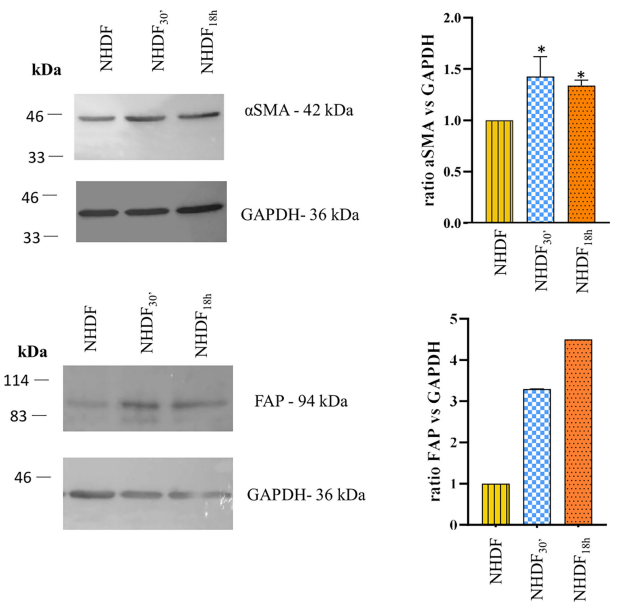

FIGURE 2 | Western blotting identification of α-smooth muscle actin (α-SMA) and fibroblast activation protein (FAP). The expression of α-SMA and FAP was increased in $NHDF_{30'}$ and $NHDF_{18h}$. Band intensity was analyzed by ImageJ and presented in the graph on the right as ratio α-SMA/GAPDH or FAP/GAPDH, in which 1 is the ratio conventionally attributed to NHDF. For α-SMA, the image on the left is representative of 1 of 3 independent experiments (all of them represented in the graph as mean ± SD; *p < 0.05). FAP assay was performed once.

to quantify this ability to close the wound, the wounded area (i.e., the area uncovered from the cells) was measured with ImageJ software at the intermediate time intervals, 24 h (**Figure 4B**) and 32 h (**Figure 4C**). The 100% value was conventionally assigned to the wounded area of the original scratch (time zero). $NHDF_{18h}$ migrated with the same trend as NHDF, while $NHDF_{30'}$ exhibited higher motility: indeed, after 24 h, the area not yet covered was quite comparable in NHDF and $NHDF_{18h}$ (respectively 69% and 71% with respect to the original wound), but it was significantly lower (53% with respect to the original wound) in $NHDF_{30'}$. After 32 h, the scratch area of NHDF and $NHDF_{18h}$ was again comparable (respectively 61% and 60% with respect to the original wound), but it was significantly lower in $NHDF_{30'}$ (44% compared to the original wound). After 48 h, the trend was substantially maintained, but since proliferative effects could begin to occur at this time interval, it was not considered (despite that the experiment conducted in the presence of a low concentration of serum has certainly avoided the scratch closure by means of cell growth) (data not shown).

The invasion assay performed with the modified Boyden chamber showed that both fibroblasts treated with $EVs_{30'}$ and EVs_{18h} showed a trend to a greater invasiveness capacity (respectively +101% and +30%) as compared to NHDF (**Figure 5A**), but only $NHDF_{30'}$ had a statistically significant greater ability if compared to NHDF. To estimate if the invasion ability induced by the EV treatment could be supported by an increased secretion of proteolytic enzymes, CMs from EV-treated fibroblasts were normalized according to the same volume and assayed to evaluate the gelatinolytic and PA activities by employing zymographic techniques. The gelatinase assay (**Figure 5B**) revealed that both $EVs_{30'}$ and EVs_{18h} induced in NHDF the expression of pro-MMP-2: +41% and +24%, respectively, in $NHDF_{30'}$ and $NHDF_{18h}$ compared to NHDF (calculated molecular weight 70 kDa). The casein–plasminogen zymography (**Figure 5C**) similarly highlighted a trend to a

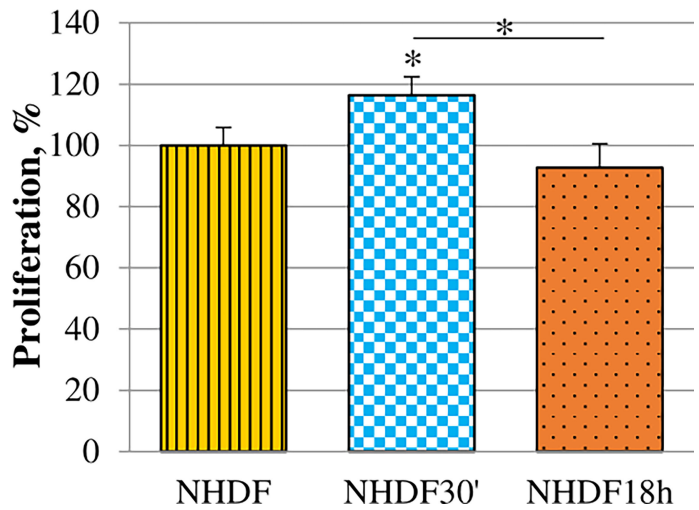

FIGURE 3 | Effects of $EVs_{30'}$ and EVs_{18h} on fibroblasts' proliferation. Proliferation was evaluated by XTT assay, on the 5th day of treatment, i.e., at 96 h from the beginning of the EV treatment. Values were calculated as mean ± SD and are expressed as percentages with respect to 100% proliferation, conventionally attributed to untreated NHDF. Experiments were performed three times in triplicate. The asterisk on the bar indicates the statistical significance with respect to NHDF, and the horizontal line refers to the statistical significance between the $NHDF_{30'}$ and $NHDF_{18h}$ (*$p < 0.05$).

higher release of the high-molecular-weight urokinase-type PA (HMW-uPA) (calculated molecular weight 48–55 kDa), particularly in $NHDF_{30'}$ (+51% in $NHDF_{30'}$ compared to NHDF).

$NHDF_{30'}$ and $NHDF_{18h}$ cell surface was also observed by SEM to verify if EV-mediated activation stimulated, in turn, the EV release, particularly of MVs from the cell surface, whilst the shedding of MVs was very sporadic in untreated NHDF; in EV-treated fibroblasts, the extent of the MV release was more evident and involved large membrane areas (**Figure 6**).

Secretome of $NHDF_{30'}$ and $NHDF_{18h}$ Affects Bystander Cells

After the end of the EV treatment, the CMs of $NHDF_{30'}$ and $NHDF_{18h}$ (representing the cell secretome and containing both EV-associated and soluble molecules) were used as stimuli to evaluate their effect on the cells normally present in the tumor microenvironment, such as fibroblasts, endothelial cells, and tumor cells. CM from untreated NHDF was used as a control.

Fibroblast proliferation rate was not at all affected by CM (**Figure 7A**). On the contrary, CM $NHDF_{30'}$ and CM $NHDF_{18h}$ exerted a considerable chemotactic effect, stimulating the migration ability of normal fibroblasts (**Figure 7B**): migration of fibroblasts toward the secretome of EV-treated fibroblasts was almost 2-fold increased with respect to the migration toward the CM NHDF (+93% and + 81%, respectively for CM $NHDF_{30'}$ and CM $NHDF_{18h}$), even if no differences were appreciable between CM $NHDF_{30'}$ and CM $NHDF_{18h}$.

The effects of activated fibroblasts' secretome on ovarian cancer cells were also analyzed evaluating the migration and invasion abilities, in addition to their proliferative capacity (**Figure 8**). CABA I cells cultured in the presence of CM NHDF, CM $NHDF_{30'}$, and CM $NHDF_{18h}$ showed no significant change in their proliferation (**Figure 8A**). On the contrary, their motility (**Figure 8B**) and invasiveness (**Figure 8C**) were significantly promoted: they were higher in response to CM $NHDF_{30'}$ and CM $NHDF_{18h}$ than in response to CM NHDF, with no significant differences between CM $NHDF_{30'}$ and CM $NHDF_{18h}$ (motility, +140% and +116% compared to CM NHDF in CM $NHDF_{30'}$ and CM $NHDF_{18h}$, respectively; invasion, +158% and +176% compared to CM NHDF in CM $NHDF_{30'}$ and CM $NHDF_{18h}$, respectively).

HUVECs, too, were stimulated by CM NHDF, CM $NHDF_{30'}$, and CM $NHDF_{18h}$ to assess their proliferation response (**Figure 9**): although the cell number appeared to increase in endothelial cells treated with CM from EV-treated fibroblasts, this increase was not statistically significant (**Figure 9A**). On the contrary, the tube formation assay highlighted the pro-angiogenic potential of CM. The test revealed that the differentiation of HUVECs into primitive capillary-like structures occurred in response to both CM $NHDF_{30'}$ and CM $NHDF1_{8h}$; the number of nodes, the total length of formed tubes, and the number of segments were significantly higher in HUVECs treated with CM $NHDF_{30'}$ and CM $NHDF_{18h}$ than with CM NHDF (**Figure 9B**) (number nodes/area: 14.6 in HUVECs treated with control NHDF CM; 56.7 and 39.6 in CM $NHDF_{30'}$- and CM $NHDF_{18h}$-treated HUVECs, respectively. Total length/area: 1,078 pixels in HUVECs treated with control CM NHDF; 1,751.6 and 1545.8 pixels in CM $NHDF_{30'}$ and CM $NHDF_{18h}$ treated HUVECs, respectively. Number segments/area: 2.12 in HUVECs treated with control CM NHDF; 19.7 and 14 in CM $NHDF_{30'}$ and CM $NHDF_{18h}$ treated HUVECs, respectively).

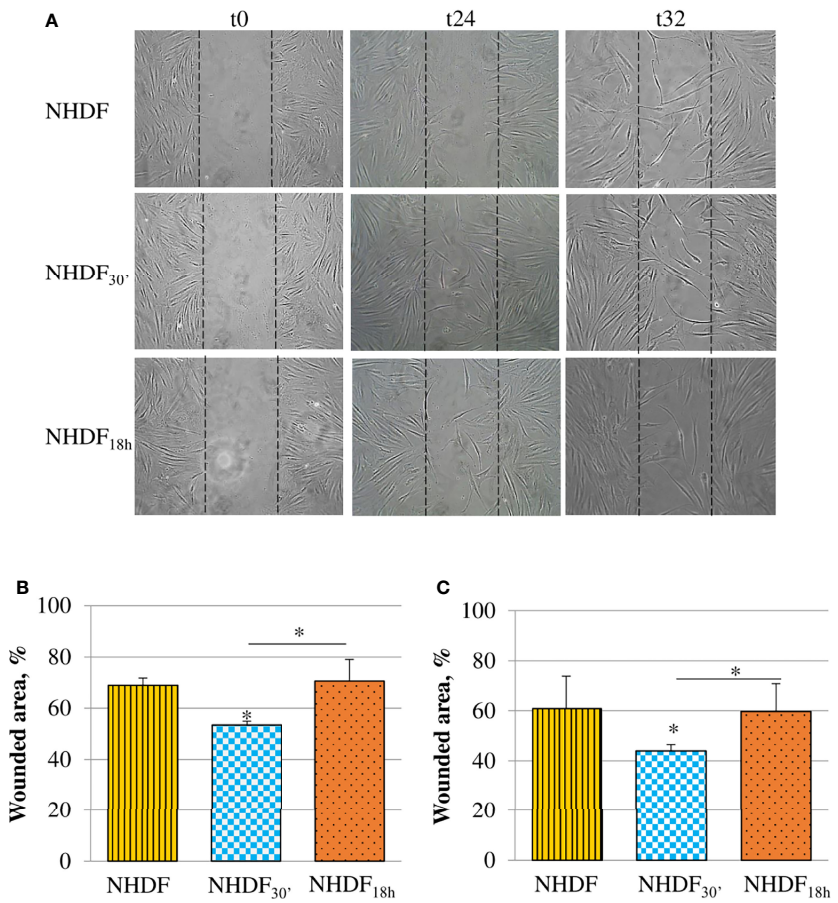

FIGURE 4 | Effects of $EVs_{30'}$ and EVs_{18h} on fibroblasts' motility. Motility was assessed by the scratch wound assay **(A)**; the panel reports representative images recorded 24 and 32 h after the scratch creation (0 h); dotted lines represent the size of the original wound. Graphs at the bottom show the percentage of the still wounded area at 24 h **(B)** and 32 h **(C)** with respect to the original wound (conventionally set as 100%). Only the migration of fibroblasts treated with $EVs_{30'}$ resulted in statistical significance compared to the migration of control fibroblasts. Data derived from three biological replicates tested individually due to scarcity of the material and are shown as mean ± SD; the asterisk on the bar indicates the statistical significance with respect to NHDF, and the horizontal line refers to the statistical significance between the $NHDF_{30'}$ and $NHDF_{18h}$ (*$p < 0.05$).

DISCUSSION

Ovarian cancer is one of the deadliest gynecological malignancies and is characterized by a poor prognosis, with an overall 5-year survival rate lower than 40%, which increases when the cancer is diagnosed at an early stage—while still confined to the ovary—and treated by surgery and chemotherapy (29, 30). Many cases of ovarian cancer, unfortunately, are diagnosed when already in an advanced stage, with metastasis to bladder, uterus, or abdomen, as ovarian cancer symptoms typically resemble gastrointestinal problems (abdominal discomfort, nausea, and bloating) (20). The traditional clinical approach to ovarian cancer relies on a combination of surgery and platinum/taxane-based chemotherapies. While initially sensitive to chemotherapeutic drugs, unfortunately, most patients develop a resistance to these pharmacological therapies (30).

So far, the used therapeutic drugs predominantly targeted the tumor cells, without taking proper account of the role of the tumor microenvironment. The latter, instead, is composed of an extracellular matrix and many cells that could actively participate in tumor progression and may serve as novel therapeutic targets for ovarian cancer patients (20, 21, 31). Among the stromal cells —besides adipocytes, endothelial cells, and immune cells— fibroblasts have been strongly reconsidered, as their ability to create a loop of intercellular communications that strengthen the cancer progression has been revealed (31).

It has been highlighted, indeed, that within the tumor microenvironment, fibroblasts, which usually constitute the most abundant population, can acquire a perpetually "activated" state, making them able to support, in turn, the cancer progression; these activated fibroblasts were identified as CAFs (20, 32–34). Besides, CAFs' supportive role in ovarian cancer has been already proved (21, 35, 36).

Generally, the activation of resident fibroblasts, induced by the cross-talk with tumor cells, may be sustained by growth factors released from tumor cells, the most important being the

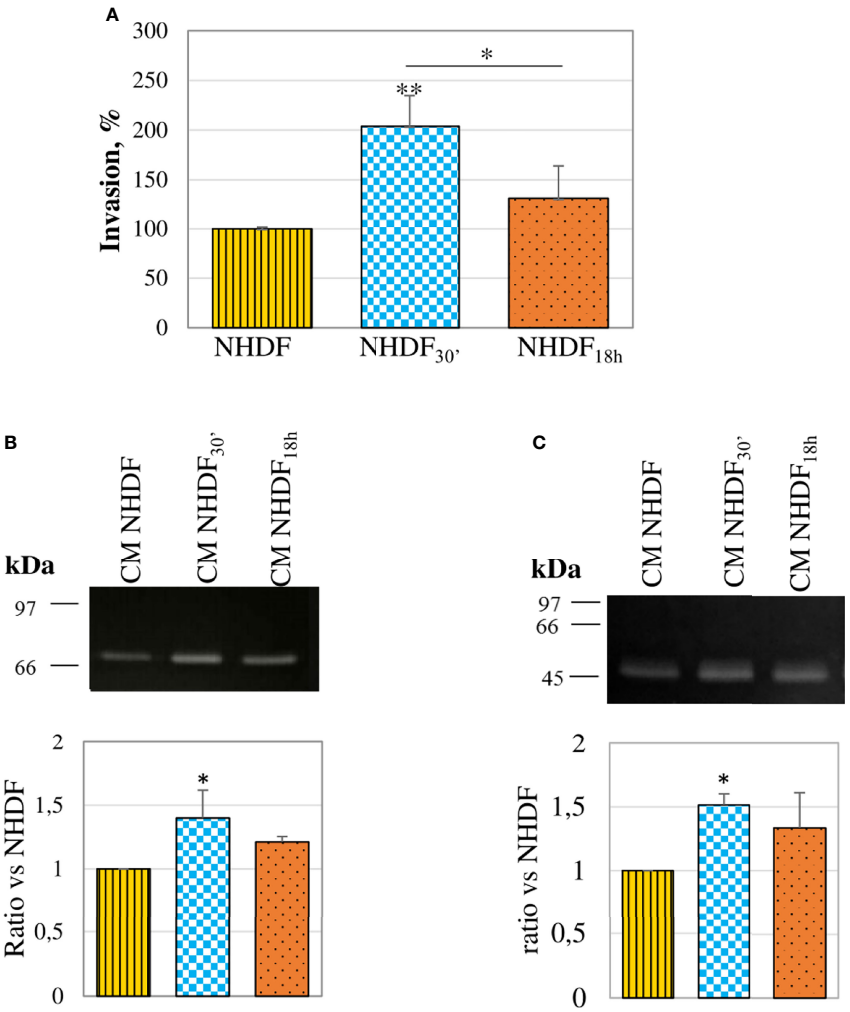

FIGURE 5 | Invasion ability and proteolytic enzymes. **(A)** In an invasion assay with a modified Boyden chamber, $NHDF_{30'}$ and $NHDF_{18h}$ invaded through the Matrigel®-coated membrane significantly more than NHDF. Data derive from 5 measures from each replicate (3 replicates in total) and are shown as mean ± SD; the asterisk on the bar indicates the statistical significance with respect to NHDF, and the horizontal line refers to the statistical significance between the $NHDF_{30'}$ and $NHDF_{18h}$ (*$p < 0.05$; **$p < 0.01$). **(B)** Gelatin zymography assay was performed to detect gelatinolytic activity in the serum-free conditioned media of NHDF, $NHDF_{30'}$, and $NHDF_{18h}$. $EVs_{30'}$ induced a more marked increase in pro-MMP-2 (~72 kDa) release than the EVs_{18h}. **(C)** Casein–plasminogen zymography assay was performed to detect plasminogen activator (PA) activity in the serum-free conditioned media of NHDF, $NHDF_{30'}$, and $NHDF_{18h}$. The bands represent the high-molecular-weight PAs (~48-55 kDa), whose release resulted in higher fibroblasts treated with $EVs_{30'}$. In both zymography assays, the densitometric values of the bands were calculated with ImageJ and reported in the graphs below as a ratio of the band $NHDF_{30'}$ or $NHDF_{18h}$ vs. NHDF, which have been conventionally assigned the value 1. The images shown in panels B and C are representative of 3 independent experiments (all of them being reported in the graphs).

TGF-β, even if many other molecules seem to be involved in the CAFs activation, such as HGF, PDGFs, FGFs, EGFs, and interleukin-1β (18, 30, 37, 38). Once activated from cancer cells, CAFs' secretome, in turn, remodels tumor stroma to become more advantageous for tumor progression, thus deeply contributing to the malignant behavior of cancer cells. CAFs, indeed, can enhance the invasive properties of cancer cells releasing several tumor-promoting growth factors and chemokines (for example, TGF-β, HGF, FGF1, and FGF2) and also molecules (like VEGF) that strongly induce angiogenesis, further supporting proliferative, migratory, and invasive abilities of cancer cells (29, 33, 35, 39–41). They can migrate along with cancer cells in the bloodstream, secreting cytokines that sustain invasive properties and growth of tumor cells at distant sites, supporting the hypothesis that they can contribute to the pre-metastatic niche formation; they also support immunosuppression and drug resistance (22–24, 37, 38, 41–43).

As for the latter, the key role of CAFs and how they exploit several mechanisms to sustain the resistance to antineoplastic drugs have emerged: they can modify the composition of the extracellular matrix so to increase the intratumoral interstitial fluid pressure, resulting in a physical barrier that prevents an efficient delivery of anticancer drugs (22, 44). CAFs can also activate signaling pathways that revert the therapeutic outcome,

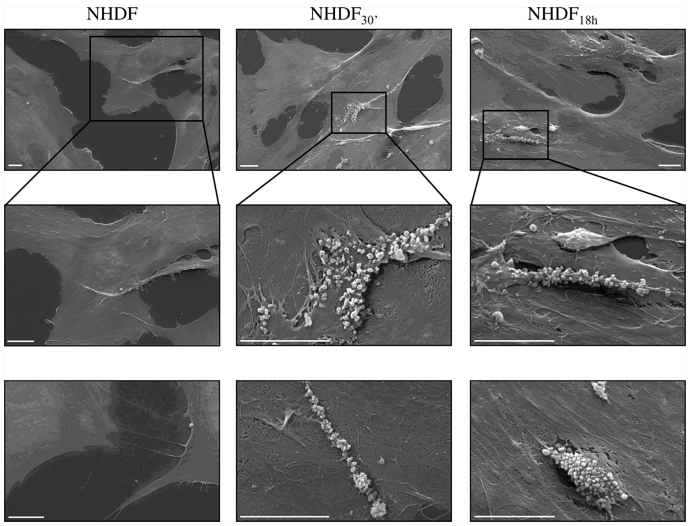

FIGURE 6 | Scanning electron microscopy (SEM). SEM images highlighted the intense shedding of microvesicles from the plasma membrane of $NHDF_{30'}$ and $NHDF_{18h}$. On the other hand, NHDF cells showed an extremely sporadic production of microvesicles. The first row shows images at low magnification; the second row shows details of the first row at higher magnification, as highlighted by the boxes; row 3 shows other independent and representative images at higher magnification. The scale bar is 10 μm in all images.

driving tumor cells to a more chemoresistant phenotype by different mechanisms (45): they can release growth factors involved in the therapy resistance (among the growth factors released from CAFs, for example, the HGF has been correlated to therapeutic resistance occurrence in melanoma) (22, 46) or factors that, stimulating tumor cells to undergo epithelial-to-mesenchymal transition, increase the resistance to chemotherapy (24, 45); they have also been shown to promote the chemoresistance by promoting the metabolic reprogramming or maintaining the stemness of cancer stem cells (23).

Being increasingly demonstrated that CAFs contribute to cancer progression and drug resistance, they are more and more considered as a pivotal target of novel anticancer therapies. In parallel, the understanding of biological processes involved in CAFs activation into the tumor microenvironment is critical to reveal mechanisms underlying cancer progression and drug resistance as well.

Since EVs are known for their role as mediators of cell-to-cell communication (3, 47–49), we wondered if EVs released from human ovarian cancer cells could activate normal fibroblasts into

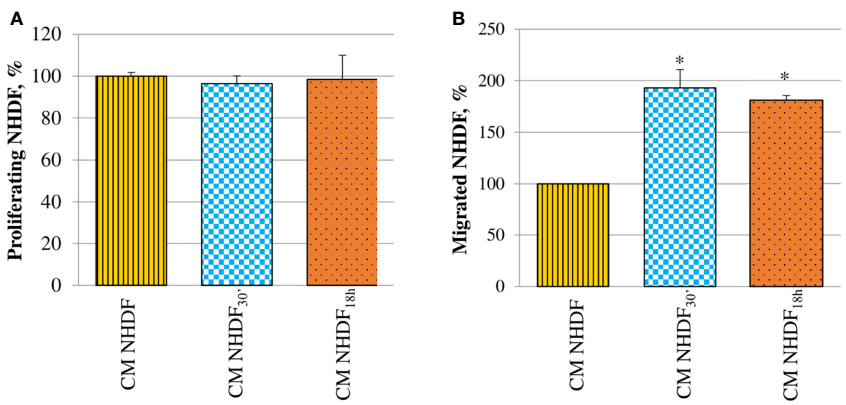

FIGURE 7 | Effect of NHDF, $NHDF_{30'}$, and $NHDF_{18h}$ secretome on normal fibroblasts. **(A)** Normal fibroblasts were cultured for 96 h with conditioned media from NHDF, $NHDF_{30'}$, and $NHDF_{18h}$. Cell proliferation rate was tested using XTT assay. Data were derived from experiments performed twice in triplicates and are shown as mean ± SD. No significant differences in the proliferation percentage were revealed. **(B)** Normal fibroblast migration assay performed twice in duplicates with modified Boyden chamber. Data are expressed as mean ± SD and are shown as a percentage with respect to 100% migration, conventionally attributed to fibroblasts migrated in response to conditioned medium of NHDF (*$p < 0.05$).

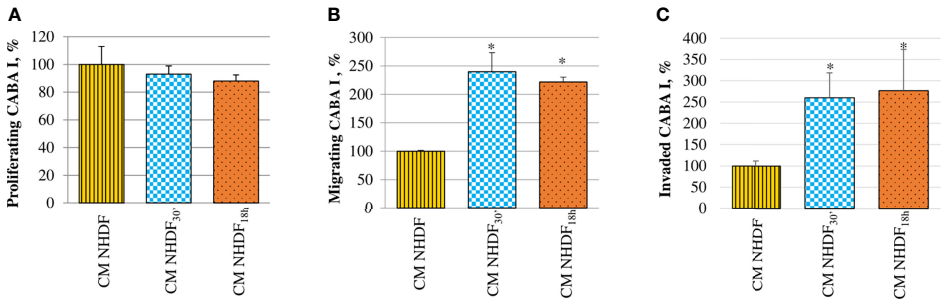

FIGURE 8 | Effect of NHDF, $NHDF_{30'}$, and $NHDF_{18h}$ secretome on CABA I cells. **(A)** CABA I cells were cultured for 72 h with conditioned media (CMs) of NHDF, $NHDF_{30'}$, and $NHDF_{18h}$. Data, derived from experiments performed twice in triplicates, are expressed as mean ± SD and shown as a percentage, and 100% proliferation was assigned to CABA I cells proliferating with CM of untreated NHDF. **(B)** CABA I cell migration was measured twice in duplicate in response to serum-free CM NHDF, CM $NHDF_{30'}$, and CM $NHDF_{18h}$, by modified Boyden chamber (*$p < 0.05$). Data (mean ± SD) are expressed as a percentage with respect to 100% migration, conventionally attributed to CABA I cells migrating toward CM NHDF. **(C)** CABA I cell invasion was tested twice in response to CM NHDF, CM $NHDF_{30'}$, and CM $NHDF_{18h}$ with a modified Boyden chamber coated with Matrigel® (*$p < 0.05$). Data (mean ± SD) are expressed as a percentage, and 100% invasion was attributed to CABA I cells migrating toward CM NHDF.

CAFs, as is the case with other types of cancer (18, 50–54) and found out that when EVs isolated from human ovarian CABA I cancer cells were administered to normal fibroblasts, they induced their activation into a CAF-like state (55).

Moreover, we had already demonstrated that it is possible to isolate two EV subpopulations from CABA I cells in a time-dependent way: starved CABA I cells, once stimulated with FBS, released a nearly pure population of EXO-like EVs or sEVs (mean size ~100 nm) after 30 min and a second population consisting of a high amount of MVs-like EVs or lEVs (size > 100 nm) combined with a low EXO-like EV contribution, i.e., lEVs +sEVs after 18 h (25). Those data highlighted that different time intervals lead to the release of different subpopulations of EVs, in terms of not only size but also amount and molecular composition, suggesting possible different cargoes and, consequently, a different biological role for the different subpopulations.

Hereinafter, these subpopulations will be indicated, respectively, as $EVs_{30'}$ and EVs_{18h} and the NHDF cells obtained by their administration as $NHDF_{30'}$ and $NHDF_{18h}$; untreated fibroblasts will be indicated as NHDF.

Based on those previous results, in the present work, we aimed to verify if the two specific subpopulations of sEVs and lEVs+sEVs released from the human ovarian cancer cell line CABA I could differentially activate fibroblasts, so as to verify if they could induce different biological processes (maybe related to a different cargo). To this purpose, NHDF were treated daily with the EV subpopulations for 5 days, in a cumulative way (i.e., adding the new dose of EVs to the previous one without replacing medium throughout the treatment), to reproduce, *in vitro*, continuous stimulation from cancer cells–EVs on stromal fibroblast that, supposedly, takes place *in vivo*.

When administered to fibroblasts, the EVs modified their morphological and molecular features, supporting the idea that EVs can induce the activation of fibroblasts into a CAF-like state: in fact, untreated cells displayed the usual elongated and spindle-shaped aspect of normal quiescent fibroblasts, while some $NHDF_{30'}$ and $NHDF_{18h}$ acquired the typical "spread" phenotype of CAFs, which is similar to that of myofibroblasts involved in the wound-healing process (**Figure 1**) (33, 41); at the same time, there was an increase in the α-SMA levels (**Figure 2**), a common marker for CAFs [along with SDF-1, FSP-1, vimentin, desmin, tenascin, and FAP (20, 33, 41, 56)]. To further confirm the activation into a CAF-like state, FAP was also analyzed, highlighting an increase in its level in EV-treated NHDF.

Even if both EV subpopulations affected the morphology and marker expression in NHDF, we found out that $EVs_{30'}$, but not EVs_{18h}, also enhanced the proliferative, migratory, and invasive abilities of NHDF (**Figures 3–5**); all these processes are typically increased in CAFs (30, 54). Since $NHDF_{30'}$ releases a higher content of proteolytic enzymes as compared to $NHDF_{18h}$ and NHDF, we can suppose that both invasion and motility were sustained by the increased levels of gelatinases and PAs (**Figures 5B, C**). The release of proteolytic enzymes could also sustain the drug resistance: CAFs have been demonstrated to be actively involved in the secretion of uPA, which can cleave and activate several MMPs that, in turn, could facilitate cancer cells migration and invasion, by degrading the extracellular matrix, as well as drug resistance (18, 24, 33, 34, 57–59). So our data confirm that EV-activated fibroblasts release both the MMPs required for these processes, in an active pro-MMP form, and their activators PAs.

The activated state of $NHDF_{30'}$ and $NHDF_{18h}$ also seems to result in an increased release of EVs (specifically MVs) from the cell surface (**Figure 6**); it is not possible to quantify the extent of MVs' release from activated NHDF through the SEM images, but the observation clearly revealed an increase in membrane shedding. It has been previously reported that the extensive production of MVs by CAFs is used as a way to move lipids and proteins to target cancer cells to support tumor growth (60). This evidence led to hypothesize that activated fibroblasts are more prone to communicate with neighboring cells; after all, several studies already suggested that CAFs can actively modulate bystander cells in the tumor microenvironment by means of soluble or EV-associated mediators [fibroblasts-derived EXOs,

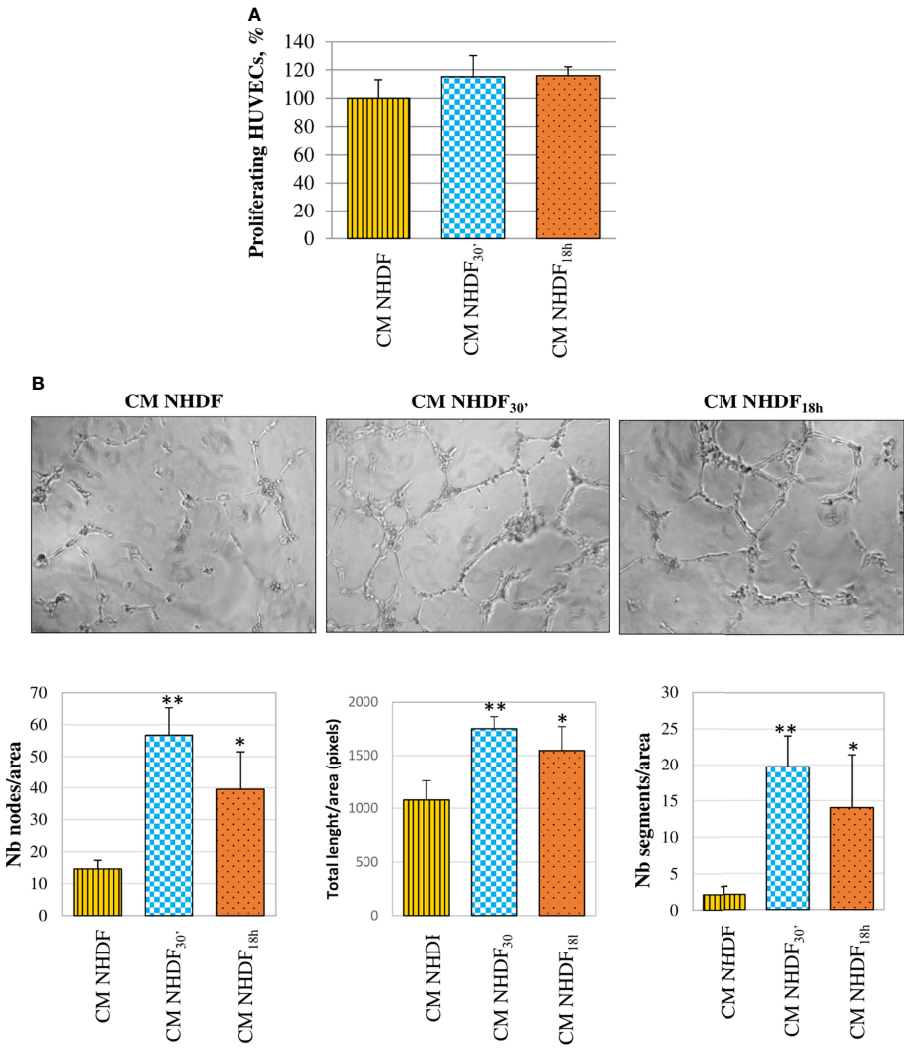

FIGURE 9 | Effect of NHDF, $NHDF_{30'}$, and $NHDF_{18h}$ secretome on HUVECs. **(A)** The effect of conditioned media (CM) NHDF, CM $NHDF_{30'}$, and CM $NHDF_{18h}$ on endothelial cell growth was assessed using the XTT assay. Proliferation was expressed as a percentage, conventionally attributing 100% proliferation to HUVECs treated with CM NHDF. Data derived from experiments performed twice in triplicates. **(B)** Representative pictures showing the formation of capillary-like structures formed by HUVECs seeded on Matrigel®-coated plates in a serum-free condition and treated with CM NHDF, CM $NHDF_{30'}$, and CM $NHDF_{18h}$; the graphs at the bottom show the number of nodes or total length of tubes or number of segments normalized per area. Data derive from experiments performed twice in duplicate and are expressed as the mean ± SD (*$p < 0.05$; **$p < 0.01$).

for example, stimulate motility in breast cancer cells (61), while CAF-derived EXOs can lead to higher drug resistance (62)].

This considered, we wondered whether our $EVs_{30'}$- and EVs_{18h}-activated fibroblasts were actually able to modulate the response of some cells usually present in the tumor microenvironment, such as tumor and endothelial cells as well as still quiescent fibroblasts. For purely technical problems, due to material shortage, we have not used the EVs isolated from activated fibroblasts but their CM (which represents their whole secretome, containing both soluble and EV-associated molecules). CMs obtained from NHDF, $NHDF_{30'}$, and $NHDF_{18h}$ are indicated, respectively, as CM NHDF, CM $NHDF_{30'}$, and CM $NHDF_{18h}$.

CM $NHDF_{30'}$ and CM $NHDF_{18h}$ did not significantly affect the proliferation, neither of normal fibroblasts (**Figure 7A**) nor tumor (**Figure 8A**) or endothelial cells (**Figure 9A**); on the other hand, instead, both CMs significantly affected the motility of fibroblasts (**Figure 7B**) and motility and invasiveness of CABA I tumor cells (**Figures 8B, C**). The CM from activated fibroblasts also exhibited a pro-angiogenic behavior, being able to stimulate the tube formation assay of HUVECs (**Figure 9B**).

These assays indicated that the secretome released by fibroblasts, being previously activated by cancer EVs, may deeply affect the behavior of neighboring cells through paracrine mechanisms; this observation parallels what is already known for tumor-derived secretome/EVs. Indeed, the

role of cancer cells in inducing the reprogramming of other neighboring cells (such as epithelial cells or mesenchymal stem cells) toward a tumor-like phenotype possibly sustaining cancer progression has been already shown (63–69).

Likewise, it is widely demonstrated that tumor EVs can move in the blood, thus contributing to the formation of the pre-metastatic niche (19, 68–70); among the processes involved in the formation of the pre-metastatic niche, a critical role is sustained by the cross-talk between cancer cells and resident fibroblasts, resulting in the activation of the latter ones (18, 71). While many studies dissected the role of tumor-derived EVs in metastatic niche modulation (70–72), only sporadic studies have explored the ability of CAF-derived EVs to promote the pre-metastatic niche formation (73), and their role remains to be further elucidated. Given that the $EVs_{30'}$- and EVs_{18h}-activated fibroblasts showed an increased ability to produce MVs and to stimulate, in turn, other normal fibroblasts, our data could support the hypothesis, to be verified, that also the EVs released by the activated fibroblasts in the primary site of the tumor, as well as those released by the tumor cells themselves, can move through the blood and prepare the pre-metastatic niche by stimulating the resident cells.

The disclosed data, overall, support the idea that ovarian cancer cells could initially modulate fibroblast behavior within the tumor microenvironment through the release of EVs, activating them to a CAF-like state, and then, in turn, these CAF-like cells can stimulate the surrounding normal and tumor cells to acquire a cancer-supportive behavior and, maybe, distant fibroblasts in the pre-metastatic niche.

It is interesting to note that the population $EVs_{30'}$ is the strongest in the activation of all described processes, aligning with some proteogenomic assays that have previously shown that EXOs and MVs are functionally distinct (74), with EXOs being more oncogenic than MVs (75); it looks like in the EVs_{18h} population, being the EXOs diluted by the simultaneous presence of MVs, oncogenic stimuli are weakened. The higher ability of $EVs_{30'}$ to activate fibroblast could rely on their higher content in TGF-β with respect to the EVs_{18h}, as demonstrated by Western blotting and ELISA (data not shown); TGF-β, along with other several molecules, is required for the induction and maintenance of CAFs by cancer cells (54, 76, 77).

That the EXOs play a crucial role in cancer biology and metastasis has widened their possible applications for cancer detection and medical diagnostics; indeed, there is a continuous evolution of techniques and applications in these fields based on EXO use, ranging from liquid biopsy to EXO-based biosensors (78–80).

There is no doubt that to understand more fully the molecular protagonists of this virtuous (from the tumor point of view) cross-talk, it will be necessary to dissect the content of the $EVs_{30'}$ and EVs_{18h} and the composition of the CAFs secretome; regarding the latter, understanding whether the molecules involved in the stimulation of neighboring cells are soluble or EV-associated could help in identifying involved pathways as well as possible specific therapeutic targets to improve clinical approaches aimed to slow down cancer progression and overcome CAF-supported drug resistance.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

IG and MDF contributed to conception and design of the study. IG wrote the first draft of the manuscript. IG, MDF, GP, LE, SDA, and VD acquired and analyzed data. IG and VD supervised. VD acquired funds. All authors listed contributed to manuscript revision, read, and approved the submitted version.

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REVIEW
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CircRNAs in Malignant Tumor Radiation: The New Frontier as Radiotherapy Biomarkers

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World Health Organization (WHO) data show that of the top 20 factors that threaten human life and health, cancer is at the forefront, and the therapeutic approaches for cancer consist of surgery, radiotherapy, chemotherapy and immunotherapy. For most highly metastatic and recurrent cancer, radiation therapy is an essential modality to mitigate tumor burden and improve patient survival. Despite the great accomplishments that have been made in clinical therapy, an inevitable challenge in effective treatment is radioresistance, the mechanisms of which have not yet been completely elucidated. In addition, radiosensitization methods based on molecular mechanisms and targets, and clinical applications are still inadequate. Evidence indicates that circular RNAs (circRNAs) are important components in altering tumor progression, and in influencing resistance and susceptibility to radiotherapy. This review summarizes the reasons for tumor radiotherapy resistance induced by circRNAs, and clarifies the molecular mechanisms and targets of action. Moreover, we determine the potential value of circRNAs as clinical indicators in radiotherapy, providing a theoretical basis for circRNAs-based strategies for cancer radiotherapy.

Keywords: circRNAs, radiotherapy, malignant tumor, biomarkers, clinical application

Abbreviations: DDR, DNA damage repair; EMT, Epithelial–mesenchymal transition; ICFs, Intron-containing fragments; RBPs, RNA-bind proteins; ADAR1, Adenosine deaminase acting on RNA 1; EGFR, Epidermal growth factor receptor; ATM, ataxia telangiectasia-mutated; ATR, ATM and Rad3 related protein; DNA-PK, DNA-dependent protein kinase; H2AX, H2A histone family member X; ZEB1, Zinc finger E-box binding homeobox 1; PDGFRα, Platelet derived growth factor α; PTEN, phosphate and tension homology on chromosome ten; CDKs, cyclin-dependent kinases; MVEs, multivesicular endosomes; RIBE, Radiation-induced bystander effect; IFG1R, Insulin-like growth factor receptor; IR, irradiation; RT, radiotherapy; CSCs, Cancer stem cells; APC, Adenomatous polyposis coli; Axin, axis inhibition; TME, Tumor microenvironment; HIF-1α, Hypoxia inducible factor-11α (HIF-1α); NPC, Nasopharyngeal Carcinoma; HPSCC, hypopharyngeal squamous cell carcinoma; NSCLC, non-small cell lung cancer; OSCC, oral squamous cell carcinoma; GBM, glioblastoma multiforme; 2-DG, 2-deoxy-d-glucose.

INTRODUCTION

Radiation therapy is one of the conventional methods in cancer treatment. It is utilized in more than 50% of oncology patients and can be applied alone or in combination with immunotherapy and chemotherapy (1). Radiation-induced DNA double-strand breaks are one of the most lethal causes of cell death. When exposed to radiation, DNA double-strands break directly or cells generate surplus free radicals that indirectly damage DNA (2). Synchronously, radiotherapy can also induce apoptosis, autophagy and cell cycle changes, thereby altering tumor cell proliferation, invasion and other properties. However, there are still some unavoidable problems in radiotherapy, for example, how to reduce side effects and implement precision radiotherapy strategies. Of these problems, radiation tolerance is a common and intertwined phenomenon that impedes therapeutic efficacy, resulting in the neoplasm recrudescence or poor prognosis after radiotherapy (3, 4). Consequently, it is of great significance to uncover the mechanisms of radiation resistance, predict sensitivity to radiotherapy in patients and formulate strategies to overcome radioresistance.

CircRNAs, a naturally occurring event of widespread and multitudinous single-stranded RNAs, were initially misinterpreted as useless products of splicing in the 1970s. Subsequently, their structures and functions have been broadly expounded by high-throughput RNA sequencing. The distribution of circRNAs is diverse, with a small fraction located in the nucleus, and they intervene in the transcription of parental genes by RNA polymerase II (5) or U1 small nuclear ribonucleoprotein (snRNP) (6). UAP56 and URH49 are responsible for the nuclear transport of circRNAs (7), and these molecules transported into the cytoplasm act as competing endogenous RNAs (ceRNAs) by sponging miRNAs. Accumulating evidence demonstrates that circRNAs have emerged as one of the central regulators of human diseases (8). In tumors, circRNAs are linked to radiation resistance and radiosensitivity. When receiving radiation, altered circRNAs regulate the radiotherapy response by activating signal pathways and targeting genes, inducing cellular process changes, such as DNA damage repair (DDR), epithelial–mesenchymal transition (EMT), apoptosis, autophagy, cell cycle, metabolism (9).

Previous studies have stated that dysregulated circRNAs are involved in chemotherapy (10, 11), showing good prospects and clinical value. However, the current systematic generalization of circRNAs in radiotherapy is limited. Here, we describe the origin and characterization of circRNAs, and systematically review the regulation mechanisms of circRNAs in radiotherapy. We also discuss targeted therapy strategies and other potential clinical values of circRNAs in irradiation (IR).

BACKGROUNDS OF CIRCRNAS: CLASSIFICATION, BIOGENESIS AND FEATURES

Classification and Biogenesis

CircRNAs, single-stranded covalently closed continuous loop RNA without a 5′ cap and 3′ poly A tail, were first reported in models of viroid in 1976 (12). Subsequently, advanced bioinformatic technology identified and classified different types of circRNAs, which are ubiquitous in various cells and tissues (13). These circRNAs are also unique in terms of their origin and structure. Previous studies have identified the following four categories based on various combinations of exons and/or introns (14): (1) Exonic circRNAs (ecRNAs) are generally thought to be products of canonical spliceosomes. When pre-mRNA splicing removes introns and retains exons (15, 16), the downstream 3′ splicing site binds to the upstream 5′ splicing site in reverse order (17). Several studies have suggested that exon-only circRNAs can act as regulators in the cytoplasm (18); (2) Exon-intron circRNAs (EIciRNAs), which are nuclear retained and can promote the transcription of parental genes (6); (3) Intron circRNAs (ciRNAs), unlike most introns that are degraded immediately after excision, some introns have the capacity to escape branching and form circRNAs containing introns instead (19, 20). CiRNAs predominantly exist in the nuclear region, indicating that they may interact with host gene transcription (19); (4) Transfer RNA intronic circular RNAs (tricRNAs). Pre-tRNA removes 5’ leader and 3’ trailer by tRNA splicing endonuclease, and then modifies the CCA structure on 3 tail ends to form mature tRNA. RtcB ligase is not only responsible for the splicing of tRNA, but also the circularization process of tricRNAs (21, 22). Interestingly, some scholars discovered a new type of circRNAs named intergenic circRNAs. Intron-containing fragments (ICFs) flanking GT-AG splicing signals, act as splice donors and splice acceptors to conduct intergenic circRNAs through circularization (23). In **Figure 1**, we briefly describe the formation and functions of circRNAs, whose functions are determined by their sequence, post-transcriptional modification, and location. Commonly proposed functional mechanisms of circRNAs include miRNA sponges, protein interactions, translation, and regulation of parental genes.

The regulation mechanisms engaged in the formation of circRNAs transcripts include cis- and trans-acting factors (24). One of the essential cis-acting elements is the external reverse complementary sequences flanking exon splicing introns (25). Alu repeats (short repetitive sequence), the first determined elements that modulate back-splicing by tightly connecting to the splice sites (26), result in multiple circRNAs produced from a single gene locus (25). The existence of Alu repeats in flanking introns is an important basis for predicting and analyzing the formation of circRNAs (25). Additionally, RNA-binding proteins (RBPs) act as activators or inhibitors to regulate the production of circRNAs, respectively. Studies have showed that some RBPs can bind to intronic regions and improve back-splicing efficiency by homo- or hetero-dimerization (27, 28) to facilitate circularization. For instance, Sam68 can interact with the Alu-rich introns in the survival of motor neuron gene (SMN) pre-mRNA to promote circRNAs biogenesis (29). On the other hand, a few proteins may inhibit the formation of circRNAs by disrupting the base pairing of introns. Adenosine-to-inosine(A-to-I) edited by adenosine deaminase acting on RNA 1 (ADAR1) antagonizes circRNAs expression through weakened inverted Alu repeats (30). Similarly, nuclear RNA helicase DHX9

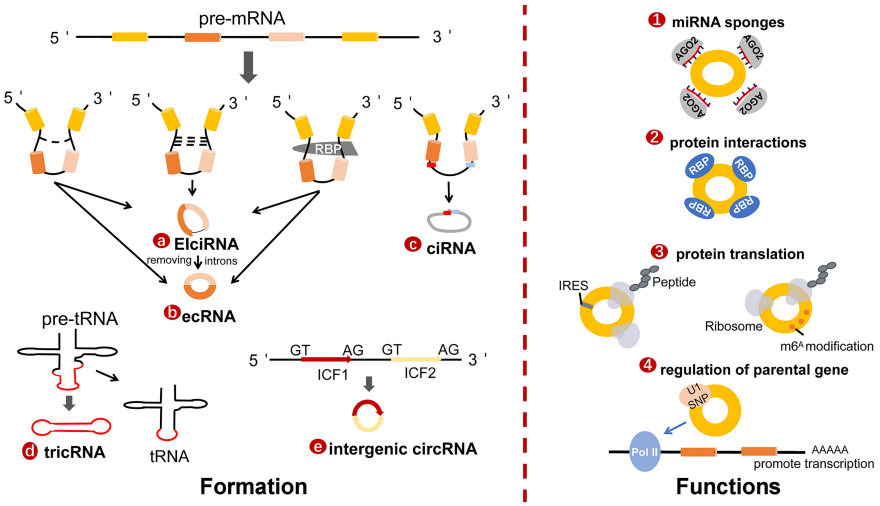

FIGURE 1 | The formation and functions of circRNAs. **(A)** Exon-intron circRNAs (EIciRNA) are the nuclear retained and promote the transcription of parental genes. **(B)** Exonic circRNAs (ecRNAs) are generally supported as products of canonical spliceosome. When pre-mRNA splicing removes introns while retaining exons, the downstream 3′splicing site binds to upstream 5′splicing site in reverse order. **(C)** Intron circRNAs (ciRNAs). The sequence close to the 5′ splicing site containing a 7 nt GU-rich element (red box) and an 11 nt C-rich element near the branch point (blue box) are essential to stable ciRNAs. **(D)** TRNA intronic circular RNAs (tricRNAs), the production of tricRNAs requires conserved tRNA sequence and several processing enzymes, such as RtcB ligase and TSEN endonuclease. **(E)** Intergenic circRNA. Intron-containing fragments (ICFs) flanking GT-AG splicing signals, acting as splice donors and splice acceptors to conduct intergenic circRNAs through circularization. The main functions of circRNAs include miRNA sponges, protein interactions, protein translation and regulation of parental gene.

threatens circRNAs processing by untwisting the reverse complementary of Alu elements flanking exons (31).

The Features of circRNAs

The alternative translation of the same genetic locus produces multiple RNA isoforms, endowing the isoforms with unique functions (32). CircRNAs and homologous linear mRNAs are derived from the same splicing precursor. Compared with the linear mRNAs, circRNAs not only have special structures, but also have independent characteristic. (1) stability: The spliced products of precursor RNAs include linear RNAs, circular RNAs and others, of which linear RNAs are completely degraded, whereas lariat and circRNAs can resist RNase R-digestion (33), owing to the lack of accessible ends. Yehoshua et al. confirmed that the great majority of circRNAs were long-lived compared with linear mRNAs (34), as the half-life of circRNAs exceed 48 h while that of linear is less than 20 h (26); (2) abundance: Next-generation RNA sequencing corrected the early false assumption that circRNAs were errors with non-function and low expression during transcription. Numerous studies have been devoted to considering the expression and function of circRNAs (26, 30, 35, 36). Generally, the expression level of most circRNAs is only 5-10% of its linear transcript (37). However, some circRNAs possess special features. In human fibroblasts, more than 14% of transcribed genes have the ability to express circRNAs, under certain circumstances, which are more abundant than the corresponding mRNAs (26). Julia Salzman et al. clarified approximately 50 circRNAs that were highly expressed compared with their parental linear transcripts in all tested cell lines (38), especially in neuronal organs (28, 39, 40). Moreover, the relative percentage of circRNAs to their canonical linear RNAs shows a higher average in human blood (41). Analysis of circRNAs genome characteristics documented that alternative back-splicing (ABS) allows a single gene to produce diverse circRNAs with the same reverse splicing site (42). This may be one of the reasons for the diversity and specificity of circRNAs; (3) unique function: CircHIPK3 is derived from exon2 of the HIPK3 gene and impairs human cell growth while being silenced. However, the linear molecule HIPK3 mRNA has no similar biological functions (43). A genome-wide analysis of 144 prostate cancer specimens identified 7,232 circRNAs; 11.3% of which were highly expressed and were related to cell proliferation; approximately 90% of their linear counterparts were not necessary for proliferation (44). In addition, circRNAs and mRNA sometimes exhibit antagonistic relationships. The Zbtb7a gene generates coding mRNAs and exerts its tumor suppressive role, but the non-coding product of Zbtb7a, circPOK, acts as an oncogene in mesenchymal cancers (32). The E-cadherin variant protein (C-E-Cad) encoded by circ E-cadherin represents antithetical functions with E-cadherin. C-E-Cad accelerates glioma stem cell tumorigenicity through the independent activation of the oncogenic epidermal growth factor receptor (EGFR) signal pathway and EGFRviii by the exceptional C -terminal (45). Studies have shown that the association between circRNAs and parental gene is weak, suggesting that circRNAs are largely independent and not just byproducts of aberrant splicing. In a word, the unique expression pattern of circRNAs suggest that they likely possess functional significance.

THE ROLE AND MECHANISMS OF CIRCRNAS IN RADIOBIOLOGY

CircRNAs have emerged as significant regulators in the progress of tumors. Radiation is a linchpin of cancer treatment, which can alleviate sufferings or even completely cure tumor patients. Notwithstanding the advances in circRNAs research, the hidden mechanisms of how circRNAs contribute to radiotherapy remain largely unexplored. Here, we generalized the current mechanisms of action of circRNAs in modulating radiotherapy **(Figure 2)**, aiming to foster new insights into circRNAs therapeutic strategies.

CircRNAs Participating in DNA Damage Repair

Correct DNA repair is one of the basic methods of maintaining cell homeostasis. Inducing DNA strand damage is the pivotal biological function of radiotherapy, while activating the DNA-repair signaling pathway may attenuate the efficacy of anticancer therapy. ATM, ATR kinase, and DNA-PK are the key indicators in detecting DNA damage. Cell cycle checkpoint kinases CHK1, CHK2 are phosphorylated and activated by ATM and ATR after DNA damage, and play an important role in S phase and G2 phase (46). It has been proved that ATM-, ATR-, and DNA-PK inhibitors benefit tumor sensitivity to IR (46). In addition, γH2AX, TP53BP1 (47), and RAD51 (48) have been identified as sensitive candidates to predict radiation damage and repair. In particular, ATM has a central role in the perception of DNA damage and initiates a series of responses to cell cycle activation and apoptosis **(Figure 3)**.

Zinc finger E-box binding homeobox 1 (ZEB1) is an EMT-inducing transcription factor, and a well-known DDR regulator, responsible for chemo- or radio-resistance (49). Previous studies have shown that ZEB1 participated in radiation response with ATM kinase. For example, miR-875-5p increased radiation reaction by suppressing ZEB1, which impeded CHK1-mediated DNA homologous recombination repair (50). Similarly, down-regulation of circZEB1 reduced ZEB1 protein expression, thereby inhibiting CHK1 protein (51).

H2AX is a member of the histone H2A family. Within a few minutes after DNA double-strand breaking, H2AX is phosphorylated by ATM, ATR and DNA-PKcs to form γH2AX, which then rapidly recruits DNA repair proteins and apoptotic proteins to the injury site. H2AX phosphorylation modification is one of the cellular DNA double-strand break stress responses, and is also the most prominent DNA-associated marker (52). When absorbed in radiation, numerous circRNAs predispose the formation of γH2AX and maintain DNA repair, which is a critical component of radioresistance in cancer. Several circRNAs were reported to respond to IR-mediated H2AX regulation. Research has disclosed that inhibiting some circRNAs could effectively reduce the recruitment of γH2AX, thereby improving the sensitivity of radiotherapy. Si-circ-METRN (53) in glioblastoma as well as si-circ_0086720 (54) in non-small cell lung cancer (NSCLC) reversed circRNAs-mediated γH2AX activation. Mechanically, circ-METRN was regulated *via* the miR-4709-3p/GRB14/PDGFRα interaction network and circ_0086720 depletion reinforced radiation sensitivity by regulating the miR-375/SPIN1 axis. Conversely, circ-AKT3 overexpression increased the number of H2AX foci. As one of the AKT transcript variants, circ-AKT3 was under-expressed in glioblastoma and had the ability to encode AKT-174aa protein. AKT thr-308 phosphorylation was blocked by AKT-174aa, which interfered with downstream signal transmission. AKT-174aa acted as a negative regulator of the PI3K/AKT pathway and ultimately increased the radiosensitivity of glioblastoma multiforme (55). This research innovatively discovered the protein translation function of circRNAs. Although generally classified as non-coding RNAs, there is growing evidence that circRNAs have the ability to translate proteins. To sum up, delineation of exact signals which induce

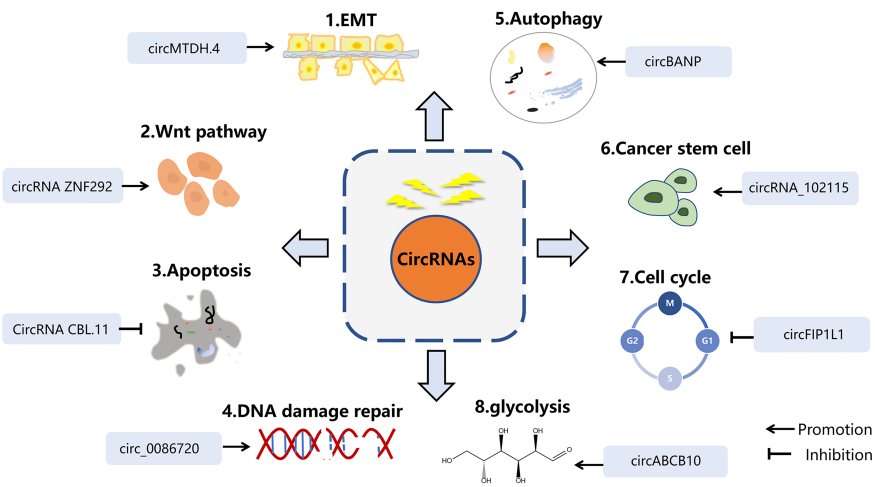

FIGURE 2 | The mechanisms of circRNAs in radiobiology. The main mechanisms of cicRNAs in radiation inculde EMT, Wnt signaling, apoptosis, DNA damage repair, autophagy, cancer stem cell, and cell cycle arrest.

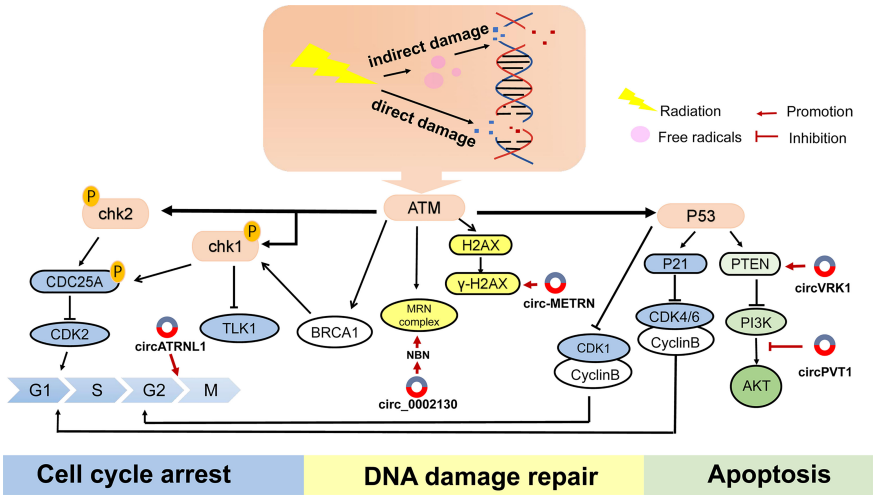

FIGURE 3 | The main reaction of cells to DNA damage and the mechanism of several circRNAs on radiotherapy. DNA double-strand breaks (DSBs) are most cytotoxic injuries in respond to radiation, leading to cells death. When receiving radiotherapy, cells produce a series of repair mechanisms to deal with these damages, resulting in resistance to radiation.

DDR during tumor treatment will undoubtedly help to provide a broader picture of how circRNAs exert their action.

CircRNAs Are Involved in IR-Mediated Apoptosis

Apoptosis is proactive spontaneous death that maintains the stability of the intracellular environment. One of the main tasks of apoptosis is cleaning up precancerous cells and arresting the development of malignancy (56). However, dysregulation of apoptosis not only leads to unchecked cell proliferation and tumor occurrence, but also resistance to therapy. Therefore, the modulation of apoptosis signaling pathways is one of the key factors in optimizing cancer treatment.

The intrinsic apoptotic pathway is started by intracellular signals at the mitochondrial level in order to overcome various stresses, such as radiation and chemotherapy. Activation of the apoptotic pathway is closely associated with the B-cell lymphoma 2 family, which contains pro-apoptotic proteins (Bax) and anti-apoptotic proteins (Bcl-2). P53, a classic tumor suppressor gene, inhibits Bcl-2 activity by increasing the transcriptional expression of pro-apoptotic proteins Bax and Bak, thereby promoting apoptosis. In addition, the caspases are a family of cysteine-dependent endoproteases that regulate cell apoptosis (57). In this regard, several circRNAs have been proved to participate in IR-mediated apoptosis by affecting the above-mentioned pathways. CircRNA CBL.11 modulated YWHAE and varied equally to YWHAE in colorectal cancer cell (CRC) under carbon ion irradiation (58). A previous study reported that YWHAE was conducive to P53 signal activation. The up-regulated YWHAE indeed increased P53-mediated Bax expression and promoted apoptosis. However, this study only briefly introduced that circRNACBL.11 is involved in IR-mediated apoptosis, and functional research is still unclear.

PI3K/AKT signaling represents one of the most influential signaling pathways that inhibit cell apoptosis and promote cell survival. The high activation of PI3K/AKT is related to radiotherapy tolerance in various cancer types (59). The expression of circPVT1 in NSCLC cells following radiotherapy was higher than that previous to radiation. Down-regulated circPVT1 promoted apoptosis by blocking the PI3K/AKT/mTOR pathway and improved the radiosensitivity (60). The above studies further consolidated the role of circRNAs in radiotherapy.

Phosphatase and tension homology on chromosome 10 (PTEN) acts as a tumor suppressor, and governs a plethora of cellular processes, such as survival, proliferation, apoptosis. PTEN antagonizes PI3K activity by dephosphorylating PIP3 and inhibits the PI3K/AKT pathway, thereby preventing IR-mediated cell apoptosis. CircVRK1 heightened radiation sensitivity by adjusting the miR-624-3p/PTEN axis and inactivating the PI3K/AKT signaling pathway by upregulating PTEN (61). CircATRNL1 decreased in oral squamous cell carcinoma (OSCC) patients treated with radiation. Over-regulated circATRNL1 intensified OSCC radiosensitivity by directly binding to miR-23a-3p and relieving endogenous inhibition of the target gene PTEN, which is essential for apoptosis and cell-cycle arrest. Furthermore, adjustment of the PI3K/Akt signaling pathway was accompanied by the alteration of circATRNL1 (62). However, it is not yet certain whether the PI3K/Akt signaling pathway is a key process in circATRNL1 affecting OSCC radiotherapy. These corresponding results show that circRNAs can be utilized as a therapeutic target of radiation-mediated apoptosis in cancer.

CircRNAs Regulate the Autophagy Response to Radiotherapy

Autophagy is a process which maintains cell homeostasis and responds to various stresses. When the cell is under external

pressure, it will initiate an autophagy program against the damaged intracellular substances (63, 64). During this process, some damaged proteins or organelles are encapsulated by autophagic vesicles with a double-layer membrane structure, and then transferred to lysosomes for degradation and recycling. Accruing reports show that circRNAs facilitate radiotherapy and chemotherapy tolerance by inducing autophagy (65, 66). The crosstalk between circRNAs and radiation-mediated autophagy provides a new strategy for the regulation of tumors.

Microtubule-associated protein light chain 3 (LC3) and p62 play essential roles in the formation of autophagy. circRNA_102115 promoted the insensitivity of CRC through the endogenous competitive combination of miR-338-3p, increasing the level of LC3II/I and reducing p62 (67). Correspondingly, circCCNB2 knockdown inhibited autophagy of prostate cancer cells by up-regulating p62 but reducing Beclin1 and LC3II/I, thus increasing radiosensitivity. This effect relied on an interaction between circCCNB2 and miR-30b-5p as shown by rescue experiments. KIF18A was identified as a direct target of miR-30b-5p, and its ectopic expression restored radioresistance and restrained autophagy upon miR-30b-5p overexpression (68). From the current reports, different kinds of circRNAs exert different roles in tumor. Some inhibit autophagy and some promote autophagy. This shows that multiple circRNAs are involved in autophagy, but it is necessary to further clarify whether more circRNAs are participated in autophagy-related radiotherapy regulation. In the future, targeting autophagic pathway may become a new and potential mean of changing radiosensitivity.

CircRNAs Adjust the Cell Cycle

Cell cycle regulation, a large and sophisticated network involving multiple factors, is essential in order to maintain normal cell growth. It includes cyclins and cyclin-dependent kinases (CDKs) that drive the cell cycle, as well as the brake system that exists to avoid unrestricted proliferation, such as CDKs inhibitory proteins (CDKIs) (69). Following damage of DNA molecules by radiotherapy (RT), related genes initiate cell cycle regulation mechanisms and retard cell cycle progression at the G1/S and G2/M checkpoints, with the G2/M period being the most sensitive to radiation, while the S phase exhibits radiation resistance. Cell cycle arrest enables damaged DNA more time to repair, or induces apoptosis on damaged cells that cannot be repaired, thus escaping IR killing effects and increasing radiation resistance (70). Several studies have shown that circRNAs affect cell cycle progression by alternatively binding to cell cycle proteins or acting on miRNA to regulate proteins. A study found that, compared with normal cells, the expression of circFIP1L1 decreased and the proportion of S phase increased in 5-8F-IR cells (radiotherapy-resistant nasopharyngeal cancer cell lines). Overexpression of circFIP1L1 rendered nasopharyngeal carcinoma (NPC) cells more sensitive to RT and resulted in accumulation of the cell cycle in G2/M phase (71). This effect on radiation and the cell cycle was mediated by the miR-1253/SFN pathway. SFN plays a negative role in the cell cycle and blocks the cell cycle at G2/M, acting as a key protein that maintains the stability of the genome (72). Similarly, in circATRNL1-increased OSCC cells, the percentage of G2 was higher and intensified OSCC radiosensitivity (61). CircRNA_014511 overexpression blocked the G2 phase and down-regulated the expression of P53 by coexisting with miR-29b-2-5p, affecting the cell cycle and apoptosis of bone marrow mesenchymal stem cells, and reducing sensitivity to RT (73). Inhibition of circPRKCI repressed viability, colony formation, cell cycle progression of esophageal cancer and elevated cell radiosensitivity through the miR-186-5p/PARP9 axis (74), showing that circPRKCI played a suppressor role in RT of esophageal cancer. As to how these circRNAs influence the biological effects of radiation, how they interfere with their expression to benefit clinical RT, etc., are attracting the attention of researchers.

CircRNAs Manipulate Wnt Pathway in IR-Response

Wnt/β-Catenin, is an indispensable signaling pathway controlling development, differentiation, homeostasis, and stemness of tissues, and is closely associated with cancer progression. Further, a growing body of evidence has demonstrated that Wnt/β-Catenin signaling pathway in clinical therapy is a critical modulator driving cell phenotypic resistance to various types of anticancer treatment (75). The expression level of circRNAs in tumor cells changes after IR, which helps to initiate or inhibit the Wnt signaling, thereby altering the sensitivity of tumor cells to radiotherapy. For instance, circRNA-microarray identified 57 increased circRNAs and 17 decreased circRNAs in radioresistant esophageal cancer cell (ESCC) compared with the parental cell line. KEGG and GO analysis found that Wnt signals may be related to radioresistance (76). In this study, circRNA microarray was used to detect differentially expressed circRNAs, providing a basis for radiotherapy biology. CircRNA ZNF292 can be induced in hypoxia-responsive human hepatoma cell. By interfering with the nuclear translocation of SOX9 protein, circRNA ZNF292 eventually activated the Wnt signaling pathway and led to radioresistance (77). Knockdown of circRNA ZNF292 caused cell cycle arrest, proliferation inhibition, decreased angiogenesis and increased DNA fragmentation levels, accompanied by a decrease in Wnt/β-catenin pathway-associated proteins, including β-catenin, adenomatous polyposis coli (APC), and axis inhibition (Axin). Equally, the Wnt signaling pathway was found to be implicated in the regulation of circRNA_100367, with a higher expression of circRNA_100367 was observed in radiation-resistant ESCC cells (78). These studies suggest that there is a close relationship between circRNAs, Wnt/β-catenin signaling and RT, and extensive testing is needed to reveal the mechanism of circRNAs in radiotherapy-related Wnt/β-catenin signaling.

CircRNAs Affect EMT in Radiobiology

Epithelial–mesenchymal transition is a biological process in which epithelial cells transform into cells with a mesenchymal phenotype, and plays a key role in cell biological behavior. After tumor cells are irradiated, the cells lose polarity, as well as tight

junctions and adhesion. The invasion, migration and carcinogenicity of tumor cells are enhanced, and the ability of cells to resist apoptosis is also increased. Li et al. discovered that circMTDH.4/miR-630/AEG-1 axis contributed to the improvement of radioresistance in NSCLC cells. Previous research illustrated that AEG-1 induce EMT remolding by Wnt/β-catenin signaling pathway. Li et al. indeed confirmed that AEG-1 silencing blocked Slug and Snail gene. In addition, knocking down circMTDH.4 or overexpressing miR-630 can improve radiosensitivity (79). In line with this, deprivation of circRNA_100367 enhanced the radiation sensitivity of radioresistant ESCC cells through competitively binding to miR-217/Wnt3 axis and mediating EMT process. CircRNA_100367 silencing increased E-cadherin, accompanied by a decrease in mesenchymal markers (78). Taking into consideration the conclusions presented, it is clear that circRNAs are involved in the EMT process as biomarkers of radiation response in different cancer types.

CircRNAs Modulate Glycolysis in Response to IR

Glycolysis is a universal method of carbohydrate catabolism in all life. In glycolysis, glucose is degraded to generate ATP, which provides energy for organisms. Studies have indicated that the accumulation of lactic acid is an indication for tumor development, and the aggravation of IR resistance in tumor cells (80). Compared with normal tissues and cells, circABCB10 was up-regulated in breast cancer (BC) samples. When circABCB10 was silenced, the proliferation, colony formation and radioresistance of BC cells were limited. This was because it sponged miR-223-3p to regulate profilin-2 (PFN2), exerting a facilitating role on glycolysis and contributing to the enhancement of glucose consumption and lactate production (81). A similar effect was shown in glioma cells where circPITX1 knockdown decreased glucose consumption and lactate production through the miR-329-3p/NEK2 axis, thereby increasing the sensitivity to RT (82). From the evidence shown above, it is therefore plausible that glycolysis might be the major reason for the decreased benefit of RT. This conclusion opens new avenues to better understand the hidden aspects of circRNAs modulating glycolysis in response to IR.

CircRNAs Influence Cancer Cell Stemness

Cancer stem cells (CSCs) are a group of heterogeneous cells with stem cell characteristics in the tumor. CSCs are endowed with the ability to self-renewal and infinite proliferation; thus, they can evade effective treatment and develop antitumor properties (83). Zhu et al. found that CD133+ cells increased after exposure to radiation in NPC CNE-2 cells, suggesting tumor stem-like cells were associated with NPC radiotherapy. They hypothesized that some dysregulated circRNAs induced NPC cells to differentiate into stem cells after irradiation. Bioinformatics suggested that the "hsa_circRNA_102115-hsa-miR-335-3p-MAPK1" interaction network was associated with CSCs, thus changing radiation sensitivity (84). The CSC-mediated mechanisms of radiotherapy resistance are multifactorial, including high DNA damage repair ability, cell cycle arrest, autophagy. Unfortunately, Zhu et al. have not yet clarified which mechanism CSC plays in NPC. The presence of CSCs has been largely demonstrated in therapy resistance. However, information on the involvement of circRNAs in CSCs research is scarce. In future, we may be able to focus more attention on the relationship between circRNAs and CSCs.

CircRNAs Are Linked to the Tumor Microenvironment

The tumor microenvironment (TME) is the location between tumor cells and adjacent normal tissues. It is mainly composed of tumor cells, surrounding stromal cells and infiltrating inflammatory cells. Emerging evidence shows that external pressure has the ability to remold physiological conditions and metabolism of cancer and stromal cells. Altering the TME may have an impact on radiotherapy outcome (85).

Inflammatory cells or cytokines are in involved in the formation of inflammatory TME. Inflammation has been shown to be a risk factor for the occurrence and development of cancers. Radiation can induce DNA damage while stimulating the release of pro-inflammatory mediators and remolding the tumor immune microenvironment (86). To date, two circular RNAs have been shown to respond to tumor radiotherapy through inflammatory signal activation. Research has revealed that methyltransferase like 3 (METTL3) was related to m6A of circCUX1 in Hypopharyngeal squamous cell carcinoma (HPSCC), which was favorable for circCUX1 expression. In radiation-resistant HPSCC samples, circCUX1 was higher and negatively correlated with Caspase1. Caspase1 inhibition reduced the release of inflammatory factors IL-1β and IL-18, conferring tolerance to radiotherapy in HPSCC (87). Silencing circTUBD1 in irradiated hepatic stellate cells (HSC) decreased the release of the pro-inflammatory cytokines IL-1b, IL-6, thereby reducing radiation-induced liver disease. The mechanism showed that circTUBD1 adsorbed miR-146a-5p to regulate the expression TLR4, IRAK1, TRAF6, and pNF- κB (88). Understanding how radiotherapy affects inflammation is critical if we are to effectively modulate these cytokines to benefit oncology treatments.

Hypoxia is a classic feature of malignant tumors. Rapid proliferation of tumor cells will accelerate the consumption of oxygen. Under hypoxic conditions, tumor cells secrete a variety of vascular growth factors to promote the formation of abnormal blood vessels. In parallel, the invasive and metastatic ability of tumor cells are further improved. Eventually, the malignant degree of the tumor is further increased, resulting in the tumor cells being counteractive to treatment. Hypoxia inducible factor-11α (HIF-1α) is the key controller in tumor hypoxic microenvironment. Su demonstrated that HIF-1α was positively correlated with circDENND2A which was essential for hypoxia-induced migration and invasion of glioma cells (89). Yang found circRNA cZNF292 was hypoxia-responsive, and its expression gradually increased with the extension of hypoxia culture time. Highly expressed circRNA ZNF292 represented radioresistance in hepatoma cell (77).

These findings provide important information to better understand the hidden aspects of circRNAs and TME.

However, research on the role of circRNAs in the TME related to radiotherapy resistance is in its infancy. A series of issues such as the impact of circRNAs on other cells in the microenvironment and how the TME in turn affects circRNAs remain to be resolved.

Roles of Exosomal circRNAs in Cancer Radiotherapy

Exosomes are derived from endolysosomal microparticles and are released by fusion with multivesicular endosomes (MVEs) (90). As important bridges for intercellular communication between cells, exosomes are naturally shed by various types of cells and circulate in biofluids such as blood, saliva, urine, as well as cerebrospinal fluid, ascites (91), and carry organ-specific bioactive molecules, including proteins, nucleic acids, growth factor, lipids, and non-coding RNAs (**Figure 3**).

Radiation affects the content and prosperity of exosomes, as well as their biological functions. There is significant growing data to show that radiation-derived exosomes accelerate tumor progression and decrease the curative rate, and is emerging as an increasingly pivotal field in the clinic. For instance, circRNAs isolated from extracellular vesicles were markedly diverse between radioresistant glioma cells and the control group, of which RNA−sequencing and bioinformatics identified 63 upregulated circRNAs and 48 downregulated circRNAs. Chen et al. identified exosomal circRNAs expression profiles of pancreatic cancer cells upon radiation, and found that circ_0002130 was highly expressed in irradiated mice plasma, and facilitated tumor cells proliferation by accelerating DNA damage repair. This effect was dependent on the interaction between circ_0002130, miR_4482-3p and targeted NBN gene (92). NBN is a member of the MRN complex family and is crucial in sensing of DNA strand break and checkpoint activation (93) (**Figure 3**). However, the above-mentioned regulation mechanism is only a prediction of the bioinformatics network and the underlying mechanism has yet to be verified by experiments. Meanwhile, the molecule has not been validated on clinical specimens. Future in-depth clinical trials will further strongly support their research. Zhao et al. found that circATP8B4 and downstream molecule miR−766 might be the decisive components in adjusting radiotherapy in glioma cells. Interestingly, circATP8B4 stems from EVs of radioresistant glioma cells and can be transferred to adjacent cells, thus promoting radioresistance of normal glioma (94). CircKIRKOS-71 and KIRKOS-73 are derived from endothelial cell exosomes and exhibit varying degrees of responsiveness following radiation in different cell lines, with a time-dependent effect. After receiving 0.25 Gy and 2.5 Gy for 24 h, KIRKOS-71 and KIRKOS-73 were down-regulated in neuroblastoma cells exosomes. In contrast, in the osteosarcoma cell line U2OS, elevated transcript levels were measured in exosomes irradiated for 24 h with low-dose irradiation and 4 h of medium-dose irradiation (95). This provides a theoretical basis for the diagnostic strategy of exosomal circRNAs in clinical radiation. The expression level of exosomal circ_0067835 was upregulated in CRC patients after radiation, while knockdown diminished CRC deterioration and enhanced radiosensitivity by down-regulating insulin-like growth factor receptor (IFG1R) expression through decoying miR-296-5p (96). However, the real mechanism between miR-296-5p/IFG1R and circ_0067835 in CRC radiotherapy is unclear. For example, it is unknown whether PI3K/Akt and MAPK pathways were mediated by IFG1R.

Of note, the mechanism of action of exosomes is different in various conditions. The studies by Dai (97) and Wang (96) indicated that aberrant expression of exosomes was linked to carcinogenesis, malignant behavior and radioresistance of glioblastoma. Nevertheless, Farias (98) proposed that the exosomes released by irradiated mesenchymal stem cells have systemic effects and could delay the growth and metastasis of melanoma. In a word, clearer mechanisms of circRNAs and further understanding of exosomes in radiation are needed.

THE CLINICAL APPLICATION OF CIRCRNAS IN RADIOTHERAPY

Data from previous studies proposed that circRNAs exhibited tissue and organ specificity, and were dysregulated in numerous human cancers. Intriguingly, the expression profiles of circRNAs are also divergent in different stages within the same type of tumors, as well. Owing to the signatures of stability, specificity and availability, circRNAs have irreplaceable potential in clinical oncology strategies. At this stage, the clinical research of circRNAs in tumor radiotherapy mainly focuses on the following two strategies: (1). Different expression patterns of circRNAs in patients, that is, to analyze the differential expression of circRNAs in radiation-sensitive or radiation-resistant patients, as the object of further research (99–101). For example, the expression of circRNA_0000285 and circCUX1 in radiotherapy-resistant patients is higher than that in radiosensitive patients. Therefore, the feasibility of radiotherapy can be predicted by analyzing circRNA expression. (2). Analysis of circRNAs expression level after irradiation (62, 102, 103). With the help of high-throughput sequencing, Yu et al. analyzed 153 differentially expressed circRNAs between control group and HeLa cells accepted radiotherapy, identified 76 increased circRNAs and 77 decreased (104). Similarly, compared with the parental cell line, 57 circRNAs were elevated and 17 circRNAs decreased in radiation-resistant esophageal cancer cells identified by cirRNA microarray (76). These altered circRNAs may participated in the regulation of radiation resistance at the transcriptional level. The above research strategies both suggest that circRNAs have differential expression profiles in radiotherapy. These circRNAs are expected to become sensitive indicators in the treatment of cancer patients and provide a basis for clinicians to formulate individualized treatments.

CircRNAs Are Expected to Become a Targeted Therapy Point in Radiation

Radiosensitizers are drugs or methods that are used simultaneously with radiotherapy to increase the sensitivity of

radiotherapy, by regulating signal pathways or target molecules involved in radioresistance. The above-mentioned studies indicate that circRNAs have the potential to become new clinical radiosensitizers. The following three methods can be utilized to regulate the radiation response involving circRNAs, thereby improving the efficacy of RT: 1. For circRNAs that act as suppressors (radiotherapy tolerance) or promotors (radiotherapy sensitivity) **(Table 1)**, regulating their expression level seems to be an effective strategy. Circ_0055625 knockdown sensitized colon cancer to irradiation and inhibited tumor malignancy (109). Silencing circRNA_000543 improved radiosensitivity in NPC, impeding proliferation and invasion, while promoting apoptosis (99). On the contrary, circ_000128 showed low expression in NSCLC cells and tissues. Circ_0001287 overexpression could up-regulate PTEN to repress the multiplication, metastasis, and radioresistance of NSCLC cells through endogenous competition with miR-21 (107). 2. In addition to directly interfering with circRNAs, focusing on the targets or pathways is another idea of improving radiotherapy. It has been demonstrated that circABCB10 and circPITX1 participated in radiation response *via* glycolysis. Hence, the glycolytic inhibitor 2-deoxy-D-glucose (2-DG) constrains radiation resistance caused by these two circRNAs. Application of the autophagy inhibitor chloroquine reversed the differential expression of LC3II/I and p62 in LoVo/R cells, and the radiotherapy sensitization ratio was higher than before. It also antagonized the autophagy of circBANP in the colon cancer RT. CircRNA_000543 could regulate the

TABLE 1 | The dysregulated circRNAs in radiotherapy of malignant tumors.

System	Cancer	Type of circRNAs	Dysregulation (after RT)	Target/pathway	Functions	Impact on RT	Ref
Respiratory system	NPC	circRNA_000543	up (compare to radiosensitive)	miR-9/PDGFRB	apoptosis, invasion, proliferation	radioresistant	(99)
		exosomal circMYC	up (compare to radiosensitive)	miR-20b-5p (predict) let-7e-3p (predict)	proliferation	radioresistant	(105)
		circRNA_0000285	up (compare to radiosensitive)	–	–	radioresistant	(100)
		circRNA_001387	up	–	–	radioresistant	(106)
		circFIP1L1	down	miR-1253/SFN	cell cycle	radiosensitivity	(71)
		circRNA_102115	up	miR-335-3p/MAPK1	cancer stem cell	radioresistant	(84)
	HPSCC	circCUX1	up (compare to radiosensitive)	Caspase1	inflammatory factors	radioresistant	(87)
	NSCLC	circ_0086720	up	miR-375/SPIN1	cell apoptosis, DNA damage repair	radioresistant	(54)
		circMTDH.4	up (compare to normal)	miR-630/AEG-1	proliferation, migration, invasion, apoptosis	radioresistant	(79)
		circ_0001287	down	miR-21/PTEN	proliferation, migration	radiosensitivity	(107)
		circPVT1	up	miR-1208 PI3K/AKT/mTOR	apoptosis	radioresistant	(60)
Digestive system	OSCC	circATRNL1	down	miR23a-3p/PTEN	apoptosis, cell cycle	radiosensitivity	(62)
	Esophageal cancer	circPRKCI	up (compare to normal)	miR-186-5p/PARP9	cell cycle, colony formation, cell viability	radioresistant	(74)
		circVRK1	down	miR-624-3p/PTEN/ PI3K/AKT pathway	apoptosis	radiosensitivity	(61)
		hsa_circ_0000554	up	miR-485-5p/Fermt1	proliferation, migration, invasion, apoptosis	radioresistant	(108)
		circRNA_100367	up	miR-217/wnt3	proliferation, migration	radioresistant	(78)
	Liver cancer	circRNA ZNF292	up (under hypoxia)	SOX9 protein Wnt/β-catenin	proliferation, angiogenesis	radioresistant	(77)
		circTUBD1	up	miR-146a-5p TLR4 Pathway	inflammatory factors	radioresistant	(88)

(Continued)

TABLE 1 | Continued

System	Cancer	Type of circRNAs	Dysregulation (after RT)	Target/pathway	Functions	Impact on RT	Ref
	Colorectal cancer	exosomal circ_0067835	up	miR-296-5p/IGF1R	proliferation, apoptosis, cell cycle	radioresistant	(96)
		circRNA CBL.11	up	miR-6778-5p/YWHAE P53 signaling pathway	proliferation, apoptosis	radioresistant	(58)
		circ_0055625	up	miR-338-3p/MSI1	proliferation, migration, invasion, apoptosis	radioresistant	(109)
		hsa_circ_0001313	up	miR-338-3p	cell viability, colony formation	radioresistant	(110)
		circBANP	up	miR-338-3p	autophagy	radioresistant	(66)
Urinary system	Prostate Cancer	circ_0062020	down	miR-615-5p/TRIP13	proliferation, metastasis, apoptosis, colony formation	radioresistant	(103)
		circZEB1	up	TR4-mediated QKI/miR-141-3p/ZEB1	DNA damage repair	radioresistant	(51)
		circ_CCNB2	up (compare to normal)	miR-30b-5p/KIF18A	autophagy	radioresistant	(68)
Central nervous system	Glioma	circCPA4	up (compare to normal)	miR-760/MEF2D	proliferation, apoptosis, migration, invasion	radioresistant	(111)
		circ_VCAN	down	miR-1183	proliferation, migration, invasion, apoptosis	radioresistant	(102)
		circPITX1	up (compare to normal)	miR-329-3p/NEK2	glycolysis	radioresistant	(82)
		circular AKT3	down (compare to normal)	AKT3-174aa/PDK-1 PI3K/AKT signal	proliferation, apoptosis	radiosensitivity	(55)
		circ-METRN	up (in low-dose radiation)	miR-4709-3p/GRB14/PDGFRα	DNA damage repair	radioresistant	(53)
Endocrine system	Cervical cancer	hsa_circ_0009035	up (compare to radiosensitive)	miR-889-3p/HOXB7	proliferation, apoptosis, migration, cell cycle	radioresistant	(101)
	Pancreatic cancer	circ_0002130	up	miR_4482-3p/NBN	DNA damage repair	radioresistant	(92)
	Breast cancer	circABCB10	up (compare to normal)	miR-223-3p/PFN2 axis	glycolysis	radioresistant	(81)

radioresistance of NPC cells by targeting the miR-9/platelet-derived growth factor receptor B(PDGFR)axis. The PDFGRB inhibitor imatinib sensitized radioresistance in NPC cells (99). 3. Some well-known bioactive compounds can enhance radiosensitivity by modulating circRNAs. Curcumin, a traditional Chinese medicine herb, has been widely studied in the field of cancer treatment in recent years. Zhu et al. discovered curcumin restored the increased expression of circRNA_102115 in NPC cells after irradiation. Adding curcumin during radiation could recover radiosensitivity through the circRNA_102115/miR-335-3p/MAPK1 axis (84).

Numerous experiments *in vivo*/vitro have shed light on the interference or overexpression of circRNAs that can antagonize tumor progression. Hence, a series of targeting circRNAs techniques as therapeutic approaches may have clinical value. The loss-of-function strategy of circRNAs includes CRISPR/Cas9 or CRISPR/Cas13-mediated knockdown, and the RNA interference (RNAi) mechanism based on short interfering RNA (siRNA) or short hairpin RNA (shRNA). In addition to lentiviral or adenoviral vectors, chemical synthesis and purification are used to overexpress circRNAs. The delivery of target circRNAs *in vivo* focuses on exocrine and nanoparticles (112). For instance, in a mouse model of nonalcoholic steatosis, the author injected high-fat diet mice with nanoparticles encapsulated circRNA SCAR, which notably alleviated symptoms of liver cirrhosis (113). Recently, some scientists synthesized novel tools named small circular interfering RNAs (sciRNAs) which are sense strands functionalized with GalNAc

ligand then annealed into antisense strands after chemical modification. The effect of sciRNAs *in vivo/vitro* is equivalent to that of clinically used siRNA (114). However, the targeted therapy of circRNAs is still in its infancy. The safety and efficacy of circRNA-based therapeutics are yet to be ensured.

CircRNAs Are Used to Predict the Effect of Radiotherapy

Predicting tumor response to radiotherapy, recurrence, and prognosis are necessary to improve the cure rate of patients. In order to study the clinical value of circRNAs in predicting NPC radiotherapy, Shuai detected the expression of circRNA_001387 in RT patients and cell lines. The patients who were tolerant to RT had higher expression of circRNA_001387 than those who were sensitive to radiotherapy. Surprisingly, the expression of circRNA_001387 in cells was apparently increased with the frequency of irradiation enforced (106). Highly expressed exosomal circMYC is related to clinicopathological parameters in NPC, such as survival rate and recurrence. Gain-functional experiments indicated that overexpression of circMYC reduced radiosensitivity, suggesting that circMYC might afford vital characteristics as a target for radiotherapy efficacy (105), to some extent. Circ_0062020 functioned as a competing endogenous RNA to restrain the radiosensitivity by modulating miR-615-5p/TRIP13 axis in prostate cancer (PCa) cells. The upregulation of circ_0062020 was detected in PCa tissues including radiosensitive and radioresistant tissues in contrast to adjacent normal tissues, especially in radioresistant tissues (103). Chen et al. found circRNA_000543 expressed at high levels in radioresistant NPC compared to radiosensitive samples (99). CircRNAs isolated from extracellular vesicles were markedly diverse between radioresistant glioma cells and the control group, of which RNA–sequencing and bioinformatics analyzed 63 upregulated circRNAs and 48 downregulation (94). It is becoming increasingly difficult to ignore the significant roles of circRNAs play in radiotherapy. Therefore, if further validated in larger-scale clinical research, circRNAs are expected to be complementary to traditional clinical assessment indicators.

CONCLUSIONS AND FUTURE PERSPECTIVES

With the development of bioinformatics and experimental techniques, the characteristic and functions of circRNAs have been widely elaborated. Particularly in the field of cancer, these remarkable new molecules exert their unparalleled status in all aspects of tumor biology. To data, only a few studies have been carried out on the actions of circRNAs in RT responses. In this article, we summarized the different underlying mechanisms involved in the multiple aspects of cellular response to IR. CircRNAs are a double-edged sword in radioresistant tumors, as they can not only promote radioresistance but also inhibit radioresistance. Functional experiments have demonstrated that regulation of circRNAs can obviously benefit radiotherapy, implying the great potential of targeting circRNAs. Research on the regulatory mechanisms of circRNAs in tumor radiation therapy will be the future research trend.

At present, the research on the role of circRNAs in tumor radiotherapy is still in its infancy, and plenty of aspects regarding their regulatory mechanisms remain undefined. Currently, the mainstream research on radiotherapy-related circRNAs is still focused on ceRNAs, and mechanisms to determine other models are strongly required. In this review, it was evident that most circRNAs acted as miRNA sponges to activate or inhibit signal

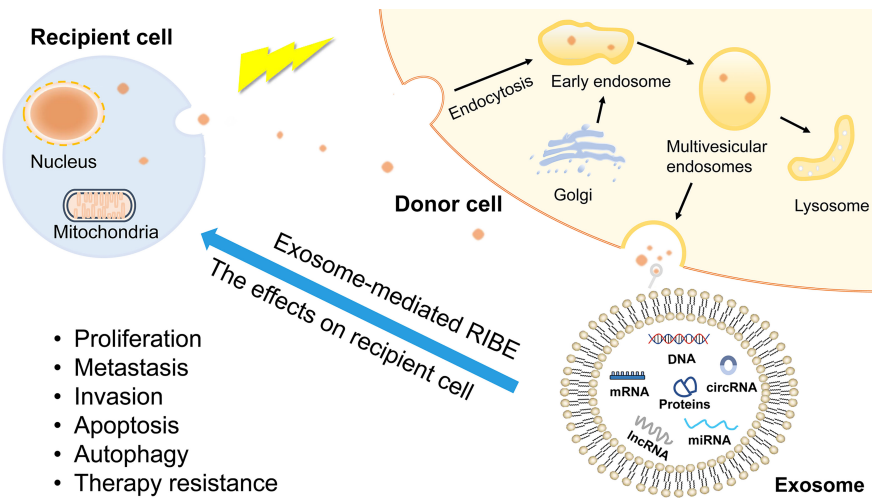

FIGURE 4 | The production of exosomes and exosome-mediated RIBE. Exosomes are derived from endolysosomal microparticles and are released by fusion with MVEs. The contents of exosomes include proteins, nucleic acids, growth factor, lipids, non-coding RNAs. RIBE is mediated by gap junction or substances released into extracellular environment. As bystander effect mediators, exosomes induce recipient cells present the same outcomes as directly exposed cells, such as proliferation, metastasis, therapy resistance.

pathways, except for circCUX1 (87) and circRNA ZNF292 (77), which functioned as regulatory proteins, and circular AKT3 (55), which played translational functions. We believe that further research can focus more on other molecular mechanisms, such as post-transcriptional modifications and translation machinery. A thorough understanding of the molecular mechanism of circRNAs will help to identify novel and effective diagnostic and therapeutic targets.

In addition to the direct effects of radiation on irradiated cells, studies have found that non-irradiated cells are also indirectly affected by radiation, a process known as radiation-induced bystander effect (RIBE) (115). RIBE is a major factor in determining the success of radiotherapy, not only because of the damage to irradiated cells, but also because it can induce cancer cells to become resistant to radiotherapy (116). Exosomes are important mediators in the bystander effect (**Figure 4**). It has been reported that miRNAs in exosomes can alter radiotherapy efficacy through RIBE (117, 118). To the best of our knowledge, circRNAs research on the benefits of RIBE has not yet been reported so far. Therefore, we infer that circRNAs can also induce RIBE through exosomes. Further research may help to discover the association between circRNAs and RIBE, and establish early interventions against RIBE to improve the efficacy of radiotherapy.

As of now, no circRNA has really been applied in clinical practice. Targeting circRNAs as a therapeutic strategy remains obstacles and bewilderments. Meanwhile, the studies of circRNAs in radiotherapy are limited to only a few solid tumors. To date, there have been no reports on the RT of hematological malignancies. The potential clinical value of circRNAs in tumor progression and drug resistance has been highlighted. Expanding the research field of circRNAs will undoubtedly broaden our horizon. These deficiencies will continue to be overcome in the future, and the value of circRNAs as biomarkers in radiotherapy is worthy of attention.

AUTHOR CONTRIBUTIONS

BW and HY designed the review. XW and JW collected the literature and wrote the manuscript. LW and WY revised the manuscript. All authors read, reviewed, and approved the final manuscript.

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Extracellular Vesicles and the Inflammasome: An Intricate Network Sustaining Chemoresistance

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Extracellular vesicles (EVs) are membrane enclosed spherical particles devoted to intercellular communication. Cancer-derived EVs (Ca-EVs) are deeply involved in tumor microenvironment remodeling, modifying the inflammatory phenotype of cancerous and non-cancerous residing cells. Inflammation plays a pivotal role in initiation, development, and progression of many types of malignancies. The key feature of cancer-related inflammation is the production of cytokines that incessantly modify of the surrounding environment. Interleukin-1β (IL-1β) is one of the most powerful cytokines, influencing all the initiation-to-progression stages of many types of cancers and represents an emerging critical contributor to chemoresistance. IL-1β production strictly depends on the activation of inflammasome, a cytoplasmic molecular platform sensing exogenous and endogenous danger signals. It has been recently shown that Ca-EVs can activate the inflammasome cascade and IL-1β production in tumor microenvironment-residing cells. Since inflammasome dysregulation has been established as crucial regulator in inflammation-associated tumorigenesis and chemoresistance, it is conceivable that the use of inflammasome-inhibiting drugs may be employed as adjuvant chemotherapy to counteract chemoresistance. This review focuses on the role of cancer-derived EVs in tuning tumor microenvironment unveiling the intricate network between inflammasome and chemoresistance.

Keywords: extracellular vesicles, inflammasome, tumor microenvironment, IL-1β, chemoresistance

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INTRODUCTION

In the complexity of cancer progression, the intricate interplay between inflammation and tumor microenvironment (TME), depicts an extraordinary multifaceted scenario in the development of acquired drug resistance and in the clinical outcome of malignant processes (1, 2). Extracellular vesicles (EVs), in particular cancer-derived (Ca-EVs), represent signal transducer or messengers in cell-cell communication (3–5), responsible for the continuous modification of TME (6, 7). TME includes cancerous and non-cancerous cellular components such as fibroblasts, stromal, immune, and endothelial cells. The cross-talk between TME components can induce a dysregulated inflammatory and immune response (1, 2). Inflammation, indeed, plays a pivotal role in tumor initiation, by dynamically and incessantly modifying TME *via* the release of cytokines and soluble mediators generating a "vicious cycle". This, in turn, endorses oncogenic plasticity toward immune-suppression, more aggressive

phenotype and reduction of therapeutic efficacy. One of the main mechanisms contributing to inflammation is mediated by cytoplasmatic complexes known as inflammasomes. Inflammasomes are activated by endogenous/exogenous danger signals and changes in cytoplasm homeostasis. Upon activation, inflammasomes act as "signal integrators" by the release of inflammasome-effectors cytokines. Inflammasomes are pivotal hubs of innate immunity and modulate immune/inflammatory responses by cross-talking with different cellular components. Inflammasome inappropriate activation, creating a pro-inflammatory TME and suppressing local immunity, appears as an emerging player in all the initiation to progression stages of cancer (8–11). Crucial novel modulators of inflammasome are EVs that, on the basis of the different nature of their cellular source, positively or negatively affect inflammasome cascade in diverse cancerous and non-cancerous recipient cells (12–14). In this scenario, EVs, with their Janus face behavior, strongly contribute to the immune/inflammation-associated modification of TME, and play a critical role in tumorigenesis and chemoresistance.

EVs: ANOTHER BRICK IN THE WALL

EVs are a heterogeneous group of membrane enwrapped spherical particles, produced by nearly all types of cells. There are no unique markers able to classify EVs on the bases on their biogenesis (ectosomes, exosomes, apoptotic bodies), for this reason the MISEV2018 guidelines suggest classifying EVs based on physical parameters, such as size (small and medium/large EVs) density or biochemical composition (15–17). EVs, found in body fluids and in cell culture media, carry various biomolecules, including proteins, lipids, metabolites, RNA, and DNA (16, 18). Upon interaction with target cell, EVs deeply impact cellular recipient cells responses, highlighting the pivotal role of EVs as signal transducers or messengers in cell-cell communication at close or distant sites. Intercellular communication is a key feature of tumor progression and metastasis. Cancer cells can release EVs that enter the circulation and reach distant organs, where they can generate favorable environmental conditions, enabling the outgrowth of disseminated tumor cells. This process, known as pre-metastatic niche formation, requires a series of predefined steps involving induction of vascular leakiness, alteration of stromal components and immune-escape (19, 20).

Cancer-derived EVs (Ca-EVs) ability to suppress immune anti-tumor activity, is guaranteed by the exchange of EVs between cancerous and non-cancerous TME-residing cells, and by the secretion of immune-modulating molecules (14, 21). Furthermore, the "exosome-immune suppression" and the Ca-EVs-mediated transfer of oncogenes or oncometabolites from one cell to other is also involved in the unrestrained cell proliferation and, subsequently, in the metastatic spread (14). On the other hand, Ca-EVs, may also carry tumor-associated antigens, damage associated molecular pattern (DAMPs), and immune-stimulating molecules, that can induce an immune anti-tumor response (22, 23) *via* the recruitment and activation of immune cells in TME (24, 25). Although the pro-inflammatory and the immune-suppressive role of Ca-EVs seem to be contrasting, pro-inflammatory EVs may still contribute to TME maintenance (26, 27).

INTERLEUKIN-1β AND CHEMORESISTANCE: AN OLD CYTOKINE WITH A NOVEL ROLE

Interleukin-1β (IL-1β) is one of the most abundant and influential cytokines of TME. IL-1β expression and secretion are induced by different stimuli such as toll-like receptors (TLRs) ligands, tumor necrosis factor-α or IL-1β itself. IL-1β production/secretion are fine-tune controlled by a two-steps transcriptional and post-translational regulation, requiring the activation of both nuclear factor kappa B (NF-κB) and nucleotide-binding oligomerization domain (NOD)-like receptor pyrin domain-containing 3 (NLRP3) inflammasome-caspase-1 platform. NF-κB activation by inflammatory stimuli induces biologically inactive pro-IL-1β production which must be proteolytically cleaved, by inflammasome-activated caspase-1 (28). Tumor cells can directly produce IL-1β or can "instruct" cells within TME, such as stromal ones, to secrete it (26, 29). An uncontrolled increase in IL-1β release exerts immune-suppressive effects and influences all the initiation-to-progression stages of many types of cancers and represents an emerging critical contributor to chemoresistance (30, 31). Depending on tumor cell types, several "*in vitro*" and "*in vivo*" models highlighted multiple mechanisms for IL-1β promoted chemoresistance. In pleural mesothelioma, the IL-8/IL-1β signaling controls chemoresistance by inducing the overexpression of ATP-binding cassette transporter (ABC) G2, that determines resistance to cisplatin and pemetrexed (32). Prostate carcinoma cells engage bone marrow adipocytes in a functional cyclooxygenase-2 (COX-2)-dependent cross-talk that promotes IL-1β expression, leading to docetaxel resistance (33). IL-1β can also induce a reinforcement of NF-κB signaling. In fact, IL-1β induces a sustained NF-κB that has been related to chemoresistance in ovarian carcinoma (34), in acute myeloid leukaemia (35) and in renal cell carcinoma (36). In pancreatic cancer, IL-1β confers chemoresistance not only by activating NF-κB (37), but also by up-regulating COX-2 (38), an enzyme linked to chemoresistance also in cervical carcinoma (39) and in colon cancer cell lines (40). In bladder cancer, cisplatin-resistance has been linked to IL-1β-induced increase in aldo-keto reductase 1C1 levels (41). In breast cancer, IL-1β-induced chemoresistance has been attributed to several mechanisms including: methylation of the estrogen receptor α, which increases tamoxifen resistance (42); activation of β-catenin signaling, which increases cisplatin resistance (43); and induction of epithelial to mesenchymal transition (EMT), which increases doxorubicin resistance (44). In melanoma cells, ABCB5 controls IL-1β/IL-8 signaling (45) which, in turn, influences chemoresistance by activating Smad/DNA binding protein 1 signaling (46).

Considering the implication of IL-1β in influencing all the initiation-to-progression stages of many tumors and chemoresistance, this cytokine is considered a promising therapeutic target for many types of cancers (30).

THE INFLAMMASOME: A DOUBLE-EDGE SWORD

As already mentioned, inflammasome activation is the mandatory event for IL-1β maturation and secretion. Inflammasomes are cytoplasmic molecular platforms devoted to detecting pathogen associated molecular patterns (PAMPs) and DAMPs, playing a key role in innate immunity (47). The inflammasome platform is composed by a danger sensor receptor, an adaptor protein (Apoptosis-associated speck-like protein containing a CARD, ASC), and an effector enzyme (caspase-1). The receptor family includes the nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) family, composed of at least 22 members, the most characterized of which is NLRP3 (47). Upon activation, NLRP3 oligomerizes and assembles into a multimeric platform including a core unit comprehending ASC and the effector pro-caspase-1. The oligomerization of inflammasome components culminates in the autocatalytic activation of caspase-1, responsible for IL-1β and IL-18 maturation (47–49). Inflammasome activation may also induce the processing of gasdermin-D (GSDMD), leading to pyroptosis, an inflammatory form of cell death (50). The physical interaction among inflammasome components is mediated by the adaptor protein ASC which holds a pyrin (PYD) and a CARD domain which, assembling into a speck, consents the connection between NLRP3 and caspase-1. NLRP3 possesses three domains: an N-terminal effector PYD, involved in ASC recruitment *via* PYD-PYD interaction, a central NACHT domain carrying an ATPase activity essential for NLRP3 activation and platform assembly, and a C-terminal leucine-rich repeats domain, possibly involved in auto-regulation, protein-protein interaction, and signal sensing (51). Inflammasome-platform assembly is also regulated by the phosphorylation of Ser-295 of NLRP3. This post-translational modification, accomplished by several protein kinases (PKs) including PKA, PKD and PKG (52, 53), impedes inflammasome platform assembly. Because component assembly is mandatory for inflammasome activation, it represents an attractive target for the development of selective NLRP3 inhibitors, as discussed later. NLRP3 involvement in cancer is currently a very debated topic. NLRP3 and NLRP3-associated pyroptosis have been defined "a double-edge sword" (54) on the basis of their capability to achieve both an anti-tumorigenic and a pro-tumorigenic activity in different types of malignancies (9–11, 54, 55). The contrasting roles of NLRP3 inflammasome can be due to multiple factors such as type, heterogeneity and stage of cancer cells, or TME characteristics (8–11). Metabolites, cytokines and EVs released by TME residing cells, represent the key drivers of NLRP3 hyper-activation. NLRP3 dysregulated activation can induce a chronic inflammatory environment that boosts tumor progression and extinguishes local immunity (9).

Recently the uncontrolled inflammasome activation has also been associated to chemoresistance. In oral squamous carcinoma NLRP3 activation promotes 5-Fluorouracil resistance "*in vitro*" and "*in vivo*" (56); and NLRP3 inflammasome has been detected in cisplatin-resistant lung cancer cell lines (57). Conversely, NLRP3-induced pyroptosis, sensitizes gastric and epatocellular carcinoma to cisplatin (58, 59).

NLRP3 inflammasome has been also linked to cardiotoxicity of anticancer agents. The inhibition of NLRP3, as well as of the oligomerization of the myeloid differentiation primary response gene 88 (MyD88), reduces the cardiotoxicity and increases the anticancer properties of sunitinib, in renal cancer-bearing mice (60). Myd88 is molecular platform which oligomerization and assembly induces NF-κB activation and the release of cytokine and factors involved in cancer cell survival and chemoresistance (60). Pharmacological reduction of NLRP3 activity has been suggested as a tool to alleviate doxorubicin-induced cardiotoxicity while preserving or even improving its anti-cancer activity (61). On the other hand, pyroptosis-associated cytokines can induce either an evasion of immune surveillance, or an effective immune response (62).

EVs AND INFLAMMASOME: TWO PIECES OF THE SAME PUZZLE

Increasing evidence highlights the pivotal role of Ca-EVs on NLRP3 activation in different types of cancers (summarized in **Table 1**). Prostate cancer derived-EVs, by inducing NLRP3 activation and IL-1β maturation, modify the inflammatory response of ME residing cells in a tumor-promoting fashion (12). Furthermore, prostate cancer tumor progression is characterized by increased inflammasome activation (62). Lung cancer-derived EVs induce NLRP3 activation in macrophages, thus providing a positive feedback loop to promote cancer progression *via* IL-1β secretion in mice (63, 64). EVs released by primary cultures of human glioblastoma, up-regulate microglial inflammasome signaling and influence both microglial cells polarization and glioma-microglia crosstalk (65). Furthermore, EVs derived from colon adenocarcinoma cells mediate radiation-induced antitumor immunity by inducing NLRP3 activation in mice (66).

The role of EVs in immune-escape and immune-stimulation also relies on their ability to modulate inflammasome cascade positively or negatively and IL-1β production in recipient cells (12–14) (summarized in **Table 1**). This different effect is both related to the nature of the EVs-releasing and -receiving cells and to the different EVs mechanisms of action (**Figure 1**). In fact, on the one hand, Ca-EVs activate NLRP3 inflammasome platform in non-immune receiving cells *via* ERK1/2-mediated pathway (12), on the other hand non-cancerous cell-derived EVs negatively modulate NLRP3 inflammasome activation in immune cells (13). This latter effect is mediated by EVs intrinsic metabolic activity that, through adenosine production, induces the activation of the adenosine A2a receptor, a member of the purinergic P1 receptor family (13). This novel mechanism of action highlights the active role of EVs in microenvironment homeostasis, *via* the autonomous synthesis of metabolic products able to alter microenvironment composition and cell behavior (13). Furthermore, the involvement of A2a receptor in this EVs effect, offers a novel point of view on the roles of EVs/purinergic receptors on cancer immunology (12). In fact, up to date, only the connection between EVs/type P2 purinergic

TABLE 1 | Roles of EVs and drugs in inflammasome modulation.

	Role and mechanism of action	References
EVs-mediated inflammasome activation		
Prostate cancer-derived EVs (PCa-EVs)	PCa-EVs induce caspase-1/IL-1β activation *via* ERK1/2-mediated lysosomal destabilization and cathepsin B activation in non-cancerous PNT2 cells	(12)
Lung cancer-derived EVs (LCa-EVs)	LCa-EVs induce NLRP3-mediated IL-1β secretion in macrophages thus promoting lung cancer development	(63, 64)
Glioblastoma-derived EVs (GMB-EVs)	GMB-EVs induce inflammasome/IL-1β activation in microglial cells thus inducing microglial cells M1 polarization	(65)
Colon adenocarcinoma-derived EVs (CCa-EVs)	CCa-EVs induce AIM2 and NLRP3 activation, and prompt IL-1β-mediated anti-tumor effect during radiation in mice	(66)
EVs-mediated inflammasome inhibition		
Amniotic fluid stem cell-derived EVs (HASC-EVs)	HASC-EVs inhibit NLRP3/caspase-1 activation *via* an intrinsic metabolic activity leading to A2a purinergic receptor activation in THP1 cells	(13)
Embryonic stem cells-derived EVs (ES-EVs)	ES-EVs reduce doxorubicin-induced NLRP3/Caspase-1/IL-1β/IL-18/Pyroptosis activation in M1 macrophages thus converting pro-inflammatory M1 into anti-inflammatory M2 macrophages	(67)
Drug-mediated inflammasome inhibition		
OLT1177	OLT1177 blocks NLRP3 oligomerization and IL-1β secretion thus enhancing anti-tumor immunity and reducing tumor growth in melanoma cells	(68)
MCC950	MCC950 inhibits NLRP3 activation and reduces tumor growth of pancreatic cancer cells; head and neck squamous adenocarcinoma; and pituitary prolactinoma	(69–71)
Oridonin and its derivate	Oridonin and its derivate impede NLRP3 assembly and prevents liver colorectal cancer metastasis	(72–75)
MM01 and Xantone	MM01 and xantone prevent inflammasome activation interfering with ASC speck formation	(76, 77)
VX-765	VX-765 inhibits caspase-1 activation, thus preventing inflammasome activation and pyroptosis	(78, 79)
Ritonavir	Ritonavir blocks caspase-1 activation in pancreatic cancer	(80, 81)
Anakinra	Anakinra blocks the binding of IL-1 to its receptors. It is under clinical investigation for the treatment of metastatic cancers	(82)
Natriuretic Peptides (NPs)	NPs interfere with NLRP3 activation by the induction of NLRP3 phosphorylation that inhibits ASC oligomerization. NPs counteract inflammasome activation in prostate cancer cell lines	(53, 62, 83, 84)

receptors (P2Rs) and tumor-inflammatory signaling has been demonstrated (27). Only few reports demonstrate that the activation of P2Rs on immune cells induces the release of: (i) EVs containing IL-1β and IL-18, exerting a pro-inflammatory action, favor tumor progression at the expense of an effective immune response; (ii) EVs presenting P2Rs on their surfaces which activation, by extracellular ATP, can lead to the release of IL-1β, IL-18 and ATP itself (27). As discussed below, inflammasome and IL-1β dysregulation are crucial players in inflammation-associated tumorigenesis and chemoresistance. In this scenario, EVs by exerting an interaction-dependent effect on the receiving cells or by releasing immune-metabolites, can be considered novel crucial players in determining tumorigenesis and chemoresistance. The functional link between NLRP3 activation and EVs is further demonstrated by the finding that embryonic stem cell-derived EVs ameliorate the cardio-toxicity induced by the antineoplastic agent doxurobcin, by inhibiting NLRP3 signaling in mice (67). Nonetheless, further research is needed to increase the knowledge in this emerging research area.

INFLAMMASOME TARGETING DRUGS: POTENTIAL ANTI-CANCER THERAPEUTIC

Inflammation sustained by inflammasome activation has been implicated in the insurgence or progression of several human pathologies, including cancer. For this reason, several efforts have been made to identify potential effective inhibitors of inflammasome to be used as new anti-cancer therapeutics (summarized in **Table 1**). Each step leading to inflammasome activation, may represent a good candidate for therapeutic targeting.

Several small molecules and natural compounds have been identified as inhibitors of the interaction between NLRP3 inflammasome monomers. As examples MCC950 and OLT1177, block NLRP3 oligomerization by inhibiting ATP hydrolysis *via* the NACHT domain, which is pivotal for receptor oligomerization and anti-cancer effects (68). MC950 inhibits LPS-induced inflammasome activation in pancreatic cancer cell lines (69), delays cell growth in a mouse model of head and neck squamous cell carcinoma (70) and inhibits pituitary prolactinoma growth and prolactin expression/secretion in rats (71). Similarly, inhibition of NLRP3 by OLT1177 enhances antitumor immunity, thus reducing melanoma growth (68). Oridonin, a natural terpenoids found in traditional Chinese herbal medicine, impedes inflammasome assembly by forming covalent bond with NLRP3 Cys279 (72). Oridonin administration effectively prevents the formation of colorectal cancer liver metastasis (73) and improves oxaliplatin efficacy (74). Oridonin derivative, with potent anticancer effects, has been very recently synthesized (75).

ASC polymerization can be another target for broad-spectrum therapeutics. MM01 (under patent procedure: application number, 20382237.4-1109) is a small-molecule interfering with ASC speck formation (76). Xantone, used in the early twentieth century as an ovicide and larvicide (85) can

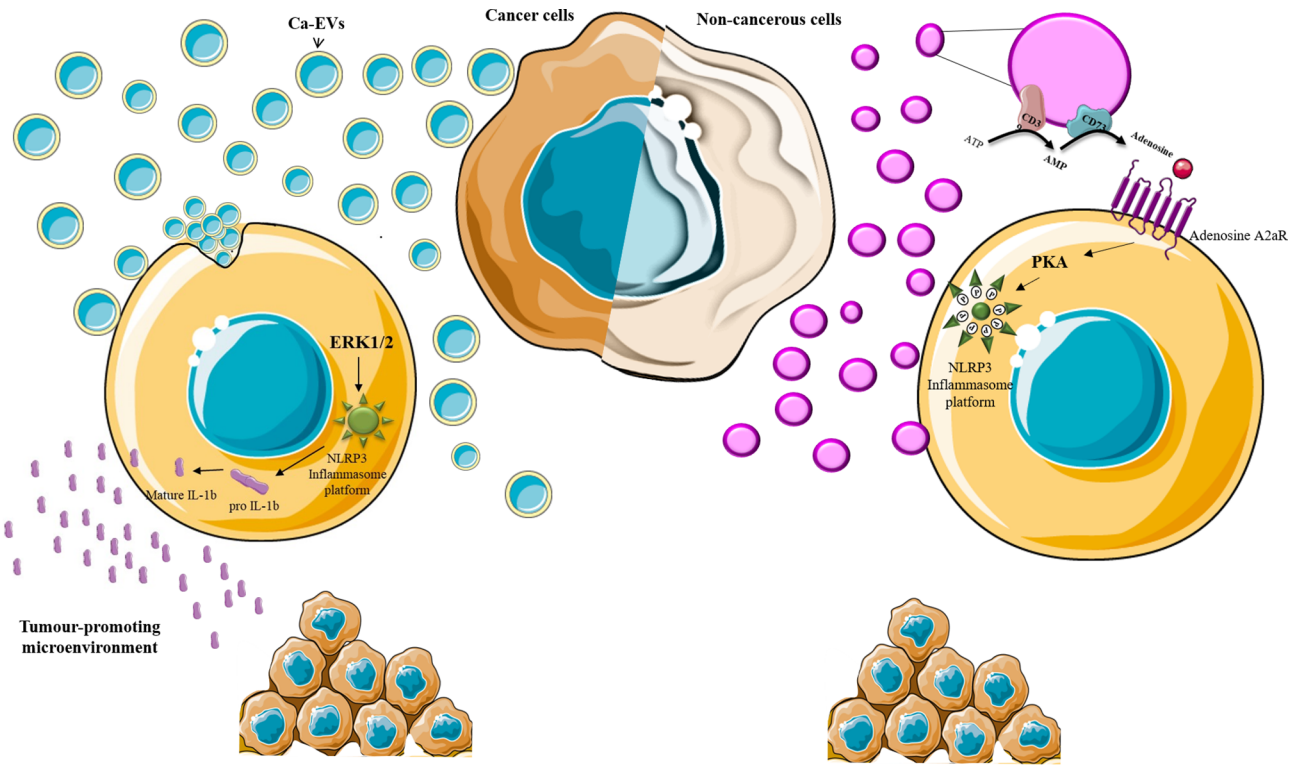

FIGURE 1 | EVs and inflammasome. Schematic representation of EVs-induced effect on inflammasome activation. Ca-EVs (light blue) are up-taken by non-cancerous cells and, *via* the induction of intracellular signaling pathways, including ERK1/2 MAPK, induce inflammasome platform assembly and the maturation of IL-1β which release affects the microenvironment in a tumor-promoting fashion. EVs released by non-cancerous cells violet may produce soluble factor (Adenosine) that, *via* receptor (adenosine A2a receptor) engagement on the target cell, activates protein kinases (PKA) which impedes inflammasome platform assembly through NLRP3 phosphorylation (P).

inhibit ASC speck formation without affecting inflammasome components expression (77). Although MM01 and xantone can be useful for the treatment of a broad range of diseases based on inflammasome dysregulation, they have not yet been tested on cancer models.

Caspase-1 activation can be targeted for impeding IL-1β maturation. Caspase-1 inhibition by the small-molecule VX-765 prevents pyroptosis in a multiple sclerosis model (78) and in monocytes and macrophages (79). Ritonavir, originally used as protease inhibitor for the treatment of HIV, effectively block caspase-1 (80), and induces apoptosis in pancreatic cancer (81). However, the quite unspecific actions of protease inhibitors should be taken into account to avoid deleterious side effects.

Specific monoclonal antibodies directed toward IL-1 receptor, including anakinra, rilonacept, canakinumab and gevokizumab have been developed to inhibit IL-1β signaling (82). Anakinra, a recombinant IL-1Ra, blocking the binding of IL-1 to IL-1 receptor, is under clinical investigation for the treatment of metastatic cancers (ClinicalTrials.gov Identifier: NCT00072111). An up-to date list of clinical trials involving IL-1 blockade has been recently published (86). Nevertheless, the blockade of IL-1 receptor, although displaying a favourable safety profile, caused a reduction in neutrophil counts with an overall increased risk for fatal infections.

Besides the possibility to inhibit inflammasome components, several strategies aimed to inhibit the pathways leading to inflammasome activation. Antioxidant compounds can inhibit ROS-mediated inflammasome platform assembly, P2X7 receptor antagonist can be used to impede K^+ efflux known to be involved in NLRP3 activation (86). A strategy, explored by our group, is the induction of NLRP3 phosphorylation. We have indeed showed that, natriuretic peptides (NPs), by binding to NPs Receptor-1, can induce an increase in cGMP levels which culminates in the activation of PKG (53, 83, 84). Moreover, we showed that EVs, isolated from amniotic fluid-derived stem cells can activate PKA *via* A2a adenosine receptor in immune cells (13). Both PKA and PKG can phosphorylate NLRP3 at Ser295, thus leading to the inhibition of inflammasome assembly and IL-1β secretion (13, 53). Furthermore, NPs are able to counteract both the constitutive and EVs-induced NLRP3 activation in cancerous and non-cancerous prostate cells (62), supporting the critical role of these molecules in prostate cancer (87). Based on the fact that NPs analogues are already in clinical use for cardiovascular diseases (88, 89) and of the growing interest toward the use of EVs as therapeutics (90), further studies are needed to better define the potential anti-cancer efficacy of NPs and EVs.

CONCLUSION

Chemoresistance represents a major challenge in the clinic. Cancer cells response to therapy is deeply influenced by immune/inflammation-associated TME modifications. Therefore, the management of TME-mediated resistance may deeply affect the efficacy of cancer therapies. The key players that trigger TME modifications are multiple and strictly interconnected *via* a complex network of cell-cell communication. Given the pivotal role of inflammasome and related cytokines in TME re-modeling, they represent promising therapeutic targets for the development of novel anticancer approaches aimed to re-educate TME toward a favorable inflammatory/immune anti-tumorigenic phenotype. EVs have been recently discovered as novel active contributors of inflammasome/IL-1β modulation. Further studies are needed to better define the potential anti-cancer therapeutic efficacy of inflammasome-modulating drugs as adjuvant chemotherapy to counteract chemoresistance in a multidrug approach.

AUTHOR CONTRIBUTIONS

LM, IB, and RR contributed to conception of the study and wrote the manuscript. VT supervised the study. All authors listed contributed to manuscript revision, read, and approved the submitted version.

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Exosomes Derived From Dendritic Cells Infected With *Toxoplasma gondii* Show Antitumoral Activity in a Mouse Model of Colorectal Cancer

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Pathogen-based cancer therapies have been widely studied. Parasites, such as *Toxoplasma gondii* have elicited great interest in cancer therapy. Considering safety in clinical applications, we tried to develop an exosome-based immunomodulator instead of a live parasite for tumor treatment. The exosomes, called DC-Me49-exo were isolated from culture supernatants of dendritic cells (DCs) infected with the Me49 strain of *T. gondii* and identified. We assessed the antitumoral effect of these exosomes in a mouse model of colorectal cancer (CRC). Results showed that the tumor growth was significantly inhibited after treatment with DC-Me49-exo. Proportion of polymorphonuclear granulocytic bone marrow-derived suppressor cells (G-MDSCs, $CD11b^+Ly6G^+$) and monocytic myeloid-derived suppressor cells (M-MDSCs, $CD11b^+Ly6C^+$) were decreased in the DC-Me49-exo group compared with the control groups *in vitro* and *in vivo*. The proportion of DCs ($CD45^+CD11c^+$) increased significantly in the DC-Me49-exo group. Levels of interleukin-6 (IL-6) and granulocyte-macrophage colony-stimulating factor (GM-CSF) significantly decreased after treatment with DC-Me49-exo. Furthermore, we found that DC-Me49-exo regulated the lever of MDSC mainly by inhibiting the signal transducer and activator of transcription (STAT3) signaling pathway. These results indicated that exosomes derived from DCs infected with *T. gondii* could be used as part of a novel cancer therapeutic strategy by reducing the proportion of MDSCs.

Keywords: *Toxoplasma gondii*, exosomes, myeloid derived suppressor cells (MDSCs), dendritic cells (DCs), colorectal cancer (CRC), immunosuppression

INTRODUCTION

Many strategies have been used in colorectal-cancer (CRC) therapy. In recent years, cancer therapies based on pathogens (viruses, bacteria, and parasites) have elicited great interest. In 2015, the first oncolytic virus was approved for melanoma therapy by the U.S. Food and Drug Administration (FDA) (1). Bacteria-based cancer therapy has been approved in clinical trials by both the FDA and the

National Medical Products Administration (NMPA). Other team and other researchers have found that infection by parasites such as *Toxoplasma gondii* and *Plasmodium* can inhibit tumor growth *in vivo*. However, although parasitic infection can change the balance of tumor tolerance, live parasites in cancer treatment increase the risk of infection.

Exosomes are extracellular vesicles with lipid bilayer molecules. They can carry proteins, biologically active lipids, and RNA from donor cells to recipient cells, thereby establishing intercellular communication and changing the functions of the recipient cells (2). Exosomes from pathogen-infected host cells have an anti-infective effect. These exosomes carry an "infection" message to immune cells, triggering them to activate the immune response (3). Dendritic cell (DC)-derived exosomes display abundant major histocompatibility complex (MHC) class I/II molecules and T cell co-stimulatory molecules, meaning that such exosomes have potential antitumoral activity (4). Researchers have discovered that pathogen-infected macrophages carry pathogen-associated molecular patterns (PAMPs), which can activate the host's immune response mechanism (5). Exosomes secreted by immature DCs (DC-exo) produce an antitumoral immune response only when co-injected with mature DCs or chemical adjuvants (6). DC-exo can be used only in combination with chemotherapeutic drugs or specific immunotherapies to achieve better effect in clinical trials (7). Previous research has also showed that intra-tumoral injection of exosomes derived from the plasma of *Plasmodium*-infected mice significantly reduces the tumor growth in Lewis lung cancer (LLC) (8).

Myeloid-derived suppressor cells (MDSCs), which are immature myeloid cells derived from bone marrow (BM), are one of the most important types of immunosuppressive cells, and can inhibit immune cell responses (9–11). Removing MDSCs from the tumor microenvironment (TME) in patients can improve the host immune system's ability to attack tumors and improve the effect of immunotherapy. Research has confirmed that *Plasmodium* infection inhibits the expansion and activation of MDSCs in a murine LLC model (12). A live, non-replicating, non-toxic *T. gondii* uracil-deficient vaccine strain (cps) reverses tumor-induced immunosuppression and promotes the M1 macrophage phenotype by activating immune cell such as DCs to suppress the role of MDSCs, thereby causing the inhibition of tumors (13). Therefore, we attempt to introduce the mechanism that activates host cell immunity by *T. gondii* to stimulate antitumoral immunity, as well as to investigate the potential of this strategy in tumor immunotherapy.

In our previous study, the DCs-derived exosomes "edited" by *T. gondii* had good cell compatibility and could interfere with immunosuppression caused by tumors. In the current study, we hypothesized that exosomes derived from DCs infected with Me49 strain of *T. gondii* could inhibit the level of MDSCs in a mouse model of CRC to achieve tumor suppressive effects. To develop an exosome-based immunomodulator instead of live parasites for tumor therapy, we evaluated antitumoral activity of exosomes isolated from DCs infected with the Me49 strain of *T. gondii* in a mouse model of CRC.

MATERIALS AND METHODS

Ethical Approvals

All animal experiments were approved by the Institutional Animal Care and Use Committee of the Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences (CAAS), Shanghai, China (IACUC approve number SHVRI–SZ–20200421-01).

Sources of Cells, Parasites and Mouse

We purchased cell line of murine colorectal carcinoma (CT26 labeled with luciferase) from the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). The cell culture medium used in this experiment was Roswell Park Memorial Institute (RPMI) 1640 supplemented with 1% penicillin-streptomycin solution and 10% fetal bovine serum (FBS), all purchased from Gibco (Thermo Fisher Scientific, Waltham, MA, USA) in a humidified atmosphere of 5% CO_2 at 37°C.

The Me49 strain of *T. gondii* was preserved at the Key Laboratory of Animal Parasitology of Ministry of Agriculture (China), Laboratory of Quality and Safety Risk Assessment for Animal Products on Biohazards of Ministry of Agriculture, China.

We obtained 6-week-old female BALB/c mice from Shanghai SPF Biotechnology Co., Ltd (Beijing, China) and kept them at the SPF Experimental Animal Center of Shanghai Veterinary Research Institute, CAAS. Animals were housed in cages at 21 ± 1°C and 50–60% humidity, on 12 h-light-dark cycles, with enrichment items located in ventilated racks.

Animal Experimental Model

We randomly divided the mice into two groups (n = 5 per group). There were no significant differences in body weight between groups. Mice were immunized with inactivated *T. gondii* Me49 strain. Then mice were subcutaneously injected in the axillary area with 5×10^6 CT26 cell suspension.

T. gondii Treatment of Mice With Colorectal Cancer

After immunizing mouse with *T. gondii* Me49 strain, mice were injected with 5×10^6 CT26 cells. Seven days later, 100 tachyzoites of *T. gondii* Me49 strain were infected in CT26+Me49 group. The control group (CT26) was injected with PBS.

qPCR Detection of *T. gondii* in Different Tissues

We performed quantitative polymerase chain reaction (qPCR) amplification of the 529-bp target gene in *T. gondii* as described below. DNA was extracted from different tissues of mice with CRC using a DNA Mini Kit (Qiagen, Hilden, Germany). We performed qPCR on QuantStudio5 PCR system (Applied Biosystems, Foster City, CA, USA). *T. gondii* from different tissues was detected by qPCR assay using the primers (forward primer: 5'-GCTCGCCTGTGCTTGGAG-3', reverse primer: 5'-ATCTTCTCCCTCTC CGACTCTC-3') and probe (probe sequence: 5'-TCGCTTCCCAACCACGCCACCC-3'). Briefly,

the 20 μL reaction mixture contained 10 μL premix, 0.8 μL forward primer, 0.8 μL reverse primer, 0.2 μL probe, 0.2 μL Rox Reference Dye II (Tokyo, Shiga, Japan), 6 μL ddH2O, and 2 μL template of DNA. The major steps of qPCR included denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15s, annealing at 60°C for 1min, and extension at 72°C for 45s. We prepared a standard curve and took measurements in triplicate for each sample.

Tumor Volume Measurement

Tumor volume = length × width^2/2, where length represents the largest tumor diameter and width represents the perpendicular tumor diameter. Mice were categorized as dead for ethical reasons when the tumor volume exceeded 1500 mm^3.

Flow Cytometric Analysis

Mice with CRC treated with *T. gondii* or exosomes were sacrificed before the flow cytometry (FCM) analysis. Then, we separated the blood, spleen, and tumor tissues. T lymphocyte infiltration and the proportion of MDSCs were detected by FCM. After washing the tumor samples in RPMI 1640, they were cut into 1 mm^3 tissue pieces and digested with RPMI 1640, containing Dispase® II (1.5 U/mL, Sigma, USA), Collagenase D (1 mg/mL, No.11088858001; Roche, Basel, Switerland) and 0.2% DNase I (Roche, USA) at 37°C for 30 min according to the previous study (14) with some modifications. The digested material was passed through a mesh (70 μM) to remove clumps and the filtrate was washed twice and then centrifuged at 400 × *g* for 8 min at room temperature (RT) (14).

We used a lysed solution to lyse blood, after which we washed it with PBS and then centrifuged at 700 g for 5 min. To prepare the single-cell spleen suspension, the whole spleen was placed in a cell strainer and crushed. We next passed the cells through the 70 μM mesh to remove clumps and washed the filtrate twice, then centrifuged it at 400 g for 8 min at RT. Cell surfaces were stained in accordance with established methods (15). A total of 1×10^6 cells were incubated for 30 min at 4°C with different combinations of the following antibodies: (FITC)-CD3, (BV421)-CD4, (PE)-CD8, (APC)-CD45, (FITC)-CD11b, (PE)-Ly6G, (APC-A)-Ly6C, (FITC)-CD45, (APC)-CD11b, (PE)-Ly6G, (APC-700)-Ly6C, (PC5.5)-CD11c, and (PB450)-F4/80. All the antibodies were purchased from Becton, Dickinson (San Jose, CA, USA). Following two washes with 1 mL staining buffer, the cells were resuspended in 200 μL staining buffer for analysis on a CytoFLEX flow cytometry (Beckman Coulter Life Sciences, Brea, CA, USA).

Isolation of DC-Derived Exosomes

We obtained bone marrow-derived dendritic cells (BMDCs) from BM suspensions prepared from mouse femurs as described previously (16). Exosomes were purified from the supernatants of DCs infected with *T. gondii* by differential centrifugation as previously described (17). Briefly, we harvested supernatants of DCs infected with *T. gondii* from the DC-Me49-exo group, and those of control DCs from the DC-exo group. The different supernatants were centrifuged at 500 g for 10 min to remove cell debris and other small particles, and the supernatants was collected. Then, we centrifuged the supernatant at 16,500 *g* for 20 min, followed by filtration through a 0.22-μm filter (Millipore Sigma). Finally, supernatant solutions were ultracentrifuged at 120,000 *g* for 90 min, and the exosome pellet was resuspended with an appropriate amount of PBS. We measured the protein content of exosomes using a BCA protein assay kit (Thermo Fisher, USA). Exosomes were stored at −80°C for future use or directly used in co-culture experiments.

Characterization of Exosomes

We observed the size, morphology, and distribution of exosomes by transmission electron microscope (TEM). Diluted exosomes were fixed for 15 min in 2.5% formaldehyde/glutaraldehyde (Solarbio, Beijing, China), and 0.1 M sodium cacodylate buffer. The samples were then placed on a 300-mesh carbon coated mesh for air drying. We stained the samples for 4 min with a negatively filtered microporous aqueous solution of uranyl acetate, and then washed them twice with 50% methanol/water. After air drying, samples were observed under TEM with accelerating voltage of 80 kV and spot size of 2. We diluted purified exosomes in PBS (10000×) and subsequently used them for size measurement and analysis on a ZetaView nanoparticle-tracking analyzer (Particle Metrix, Inningam Ammersee, Germany) to determine concentration (particles/mL) and particle size (nm). Exosomes were mixed with loading buffer and heated at 100°C for 10 min. We then loaded exosome samples on 10% SDS-PAGE gel, transferred the samples to PVDF (Billerica, MA, USA), and incubated them overnight in blocking buffer (1× PBST, 5% milk). After five washes with washing buffer (PBST), membranes were incubated for 1 h with monoclonal antibodies of CD63, CD9, TSG101 (Cambridge, MA, USA) in a buffer containing PBST, and 1% milk. After washing them with washing buffer, we incubated membranes for 1 h with secondary antibodies and detected signals on a ChemiDoc Touch Imaging (Bio-Rad, USA).

Co-Culture Assays *In Vitro*

Mouse BM–derived MDSCs were obtained as previously described (18, 19). In brief, we flushed BM cells from the femurs and tibias of approximately 5-week-old BALB/c mice. Red blood cells (RBCs) were lysed with lysis buffer (Thermo Fisher). The BM cells were then cultured in RPMI-1640 supplemented with 10% FBS, 1% penicillin-streptomycin solution and stimulated with 40 ng/mL interleukin-6 (IL-6) and granulocyte-macrophage colony stimulating factor (GM-CSF) (Rocky Hill, NJ, USA) at 37°C for 4 d in a 5% CO_2-humidified atmosphere. Then, we detected unique M-MDSCs and G-MDSCs using (FITC)-CD45, (APC)-CD11b, (PE)-Ly6G, and (APC-700)-Ly6C. Uniquely DCs and macrophages were detected by (FITC)-CD45, (PC5.5)-CD11c, and (APC)-CD11b, (PB450)-F4/80. All the antibodies were purchased from Becton, Dickinson (San Jose, CA, USA). After induction and maturation, MDSCs were harvested. Then, MDSCs incubated with exosomes of DC-exo, DC-Me49-exo, and PBS, respectively. After 24 h, MDSC was collected for flow cytometry and Western blot. All cells were lysed using RIPA buffer (Beyotime Institute of Biotechnology, China) with 1 nM PMSF, and total

proteinconcentration was determined using a BCA protein quantification kit (Thermo Fisher). Next, we loaded 5 μg total proteins per lane and resolved them on 0.6–0.8% gels by SDS-PAGE. Proteins were transferred onto PVDF membranes and blocked for 1 h with 5% non-fat milk at RT, after which the membranes were washed three times with PBST. Membranes were incubated for 1 h with monoclonal antibodies of P-JAK2, JAK2, P-STAT3, STAT3 and tubulin in a buffer containing PBST and 1% milk. All primary antibodies were purchased from CST. After washing them with PBST, we incubated membranes for 1 h with goat anti-mouse IgG-HRP (Santa Cruz, CA, USA) and detected signals on a ChemiDoc Touch Imaging (Bio-Rad, USA).

Indirect Immunofluorescence

Exosomes were labeled by ExoSparker Exosome Membrane Labeling Kit-Green (Kumamoto, Japan), MDSCs were seeded into a 6-well plate at a density of 3×10^5 cells per well and 5 μg/mL exosomes from each of the different groups were added to the MDSC culture medium. Control wells contained cells but no exosomes. After incubation for 24 h, we washed cells three times with PBS and stained them with DAPI (Beyotime Biotechnology, China). All cells were sealed and imaged using Zeiss LSM 880 confocal microscopy.

Treatment of Colorectal Cancer-Bearing Mice With Exosomes

BALB/c female mice (6 weeks old) were weighed, randomly divided into three groups (n = 10 each group), and injected subcutaneously with 5×10^6 CT26 cells. When the tumor was visible, treatment was carried out according to the following design: an intra-tumoral injection with 10 μg DC-exo (DC-exo group), 10 μg DC-Me49-exo (DC-Me49-exo group), and 10 μL PBS (PBS group). Two injections were performed on day 1 and 3 after tumor visualization (8). At day 19 post inoculation, the tumor progression was monitored by using an IVIS Spectrum imaging system (IVIS Spectrum, USA). Mice were sacrificed, and blood, spleens and tumors were collected for flow-cytometry analysis. Cytokines in serum were determined by using IL-6 and GM-CSF ELISA kits (Neobioscience Technology Company).

Statistical Analysis

All data were analyzed using GraphPad Prism software (Version 8.0.2) and presented as mean ± standard deviation. The significant differences between experiment group and control group were analyzed using Student's *t*-test or one-way ANOVA with Dunnett's multiple comparison.

RESULTS

T. gondii Infection Reduced Mortality and MDSC Levels in Tumor-Bearing Mice

In order to determine the antitumoral activity of *T. gondii*, we established the mouse models of CRC. After being vaccinated three times with the heat-killed *T. gondii*, each mouse was inoculated with 5×10^6 CT26 cells. Seven days later, the mouse was infected with the Me49 strain of *T. gondii* in the CT26 + Me49 group, and the CT26 group was injected with PBS (**Figure 1A**). Mice in the CT26 + Me49 group had a 60% survival rate at day 35 versus 0% for the untreated group (**Figure 1B**).

$CD3^+CD4^+$ and $CD3^+CD8^+$ T cells were evaluated using CytoFLEX. We used an FACS strategy (**Supplementary Figure 1A**) to isolate these cells from different tissues of tumor-bearing mice. In peripheral blood, spleen and tumor tissues of the CT26 + Me49 group, as compared with the control group, the mean frequency of $CD4^+$ T cells was significantly reduced ($p < 0.0001$) (**Figure 1C**). In peripheral blood, no differences were found in the mean frequency of $CD3^+CD8^+$T cells between the CT26 group and the CT26 + Me49 group (**Figure 1D**). Compared with the CT26 group, the mean frequency of $CD3^+CD8^+$ T cells in both spleens and tumors was significantly increased in the CT26 + Me49 group ($p < 0.0001$) (**Figure 1D**). These data indicated that *T. gondii* infection could significantly increase the $CD3^+CD8^+$T cell infiltration into tumor tissue.

MDSCs demonstrate immune evasion, and promote tumor progression by inhibiting the proliferation and functions of T cells (20). In order to investigate whether infection with the Me49 strain of *T. gondii* could affect the level of MDSC, we prepared single-cell suspensions from the peripheral blood, spleen and tumor tissues, stained them with MDSCs-specific markers, and analyzed the cells using CytoFLEX. After $CD45^+CD11b^+$ gating (**Supplementary Figure 1B**), G-MDSCs and M-MDSCs were analyzed (n = 5/group). Compared with CT26 group, the G-MDSCs from peripheral blood ($p <0.0001$), spleens ($p < 0.001$), and tumors ($p < 0.01$) were significantly decreased in the CT26 + Me49 group (**Figure 1E**). Compared with CT26 group, percentage of M-MDSCs in peripheral blood ($p < 0.001$), spleens ($p < 0.05$) and tumors ($p < 0.05$) were also significantly reduced in the CT26 + Me49 group (**Figure 1F**). These data indicated that *T. gondii* infection decreased the proportions of G-MDSCs and M-MDSCs in tumor-bearing mice.

In order to investigate whether the Me49 strain of *T. gondii* could directly infect tumor cells *in vivo*, we detected *T. gondii* in different tissues in the CT26 + Me49 group. Results showed that *T. gondii* was detected in the spleen, lung, liver, and brain, but not in tumor tissues (**Supplementary Figure 2**). These results suggested that the increase in $CD3^+CD8^+$ T cells and decrease in $CD3^+CD4^+$ T cells and MDSC *in vivo* might be involved in the antitumoral response of *T. gondii* infection in the mouse model of CRC.

Characterization of Exosomes From Different Sources

Because direct infection with *T. gondii* for cancer therapy can increase the risk of infection, we developed an alternative exosome-based method for CRC treatment. We used ultracentrifugation to obtain exosomes from the culture supernatant of DCs or DCs infected with *T. gondii*. *Via* electron microcopy, we observed the exosomes derived from both kinds of DC to be cup-shaped, with a typical bilayer membrane

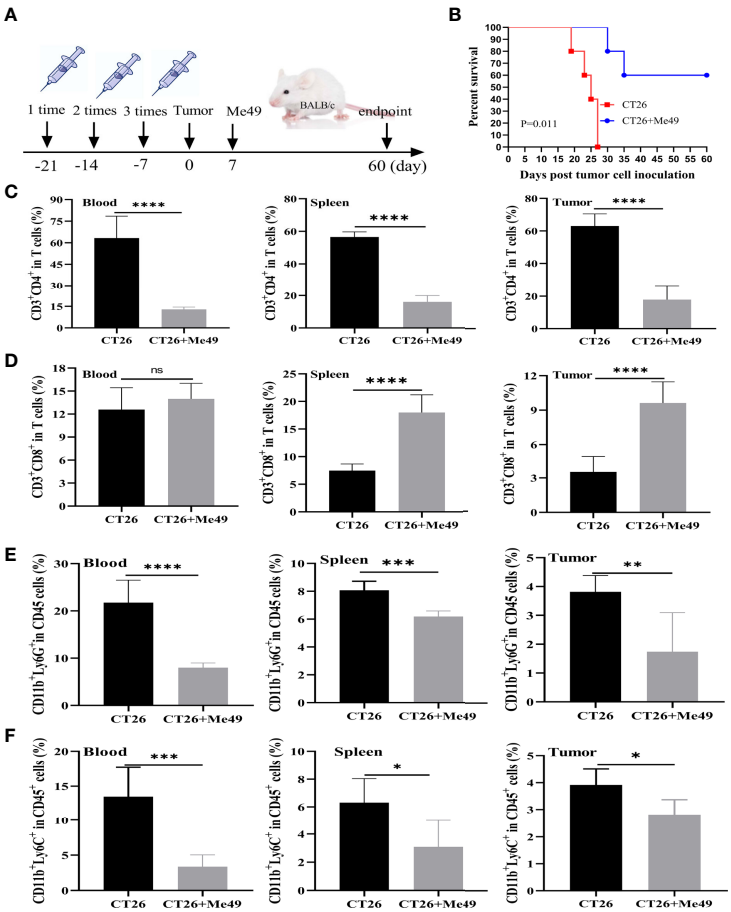


FIGURE 1 | *T. gondii* induced a protective immune response against tumor growth of CRC. **(A)** Mice were immunized with inactivated Me49 strain of *T. gondii*, and then vaccinated with CT26 cells. After 7 d, mice were reinfected with the Me49 strain of *T. gondii*. The control group was not infected after tumor inoculation (n = 5). **(B)** Survival rate of CT26–bearing mice after *T. gondii* infection. FCM analysis of $CD3^+$ $CD4^+$ T cells (anti-CD3-FITC, anti-CD4-PB450) **(C)**, $CD3^+$ $CD8^+$ T cells (anti-CD3-FITC, anti-CD8-PE) **(D)**, G-MDSCs (anti-CD11b-FITC, anti-Ly6G-PE) **(E)** and M-MDSCs (anti-CD11b-FITC, anti-Ly6C-APC-A) **(F)** in peripheral blood, spleen and tumors from different groups. ns, ($p \geq 0.05$), $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

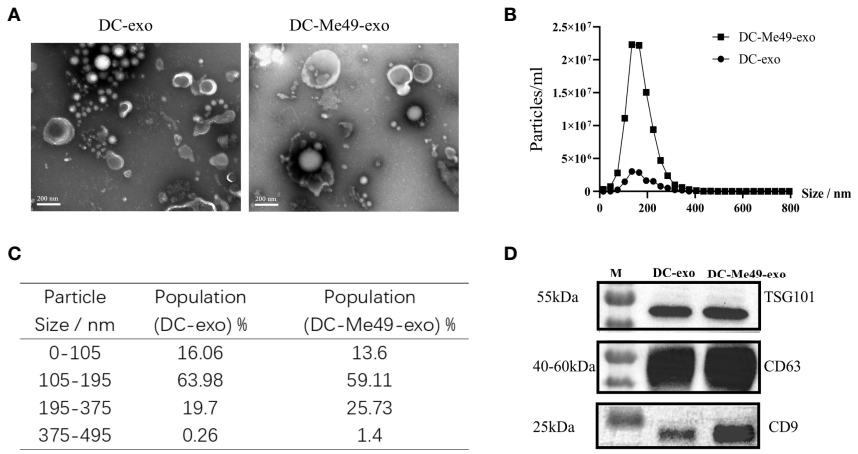
(**Figure 2A**). DCs infected with Me49 secreted more exosomes at 12 h than uninfected cells (**Figure 2B**). The diameter range of exosomes from different samples was 100–200 nm (**Figure 2C**). The presence of three exosome-enriched proteins, CD63, CD9, and TSG101 (**Figure 2D**), was confirmed by Western blot.

Exosomes Inhibited Tumor Growth

To evaluate the efficacy of exosomes isolated from DCs infected with *T. gondii*, we treated tumor-bearing mice with exosomes. We monitored tumor growth progression using the IVIS Spectrum. Compared with the DC-exo and PBS groups, signal intensity on tumor imaging was decreased significantly in the DC-Me49-exo group ($P< 0.01$), while no differences were found between the DC-exo and PBS groups (**Figures 3A, B**). DC-Me49-exo group had a 40% survival rate at day 42 versus 0% for the other two groups (**Figure 3C**). These results indicated that DC-Me49-exo could significantly inhibit CRC growth *in vivo*.

DC-Me49-exo Reduced MDSC Proportion in Tumor Bearing Mice

To evaluate the role of exosomes in regulating MDSC, we analyzed the level of MDSC in blood and tumors *via* FCM. After $CD45^+CD11b^+$ gating, G-MDSCs, M-MDSCs, DC and macrophage were analyzed (n = 5/group) (**Supplementary Figure 1C**). Results showed that in peripheral blood, the relative proportion of G-MDSCs was reduced after treatment with DC-Me49-exo compared with the DC-exo and PBS groups ($p < 0.001$) (**Figure 4A**). The ratio of M-MDSC was significantly increased ($p < 0.01$) in DC-Me49-exo group (**Figure 4B**). Similarly, compared with the DC-exo and PBS groups, the mean frequency of DCs ($CD45^+CD11C^+$) was significantly increased in the DC-Me49-exo group ($p < 0.05$) (**Figure 4C**). No significant change occurred in the mean frequency of macrophages ($CD11b^+F4/8^+$) in any of the three groups (**Figure 4D**). These data indicated that a significant decrease in G-MDSCs was accompanied by an increase of DCs in the



Particle Size / nm	Population (DC-exo) %	Population (DC-Me49-exo) %
0-105	16.06	13.6
105-195	63.98	59.11
195-375	19.7	25.73
375-495	0.26	1.4

FIGURE 2 | Isolation and characterization of exosomes. **(A)** Exosomes were stained with uranyl acetate and analyzed three times *via* TEM (original magnification, × 100,000; scale bar = 200 nm). **(B)** Nanoparticle-tracking analysis of size distribution of purified exosomes. **(C)** Percentages of purified exosomes in various size ranges. **(D)** Western blot identification of exosome markers CD63, CD9 and TSG101.

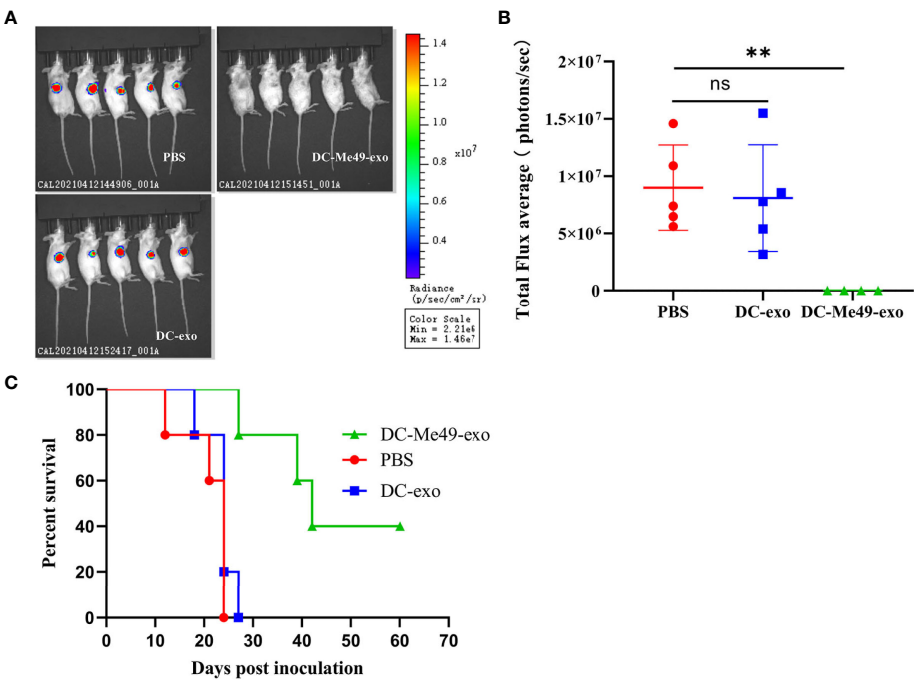


FIGURE 3 | DC-Me49-exo inhibited tumor growth in a mouse model of CRC. CT26 cells were injected subcutaneously, and DC-exo, DC-Me49-exo and PBS from different groups were injected into the tumor. **(A)** Fluorescence intensity in tumor-bearing mice after exosome treatment detected using an IVIS imaging system. **(B)** *In vivo* fluorescence imaging of tumor-bearing mice treated with exosomes. PBS, DC-exo and DC-Me49-exo represent control group, DC-exo treatment group and DC-Me49-exo treatment group, respectively. **(C)** Survival curve of a mouse model with CRC after exosome treatment (n=10). ns, ($p \geq 0.05$), **$p < 0.01$.

peripheral blood of tumor-bearing mice treated with DC-Me49-exo.

We also analyzed MDSC counts in tumor tissues. We found low proportions of G-MDSCs ($p < 0.001$) (**Figure 4E**) and M-MDSCs ($p < 0.05$) (**Figure 4F**) in the DC-Me49-exo group compared with the DC-exo and PBS groups. Mean frequencies of DCs were significantly increased in the DC-Me49-exo group ($p < 0.0001$) (**Figure 4G**). Compared with the DC-exo and PBS groups, the mean frequency of macrophages was significantly decreased in the DC-Me49-exo groups ($p < 0.05$) (**Figure 4H**).

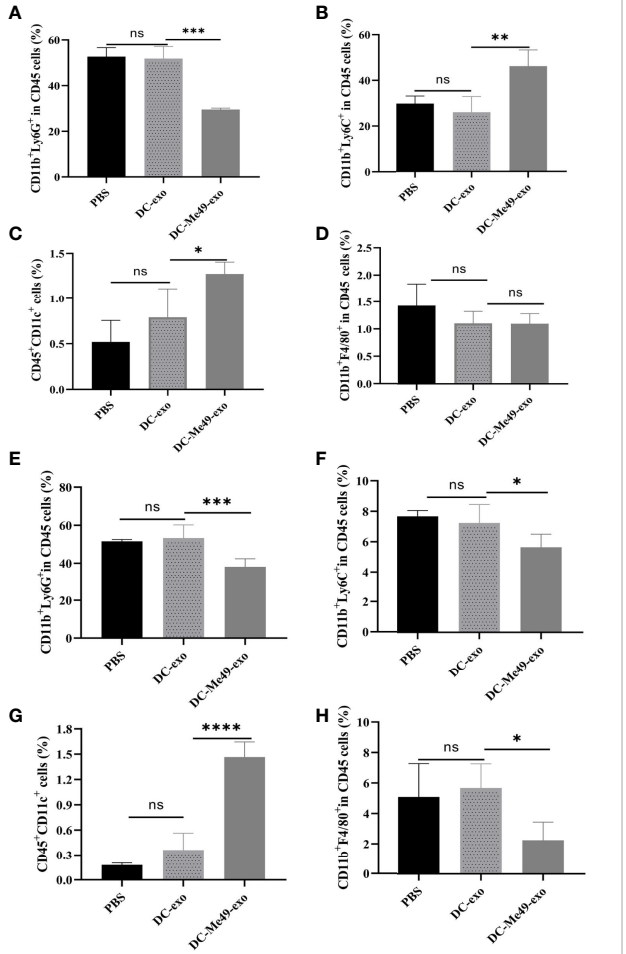


FIGURE 4 | DC-Me49-exo decreased the proportion of MDSCs in a mouse model of CRC. FCM analysis of G-MDSCs (anti-CD11b-APC, anti-Ly6G-PE) **(A)**, M-MDSCs (anti-CD11b-APC, anti-Ly6C-APC-700) **(B)**, DCs (anti-CD45-FITC, anti-CD11c-PC5.5) **(C)** and macrophages (anti-CD11b-APC, anti-F4/80-PB450) **(D)** in the blood of tumor-bearing mice treated with DC-exo, DC-Me49-exo and PBS. FCM analysis of G-MDSCs **(E)**, M-MDSCs **(F)**, DCs **(G)** and macrophages **(H)** in the tumor tissues of tumor-bearing mice treated with DC-exo, DC-Me49-exo and PBS. ns, ($p \geq 0.05$), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

These results indicated that DC-Me49-exo inhibited the accumulation of MDSCs in tumor tissues and peripheral blood, and promoted MDSC ($CD11b^+Ly6G^+$) differentiation.

DC-Me49-exo Promoted Differentiation of MDSCs *In Vitro*

Exosomes are taken up by recipient cells and the packaged contents are unloaded to regulate the function and activity of recipient cells. To verify the effect of exosomes on MDSCs, we added labeled DC-exo and DC-Me49-exo to MDSC. After 12 h of coculture, we observed labeled exosomes (green) gathered around the nuclei (blue) of MDSCs (**Figure 5A**), and the proportions of G-MDSCs ($p < 0.01$) (**Figure 5B**) and M-MDSCs ($p < 0.01$) (**Figure 5C**) were decreased in the DC-Me49-exo group. Compared with the DC-exo and PBS groups, the mean frequency of DC was significantly increased in the DC-Me49-exo group ($p < 0.0001$) (**Figure 5D**). We saw no significant change in macrophage among the three groups (**Figure 5E**). All these data indicated that the DC-Me49-exo directly regulate the MDSC differentiation and increase the level of DCs *in vitro*.

DC-Me49-Exo Regulated MDSCs by Inhibiting the STAT3 Pathway

Previous studies have confirmed that phosphorylation of JAK2 and STAT3 is associated with the differentiation and expansion of MDSCs (21, 22). We evaluated the levels of IL-6 and GM-CSF, which regulate JAK2–STAT3 activation, in serum of tumor-bearing mice after treatment with different exosomes. Compared with DC-exo and PBS groups, both the levels of IL-6 and GM-CSF significantly decreased in the DC-Me49-exo group ($p < 0.01$) (**Figures 6A, B**). To further confirm the correlation between JAK2-STAT3 signal transduction and MDSC differentiation, we detected phosphorylation of JAK2 and STAT3 in MDSC treated with exosomes using Western blot. The results showed that the phosphorylation level of JAK2 was not affected by exosomes in any of the three groups (**Figures 6C, D**), while that of STAT3 in the DC-Me49-exo group was greatly decreased compared with the DC-exo groups ($p < 0.05$) (**Figures 6C, E**). All these data indicated that DC-Me49-exo promoted MDSC differentiation by inhibiting the phosphorylation level of STAT3.

DISCUSSION

The oncolytic function of bacteria and viruses has been well studied in cancer therapy. Parasite-based cancer therapy has recently elicited great interest. Previous studies (9, 23) and our own research have found that infection with the single-celled parasite *T. gondii* can inhibit tumor growth. In this study, we detected the distribution of *T. gondii* in tumor-bearing mice, but no parasites were found in tumor tissues. Our hypothesis was that *T. gondii* inhibited tumor growth by rebalancing immune homeostasis. Considering the risk of direct infection with *T. gondii* in tumor treatment, we developed an exosome-based strategy instead of live *T. gondii* infection for cancer therapy. Our data showed that the Me49 strain of *T. gondii* inhibited tumor growth. Compared with the control groups, infiltration of $CD3^+CD8^+$ T cells was increased and the level of MDSC decreased in the CT26 + Me49 group.

Exosomes are secreted by all types of cells including normal, cancer or host, and infected cells, and their functions depend on their cellular origins. Those isolated from different immune cells have been identified, these exosomes have immunomodulatory properties, which encourages research on their clinical applications in disease treatment. At present, the main application of exosomes is tumor prevention. Exosomes derived from immature DCs have limited function, they require activation by antigens or cytokines to exert antitumoral effects (24). Previous studies showed that patients could obtain clinical benefit from exosomes isolated

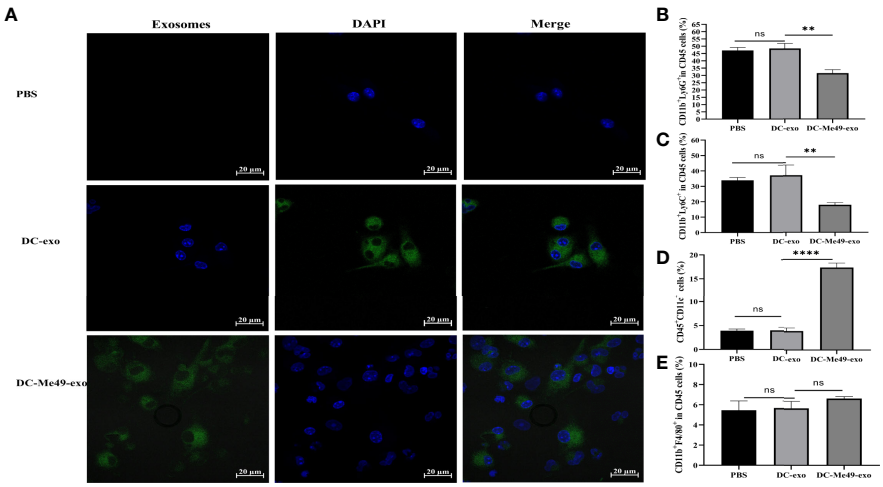


FIGURE 5 | DC-Me49-exo inhibited the proportion of MDSCs *in vitro*. **(A)** Uptake of exosomes by MDSCs. Exosomes were stained green with ExoSparker Exosome Membrane Labeling Kit-Green dye and nuclei were stained blue with DAPI after 24 h co-culture with MDSCs. Scale bar: 20 μm. FCM analysis of G-MDSC (anti-CD11b-APC, anti-Ly6G-PE) **(B)** M-MDSC (anti-CD11b-APC, anti-Ly6C-APC-700) **(C)**, DCs (anti-CD45-FITC, anti-CD11c-PC5.5) **(D)** and macrophages (anti-CD11b-APC, anti-F4/80-PB450) **(E)**. ns, ($p \geq 0.05$), $^{**}p < 0.01$, $^{****}p < 0.0001$.

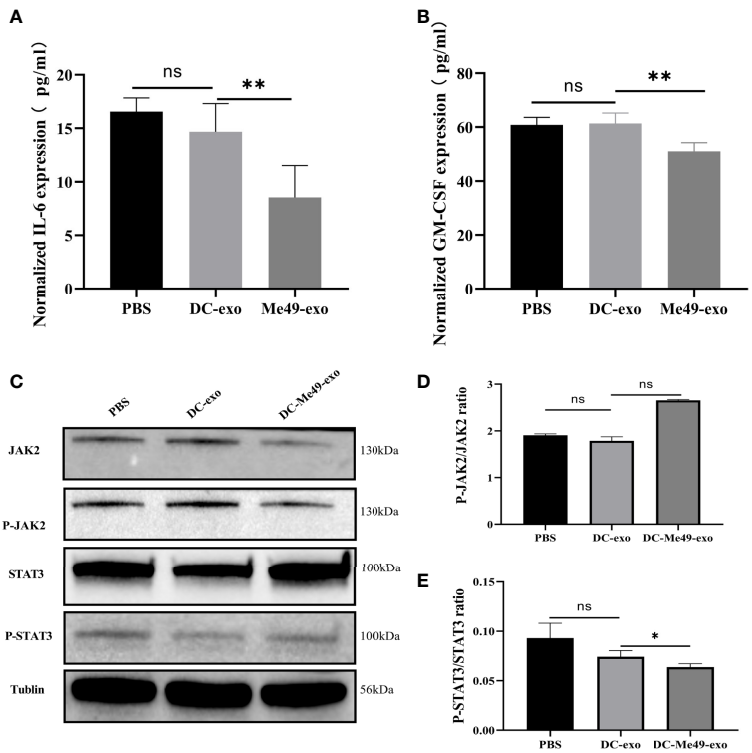


FIGURE 6 | DC-Me49-exo regulated MDSCs by affecting the STAT3 pathway. Concentrations of IL-6 **(A)** and GM-CSF **(B)** in sera of tumor-bearing mice treated with DC-exo, DC-Me49-exo and PBS. **(C)** Protein expression levels at the JAK2/STAT3 pathway. The grayscale analysis of the ratio of P-STAT3/STAT3 **(D)** and P-JAK2/JAK2 **(E)**. ns, ($p \geq 0.05$), $^{*}p < 0.05$, $^{**}p < 0.01$.

from DCs that were loaded with antigen peptides identified in melanoma and prostate cancer cells (25, 26). Exosomes isolated from DCs co-cultured with human breast adenocarcinoma cells (SK-BR-3) strongly activate tumor-specific T cells (26). These studies show that exosomes derived from DCs are an important new strategy in tumor immunotherapy. Unfortunately, exosomes derived from peptide-loaded DCs have so far failed to induce tumor-specific T cell responses and therefore have no clinical efficacy (7). Compared with exosomes derived from peptide-loaded DCs, exosomes from pathogen-infected DC have a rich variety of antigens, which can bind more effector T cells specifically and produce stronger immune response. *In vivo* experiments, exosomes derived from DC directly loaded with OVA antigen peptide were significantly less able to induce effective antigen-specific T cell response than exosomes derived from DC treated with pulsed OVA. Ova-treated DC exosomes are more sensitive to MHC than exosomes loaded with OVA polypeptides, and have a relatively high affinity for TCR (27). Previous studies (28) and our data proved that *T. gondii* infection could increase $CD8^+$ T cell infiltration. DC-derived exosomes display abundant MHC class I/II molecules and T cell co-stimulatory molecules, which mainly perform direct antigen presentation to activate T cells. Therefore, we treated colorectal cancer tumor-bearing mice with *T. gondii* infected DC-derived exosomes. Our results showed that DC-Me49-exo inhibited tumor growth by reducing the level of MDSC in our CT26 mouse model. All these data indicated that exosomes isolated from DCs infected with *T. gondii* could be a potential candidate treatment in cancer therapy. It is a pity that the components of exosomes remain unknown, but we will attempt to clarify them in the future research.

MDSCs, which are major immunosuppressive cells, accumulate in tumor site and promote cancer progression, therefore, targeting them is an attractive strategy for cancer therapy. Our data showed that DC-Me49-exo decreased the level of MDSC both *in vivo* and *in vitro*. A previous study showed that gemcitabine reduced residual G-MDSC in the lung of tumor-bearing mice and inhibited the subsequent metastatic growth (29). In this study, MDSCs and macrophages were significantly reduced in tumor tissues after DC-Me49-exo treatment, and the proportion of DCs increased significantly. These results suggest that this exosome inhibits tumor growth by reducing MDSC at the tumor site. JAK and STAT3 are activated by cytokines and chemokines in the TME, and promote the development of MDSCs (30). *Plasmodium* infection significantly reduces the proportions of MDSCs and regulate T cells (Tregs) in lung tumor tissues of mice by inhibiting phosphorylation of STAT3 and other STAT pathways (12). These findings indicate that inhibiting the proportion and function of MDSCs during tumor progression is essential for tumor treatment. Our data showed that exosomes isolated from *T. gondii*–infected DC significantly inhibited the proportions of MDSCs *in vivo* and *vitro*. However, we focused on MDSC regulation in this study, other immunosuppressive cells such as Tregs, and tumor associated macrophages (TAMs) will be investigated in the future.

In summary, in this study we developed novel pathogen-based exosomes for cancer therapy to replace live-pathogen infection. Exosomes derived from *T. gondii*–infected DCs, therefore, could be a promising therapeutic strategy to inhibit the progression of CRC. The pathogenic infection and the tumor "infection" might competitively regulate the immune system. Exosomes isolated from *T. gondii*–infected DCs, as messengers, could stimulate the immune system and change the "cold" tumor to a "hot" tumor. At the same time, these exosomes could be further modified into carriers for both drug and antibody delivery.

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 authors.

ETHICS STATEMENT

The animal study was reviewed and approved by SHVRI–SZ–20200421-01.

AUTHOR CONTRIBUTIONS

JL performed experiments, analyzed data and wrote the manuscript. NW conceived the study, performed experiments, analyzed the data. SZ and XC performed experiments. NW, JL, SZ, and XC performed flow cytometry, and conceived and designed the research. HG, RM, and YH suggested the study. GL, NW, and ZC analyzed data, revised manuscript and supervised the study. ZC were responsible for the collection and collation of clinical data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.899737/full#supplementary-material

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REVIEW
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Role of the Pro-Inflammatory Tumor Microenvironment in Extracellular Vesicle-Mediated Transfer of Therapy Resistance

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Advances in our understanding of cancer biology have contributed to generating different treatments to improve the survival of cancer patients. However, although initially most of the therapies are effective, relapse and recurrence occur in a large percentage of these cases after the treatment, and patients then die subsequently due to the development of therapy resistance in residual cancer cells. A large spectrum of molecular and cellular mechanisms have been identified as important contributors to therapy resistance, and more recently the inflammatory tumor microenvironment (TME) has been ascribed an important function as a source of signals generated by the TME that modulate cellular processes in the tumor cells, such as to favor the acquisition of therapy resistance. Currently, extracellular vesicles (EVs) are considered one of the main means of communication between cells of the TME and have emerged as crucial modulators of cancer drug resistance. Important in this context is, also, the inflammatory TME that can be caused by several conditions, including hypoxia and following chemotherapy, among others. These inflammatory conditions modulate the release and composition of EVs within the TME, which in turn alters the responses of the tumor cells to cancer therapies. The TME has been ascribed an important function as a source of signals that modulate cellular processes in the tumor cells, such as to favor the acquisition of therapy resistance. Although generally the main cellular components considered to participate in generating a pro-inflammatory TME are from the immune system (for instance, macrophages), more recently other types of cells of the TME have also been shown to participate in this process, including adipocytes, cancer-associated fibroblasts, endothelial cells, cancer stem cells, as well as the tumor cells. In this review, we focus on summarizing available information relating to the impact of a pro-inflammatory tumor microenvironment on the release of EVs derived from both cancer cells and cells of the TME, and how these EVs contribute to resistance to cancer therapies.

Keywords: extracellular vesicles, exosomes, inflammation, therapy resistance, tumor microenvironment

INTRODUCTION

Due to its high prevalence and mortality, cancer is now considered as the leading cause of death worldwide as defined by the World Health Organization (WHO) in 2019 (1). This multifactorial disease is characterized by the presence of cells that constantly proliferate in a rapid and uncontrolled manner (2). Currently, several methods exist for the treatment of cancer, including radiation therapy, surgery, immunotherapy, endocrine therapy, gene therapy and chemotherapy, the latter being the most commonly employed therapeutic approach (3).

While cancer treatments are initially quite successful, the long-term of success of such interventions is often limited by the development of drug resistance. As an example, in chemotherapy, 90% of cancer patient mortality is attributable to drug resistance (3). Processes leading to resistance can be segregated into two major categories, referred to as intrinsic or extrinsic, depending on whether the resistance was pre-existing in cancer cells, or subsequently acquired in response to treatment, respectively (4). Nevertheless, both types of resistance share common mechanisms that permit escaping cancer therapy, such as enhanced drug efflux, changes in drug targets, metabolic adaptations, dysregulation of the DNA damage repair machinery, defective apoptotic signaling, activation of pro-survival signaling, and other adaptive cellular responses (3, 5, 6).

Solid tumors display great cell heterogeneity and, together with non-cellular components, are referred to as the tumor microenvironment (TME) (7). The bidirectional communication between tumor cells and the surrounding stromal components plays a critical role in the regulation of tumor progression by favoring processes, such as metastasis and therapeutic resistance (8). The TME consists of non-cellular components, such as the extracellular matrix, and stromal cells, including cancer-associated fibroblasts (CAFs), mesenchymal cells, endothelial cells, adipocytes, and immune cells like the tumor associated macrophages (TAMs) (8). The TME is described as a pro-inflammatory microenvironment given that many of the cells present are inflammatory cells, and many cells of the TME have the ability to secrete pro-inflammatory molecules in response to different conditions including, but not limited to, hypoxia or chemotherapy (9–13). Pro-inflammatory processes also contribute to tumor progression, making the ability to suppress such events highly desirable for the successful outcome of treatments (9, 14). Thus, although cancer therapy has focused for many years primarily on tumor cells as the targets, the importance of the TME and interactions between tumor cells and the stromal components in promoting tumor development and progression, makes targeting these interactions an increasingly interesting option for cancer treatment (7, 15).

Intercellular communication in the TME is mediated by soluble factors such as cytokines, chemokines, growth factors, and extracellular vesicles (EVs) (16). EVs are a heterogeneous group of cell-derived membranous structures that are released to the extracellular space and are involved in multiple physiological and pathological processes, given that they represent vehicles for the transfer of a large variety of molecules to recipient cells, including DNAs, mRNAs, proteins, microRNAs (miRNA), long non-coding RNAs (LncRNAs), lipids, and metabolites. These days, the release and uptake of EVs is considered an important mechanism of intercellular communication and EVs are classified into two main groups according to their origin, namely exosomes that are of endosomal origin (30–150 nm in diameter), and microvesicles that are liberated directly from the plasma membrane (MVs, 50–500 nm in diameter), including apoptotic bodies (17). The content of the EVs is decisive in determining the phenotypic changes that may be triggered in recipient cells, and this in turn depends on the origin and the state of the cell when the vesicles are generated (18). For instance, EVs control several physiologically important functions such as immune surveillance, blood coagulation, stem cell maintenance and tissue repair. On the other hand, in some contexts, EVs have a pathological role. For example, EVs can favor the development of cancer, autoimmune diseases, prion diseases, neurodegeneration and HIV infection (19). Furthermore, EVs have been implicated in the acquisition of the hallmarks of cancer and driving tumor progression by promoting communication between cancer cells and the tumor microenvironment (20).

To contextualize the concept of EVs, the International Society for Extracellular Vesicles (ISEV) suggests minimal requirements to define vesicles as EVs (21). In general, EVs are structures with a lipid bilayer that are unable to replicate and lack a functional nucleus. In terms of specific markers, there is no consensus that permits clearly defining EVs of endosomal origin (exosomes) or those derived from the plasma membrane (ectosomes, microparticles, or microvesicles). Moreover, experimental limitations generally do not allow separating the different EV subpopulations. However, the ISEV recommends the use of size to define such subpopulations, and following those guidelines they can be separated into two main groups, small EVs (sEVs) (<200 nm in diameter, and medium/large EVs (m/lEVs) (>200 nm in diameter). Besides size, EVs also should be characterized by the presence of at least three positive protein markers of EVs and one negative marker to evaluate contamination by vesicles from other subcellular compartments. If an EV preparation does not meet these minimal requirements, the use of the term extracellular particles (EPs) is recommended. Therefore, the processing of samples, depending on the source of the EVs (conditioned medium or biological fluids), the experimental conditions (hypoxia or serum concentration for example), and the methods used to separate and concentrate the EVs (ultracentrifugation, size exclusion chromatography, among others) are crucial to achieve the minimal requirements to obtain vesicles considered as EVs. In this context, there are several methods to separate and concentrate EVs, but each one is different in terms of recovery and specificity. Therefore, to evaluate a biological effect of EVs, such as transfer of therapy resistance, it is important to consider which method is used. In this review, we summarize the main results of several articles which isolate, characterize and describe the role of vesicles in

therapy resistance. In most, but not all cases these can be defined as EVs by the aforementioned criteria.

The important role of EVs in the communication between cancer cells and the TME, and their contribution to the development of different hallmarks that drive tumor progression is well established (20). Moreover, the biogenesis of EVs and their content are modulated by the different stimuli and conditions present in the TME. In this context of note is the ability of pro-inflammatory conditions to promote the release of EVs, which endow cancer cells with traits that permit developing resistance to anti-cancer therapies (22). With this in mind, we will focus in this review on summarizing how the pro-inflammatory tumor microenvironment and EVs generated in this milieu contribute to the acquisition of cancer therapy resistance.

EVS IN CANCER THERAPY RESISTANCE INDUCED BY HYPOXIA AND GLYCOLYSIS

Hypoxia generates a pro-inflammatory TME that promotes resistance to cancer therapy (23). Several cell types are affected by hypoxic conditions that promote tumor cell survival, migration, invasion, and metastasis (24). Glycolysis appears as an important mechanism in this context. Indeed, a well-established hallmark of cancer that enhances tumor cell aggressiveness is metabolic reprogramming (25). Cancer cells impair mitochondrial respiration and convert to a glycolytic metabolism to obtain energy and intermediate metabolites required for tumor growth and metastasis (26). Consistent with the relevance of this switch, some drugs that prevent hypoxia-induced therapy resistance, like dichloroacetate, wogonin and baicalein, also inhibit glycolytic enzymes such as HKII, PDHK1, and LDHA (27–29). Moreover, inhibiting glucose uptake or the glycolytic pathway prevents hypoxia-induced therapy resistance (27, 30) due to HIF-1α downregulation mediated by the PTEN/PI3K/Akt/mTOR signaling pathway (28, 29, 31).

On the other hand, there is evidence suggesting that hypoxia-induced therapy resistance is independent of HIF-1α (32). Indeed, STAT3, rather than HIF-1α, appears as a key regulator in this process (33, 34). Circular RNA AKT3 (CircAKT3) is upregulated in cancer and inhibits miR-516b-5p, an inhibitor of STAT3, thereby promoting STAT3 activation and therapy resistance (35). One of the effects of the STAT3 activation is the downregulation of PTEN (36). Indeed, some authors have observed that activation of STAT3/Akt/MAP2K and PKM2/glycolysis are relevant in drug-resistant cells (35, 37). In addition to these cell intrinsic pathways, it has more recently become clear that cell extrinsic events involving EVs are important in events leading to therapy resistance.

Since hypoxia promotes EV production, several studies suggest that the hypoxia-related effects may be dependent on the delivery of proteins and nucleic acids present in EVs, which induce therapy resistance in recipient cells. Indeed, therapy-resistant cells are known to deliver EVs to therapy-sensitive cells and induce therapy resistance under hypoxic conditions (**Supplementary Table 1**). For instance, ovarian cancer cells exposed to hypoxia increase EV release by upregulating Rab27a and downregulating Rab7, LAMP1/2 and NEU-1. In this way, cisplatin-resistant cells deliver EVs containing STAT3 and FAS to sensitive cells and promote invasion through MMP2 expression and chemotherapy resistance under hypoxic conditions (38). Another mechanism observed in cancer cells exposed to hypoxia is the release of PKM2-containing EVs, which promote therapy resistance by stimulating glycolysis, ROS production and inhibiting apoptosis (39). In addition, EVs from oxaliplatin-resistant cancer cells deliver circR-122 to drug-sensitive cells. Here, circR-122 acts as a sponge for miR-122, the inhibitor of PKM2, thereby promoting PKM2 expression, glycolysis, and therapy-resistance (40). Moreover, other glycolytic enzymes, such as ALDOA and ALDH3A1, are detected in EVs of radiation-resistant cells. The transfer of these enzymes promotes glycolysis and aggressiveness in recipient cells (41).

HSP70 and Osteopontin are stress proteins that participate in hypoxia-induced radio- and chemotherapy resistance. HSP70 is present at the plasma membrane and naturally released in EVs. As hypoxia stimulates EV production, an increment in HSP70 levels in plasma is observed that promotes therapy resistance. Osteopontin expression also increases under hypoxic conditions. In fact, increases in HSP70 and Osteopontin are associated with decreased overall patient survival (42). Furthermore, small EVs from adriamycin-resistant cells contain HSP70 which directly targets mitochondria in recipient cells. In this way, HSP70 impairs mitochondrial function, promotes glycolysis, and induces therapy resistance in recipient cells (43). Taken together, these data suggest that therapy-resistant cells release EVs which promote glycolysis and therapy resistance in therapy-sensitive cells under hypoxic conditions. In this way, controlling EV content and/or glycolysis may represent a possible novel approach to target resistant tumor cells.

EV RELEASE IN RESPONSE TO CHEMOTHERAPY AND ACQUISITION OF THERAPY RESISTANCE

Chemotherapy is another factor that contributes to generating an inflammatory TME by increasing the production of inflammatory cytokines or modulating cellular components of the TME, including the immune system (12). To date, chemotherapy remains the most frequently employed treatment for cancer. However although initially effective in a large percentage of patients, relapse often occurs within a few years following the treatment and patients die due to the development of drug resistance (44). A wide range of molecular and cellular mechanisms have been identified as important in contributing to the development of chemoresistance: 1) increased rates of drug efflux; 2) activation of survival signaling and inactivation of death signaling pathways; 3) epigenetic changes and 4) effects of the local

tumor microenvironment (6). In this context, inflammation of the TME enhanced by chemotherapy also can contribute to the failure of therapy (13). Moreover, this microenvironment can promote the release of EVs from tumor cells that contribute to therapy resistance. Indeed, several reports show that chemotherapeutic agents induce the biogenesis and release of EVs from tumor cells with pro-tumorigenic activity, including the ability to transfer chemoresistance (45–49).

For example, cisplatin and paclitaxel based chemotherapy is widely used as first-line therapy in several cancers and leads to a significant reduction in the tumor size (50–52). However, the use of cisplatin for the treatment of ovarian cancer (OC) promotes the release of EVs that induce drug resistance in bystander cells by modulating the p38 and JNK signaling pathways to increase cisplatin resistance (53). Furthermore, EVs released from chemosensitive bladder cancer cells, in particular the non-stem cancer cell (NSCCs) population, in response to cisplatin or gemcitabine, another chemotherapeutic agent, also promote therapy resistance and additionally favor cancer stem cell (CSC) survival in response to chemotherapy (54). A proteomic analysis of the EV cargo implicated the transfer from NSCCs to CSCs present in the TME of protein synthesis/degradation machinery components, which are critical for CSC survival, maintenance, and plasticity. Even though, the large majority of NSCCs die in response to chemotherapy, they release EVs containing ribosomal proteins that are taken up by CSCs and induce protein synthesis, aiding CSCs in adapting to the post-therapy TME, ultimately resulting in resistance and disease (54).

Chemotherapy with paclitaxel also modulates EV biogenesis, thereby contributing to therapy resistance in recipient cells. In breast cancer cells, treatment with paclitaxel induces the release of exosomes highly enriched in the protein Survivin, a member of the inhibitor of apoptosis (IAP) protein family that blocks cell death (55), and the transfer of these exosomes to breast cancer cells promotes cell survival in a Survivin-dependent manner (56). Recent studies show that paclitaxel and doxorubicin chemotherapy increases the levels of miR-378a-3p and miR-378d, microRNAs associated with chemoresistance, in EVs derived from patients and preclinical models. The uptake of such EVs by recipient breast cancer cell promotes cancer stemness and chemoresistance *via* enhanced EZH2/STAT3 signaling (57). Paclitaxel and doxorubicin also promote the secretion of EVs from breast cancer cells, which contain several microRNAs that target the transcription factor One Cut Homeobox 2 (ONECUT2), a protein involved in the induction of CSC-like properties that allows cancer cells to survive in response to cytotoxic treatment and therefore contributes to chemoresistance (58). Doxorubicin also has been described to promote the release of EVs by another mechanism. Cancer cells treated with Doxorubicin stimulate the secretion of EVs enriched in the protein ATP-binding cassette sub-family B member 1 (ABCB1), a transporter involved in promoting the efflux of chemotherapeutic drugs (59), by the upregulation of Rab8B and downregulation of Rab5 proteins. Moreover, these EVs transfer ABCB1 to sensitive cancer cells and confer a transient drug-resistant phenotype by downregulation of Rab5 in the recipient cell (46).

In pancreatic cancer cells, following treatment with gemcitabine the acquisition of chemoresistance mediated by EVs has been described. In response to drug treatment, exosomes transfer to neighboring cells superoxide dismutase 2 (SOD2) and catalase (CAT) transcripts, which encode ROS-detoxifying enzymes, that improve cell viability in response to the chemotherapy (60). Furthermore, downregulation of the gemcitabine-metabolizing enzyme, deoxycytidine kinase (DCK) is in part responsible of chemoresistance acquisition *via* an indirect mechanism involving the transfer of its targeting miRNA (miR-155). Indeed, when pancreatic cells stimulated with the exosomes containing miR-155 were treated with anti-miR-155 to block the effect, the cells became more sensitive to gemcitabine. These findings show that DCK downregulation mediated by exosomes from gemcitabine treated cells provides a survival advantage to gemcitabine-treated pancreatic cells (60). Thus, chemotherapy has two major EV-related effects, on the one hand increasing EV production and on the other hand including pro-tumorigenic cargos, which when transferred to sensitive cells promote chemoresistance (**Supplementary Table 2**).

MACROPHAGE-DERIVED EXTRACELLULAR VESICLES IN CANCER DRUG RESISTANCE

Tumor-associated macrophages (TAMs) are the major cellular component from the immune system in the TME (61) and key mediators of inflammation that contributes to many of the hallmarks of cancer (25). In fact, the high presence of TAMs in the tumor stroma is associated with tumor progression and poor prognosis, since they participate in tumor angiogenesis, matrix remodeling, invasion, metastasis, immunosuppression, and drug resistance (62–65).

As the main participants in the inflammatory response in the TME, macrophages mediate drug resistance in cancer cells through various molecular mechanisms. One of them involves the polarization of macrophages, whereby TAMs acquire characteristics similar to those of M2 macrophages. In breast cancer cells, SGLT1 overexpression drives glucose uptake and lactic acid secretion, which promotes macrophage polarization to M2-like TAMs that then activate the EGFR/PI3K/Akt/SGLT1 signaling pathway in the tumor cells to induce resistance to tamoxifen (66). Likewise, M2 macrophage polarization induces resistance to fluorouracil (5FU) treatment in gastric cancer cells by promoting cell survival *via* the PI3K/Akt/NF-κB pathway and inducing cell invasion through increasing the expression of integrin β3, FAK, and cofilin (67). Another report describes a similar mechanism whereby M2-polarized TAMs secrete CC chemokine ligand 2 [CCL2 also known as MCP-1)], which activates the PI3K/Akt/mTOR signaling pathway and promotes tamoxifen resistance in endocrine-resistant breast cancer cells (68). Moreover, it has been observed that TAMs might be able to induce epithelial to mesenchymal transition (EMT) and consequently decrease sensitivity to the chemotherapeutic

agent gemcitabine in pancreatic cancer cells (69). In addition, M2 macrophages induce the release of pyrimidine nucleosides, such as deoxycytidine, that confer resistance to gemcitabine in pancreatic cancer cells, by a mechanism of molecular competition at the level of drug uptake and metabolism (70).

However, the mechanisms responsible for cancer progression and drug resistance are currently being re-evaluated with the discovery of EVs as new players in this process. One of the principal mechanisms described is the exosomal transfer of miRNA from macrophages to tumor cells. For instance, it has been reported that TAM-derived EVs containing miR-365 induce resistance to gemcitabine in pancreatic adenocarcinoma cells, through a mechanism that involves an alteration in the metabolism of pyrimidine and an increase in cytidine-deaminase, the enzyme responsible for the inactivation of gemcitabine in humans (71). Similarly, EVs derived from a population of anti-inflammatory human macrophages contain proteins such as chitinase 3-like-1 and fibronectin, which decrease the sensitivity of pancreatic adenocarcinoma cells to gemcitabine by activating ERK (72). In oral squamous cell carcinoma (OSCC), EVs released by macrophages attenuate the susceptibility of cells to chemotherapeutic drugs, like 5-fluorouracil and cis-diaminedichloroplatinum, by activating the AKT/GSK–3β pathway (73). A similar mechanism has been reported in gastric cancer cells, where exosomal miR-21 is delivered by macrophages to cancer cells and prevents cisplatin-triggered apoptosis *via* inhibition of PTEN and subsequent activation of the PI3K/AKT pathway (74). Similarly, EVs shed from hypoxic macrophages transfer miR-223 to ovarian cancer cells to elicit a chemoresistant phenotype through the down-regulation of PTEN and activation of PI3K/AKT (75). Finally, crosstalk between neuroblastoma cells and human monocytes induces resistance to cisplatin through two exosomal signaling pathways involving the miR-21/TLR8-NF-κB and miR-155/TERF1 pathways (76).

Interestingly, the EV-mediated crosstalk between cancer cells and macrophages is bidirectional. EVs derived from ovarian cancer cells abundantly express exosomal miR-1246, which confers resistance to paclitaxel through inhibition of Caveolin-1 (CAV-1) and increased levels of multidrug resistance protein 1 (MDR1). Furthermore, ovarian cancer cells can also transfer their exosomal miR-1246 selectively to M2-type macrophages, which then produce lower CAV-1 mRNA levels. These results suggest that TAMs may indirectly play an important role in drug resistance mechanisms (77). Additionally, umbilical cord blood-derived M1 macrophage exosomes could be employed as vehicles for the administration of drugs in the treatment of platinum-resistant ovarian cancer cells (78). Taken together, these observations identify macrophages as important players in contributing to drug resistance. Furthermore, they uncover multiple signaling pathways involving the interaction between TAMs and cancer cells, whereby the pathway of choice appears to vary depending on the type of cancer cell and antitumor therapy (**Supplementary Table 3**). Importantly, they identify macrophage-derived EVs within the TME as promising molecular targets for restoring drug sensitivity, identifying potential drug response biomarkers and improving the efficacy of cancer therapies.

ADIPOCYTE-DERIVED EXTRACELLULAR VESICLES IN DRUG RESISTANCE

Obesity-associated adipose tissue dysfunction is characterized by several local and systemic changes, such as elevated levels of pro-inflammatory factors, sex hormones, lipid metabolites and altered levels of adipokines, which are implicated in carcinogenesis, tumor progression, metastasis, and alterations in therapy responses (79).

Several studies have reported on the mechanisms by which adipocytes contribute to resistance to anticancer drugs. For instance, adipocytes induce FABP4 expression by promoting metastasis and mediating Carboplatin resistance in ovarian cancer cells. Alternatively, the inhibition of FABP4 leads to increased levels of DNA demethylation, impairs metastasis and sensitizes cancer cells to Carboplatin chemotherapy (80). Also, adipocyte-conditioned medium reduces the sensitivity of HER2+ breast cancer cells to the cytotoxic activity of Lapatinib and other tyrosine kinase inhibitors. Soluble factors released from adipocyte lipolysis are likely to be responsible for the reduced activity of Lapatinib on breast cancer cells exposed to the adipocyte-conditioned medium (81). Similarly, it has been reported that the conditioned media from adipocytes contribute to the resistance of melanoma cells to chemotherapeutic drugs (Cisplatin and Docetaxel) and therapeutic agents targeting the PI3K/Akt and MEK/ERK pathways (82). Along the same line, another study shows that adipocytes secrete soluble factors that increase resistance to chemotherapeutic drugs in ovarian cancer cells by activating the Akt pathway (83). Interestingly, adipocytes reportedly protect acute lymphoblastic leukemia (ALL) cells from chemotherapy drugs (84) and even sequester and metabolize Daunorubicin (DNR) to an inactive form, allowing nearby ALL cells to avoid DNR-induced cytotoxicity (85).

While the effects of adipocytes are well-documented, studies implicating adipocyte-derived EVs in drug resistance are limited. One study reported that EVs from cancer-associated adipocytes (CAAs) delivered the miR21 to ovarian cancer cells, where it suppresses apoptosis and induces Paclitaxel resistance, as well as an aggressive phenotype by binding directly to a novel target APAF1 (86). Also, crosstalk mediated by EVs between multiple myeloma (MM) cells and adipocytes has been described, whereby exosomal adipocyte LncRNAs contribute to MM therapy resistance and in turn, MM cells educate adipocytes through the EZH2/METTL7A/LncRNA axis (87). Finally, adipocytes confer a multidrug resistance phenotype to breast cancer cells by increasing the nuclear efflux of Doxorubicin (DOX) through a major vault protein (MVP)-dependent process and its expulsion from breast cancer cells *via* EVs (88). In summary (**Supplementary Table 4**), multiple mechanisms have been shown to be involved in adipocyte-mediated drug resistance in various cancers. However, less is known about the

role of adipocyte-derived EVs in the mechanisms leading to drug resistance. One may anticipate that greater insight in this respect could contribute to the development of new strategies to prevent the development of drug resistance.

EVS FROM CAFS IN CANCER THERAPY RESISTANCE

Cancer-associated fibroblasts (CAFs) are naturally resistant to cancer therapy. Moreover, CAFs contribute to therapy resistance through their crosstalk with cancer cells in several ways. Soluble compounds, such as cytokines and growth factors, have been implicated in this type of intercellular communication. For instance, therapy resistant CAFs produce and secrete IL-6, which has paracrine effects in cancer cells, thereby promoting chemotherapy resistance. Indeed, IL-6 upregulation is associated with poor prognosis in gastric cancer patients (89). IL-6 activates the JAK1/STAT3 signaling pathway in cancer cells (89, 90), and increases MDM2 expression, thereby promoting p53 polyubiquitination and degradation, which enhances cancer cell survival following drug treatment (91). In addition, IFN-β1 expression by CAFs is induced after the chemotherapy, leading to paracrine effects in breast cancer cells. The expression of IFN-β1 is related to reduce survival after chemotherapy (92). Furthermore, IL-1, in association with TGF-β1, induces the recruitment and transformation of normal fibroblasts to CAFs, which subsequently secrete pro-inflammatory factors that activate JAK/STAT and PI3K/Akt pathways in cancer cells, finally promoting therapy resistance (93). Moreover, patient-derived xenografts (PDX) resistant to cetuximab express higher levels of TGF-β1 in CAFs than xenografts sensitive to drug treatment (94). TGF-β1 secreted by CAFs upregulates the expression of ATF4 in cancer cells *via* the SMAD2/3 pathway. ATF4 promotes the expression of ABCC1 which favors the development of multiple drug resistance in cancer cells by extrusion of chemotherapy drugs (95). Also, CAFs secrete IGF-1 and HGF, as well as induce ANXA2 expression, which is required for CAF-induced EMT and therapy resistance (96). Also, CAFs secrete stromal cell-derived factor 1 (SDF-1 also known as CXCL12) which induces cancer cell drug resistance *via* a CXCR4, NF-κB and Bcl-xL-mediated signaling pathway (97). Finally, BDNF released from CAFs promotes therapy resistance *via* the TrkB/Keap1-Nrf2 pathway. Cancer cell-derived lactate upregulates BDNF expression in CAFs *via* the NF-κB pathway, thereby promoting a feedback amplification loop (98).

Soluble factors are however not the only components released by CAFs. Indeed, many molecules implicated in conferring drug resistance are transferred from CAFs to cancer cells in EVs. Moreover, there is strong evidence highlighting the relevance of EVs derived from CAFs in promoting cancer cell survival, proliferation, and subsequently drug resistance. Furthermore, the transfer of miRNAs in EVs from CAFs to cancer cells is commonly observed in connection with therapy resistance. Indeed, controlling the expression of pumilio homolog 2 protein (PUM2), an RNA-binding protein, appears to represent a novel mechanism to prevent therapy resistance. This protein is responsible for the packaging of miRNA-130a into exosomes, which are delivered from CAFs to lung cancer cells and promote cisplatin resistance (99). Another miRNA delivered by CAFs to cancer cells is miR-196a, which targets CDKN1B and ING5 in head and neck cancer cells and also confers cisplatin resistance (100). Moreover, gemcitabine resistant CAFs transfer miR-106b-containing EVs to pancreatic cancer cells, thereby promoting therapy resistance by targeting TP53INP1 (101), also known to be implicated in inducing drug-resistance in GC and BC (102, 103). In OC, paclitaxel-resistant CAFs transfer miR-21 containing EVs to cancer cells targeting APAF1 and apoptosis, thereby promoting therapy resistance (86). The latter mechanism has also been shown to be relevant in melanoma (104). Another miRNA delivered from CAFs to cancer cells related with paclitaxel resistance is miR-148b-3p, which induces the PTEN/Wnt/β-catenin pathway (105). This signaling pathway is also targeted by miR-92a-3p-containing EVs from CAFs in chemoresistant colorectal cancer cells (106). Also, miR-24-3p is transferred from CAFs to colon cancer cells targeting CDX2 and HEPH and promoting methotrexate resistance (107). Finally, prostate cancer cells acquire therapy resistance after miR-423-5p transfer in EVs from CAFs, which activates the TGF-β signaling pathway and controls Gremlin-2 expression (108).

However, miRNAs are not the only molecules relevant in therapy resistance delivered from CAFs to cancer cells. EVs containing Annexin-6 are transferred from CAFs to gastric cancer cells, thereby promoting therapy resistance though β1 Integrin/FAK-YAP activation (109). Moreover, lncRNA are delivered from CAFs to cancer cells. In fact, the lnc-RNA AFAP1-AS1 is present in CAF EVs and enhances the translation of ERBB2 mRNA by binding to AUF1, to induce the upregulation of HER-2 protein levels and subsequently trastuzumab resistance in breast cancer cells (110). Also, colorectal cancer associated lncRNA is transferred from CAFs to cancer cells through EVs and interacts with the mRNA stabilizing protein HuR (human antigen R) to increase β-catenin mRNA and protein levels, thereby promoting oxaliplatin resistance (111).

In summary (**Supplementary Table 5**), CAFs are resistant to therapy, and transfer proteins, miRNAs and lncRNAs in EVs to cancer cells. In doing so, CAFs induce therapy resistance. Thus, modulating either EV production by CAFs or their content could represent a novel therapeutic option for the treatment of non-sensitive tumors.

CSC-DERIVED EVS IN THERAPY RESISTANCE

In the TME, there are different types of cells that contribute to tumor progression, and specifically within tumors there is a small population with referred to as cancer stem cells (CSCs), which display the capacity of self-renewal, the ability to differentiate to other cell types and thereby to initiate, as well as maintain tumor

growth (112). These cells are held responsible for generating drug resistance in many types of tumors because they display several properties that permit escaping from the consequences of chemotherapy. Moreover, they also can convert into many cell types associated with drug resistance, as mentioned previously (6, 112–114). Consistent with the relevance of the TME, CSCs are considered a component of this pro-inflammatory network because CSCs express different cytokine receptors, which bind to inflammatory cytokines, such as interleukin (IL)-1, IL-6, and IL-8, present in the TME (115). Since drug resistance is one of the main properties of CSCs, EVs released by these cells can transfer therapy resistance to sensitive tumor cells by delivering specific molecules that activate a drug resistance phenotype in the recipient cells.

For example, in a hepatocellular carcinoma (HCC) model, CSCs were found to release larger amounts of exosomes, a subtype of EVs, in comparison with the non-CSC population of the tumor cells, and the secretion was mediated by Rab27a (116). Interestingly, the exosomes derived from the CSCs upregulate the expression of Nanog in recipient tumor cells and the acquisition of regorafenib resistance (116). To identify cells with CSC properties in the TME, several markers have been identified. A protein typically identified in several types of cancers is the transmembrane glycoprotein CD133 (117). For instance, Kang et al. reported that colon cancer cells release EVs containing CD133 in response to epidermal growth factor (EGF). In addition to activating the NF-κB signaling pathway, these EVs transfer the oncogenic protein KRAS to the recipient cells, thereby promoting the development of resistance against anti-EGF receptor (EGFR) drugs (118).

The CSCs are commonly found in hypoxic niches in tumors and hypoxia promotes CSC survival (119). In this context, Yin and colleagues observed that EVs derived from hypoxic glioma stem cells (GSCs) transfer temozolomide resistance to glioblastoma cells by delivering the miR-30b-3p, which targets RHOB to avoid apoptosis induced by the drug (120). Another study suggested that exosomes secreted by hypoxic glioma cells, which are enriched in CSCs, transfer the miR-301a and activate the Wnt/β-catenin signaling pathway by targeting TCEAL7 in glioblastoma cells, thereby promoting radiotherapy resistance (121).

A study in pancreatic cancer (PC) identified another miRNA responsible for therapy resistance mediated by CSC-EVs. Yang et al. reported that exosomes derived from pancreatic CSCs, which are resistant to gemcitabine, have high levels of miR-210. Transfer of this miRNA in exosomes to sensitive cells activates the mammalian target of rapamycin (mTOR) signaling pathway conferring resistance to gemcitabine-sensitive pancreatic cancer cells (122). In addition, CSC-EVs derived from OSCC contain miR-21-5p, another microRNA that activates mTOR. Such EV-mediated delivery of miR-21-5p and activation of the PI3K/mTOR/STAT3 signaling pathway in OSCC cells, leads to cisplatin resistance, increased clonogenicity and tumor sphere formation potential (123).

Another mechanism favoring the development of tumor cell resistance to anti-cancer therapies is activation of the EMT, because cells which activate this process acquire CSC properties (124). In this context, the role of exosomes as regulators of EMT has been investigated in many studies (125). Thus, by triggering this mechanism in recipient tumor cells, CSC-EVs also could transfer resistance to therapy. For example, it has been reported that miR-155 is an important regulator of EMT (126). Therefore, horizontal transfer of this miRNA mediated by EVs could confer resistance to therapy. Santos et al. demonstrated that exosomes derived from breast CSCs contain high levels of miR-155, and transfer of this miRNA to sensitive breast cancer cells reduces c/EBP-β activity, downregulate TGF-β and targets directly FOXO3a genes, resulting in the activation of EMT and acquisition of a chemoresistance phenotype against doxorubicin- and paclitaxel (127). In glioblastoma there is subtype of GSC called proneural (PN)-GSC and a more aggressive subtype called mesenchymal (MES)-GSC which display increased radio and chemoresistance. EVs derived from such MES-GSC cells increase stemness of normal PN cells, as well as therapeutic resistance to temozolomide, by inducing EMT through activation of the NF-κB/STAT3 signaling axis (128). Another example in which EMT is triggered by exposure to CSC-EVs has been reported for colon CSC-derived exosomes. These EVs contain Claudin-7, which induces EMT in low metastatic recipient cells, and likely also therapy resistance (129). Like CD133 in pancreatic cancer, CD44v6 is a marker of CSCs that promotes EV secretion. The transfer of such exosomes promotes resistance to apoptosis, as well as EMT in recipient cells by G protein-coupled receptor (GPCR) and integrin activation, transcription of EMT factors, and reduction of miRNA which target mRNAs from genes that contribute to self-renewal potential and migratory activity (130).

Finally, therapy resistance can be promoted indirectly by modulating the TME (131). CSC-derived EVs potentially modify the phenotype of many different types of cells in the TME and contribute thereby to therapy resistance. For instance, EVs liberated by renal CSCs promote *in vitro* the formation of capillary-like structures in matrigel (a proxy for vasculogenesis) and prevent doxorubicin-induced apoptosis in endothelial cells, which are required for tumor growth (132). In summary (**Supplementary Table 6**), CSCs display intrinsic properties that permit escaping from different types of anti-cancer treatments. Moreover, and quite importantly, they can transfer these properties *via* EVs to different cells present in the TME, which thereby become therapy resistant and this contributes to tumor progression.

EVS IN ANTIBODY-BASED CANCER THERAPY RESISTANCE

Several soluble pro-inflammatory factors released from cellular components of the TME activate signaling pathways in target cells that contribute to the tumor progression. Therefore, different therapies which block the interaction between such soluble factors and their receptors in cells have been developed. Antibody-based cancer therapy is one of the technologies used to

block such interactions. The antibodies either bind specifically to the soluble factor neutralizing its effect or can target the surface receptor of the soluble factor and block its interaction with the ligand, therefore precluding triggering pro-tumorigenic signals (133). Among the different antibody-based cancer therapies, antibodies are commonly employed which block signaling pathways that promote development of the pro-inflammatory TME, such as those against vascular endothelial growth factor (VEGF), epidermal growth factor receptor (EGFR) or human epidermal growth factor receptor 2 (HER2) (134). Unfortunately, although antibody-based cancer therapy has proven to be successful, some patients also develop resistance to these types of treatment by different mechanisms (135, 136).

In this context, there is evidence demonstrating that EVs also participate in the development of resistance to antibody-based cancer therapy (**Supplementary Table 7**). One example is the antibody therapy against HER2, a receptor of the EGFR family, that promotes pro-tumorigenic properties by triggering different signaling pathways and is overexpressed in the 25-30% of BC (137, 138). HER2 triggers the IL-1α pro-inflammatory signaling pathway, which is important for maintenance of the CSC phenotype in HER2-positive breast cancers (139). Trastuzumab is a monoclonal antibody against HER2 which has yielded positive results in the treatment of metastatic breast cancer in patients with tumors overexpressing HER2 (140). Ciravolo et al. observed in the serum of HER2 breast cancer patients and in conditioned medium of HER2-overexpressing breast cancer cells the presence of exosomes containing functional HER2 protein. Importantly, release of these exosomes is modulated by the activation of HER2 in response to two different ligands (141). Moreover, these exosomes containing HER2 have the capability to bind trastuzumab *in vitro*, suggesting they act as antibody sponges and contributing therapy resistance by reducing trastuzumab availability for therapeutic purposes (141). Another way in which EVs can contribute to antibody-based cancer therapy resistance was observed using EGFR as a target. In cancer, EGFR activity drives tumorigenesis in different types of cancer since sustained activation triggers signaling pathways favoring cell survival, proliferation and migration that all contribute to tumor progression (142). Like HER2, the EGFR promotes CSC-like activity and tumor progression by activation of pro-inflammatory signaling (143). For this reason, the EGFR is considered a good candidate for targeted therapy. At least four EGFR-specific antibodies are used in clinical settings, namely cetuximab, panitumumab, nimotuzumab and necitumumab (144). Unfortunately, here too cases have been reported where cancer patients develop resistance to the treatments involving these antibodies (145, 146). For instance, OSCC is one of the cancers typically treated with the anti-EGFR antibody cetuximab; however resistance to this drug has been observed, since OSCC release EVs containing EGFR in response to EGF or cetuximab. These EVs can bind to and sequester cetuximab providing thereby a mechanism to explain how resistance against therapeutic anti-EGFR antibodies can develop (147).

Tumor progression depends on multiple cellular process, but angiogenesis is considered one of the most important due to its relevance in supplying the primary tumor with oxygen and nutrients that promote growth, facilitate the dissemination of tumor cells to generate metastasis, and contribute to inflammation in cancer (148, 149). Therefore pro-angiogenic factors are excellent therapeutic targets for antibody-based cancer therapy. Particularly VEGF and its receptor are the most common angiogenic signaling molecules used as targets in the treatment of several types of cancer (150). Again, although such antibody-based treatments have a favorable impact on cancer patient survival, the effects are not permanent due to the development of resistance (151). In this context, EVs also contribute to the acquisition of resistance to therapies that target VEGF signaling. Bevacizumab is a humanized monoclonal anti-VEGF antibody used to treat several solid tumors (152). In glioblastoma, bevacizumab is used as a therapeutic agent to block angiogenesis (153). However, glioblastoma cells have the ability to internalize and sort the antibody to the surface of the EVs produced by these cells, as well as change the proteome of the EVs released, which in combination is associated with therapeutic resistance (154). VEGF also can be sorted to the surface of tumor cell EVs. An isoform of VEGF ($VEGF_{189}$) is preferentially found on the surface of the EVs, where in conjunction with heparin, it can sequester bevacizumab, thereby contributing to therapy resistance (155). Recently, other EV-specific mechanisms relating to anti-VEGF therapy resistance have been described. VEGF produced by tumor cells is captured by the protein CD63 present on the surface of EVs and packaged within the EVs in response to anti-VEGF therapy. This process reduces the accessibility of bevacizumab to the VEGF (156). On the other hand, the VEGF loaded inside the EVs can be internalized by endothelial cells where it triggers intracellular signaling events that promote angiogenesis and therefore generate resistance to the anti-VEGF therapy (156).

CONCLUSIONS

During the past decades our understanding of the mechanisms leading to therapy resistance has evolved from focusing exclusively on intrinsic properties of tumor cells to implicating also the inflammatory TME. Indeed, cells of the inflammatory TME are resistant to therapy and transfer this ability to tumor cells. EVs are relevant mediators of signaling between cells. In different contexts, EVs participate in physiological and pathological events. In cancer, EVs have been implicated in transformation, progression and metastasis, due to their ability to communicate between cancer cells and the tumor microenvironment. However, the role of EVs in transferring therapy resistance from stromal to tumor cells has only become apparent in more recent years. In this review, we summarized the studies describing the relevance of vesicles (generally defined as EVs following the ISEV guideline) in the development of therapy resistance following chemotherapy. In this context, EVs have been shown to transfer protein/miRNA/lncRNA cargos from the TME to tumor cells, to modulate survival, metabolism and EMT in these recipient cells (**Figure 1**).

After cancer therapy, the resulting inflammatory microenvironment contains tumor-resistant cells, hypoxic cells, CSCs, macrophages, adipocytes, and fibroblasts, which transfer

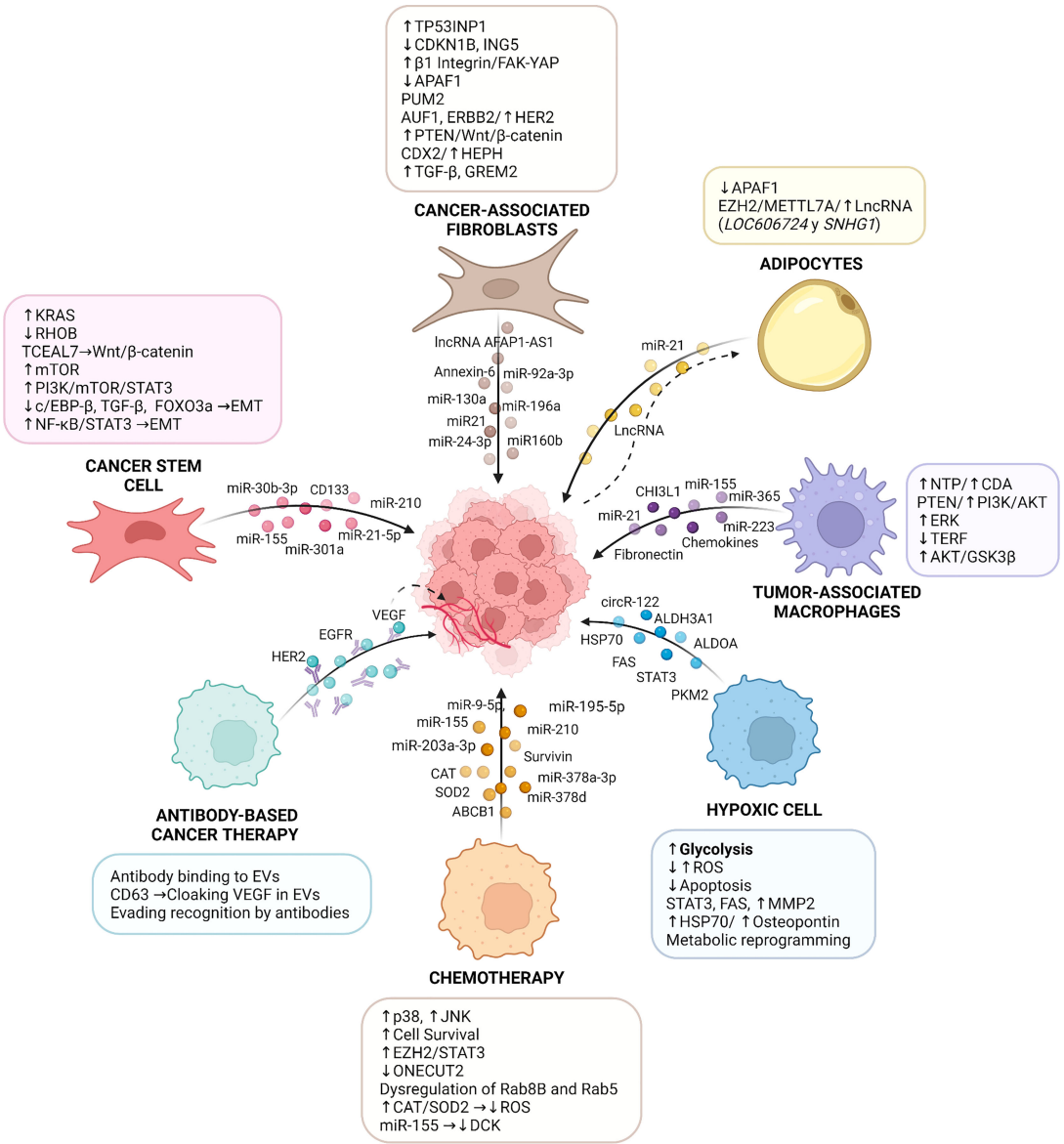

FIGURE 1 | Role of Extracellular Vesicles in Cancer Therapy Resistance. The tumor microenvironment (TME) is involved in the initiation and maintenance of resistance to therapies by multiple molecular mechanisms. Specifically, extracellular vesicles derived from TME cells (e.g., cancer-associated fibroblasts, endothelial cells, cancer stem cells, immune cells, and adipocytes) transfer a variety of bioactive molecules, including mRNA, miR, lncRNA and proteins, which all play important roles in the communication between stromal components and tumor cells, activating in the latter signaling pathways that lead to cancer therapy resistance. In addition, resistance to therapy can be triggered by an inflammatory TME caused by conditions, such as hypoxia, or following chemotherapy, which modulate the content and release of EVs and alter the responses of tumor cells to cancer therapies. EVs also participate in resistance to antibody-based cancer therapy where cancer-derived extracellular vesicles package elevated amounts of validated targets for cancer treatment (e.g., VEGF, EGFR, and HER2), which are recognized by therapeutic antibodies and compromise the response of cancer cells to these therapies. TP53INP1: tumor protein p53 inducible nuclear protein 1; CDKN1B: cyclin-dependent kinase inhibitor 1B; ING5: inhibitor of growth family 5; FAK: focal adhesion kinase; YAP: yes-associated protein 1; PUM2: pumilio homolog 2 protein; AUF1: AU-binding factor 1; HER2: human epidermal growth factor receptor 2; CDX2: caudal-related homeobox 2; HEPH: hephaestin; TGF-β: transforming growth factor β; GREM2: gremlin 2; APAF-1: apoptotic peptidase activator factor 1; RHOB: ras homolog family member B; TCEAL7: transcription elongation factor A-like 7; mTOR: mammalian target of rapamycin; PI3K: phosphoinositide-3-kinase; STAT3: signal transducer and activator of transcription 3; c/EBP-β: CCAAT enhancer binding protein-β; FOXO3a: forkhead box O3a; EMT: epithelial-mesenchymal transition; AFAP1-AS1: actin filament associated protein 1 antisense RNA 1; EZH2: enhancer of zeste homolog 2; METTL7A: methyltransferase like 7A; LncRNA: long noncoding RNA; SNHG1: small nucleolar RNA host gene 1; NTP: triphosphate-nucleotide; CDA: cytidine-deaminase; TERF: telomeric repeat-binding factor 1; GSK3β: glycogen synthase kinase 3 β; ROS: reactive oxygen species; FAS: fatty acid synthase; HSP70: heat shock 70 kDa protein; ONECUT2: factor One Cut Homeobox 2; ABCB1: ATP-binding cassette sub-family B member 1; CAT: catalase; SOD2: superoxide dismutase 2; DCK: deoxycytidine kinase; EGFR: epidermal growth factor receptor; VEGF: vascular endothelial growth factor; ALDH3A1: aldehyde dehydrogenase 3 family member A1; ALDOA: aldolase A; CHI3L1: chitinase 3-like-1. The figure was created with BioRender.com.

EVs to treatment-sensitive cells and promote therapy resistance. Several proteins (such as STAT3, fibronectin, Survivin), miRNAs (such as miR21, miR155, miR210), LncRNAs and circRNAs are common cargos of EVs involved in conveying resistance. These cargos activate signaling pathways (such as PI3K/Akt, ERK, RAS, FAK) in tumor cells, thereby inducing changes in metabolism, survival, metastatic potential, and subsequently therapy resistance. Moreover, another direct mechanism involved in therapy resistance is the transfer of the protein ABCB1 in EVs from therapy-resistant to sensitive cells. Uptake of ABCB1 by recipient cells enhances drug efflux and the acquisition of resistance to the cancer treatment. In addition, EVs can act as sponges that sequester antibodies used in antibody-based cancer therapy. An example here is the recruitment of trastuzumab which reduces its effects on cancer cells (**Figure 1**).

Taken together, this review highlights the relevance of EVs in the acquisition of therapy resistance after the development of an inflammatory tumor microenvironment following cancer treatment. By summarizing this literature, we hope to encourage the search for novel cancer treatments that also consider controlling EV production in the TME.

AUTHOR CONTRIBUTIONS

LS, SS, BG-R, MV-G, and AQ organized the entire manuscript, wrote the draft, and revised the last version of the manuscript. Figure was designed by SS. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.897205/full#supplementary-material

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Statins Lower Lipid Synthesis But Promote Secretion of Cholesterol-Enriched Extracellular Vesicles and Particles

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Lipid droplets are lipid-rich cytosolic organelles that play roles in cell signaling, membrane trafficking, and many other cellular activities. Recent studies revealed that lipid droplets in cancer cells have various biological functions, such as energy production, membrane synthesis, and chemoresistance, thereby fostering cancer progression. Accordingly, the administration of antilipemic agents could improve anti-cancer treatment efficacy given hydrophobic chemotherapeutic drugs could be encapsulated into lipid droplets and then expelled to extracellular space. In this study, we investigated whether statins could promote treatment efficacy of lipid droplet-rich ovarian SKOV-3 cells and the potential influences on generation and composition of cell-derived extracellular vesicles and particles (EVP). Our studies indicate that statins can significantly lower lipid biosynthesis. Moreover, statins can inhibit proliferation, migration, and invasion of SKOV-3 cells and enhance chemosensitivity *in vitro* and *in vivo*. Furthermore, statins can lower EVP secretion but enforce the release of cholesterol-enriched EVPs, which can further lower lipid contents in parental cells. It is the first time that the influence of statins on EVP generation and EVP-lipid composition is observed. Overall, we demonstrated that statins could inhibit lipid production, expel cholesterol to extracellular space *via* EVPs, and improve chemosensitivity.

Keywords: extracellular vesicles and particles, statins, cholesterol, lipidomics, ovarian cancer

INTRODUCTION

Lipid droplets (LD) are highly dynamic organelles in almost all kinds of mammalian cells, which play important roles in cell activities (1), including but not limited to energy storage, ATP production, membrane expansion, and signaling (2). The components of LDs are complex, which store thousands of kinds of lipids, lipoproteins, and relevant precursors (3–5). These

molecules participate in lipid metabolism and various biological behaviors of cells and tissues. Nevertheless, the biofunctions of these cargos are not fully understood yet. In cancer, metabolism of cancer cells is vigorous as cancer cells have increased energy requirements in comparison to normal cells (6). Moreover, LDs modulate the availability of proteins and signaling lipids, and their dysfunction may lead to disruption of cellular membranes or inappropriate nuclear signaling. Furthermore, LDs function as a place for detoxification. They isolate lipophilic anti-cancer drugs, and thus may contribute to chemoresistance (7–10). Correspondingly, damage or depletion of cytosolic lipid droplets could enhance anti-tumor efficacy through reduction of energy supply, blocking signaling pathways, improving chemosensitivity, and other mechanisms (11). Therefore, it was assumed that LD inhibitors could promote anti-cancer efficacy (10, 12, 13).

Statins are a group of lipid-regulating drugs that can significantly downregulate total cholesterol (TC) (14), low-density lipoprotein (LDL) (15), and triacylglycerol (TG), while upregulating high-density lipoprotein (HDL) (16). Currently, statins are mainly used to treat hyperlipemia and cardiovascular diseases. However, statins have also shown promising anti-tumor efficacy in combination with chemotherapy and immunotherapy (17–21). Although the exact anti-tumor mechanism of statins remains unclear, it might be associated with their lipid-regulation effect. For example, statins are β-hydroxy β-methylglutaryl-CoA (HMG-CoA) inhibitors that can block the mevalonate (MVA) pathway (22). Geranylgeranyl diphosphate, an intermediate product of the MVA pathway, can thereby be down-regulated by statins, which further inhibit the phosphorylation of Ras family proteins (23). The ripple effect may benefit cancer treatment through inhibition of cancer cell proliferation. Furthermore, statins can also inhibit adhesion and invasion of cancer cells through the downregulation of membrane proteins, such as VCAM-1 and integrin-β (24–26). In clinical treatment, the repurposing of well-tolerated and low-toxic statins in combination with chemotherapies have been reported to extend the overall survival of patients with breast cancer (27), ovarian cancer (28), colorectal cancer (29), and other cancers without a resulting increase in cytotoxicity to normal cells. Altogether, combination therapy with statins has been considered as a promising strategy for cancer treatment. It is noteworthy that concern has been expressed regarding the over-prescription of statin drugs as well as the potential for severe adverse effects from statin therapy at high doses (30). The adverse reactions of statins can affect a variety of organs. The most commonly affected ones are musculoskeletal, nerve, skin, gastrointestinal tract, liver, and gallbladder (31). Atorvastatin is a moderately lipid soluble statin with high potent and low toxicity, which can last longer in the body in comparison with other statin drugs (32). For example, atorvastatin cannot cross the blood-brain barrier and may prevent Alzheimer's disease in the long term without significant adverse effects (32). Therefore, atorvastatin with low dose was investigated in this study.

Small extracellular vesicles and particles (EVP) are lipid-bilayer enclosed particles with a size in the range of 30-300 nm (33). EVPs act as fingerprints of parental cells and they carry proteins, nucleic acids, and lipids (33). They can efficiently deliver these cargos to nearby or distant recipient cells (34). Numerous studies demonstrate that EVPs have a close relationship with tumor development, metastasis, and therapeutic resistance (35). Currently, EVP-derived proteins and nucleic acids are under intense investigation due to potential contributions in cancer liquid biopsy and the molecular mechanisms of cancer. On the other hand, EVPs are also rich with lipid contents, including cholesterol, ceramide, sphingomyelin, and phosphatidylserine (35), which are irreplaceable ingredients in the formation and function of EVPs. For instance, prostate hormone can be delivered to recipient cells *via* EVPs (36), and EVP-derived lipid molecules also participate in intercellular communication (37). Nevertheless, in comparison with proteins and nucleic acids, lipid cargo of EVPs has rarely been investigated thus far. In this study, we investigated the effect of atorvastatin on lipid-enriched ovarian SKOV-3 cells and analyzed lipid contents of EVPs derived from SKOV-3 cells. We found atorvastatin can significantly inhibit SKOV-3 cell proliferation, migration, invasion, and lipid synthesis without obvious cytotoxicity. Moreover, atorvastatin can significantly increase cellular chemosensitivity to paclitaxel (PTX) *in vitro* and *in vivo*. Furthermore, lipidomic sequencing data reveals that atorvastatin can inhibit EVP secretion and enforce the release of cholesterol-enriched EVPs to the extracellular space. It is the first time to observe the influence of statins on EVP generation and lipid composition of EVPs. Our findings reconfirmed statins can enhance anti-cancer treatment efficacy, preliminarily revealed the composition of EVP lipids derived from cancer cells, and may pave a new way for investigating the biologic functions of lipids in cancer biology and drug resistance.

RESULTS

Characterization of Cells

Ovarian cystadenocarcinoma SKOV-3 cell line was reported owning high cytosolic LDs (38). Stimulated Raman scattering (SRS) images also indicated that SKOV-3 cells own the highest LDs in comparison with that of lung adenocarcinoma H1975 cells, colorectal adenocarcinoma HT29 cells, and pancreas ductal adenocarcinoma PANC-1 cells (**Figure 1A**). Therefore, SKOV-3 cells were selected for the following studies. First, we optimized the dose of atorvastatin to avoid direct atorvastatin-induced cytotoxicity. Based on the result of CCK-8 assay, the optimal atorvastatin dose was determined to be 6 μM, at which the viability of SKOV-3 cells was over 90% (**Figure 1B**). After atorvastatin treatment, the morphology of SKOV-3 cells changed from a well-spread shape to a thin and filamentous shape (**Figures 1C, D**). The SRS images of LDs shown the overall signal intensity (average intensity by area) of LDs (bright dots in cytosols) decreased ~16.6% ($p<0.05$) after atorvastatin treatment (**Figure 1C**). The fluorescence images of dye-stained LDs in atorvastatin treated or untreated SKOV-3 cells further confirmed the decrease of LDs in

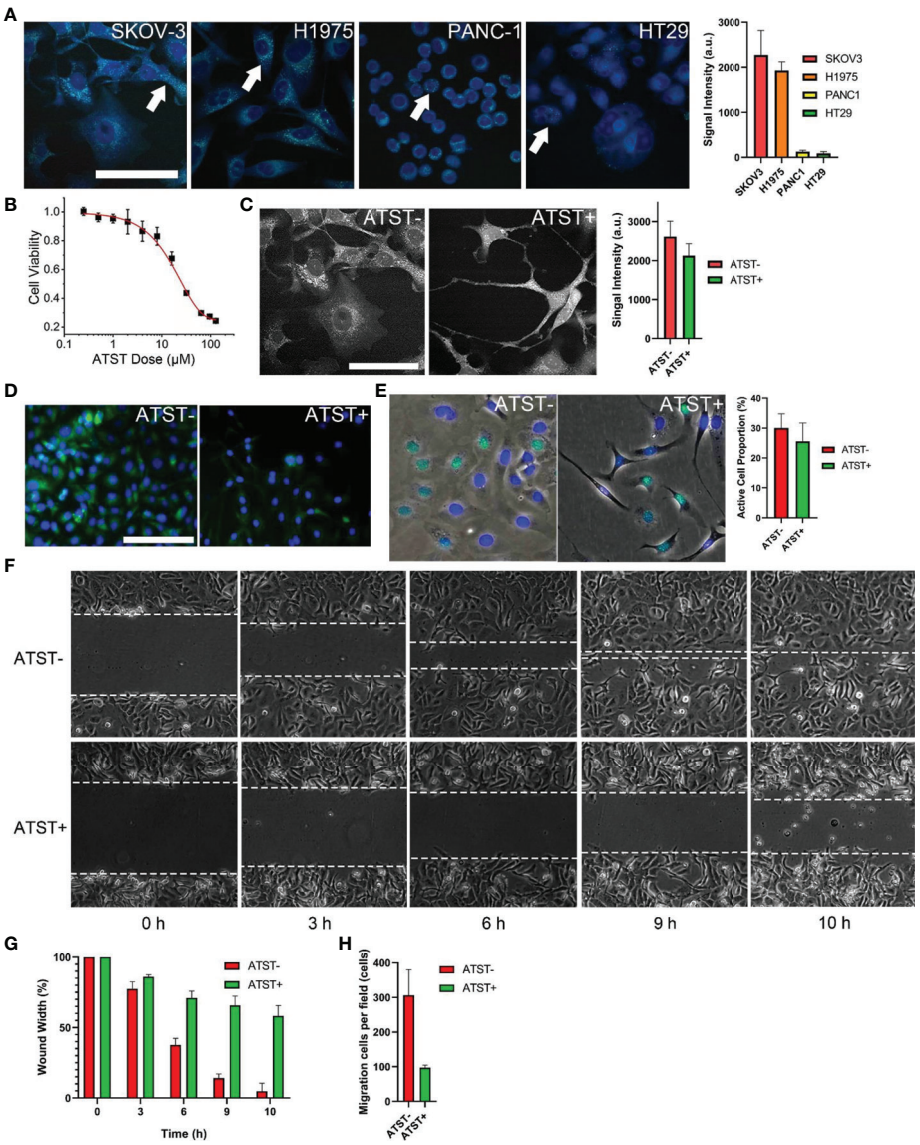

FIGURE 1 | Cell characterization **(A)** Lipid droplets (LD) in four cell lines and quantified signal intensity (scale bar is 10 μm, pseudo color generated by ImageJ). Arrows indicate LDs in cytosol. **(B)** IC_{50} of atorvastatin (ATST) for SKOV-3 cells. **(C)** SKOV-3 LDs under SRS microscopy and quantified signal intensity (scale bar is 10 μm). **(D)** Fluorescence imaging of dye-stained LDs in SKOV-3 cells (scale bar is 100 μm, PKH67: green; DAPI: blue). **(E)** EdU image of SKOV-3 cells (EdU: green; DAPI: blue). **(F)** Representative image of wound healing assay (n=3). **(G)** Quantification of *in vitro* wound healing assay. **(H)** Quantification analysis of trans-well migration assay.

cytosols (**Figure 1D**). Atorvastatin also inhibits SKOV-3 proliferation. EdU cell proliferation assay revealed the average proportion of SKOV-3 cells with active DNA synthesis dropped from 30.1% to 25.6% ($p<0.05$) after treatment with atorvastatin (**Figure 1E**). Wound healing assay demonstrated that atorvastatin could inhibit SKOV-3 cell migration. The average wound gap of untreated cells decreased to 14.1% at the 9-h time point, while the average wound gap of atorvastatin treated SKOV-3 cells remained at 65.7% ($p<0.01$; **Figures 1F, G**). Trans-well assay showed cell trans-well migration decreased ~3.2-fold after atorvastatin treatment ($p<0.05$; **Figure 1H**).

Combination Therapy *In Vitro* and *In Vivo*

SKOV-3 cells in the logarithmic growth phase were used to determine the IC_{50} of PTX. In the atorvastatin treated group, SKOV-3 cells were treated with 6 μM atorvastatin every 12 h for 48 h given the half-life of atorvastatin is ~7 h (39). CCK-8 assay revealed that the IC_{50} of PTX in the statin+ group was 0.43 nM (95% confidence interval: 0.31-0.59 nM), while that of the statin- group was 9.62 nM (95% confidence interval: 7.09-13.21 nM), which suggests the treatment efficacy of PTX in combination with atorvastatin was ~22.4-fold higher than monotherapy with PTX only (**Figure 2A**). Notably, few SKOV-3 cells survived even

though PTX dosage was high enough, while almost no cells survived under stress of PTX in combination with atorvastatin, indicating combination therapy could enhance cellular chemosensitivity. Next, the anti-tumor effect of PTX-atorvastatin combination was investigated *in vivo*. On the 28th day, the average tumor volume was 1289.3 mm^3 in the NC group. In contrast, tumor volume in the PTX only, atorvastatin only, and PTX-atorvastatin groups was 1165.7, 345.8, and 90.0 mm^3, respectively (**Figures 2B, C**). A significant difference in tumor size was found between the PTX treated groups and the non-PTX treated groups ($p<0.05$). Moreover, a significant difference in tumor size was found between the PTX only group and the PTX-atorvastatin group ($p<0.05$). There was no significant difference in mouse body weight during the 3-week administration period (**Figure 2D**).

Characterization of EVPs

The SKOV-3 derived EVPs were characterized by TEM after isolation and purification. EVPs showed a typical saucer shape under microscope (**Figure 3A**). EVP size ranged from 30 nm to ~300 nm, measured by nanoparticle tracking analyses. The average size of EVPs in the control group and EVPs in the atorvastatin treated group were 109.9 nm and 102.1 nm ($p<0.001$), respectively (**Figure 3B**). Moreover, atorvastatin decreased EVP generation rate to 1.53×10^4 EVP/cell/h compared to 3.29×10^4 EVP/cell/h in the control group ($p<0.001$). The internal reference protein, GAPDH, as well as classical EVP protein markers, including TSG101, CD81, and CD63 extracted from EVP protein lysates and cell lysates were detected by Western blot (**Figure 3C**). The expression level of these proteins did not show significant alterations (<1.3-fold; $p>0.05$).

Lipidomic Sequencing

Lipidomic sequencing was used to analyze SKOV-3 cells derived lipids (statin-), atorvastatin treated SKOV-3 cells derived lipids (statin+), SKOV-3 EVPs derived lipids (EVP-), and atorvastatin treated SKOV-3 EVPs derived lipid (EVP+). A total of 2608 different lipids were identified from cells, and 2124 lipids were identified from EVPs based on untargeted lipidomic analysis. The differences in lipid profiles between four groups was visualized by principal component analysis (PCA) which revealed significant intergroup difference (**Figure 4A**). The intra-group variation in lipids derived from cells was lower than that derived from EVPs (**Figure 4A**). The highest batch-to-batch variation in lipid contents was observed in the EVP+ group. In two cell groups (statin+ vs. statin-), volcano plot shows 891 downregulated lipids and 275 upregulated lipids after statins treatment (fold change >2). In two EVP groups (EVP+ vs. EVP-), there were 1430 upregulated lipids and 15 downregulated lipids after statins treatment (fold change >2) (**Figure 4B**). The result of lipid abundance analysis showed the expression level of common high abundance lipids, such as sterol, diacylglycerols (DG), ceramide, and phosphorylated esters, significantly decreased after atorvastatin treatment in SKOV-3 cells, but significantly increased in EVPs (**Figure 4C**). Moreover, in terms of sterol, cholesteryl easter (CE) 18:1 and CE 18:2 showed significant differences both in cells and EVPs (**Figure 4D**). TG was slightly increased, while DG was significantly decreased in cells (**Figures 4E, F**).

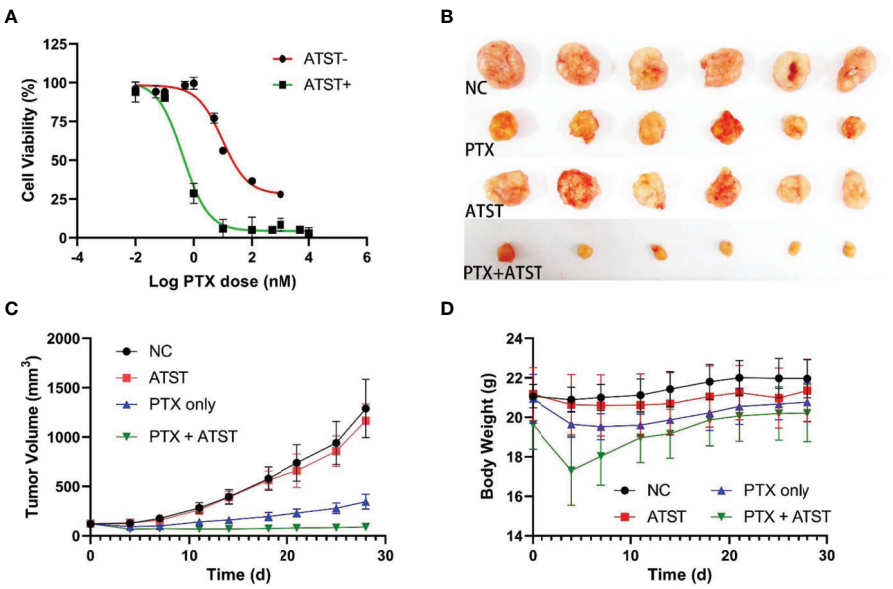

FIGURE 2 | **(A)** IC_{50} of paclitaxel (PTX) in atorvastatin (ATST) treated and untreated SKOV-3 cells, respectively. **(B)** Tumor volume in mice treated with PBS, PTX only, atorvastatin only, and PTX-atorvastatin combination at Day 28. **(C)** Dynamic changes of tumor volume in each group for 28 days. **(D)** Dynamic changes in body weight of mice in 28 days.

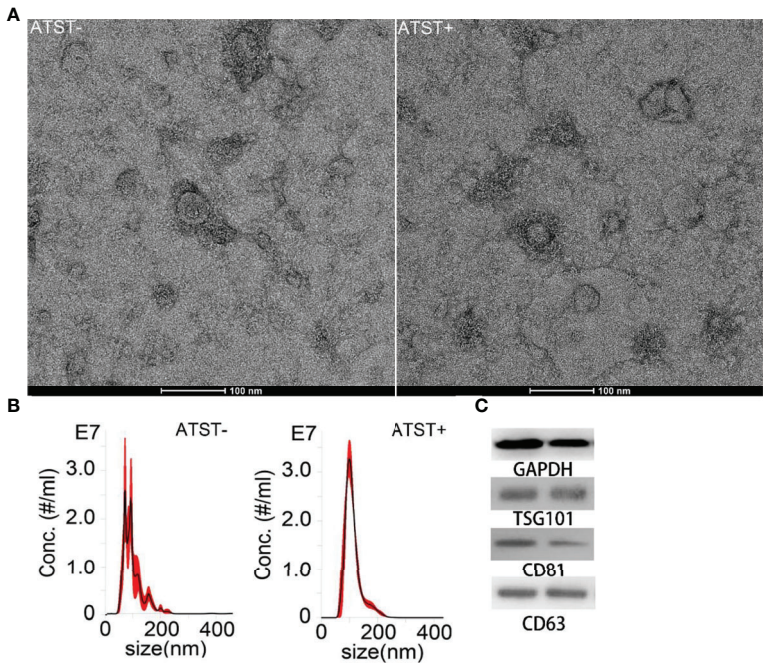

FIGURE 3 | EVP characterization. **(A)** TEM image of EVP derived from cells treated or untreated with atorvastatin (scale bar is 100 nm). **(B)** Size distribution of EVPs derived from cells treated or untreated with atorvastatin. (Left: Atorvastatin- (ATST-) EVPs; Right: ATST+ EVPs). **(C)** Western blotting analysis of EVPs derived from cells treated (right) or untreated (left) with atorvastatin.

DISCUSSION

Lipid metabolism is closely involved in cellular functions (40). However, the relationship between lipid metabolism and tumors is overly complex with very limited understanding of relevant mechanisms thus far. Undeniably, altered lipid metabolism is among the most prominent metabolic alterations in cancer. Enhanced uptake or synthesis of lipids contributes to rapid cancer cell growth, tumor formation, and drug resistance. It has been reported that many tumor cells, including ovarian cancer, showed increased cholesterols uptake and synthesis compared with normal cells (41). Naturally, LDs were accumulated in cytosol of tumor cells as energy source under stress. On the other hand, cholesterol metabolism depends on the MVA pathway which is heavily involved in the synthesis of various cellular membrane components and organelles. In tumor cells, the MVA pathway loses its feedback inhibition, and thus a large amount of cholesterol is synthesized. The synthesized cholesterol is further used for membrane formation, which supports fast division of tumor cells. Moreover, cholesterol is enriched in lipid raft (42). The massive production of cholesterol thereby facilitates the expression of several tumor related signaling proteins, such as CD24 (promoting angiogenesis) (43), TGFβ (promoting epithelial-mesenchymal transition) (44), matrix metallopeptidase (promoting migration) (45), and CD44 (promoting adhesion) (46), which need to anchor on a lipid raft. In addition, studies found that lipids reduce chemosensitivity of tumor cells. For instance, tyrosine kinase inhibitor (TKI) resistant lung cancer cell lines, HCC827GR, H1975, and PC9GR, have more LDs than TKI sensitive cell lines, and cancer cells treated with oleic acid can restore TKI sensitivity (47). Overall, cancer cells demand lipids for proliferation, migration, invasion, and drug resistance. Correspondingly, we hypothesized that reducing or altering lipid metabolism could inhibit cancer development and restore drug sensitivity.

Repurposing of statins for cancer treatment may achieve the above-mentioned goals. First, statins can inhibit HMG CoA reductase, further restrain MVA pathway and lipid synthesis, and finally reduce cellular activities (48). Second, the expression of certain proteins, e.g., hormone receptors, can be downregulated by statins (49, 50), and thus affect cancer cells. Third, at the nucleic acid level statins can induce the expression of certain small RNAs that can down-regulate the expression of LDL receptors and thus inhibit cancer cells *via* reduced cholesterol intake (51). Fourth, statins may also lower ATP production and inhibit efflux pumps on membranes. Efflux pumps require ATP to transport foreigners, including chemical drugs, from cytosol to the extracellular space (52). The downregulation of intracellular cholesterol level induced by statins can activate sterol regulatory element-binding protein-2 (SEBP2) gene which can further promote lipoprotein uptake. Meanwhile, the SEBP2 can downregulate the expression of efflux pumps (53), and thus statins can retain chemotherapy drugs within the cytosol. Last, statins can reduce the number of cytosolic LDs. Instead of being trapped in LDs and expelled to extracellular space through exocytosis, lipophilic anti-cancer drugs can efficiently stay within cytosols, interact with target molecules, and exert therapeutic functions. In this study, we observed that

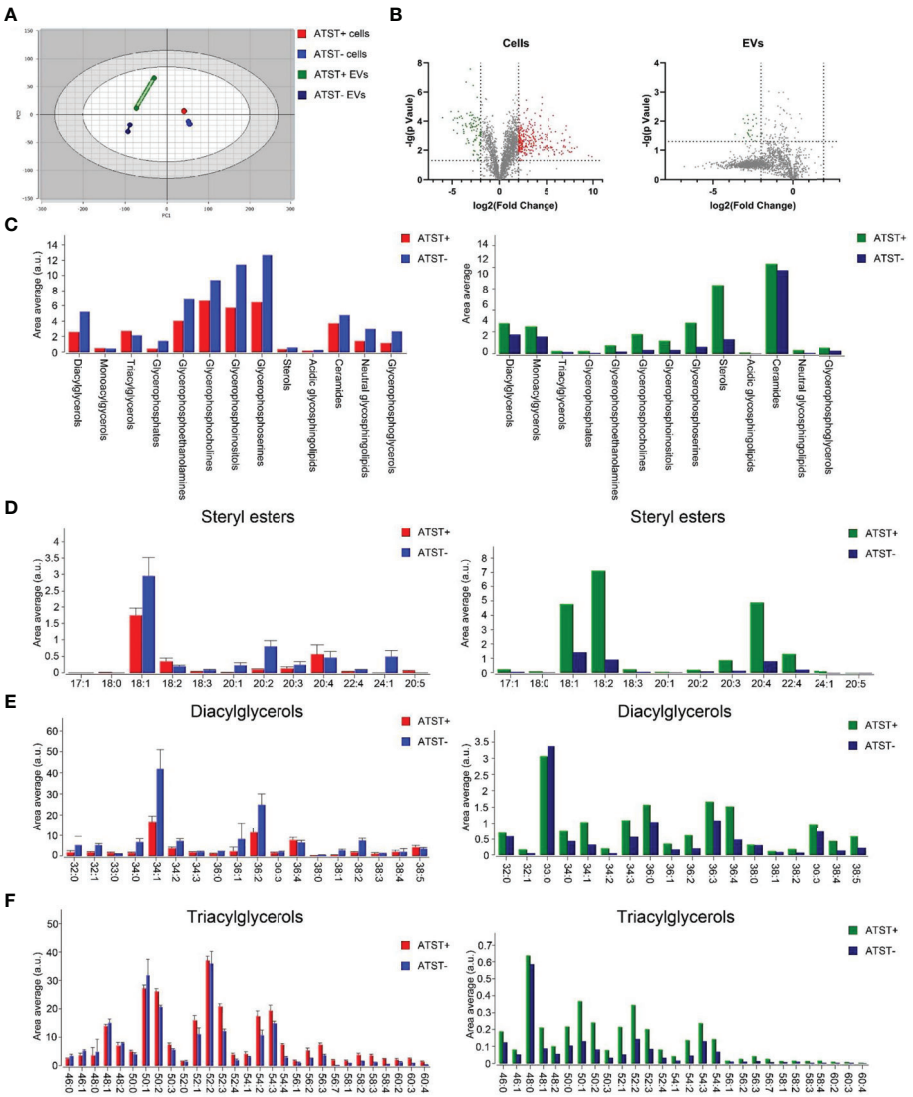

FIGURE 4 | **(A)** PCA plot lipid feature of each group (Red: Atorvastatin+ (ATST+) cells; Blue: ATST- cells; Green: ATST+ EVPs; Blue: ATST- EVPs). **(B)** Volcano plot comparing the lipid composition in cell samples (left) and EVP samples (right). **(C)** Lipid abundance plot by lipids in cell samples and EVP samples. **(D)** Difference in abundances of stearyl ester between cell samples (left) and EVP samples (right). **(E)** Difference in abundances of DG between cell samples (left) and EVP samples (right). **(F)** Difference in abundances of TG between cell samples (left) and EVP samples (right).

atorvastatin can significantly inhibit SKOV-3 cells and increase the chemosensitivity *in vitro* and *in vivo*, which is in line with previous studies (54–57). Notably, we optimized atorvastatin dose and ensured atorvastatin did not show significant cytotoxicity. Therefore, the improved cytotoxicity of PTX was not contributed by the toxicity of atorvastatin itself. We speculated the aforementioned statins' functions might be exerted simultaneously in assisted chemotherapy, although the exact mechanisms are unclear.

We noticed the EVP generation rate was significantly reduced by atorvastatin. Given EVP cargos derived from cancer cells can promote cancer metastasis, this finding indicates atorvastatin may inhibit the formation of premetastatic niche, influence tumor microenvironment, and decrease organotropic metastasis. As to the decreased EVP generation rate, it might be caused by altered cholesterol level in SKOV-3 cells. Cholesterol is an essential component of mammalian cells. The concentration of cholesterol on plasma membranes is much higher than that in other cellular compartments (58). Because atorvastatin directly inhibits synthesis and uptake of cholesterol, inherently membrane synthesis can be inhibited. Subsequently, EVP production is affected due to inadequate membranes for EVP assembly and release. Moreover, atorvastatin participates in G protein modification, which negatively influences the self-assembly of cytoskeletal components and the transportation of

lipoproteins (59). Consequently, the EVP generation can be decreased in a non-lipid dependent way.

Lipid analysis of EVP reported that EVP-derived lipid contents are different from that of parental cells (60), which may relate to the biogenesis of EVPs (61). Therefore, we further performed lipid sequencing. Overall, lipid sequencing data validated the atorvastatin lowered the lipid abundance in cells. But atorvastatin did not always downregulate lipids. For example, the abundance of DG decreased while that of TG raised (**Figure 3C**). Although both TG and DG are the key components of LDs, they play different roles in cellular functions. TG often functions as energy storage and is more related to maintaining cell survival. When the cell is under stress, TG will be upregulated to maintain a relatively mild metabolic environment, which was exactly in line with our lipid sequencing data. Moreover, TG in LD can be hydrolyzed to form DG. In contrast, phosphorylated DG can form a series of new second messengers and participate in various signaling pathways. DG itself can also bind to a variety of receptors which are mainly related to cell proliferation. In living cells, DG and TG regulate each other, and thus cells can keep a balance between growth and proliferation. Under the pressure of atorvastatin, the imbalance of the ratio of TG and DG can significantly alter the cell cycle of SKOV-3 cells. The altered cellular cycle may convert a cold tumor to a hot tumor, which can increase sensitivity to immunotherapy to a certain extent.

On the contrary, the abundance of lipids was significantly increased in EVPs derived from atorvastatin-treated SKOV-3 cells (**Figure 3C**). It was reported that cholesterol in excess of the current cellular demand is either exported from the cell by ATP-binding cassette transporters, or converted to less toxic cholesteryl esters and then stored in lipid droplets or secreted within lipoproteins (62). Therefore, we assume surplus lipid components, especially stearyl esters, can attach onto lipoproteins, and the complex can be further encapsulated into EVPs for extracellular secretion. Moreover, the large number of lipid rafts composed by sphingolipids and cholesterol may promote lipid raft-mediated EVP endocytosis of recipient cells (63). Given macrophages are primarily responsible for the rapid clearance of EVPs from the bloodstream, which drastically limits the amount of EVPs that are available to reach the recipient cells and tissues (64), we speculate that atorvastatin-induced lipid-enriched EVPs could be efficiently cleared by macrophages (65). The risk of cancer metastasis thereby can be further reduced. On the other hand, because many receptors are anchored on the lipid raft, we assume that it would be more feasible to screen tumor biomarkers by analyzing atorvastatin-induced lipid-enriched EVPs derived from cancer cells. The results of untargeted lipid sequencing also confirmed that parental cells and EVPs have a significantly different lipid composition. The abundance of lipids other than cholesterol esters in cells was significantly higher than that in EVPs, suggesting that EVPs are rich with lipid rafts but poor with energy storage.

In conclusion, atorvastatin can significantly inhibit ovarian SKOV-3 cell proliferation, migration, and invasion. Meanwhile atorvastatin increases chemosensitivity of SKOV-3 cell *in vitro* and *in vivo*. Moreover, atorvastatin can reduce EVP generation, which may lower the risk of cancer metastasis. Lipid sequencing data revealed the significant differences in lipids derived from parental cells and respective EVPs. Only TG level was upregulated after atorvastatin treatment. In contrast, all lipids were upregulated in EVPs derived from atorvastatin treated SKOV-3 cells. The potential influence of these changes is unclear yet, but we speculated these ripple effects may benefit atorvastatin treated patients from chemotherapy and immunotherapy. In our future work, we will further explore the association between EVP-derived lipids and cancer progression, screen potential EVP lipids as diagnosis or prognosis markers, and investigate the treatment efficacy of immunotherapy in combination with statins.

METHODS AND MATERIALS

Cell Culture

The human cancer cell lines, including SKOV-3 cells, H1975 cells, HT29 cells, and PANC-1 cells, passed the mycoplasma test throughout the whole experiments. Cells were cultured in DMEM with 5% FBS and 100 IU/mL penicillin-streptomycin at 37°C, 5% CO_2, and 95% humidity. Before collecting EVPs, the cells were kept in FBS-free medium for at least 24 h.

Optimization of Atorvastatin Dose

Approximately 1000 SKOV-3 cells were seeded into each single well in a 96 well plate. The plate was incubated at 37°C, 5% CO_2, and 95% humidity for 24 h. Then, 100 µl of atorvastatin with concentration gradient was added to each well. After incubation for 48 h, 10 µl of CCK8 solution was add to each well followed by 1 h incubation at 37°C. The absorbance at 450 nm was read to calculate cell viability. Three biological replicates were prepared.

Lipid Droplet Imaging

The cells were divided into two groups. In the experimental group, cells were treated with 6 µM atorvastatin for 48 h followed by fixation with 4% paraformaldehyde. Images were taken by homemade SRS microscopy which was built by Biophotonics and Translational Optical Imaging Lab. Signal intensities of LDs were analyzed with ImageJ.

Cell Proliferation Assay

The protocol of EdU can be found elsewhere. In short, an appropriate number of cells at logarithmic phase were seeded in two 60-mm petri dishes. In the experimental group, 6 µM of statin was supplied while normal DMEM was supplied in the control group. After 48 h incubation, prewarmed 37°C EdU working solution was added. The dishes were continually incubated for 2 h before fixing by 4% paraformaldehyde. Then, the dishes were washed by PBS 3 times followed by treating with 0.3% Triton X-100 solved in PBS. Triton X-100 was removed, and the dishes were washed another 3 times by PBS. Before imaging, click reaction buffer, $CuSO_4$, biotin azide, and click additive solution were added to each dish based on the manual. The cells were further stained with DAPI. The image was analyzed by ImageJ.

Cell Wound Healing Assay

Approximately 5×10^5 cells were seeded in a 6 well plate and incubated overnight for firm surface attachment. The wound was created by scratching with a tip. The plate was washed 3 times with PBS to remove detached cells and debris. Images were taken at 0-, 3-, 6-, 9-, and 10-time points under microscope. The data were analyzed by MATLAB.

EVP Harvest and Characterization

In total, 200 ml of cell culture supernatant was collected followed by centrifugation to remove intact cells (500 g for 5 min) and cellular debris (20,000 g for 15 min). EVPs were isolated by ultracentrifugation following MISEV2018 EVP isolation protocol. The EVP pellets were resuspended in 30 μl of PBS and cryopreserved at -80°C. EVP concentration and size distribution were determined with Nanosight NS300. Five microliters of EVP samples was placed on 300 mesh grids and incubated for 3 min at room temperature (RT). Excess samples were blotted with filter paper and stained with 1% uranyl acetate for 5 min. Samples were then examined under TEM (Hitachi). The Western blot was routinely performed. After lysis with RIPA buffer, Mini-PROTEAN Tetra Handcast System (BioRad) and Trans-Blot Turbo Transfer System (BioRad) were used for electrophoresis and subsequent transferring. The protein blot was blocked for 1 h with 5% skimmed milk in PBS/0.05% Tween 20 and incubated for 6 h at 4°C with Santa Cruz Biotechnology HRP conjugated antibodies against TSG-101 (sc-7964, 1:500), CD81 (sc-166029,1:500), CD63 (sc-100304, 1:500), and GAPDH (sc-47724, 1:1000). Samples were washed with PBS/0.05% Tween for 10 min 3 times. Blots were developed with chemiluminescence (BioRad).

Lipid Extraction

Two milliliters of mixture containing chloroform, methanol, and water (2:1:1) was added to sedimented cells followed by vortex and centrifugation (20,000 g for 10 min). The lower organic phase was collected. Approximately 50 μL of formic acid and 1 mL of chloroform-methanol-water mixture were added to the remaining aqueous phase solution followed by vortex and centrifugation (20,000 g for 10 min). The organic phases obtained twice were mixed and dried. EVP derived lipids were extracted following the same protocol. Lipid sequencing was performed by Cayman Chemical Company.

In Vitro Therapy

All animal experiments were approved by and performed in accordance with guidelines from the Institutional Animal Care and Use Committee (IACUC) of the Model Animal Research Center of the Wuxi People's Hospital Affiliated with Nanjing Medical University (Wuxi, China). The approval number was 2021-0168. The BALb/c nude mice age in 6-8 weeks with weights of 18-22 g were used. To establish a xenograft model, adult female BALb/c nude mice were s.c. injected under anesthesia with 5×10^6 SKOV-3 cells resuspended in mixture of Matrigel (Corning) and PBS (1:1). The mice were divided into 4 groups and were respectively injected with PBS (negative control, NC group), atorvastatin (ATST), PTX (PTX group), and a mixture of PTX and atorvastatin (PTX+ATST group). The body weight and tumor size of each group were measured every 84 h.

Statistics

Data analyses were carried out using SPSS 23 software program. The statistical significance was determined by Student's t-test and ANOVA test. All tests were two-sided, and p-values <0.05 were considered statistically significant.

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 authors.

AUTHOR CONTRIBUTIONS

YC, WM, and YW designed the research. YC and YX conducted experiments and analyzed data. JW, PP, YY FL and MZ assisted in experiments. YC, YX, and YW wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Extracellular Vesicles and Hepatocellular Carcinoma: Opportunities and Challenges

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The incidence of hepatocellular carcinoma (HCC) is increasing worldwide. Extracellular vesicles (EVs) contain sufficient bioactive substances and are carriers of intercellular information exchange, as well as delivery vehicles for nucleic acids, proteins and drugs. Although EVs show great potential for the treatment of HCC and their role in HCC progression has been extensively studied, there are still many challenges such as time-consuming extraction, difficult storage, easy contamination, and low drug loading rate. We focus on the biogenesis, morphological characteristics, isolation and extraction of EVs and their significance in the progression of HCC, tumor invasion, immune escape and cancer therapy for a review. EVs may be effective biomarkers for molecular diagnosis of HCC and new targets for tumor-targeted therapy.

Keywords: hepatocellular carcinoma, tumor microenvironment, hypoxia, vesicle drug delivery, extracellular vesicles

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1 INTRODUCTION

China is the world's top liver cancer country, and the 2020 Global Oncology Report showed that 906,000 patients of liver cancer occurred worldwide, of which 410,000 new cases occurred in China, accounting for >45% (1, 2). HCC is a common and fatal cancer, accounting for approximately 90% of all liver cancer cases (3). Although much progress has been made in diagnostic and treatment of HCC, such as liver excision, chemotherapy embolism and Sorafenib, it remains a health problem worldwide, with the incidence expected to exceed one million cases in a few years, due to its metastatic nature, high recurrence rate and low long-term survival (4, 5). EVs exist in tissues, various body fluids and supernatant, such as saliva (6), pleural effusion (7, 8), plasma (9, 10), urine (11), breast milk (12, 13), cerebrospinal fluid (14) and ascites (15, 16), which are greatly released by a variety of cells in a constitutive or inducible manner. EVs can regulate many biological processes, such as migration and extracellular matrix remodeling (17). Recently, some studies have shown that EVs play an important part in regulating cell signaling. Particularly, HCC cell-derived EVs may lead to local spread, distant metastasis and multifocal growth (18). HCC cell can secrete more EVs and promote tumor metastasis. After exposure to anti-tumor drugs, the release of EVs from hepatoma

cell also increased, which activate natural killer cells and induce anti-tumor immunity. Besides, tumor cell-derived EVs can produce direct immune effects to stimulate target cells. It has been reported that EVs-mediated intercellular transfer may promote the invasion of HCC by affecting the tumor microenvironment (TME) (18, 19). EVs-mediated signaling in liver disease makes them a unique therapeutic tool that can provide targeted delivery of tissue siRNAs, miRNAs and circRNAs to affect gene expression (20). Notably, EVs are natural nanomaterials. Compared with drugs, modified EVs have many advantages, which significantly improve the specificity, efficacy, and safety of EVs-based cancer therapies and become ideal candidates for drug development and delivery (20). Nowadays, the use of biogenic EVs as drug delivery has become a research hotspot, and its complex phospholipid membrane structure may be conducive to immune escape, site-specific transmission, cell uptake and intracellular transport (21). In addition, some microRNAs in EVs have also been introduced as potential biomarkers, and their expression level is related to the invasiveness of HCC (22). It has been reported that EVs play a key role in biological functions, including intercellular transfer, angiogenesis, immune response, tumor growth and metastasis of HCC (23–25).

2 INTRODUCTION OF EVS

2.1 Biogenesis and Morphological Characteristics of EVs

It is known that EVs can be a key role in human physiological and pathological diseases with various subtypes of cell-released membrane structures. EVs of particle diameters <200 nm are referred to as small EVs (sEVs) and medium-to-larger particles of diameters >200 nm are referred to as m/lEVs (26). Depending on the description of conditions or cell of origin, EVs can also be classified as apoptotic body, large oncosome, hypoxic EV, podocyte EV, etc, which are showed as follows (22, 26–28) (**Table 1**).

The sEVs (<200nm) originate from the inward outgrowth of endosomal membranes, are one of subpopulations of EVs (30), which can be produced from different cells such as hepatocytes (40), NK cells (41), T cells (42), and B cells (43), and surface markers of sEVs include CD9, CD63, CD81, and CD82 (44). sEVs are formed by the endonuclear body system and transmit information to the recipient cell through three main processes: First, the cytoplasmic membrane is initially invaginated by lipid raft-mediated endocytosis to form endocytic vesicles, which fuse with each other to form early endosomes (Endocytosis) (45); Second, early intranuclear bodies regenerate and invaginate, and intracellular material forms multiple intraluminal vesicles (ILVs), which are further transformed into late intranuclear bodies and multivesicular bodies (MVBs). This process also involves the inversion of cytoplasmic contents, transmembrane proteins, and peripheral proteins (Receptor-ligand Interaction) (46). Finally, MVBs fuse with the cytoplasmic membrane to form sEVs (Fusion With the Plasma Membrane) (5, 23, 47). In addition, MVBs have also been reported to fuse with lysosomes and promote the degradation of vesicle contents (27, 44, 48). The formation, release and sorting of sEVs are a series of regulated processes, which mainly require the endosomal sorting complex required for transport (ESCRT), members of the ESCRT family [apoptosis contiguous gene 2-interacting protein X (ALIX), also called PDCD6IP (49), tumor susceptibility gene 101 (TSG101)] (50, 51), four transmembrane proteins family (49, 52) and lipid raft-associated proteins (53, 54) and many substances are involved. As we all know, ESCRT is composed of ESCRT-0, ESCRT-I, ESCRT-II and ESCRT-III (55), and is associated with delivery of ubiquitinated proteins, degradation of lysosomes and recycling of proteins (20). Moreover, ESCRT plays an important part in luminal vesicle biogenesis and cargo aggregation (49). ESCRT-independent processes also seem to be involved in the formation and secretion of sEVs in an intertwined manner (56). Intracellular transport of sEVs involves many molecular switches, such as RAB GTpase proteins, membrane linked proteins, actin and microtubulin (23). Besides, Rab family proteins,including Rab7, Rab11, Rab27, and Rab35, also play a crucial role in the process of sEVs secretion (25). The secretion of sEVs also requires the involvement of the SNARE complex and the synaptic binding protein family (30). Furthermore, the involvement of sphingomyelinase in vesicle release was confirmed by the elevated ceramide levels in sEVs and less release of sEVs after sphingomyelinase inhibition (20, 56). Overall, sEVs regulate signaling pathways in receptor cells, coordinate TME and communication between different cells.

The m/lEVs (>200nm) are released by the plasma membrane to the outgoing buds, so the membrane composition of the m/lEVs is extremely close to the plasma membrane. The cell membrane surface is full of phosphatidylserine and most of the membrane-associated proteins, which can regulate the intercellular information exchange and affect the functions of target cells (30). The mechanism of m/lEVs formation is related to intracellular calcium signaling stimulation (23, 57), membrane bending proteins and the asymmetric distribution of phospholipids. The inward flow of calcium ions in the cytoplasm activates phospholipid crawling enzymes to disrupt phospholipid asymmetry, leading to redistribution of phospholipids in the cell membrane bilayer (58). The junctional protein ARRDC1 recruits ESCRT proteins and VPS4 (an ATPase) to the cell membrane (59); ESCRT-1 protein interacts directly with inhibitory proteins; pro-caspase3 stimulates Rho-related protein kinase 1 to promote apoptogenesis and induces myocardin contraction, contributing to the release of m/lEVs.

Apoptotic body (50-2000 nm), also known as apoptotic vesicles, are produced by debris cells that undergo apoptosis (60, 61). When cells undergo apoptosis, the cell membrane folds inward and wraps around the cytoplasm, organelles and nuclear fragments to form vesicles, which are the largest subpopulation of EVs. Apoptotic vesicles have surface markers and are enriched in caspases-3 and caspases-7, caspases-3 and Rho/Rock pathway taking part in membrane blistering (30, 32, 62). Moreover,

TABLE 1 | The types of EVs.

Classification	Subtypes	Diameter	Source	Marker	Ref
Physical characteristics/ Size	sEV	< 200nm	Originates from the inward outgrowth of multivesicular bodies (MVB), endosomal system Derived from hepatocytes, macrophages, NK cells, T cells, B cells	Transmembrane proteins CD9, CD63 and CD81; ALIX; TSG101	(5, 22, 29)
	m/lEV	> 200nm	Plasma membrane outward budding production Derived from almost all healthy living cells.	Integrin; Selectin; CD40; Most membrane-associated proteins in source cells	(30, 31)
Descriptions of conditions/Cell of origin	Apoptotic body	1-5μm	generated from cell fragments undergoing apoptosis	Phosphatidylserine; Genomic DNA; It is similar to the surface markers of its derived cells and rich in caspases-3 and caspases-7	(30, 32)
	Large oncosome	1-10μm	originates from the shedding of the membrane bubbles released by Invasive prostate cancer cells, urinary bladder, and glioblastoma	CK18	(33–36)
	Hypoxic EV	–	Hypoxic cell	include mRNA and proteins (MMPs, IL-8, PDGFs, caveolin 1, and lysyl oxidase, etc)	(37, 38)
	Podocyte EV	–	from the tip of the microvilli of the podocytes	—	(39)

apoptotic vesicles play a key role in attracting phagocytes, promoting the clearance of apoptotic cell debris, and regulating antigen presentation and immune cell responses (30). Apoptotic cells have been reported that can facilitate the encapsulation of chemotherapeutic drugs or nanoparticles into EVs (22). In addition, apoptotic vesicles from apoptotic cells can be preferentially taken up by macrophages and produce antitumor effects (22). Thus, apoptotic vesicles may also be an ideal delivery system, but the use of apoptotic vesicles as therapeutic nanovesicles (NVs) has been less studied, which may be related to their large cell size and uneven distribution.

Large oncosomes(1-10 μm) are released by cancer cells and may play a role in the tumor microenvironment. It has been shown that CK18 is a marker of large oncosomes and can be identified in circulation and tissues (63).

The mechanism of production of hypoxic EVs may depend on hypoxia-inducible factors and RAB22A, which in a hypoxic environment relies on the mediating action of the small GTPase RAB22A to dislodge hypoxic EVs from the cells (38). Hypoxic EVs are influenced by the environment and containing biomarkers such as mRNA and proteins, among which proteins include MMPs, IL-8, PDGFs, caveolin 1, and lysyl oxidase (37).

Podocyte EVs (100-200 nm) derived from the tip vesicles of podocyte microvilli (39). It can be expressed before other markers of nephropathy and therefore may serve as a new marker of glomerular and tubular injury.Medeiros et al. have shown that EVs can be produced by podocyte cells after exposure to high glucose and expressed before proteinuria (64). It remains to be proven about the biomarkers contained in EVs produced by podocytes.

2.2 Contents of EVs

EVs are usually secreted under physiological conditions and rich in nucleic acids, proteins, lipids, and metabolites (31) (**Figure 1**). In response to stimuli such as differentiation, neuronal signaling or immune response, the secretory content varies depending on the cells of EVs origin and their function. Surface proteins were abundant, with high enrichment of tetraspanins (CD9, CD63) and lysosome-associated membrane protein 2b (Lamp2b) (20). Besides, RNA is presented in EVs, including miRNA, long non-coding RNA (lncRNA), transfer RNA (tRNA), etc, which range from approximately 25 to 700 nucleotides in length and vary in content depending on the different origin of EVs (5). To be interest, EVs from tumor cells are particularly rich in RNA. According to the Vesiclepedia database, 213 unique proteins were identified in HCC cell-derived EVs. The sEVs proteins include cargo proteins and membrane proteins, the latter being associated with exocytosis of recipient cells and target organ selection (65, 66). The composition of cargo proteins in sEVs varies across tumor cells (5). Studies have found that the ultraconserved lncRNA (ucRNA) expression is dramatically altered within EVs as compared to donor cells. For example, HCC cell-derived EVs transfer ultraconserved lncRNA TUC339 enrichment to neighboring cells in the microenvironment, which is transcribed in host cells and promotes HCC proliferation and diffusion (66). In addition, Yang, B et al. suggested that EVs promote hepatocellular carcinoma metastasis because some substances in EVs are involved in epithelial mesenchymal transition (EMT) (40).

Moreover, many studies have reported that mitochondrial proteins are also cargoes of EVs (67–70). EVs can carry mitochondria, mitochondrial proteins, or mitochondrial DNA to travel between organelles (67, 71). Kiran Todka et al. found that mitochondrial proteins are selectively enriched in EVs and that delivery of mitochondrial proteins to EVs requires sorting nexin 9(SNX9)-dependent mitochondria-derived vesicles (MDVs). MDVs are responsible for carrying mitochondrial proteins between mitochondria and other organelles (72). Intercellular transfer of mitochondria (including mtDNA) results in altered mitochondrial function. If mitochondria are localized within the mitochondrial network of the recipient cell,

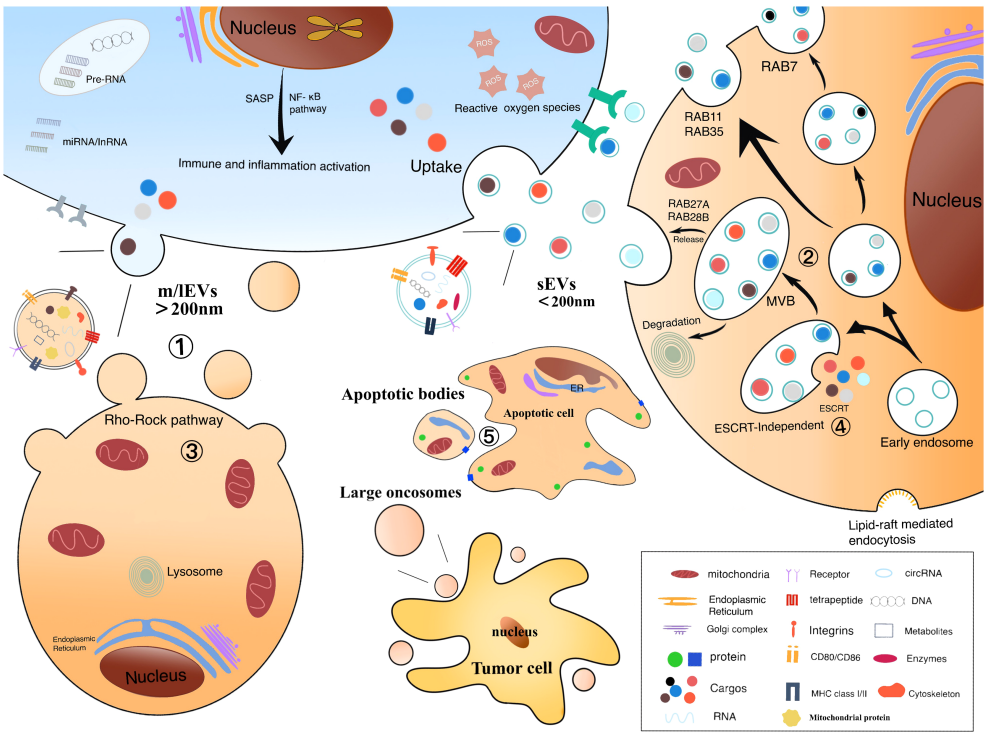

FIGURE 1 | Biological origin of electric vehicles: ① m/lEV formation is the result of mass membrane foaming. Calcium relies on the cellular scale of protein hydrolysis degrading membrane binding, which can help cell membranes germinate and promote their secretion. ② Formation of sEV includes endocytosis, the formation of nucleosomes and MVBs, and the release of sEVs. The vesicles contained in MVBs fuse with the plasma membrane, causing their release. ③ Refactoring is related to the Rho/Rock pathway. ④ Composition of ESCRT is related to the biological occurrence of sEV and MVB. Rab protein facilitates the transport and docking of MVBs over the plasma membrane, leading to cytoplasmic vomiting and the release of sEVs. ⑤ Extensive membrane vesicles occur on the membrane of apoptotic cells to form apoptotic body.

it may elevate the intracellular ATP levels, further generate metabolic stress and ROS to regulate innate immunity, which may have a significant impact on the tumor microenvironment (73–75). For example, it has been found that mitochondrial DNA (12S rRNA (RNR1) G709A) play an important role in the development of HCC (76). However, whether the process of mitochondrial can influence the hepatocellular carcinoma progression associated with EVs needs to be further explored.

2.3 Specific Mechanisms of Uptake and Internalization Between EVs and the Target Cells

Since our current knowledge about the physiology, diversity, internalization, and cargo delivery of EVs is still somewhat limited, it remains impossible to derive a clear mechanism about how EVs interact with and modify receptor cells. However, determining the intracellular pathways and mechanisms of their cargo delivery could help us to utilize EVs as therapeutic agents appropriately (77).

The uptake pathways of EVs are known to be greatly diverse by cells and EVs type, which may be more dependent on the receptor cell type than EVs itself (22, 78, 79). EVs can translocate their contents to recipient cells by different mechanisms such as direct fusion, direct binding, endocytosis or phagocytosis (22). Although the mechanism of EVs uptake and cargo translocation into the cytoplasm of the receptor cell is still not fully defined, it mainly occurs in three steps: targeting the receptor cell, entering point into the receptor cell, and delivering the contents to the receptor cell. However, the end point of EVs internalization is still uncertain, and the function of EVs-mediated cargo transfer cannot being well defined (78).

The pathway of EVs internalization determines the functional response and efficiency of cargo delivery, while the internalization of EVs is mediated by a variety of mechanisms (80), including grid protein dependence and endocytosis of grid protein non-dependent pathways (78). In general, endocytosis is usually divided into two main subgroups: phagocytosis and cytokinesis. Phagocytosis is a type of endocytosis of relatively large (>1μm) particles and is usually restricted to specialized professional phagocytes. In contrast, all cells are capable of cytokinesis (81–83). Grid protein-mediated endocytosis is a recognized pathway for extracellular substance uptake (84). Meanwhile, studies have shown that EVs enter cells mainly through grid protein-independent endocytosis and macrocytosis (83). Non-dependent endocytosis of grid proteins, including the formation of inverted influxes of

vesicle-coated cells on cell membranes (77, 84, 85). Alternatively, fusing with the exoplasmic membrane, EVs can enter cell directly, thereby release their contents into the cytoplasm (80).

3 SEPARATION METHODS OF EVS

The isolation and collection of EVs is a necessary condition for biomedical research and clinical transformation. Researchers have developed many methods to separate EVs, and it is particularly significant to use the proper isolation method under different conditions. For better clinical applications, improving existing technologies for the isolation and storage of EVs are facing great challenges (20). Efficient access to EVs is extremely important for research, and in addition to the use of suitable isolation techniques, promoting the production and release of EVs is also of great value. Upon increased release of EVs, cargo and surface marker proteins may cause altered biological functions (86, 87). Notably, EVs induced by tapping membrane complexes have been reported to play important physiological roles in enhancing immunity, promoting coagulation, wound healing and growth (88, 89). Hirsova, P found that toxic lipids induce the release of EVs from hepatocytes and can activate the pro-inflammatory response of macrophages, which also suggests that inhibiting the release of EVs could be a therapeutic strategy for patients with NASH (90). Based on the therapeutic potential of EVs, we believe that it is of great interest to select suitable methods to facilitate or inhibit the release of EVs depending on the purpose. Thus, some approaches to promote the release of EVs are summarized in **Figure 2**.

3.1 Traditional Methods

3.1.1 Ultracentrifugation

Ultracentrifugation is considered as the "gold standard" for the separation of EVs (102). Due to the different particle size and density, its settling speed is also different, using gradually increasing centrifugal speed or low speed and high speed alternate centrifugation, can be separated in batches at different separation speeds and centrifugal time (30). Cellular impurities were removed with a low speed of 300 g, and high centrifugal force of 16,000 g can be used to separate apoptotic bodies, 20,000 g to separate m/lEVs, and 100,000 g to precipitate and concentrate sEVs (103, 104). This method is widely used, but the purity of sample is not satisfied for the supernatant will contain 40% EVs, which leads to protein contamination and lower yield. There is an overlap in the size of sEVs and m/lEVs, and slightly larger sEVs and smaller microvesicles are difficult to isolate (105). In addition, it generally requires multiple centrifugation processes to achieve better separation, but it is prone to vesicle destruction and also has many disadvantages such as the large size of the instrument, high cost, lengthy and laborious processing, and few samples (106).

3.1.2 Gradient Ultracentrifugation

The requirements of gradient ultracentrifugation are more stringent, when there is a small difference in settling velocity between different particles, they are placed on the top of a medium with different density gradient. Under the action of a certain centrifugal force, the particles are separated by aggregating into the layer of the medium with a similar density to theirs, and the commonly used medium is sucrose (107). Sucrose gradient centrifugation can be used to isolate sEVs (108, 109). This method is popular because of good separation effect, high purity, no extrusion and deformation of the particles, and the ability to maintain the activity of the particles. However, it needs to prepare inert gradient media solution, be complicated to operate, not easy to master, time-consuming and labor-intensive (20-24 h), and high cost. What's more, the density of EVs and high-density lipoprotein particles (HDL) is similar and they can be separated out together, so the samples are prone to contamination (110). Besides, the use of newer isotonic gradients contribute to better maintenance of the physical properties of the vesicles (111).

3.1.3 Precipitation Method

The precipitation method mainly includes polymer precipitation and organic solvent precipitation. Commercial kits that rely on polymer co-precipitation have been reported being used for the isolation and purification of EVs, decreasing solubility and promoting precipitation. The precipitated EVs can be easily and reproducibly separated and avoid prolonged ultracentrifugation (112, 113). Unfortunately, the main problems with this method are that co-precipitation is susceptible to contamination by non-EVs substances and that mechanical forces or chemical additives can damage EVs (114). In addition, the method relies more on manual manipulation with low throughput and recovery, and purification of polymers from EVs may interfere with downstream analysis. Therefore, co-precipitation is not suitable for most research and clinical applications.

3.1.4 Molecular Exclusion Chromatography

The principle of molecular exclusion chromatography is that different solute molecules, such as EVs and protein impurities, are separated from each other as they pass through porous packings due to differences in size resulting in different rates of passing through the pores (115, 116). This approach yields purified EVs from complex biological media (117–119), removed soluble plasma proteins and HDLs effectively, preserved the biological activity and integrity of EVs and also reduced aggregation (115). A variety of influencing factors such as media type, pore size, column size, and flow rate should be considered for EVs separation (20, 116). This method is efficient and inexpensive, and it is more suitable for small volumes of blood samples because of the upper sample volume limitation.

3.1.5 Asymmetric Flow Field Flow Classification Method

Asymmetric flow field flow fractionation (AF4) is a technique in which a force field is applied to achieve the separation of EVs with different sizes and molecular weights (120). AF4 contains permeable plates and when a vertical force field is applied, the analytes in the sample will be moved to the boundary by the force and smaller particles will undergo Brownian motion to reach a new equilibrium

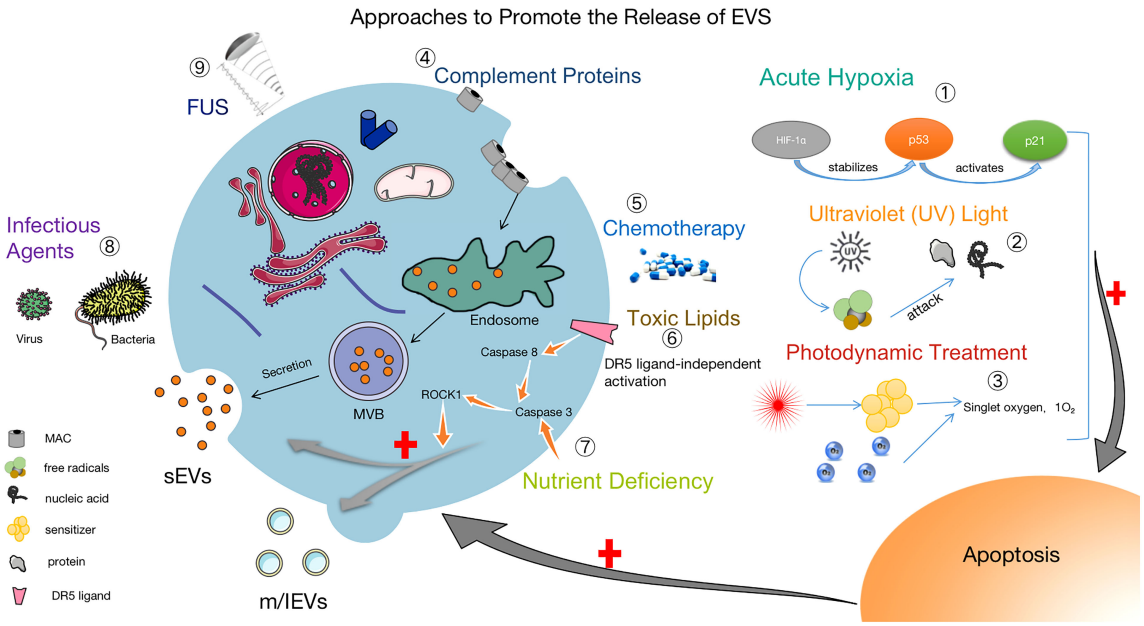

FIGURE 2 | Methods to facilitate the release of EVs. ① Acute Hypoxia: Catabolism of HIF-1α is inhibited by acute hypoxia, which stabilizes the P53 gene and activates the P21 gene, leading to apoptosis and promoting the release of EVs (91–93). ② UV: After UV irradiation, a large number of free radicals are generated to attack nucleic acids and proteins, causing apoptosis and increasing the release of EVs (94). ③ Photodynamic Treatment: Laser irradiation at a specific wavelength excites the tissue-absorbing photosensitizer, and the excited state of the photosensitizer transmits energy to the surrounding oxygen, generating strongly reactive monomorphic oxygen, which may reacts oxidatively with the surrounding neighboring biomolecules, resulting in a cytotoxic effect that causes apoptosis and also promoting the release of EVs (95). ④ Complement Proteins: The membrane attack complex (MAC) is composed of complement proteins (C5b, C6, C7, C8 and C9). MAC is cleared from the cell surface by cytosolic or cytocytic action to help release EVs (96, 97). ⑤ Chemotherapy: The use of chemotherapeutic agents (e.g., doxorubicin, methotrexate, and cisplatin) causes cellular damage and EVs release (95, 98). ⑥ Toxic Lipids: Toxic lipids activates the DR5 pro-apoptotic signaling cascade, which in turn activates ROCK1 and promotes the release of EVs from hepatocytes (90). ⑦ Nutritional Deficiency: Activation of Caspase 3, ROCK1 signaling pathway and promotion the release of EVs (99). ⑧ Infection factors (100) and ⑨ focused ultrasound (101) can also promote the release of EVs.

position (121). The advantages of this method are rapid (<1 h), high resolution, gentle, label-free, and reproducible. It can be applied to a variety of eluates, contributing to the successful separation of different subpopulations of EVs.

3.2 New Methods

3.2.1 Immunoaffinity Capture

Obviously, EVs are rich in proteins. Immunoaffinity capture is the specific binding of antibodies to the corresponding antigens on the surface of EVs such as adhesion proteins, tetra-transmembrane proteins and integrins, achieving the separation of EVs by immune reactions (122, 123). Magnetic beads provide a large surface area to capture EVs, targeting antigens on the surface of EVs to select specific subgroups, improving separation efficiency, specificity and purity, making it more suitable for marker detection of EVs and clinical diagnostic studies (124). However, the expensive antibody reagents, stringent reaction conditions, reduction of isolation yields, and the vulnerability of the biological activity of the EVs contents to PH and salt concentration have made it inappropriate to isolate large volume samples (125).

3.2.2 Microfluidics

Based on different molecular size, microfluidics can isolate EVs from large cellular debris (126). Compared to conventional separation methods, with smaller sample volumes (50μL - 500μL), microfluidic techniques are faster (30 min-2 h), portable, cost effective and automated, resulting in high purity of EVs. However, some microfluidic technologies allow only small sample input, lack method validation and standardization, which may influence the application of downstream analysis.

3.2.3 Contactless Classification

The use of acoustic waves for contactless separation of EVs has recently been proposed by some researchers. This separation method applies forces based on the size and density of vesicles (127). Particles in the acoustic region migrate toward the pressure nodes after the force is applied. Acoustic interaction forces are proportional to vesicle volume, with larger vesicles moving more rapidly. This method can separate EVs very quickly and without contact.

4 QUANTIFICATION METHODS OF EVs

Currently, the quantification of EVs has been challengig. It is suggested that for conditioned medium, the number of cells at the time of initiation and collection should be clearly indicated.

In addition, proper characterization of EVs at the time of separation helps to understand their properties. Several techniques for measuring the size of EVs are being investigated, including lateral-flow immunochromatographic assay (LFIA), nanoparticle tracking analysis (NTA), and nanopore tunable resistive pulse sensing techniques(TRPS), high resolution flow cytometry, multi-angle light scattering coupled to asymmetric flow field-flow fractionation (AF4), fluorescence correlation spectroscopy (FCS), enzyme linked immunosorbent assay (ELISA) and Raman spectroscopy, etc. Here, we talk about some advantages and disadvantages of some techniques. LFIA, with its high degree of flexibility, is a good tool for cost-effective field detection, but the assay lacks sensitivity (128). The AF4 system is highly repeatable (120), however, it requires skilled operators. NTA and TRPS can be used for particle size analysis of EVs, and their detection sensitivity is 70-90 nm and 70-100 nm, respectively. NTA technology allows one-time measurement and quantification of EVs, but the equipment is expensive and difficult to operate (129, 130). The ELISA technique is greatly flexible and can be modified appropriately for the analyte, but it is also time-consuming.

In addition, EVs are rich in proteins, lipids, nucleic acids and other biomolecules, and it can be quantified by quantifying these specific molecules. For example, total protein amounts were determined by using Bradford, micro-bicinchonic acid (BCA), fluorimetric assays, global protein stainon sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), etc. However, due to the possible presence of protein contaminants, the measurements are on the high side. The amount of total lipids can be measured by sulfofphosphovanilin assay (131) and total reflection fourier-transform infraredspectroscopy (132). RNA can be quantified by global RNA assays (133). In conclusion, the quantification of EVs is a critical topic that still lacks consensus and standardization both domestically and internationally, and we expect more studies to be reported in the future.

5 INTERACTIONS BETWEEN HCC AND HCC CELL-DERIVED EVs

In the microenvironment where tumor cells and normal cells are located, HCC cell-derived EVs build a bridge to communicate with each other and promote HCC proliferation, invasion and distant metastasis, etc. EVs origined from HCC often regulate tumor progression through autocrine and/or paracrine cellular communication. HCC cell-derived EVs stimulate recipient cells to produce cytokines and promote the migration of HCC, such as matrix metalloproteinase 2 (MMP2) and matrix metalloproteinase 9 (MMP9) (134). Meanwhile, HCC is a typical hyperangiogenic tumor. HCC cells secrete EVs loaded with different miRNAs, LncRNAs, circRNAs that can activate signaling pathways in the recipient cells, thus causing the recipient cells to respond, promoting HCC migration or inhibiting HCC proliferation, which have an impact on tumor angiogenesis (47). For example, HCC cell-derived EVs carry oncogenic RNAs and proteins, which allows EVs to activate the PI3K/AKT and MAPK signaling pathways and promote distant tumor metastasis (46).

EVs secreted by HCC cells containing some specific miRNAs will play a specific role in HCC. For example, hypomethylation causes increased expression of mir -429 in HCC cells, and these large EVs mediated by mir -429 are shed and bind to Rb-binding protein 4 (RBBP4) in surrounding target cells, promoting the transcriptional activity of E2F1 and ultimately upregulating the expression of POU class 5 homeobox 1 (POU5F1) in target cells, thereby promoting HCC development (46). Meanwhile, EVs-loaded miR-221 binds to the 3'-UTR target site of the p27/Kip1 oncogene and promotes HCC proliferation and migration (135). EVs containing protein CD147 released by HCC cells activate the NF-κB pathway of surrounding fibroblasts, induce MMP-9 expression, and stimulate the ERK1/2 and p38 MAPK pathways, leading to extracellular matrix degradation and tumor invasion (136, 137). In addition, EVs containing miR-25 released from HCC cells inhibited p53 expression in surrounding HCC cells, thereby restoring FOXM1 (a key regulator of cell cycle progression) expression, activating the HGF/Ras pathway, reversing the expression of sorafenib-induced apoptotic markers BCL2 and BAX, making HCC cells resistant to sorafenib (138). miR-34a is reduced in the large EVs released by CHB or HCC cells, resulting in increased levels of mRNA and protein in c-Mets in surrounding cells, promoting phosphorylation of c-Met-induced extracellular signal- regulated kinases 1 and 2 (ERK1/2), thereby facilitating CHB conversion to HCC (139, 140). Intracellular TLR4 signaling in HCC cells is transduced to the actin cytoskeleton *via* the MyD88 pathway, leading to the release of large EVs. Peripheral tumor macrophages take up large EVs containing microRNA let-7b, which attenuates tumor inflammation by targeting the pro-inflammatory cytokine IL-6 (141). Upregulation of ANXA2 expression in HCC cells promotes the shedding of CD147-containing large EVs and the production of MMP-2 in surrounding fibroblasts, thereby promoting HCC development (142). Thus, HCC cell-derived EVs can also act as a bridge between surrounding tumor cells or other cells, and their loaded cargo can have an impact on HCC progression when taken up by target cells.

5.1 HCC Cell-Derived EVs Promote HCC Migration by Directly Activating or Inhibiting Signaling Pathways

HCC cell-derived EVs-loaded cargoes can promote cancer cell migration by directly activating or inhibiting signaling pathways. For example, EVs-miR-1247-3p secreted by HCC cells directly transferred to lung pre-metastasisniche fibroblasts, decreased the expression of β-1,4-galactosyltransferases III (B4GALT3, a protein mediating glycosylation), thereby converting them into CAFs, and then activated the β1-integrin-NF-κB signaling pathway to promote EMT, thereby promoting the metastasis of hepatocellular carcinoma to the lung, and IL-6 and IL-8 secreted by CAFs to promote the development of HCC (**Figure 3**.①) (143). Meanwhile, EVs-miR92a-3p can promote HCC metastasis and EMT by inhibiting PTEN activation of the Akt/Snail signaling pathway (**Figure 3**.②) (40). Besides, HCC cells can also secrete EVs-miRNA-21 that directly targets PTEN and

activates the PDK1/AKT signaling pathway. Moreover, it transforms hepatic stellate cells (HSC) into activated cancer-associated fibroblasts(CAF), which can further promote HCC growth by secreting vascular growth factors(VEGF, MMP2, MMP9 and TGF-β) (**Figure 3**.③) (144). Under endoplasmic reticulum stress, HCC cells inhibit PTEN and activate the PI3K-AKT pathway by delivering EVs-miR-23a-3p to macrophages, increasing macrophage PD-L1 expression and inhibiting T-cell function, promoting immune escape (**Figure 3**.④) (145). In addition, EVs-lncRNA TUC339 can be taken up by THP-1 cells, resulting in reduced production of pro-inflammatory cytokines, reduced expression of costimulatory molecules, impaired phagocytosis, and promotion of macrophage M (IL-4) polarization (**Figure 3**.⑤) (146). EVs-miR-93 promotes HCC tumorigenesis by affecting CDKN1A, TP53INP1, and TIMP2, and sEVs-miR-93 overexpression predicts poor prognosis (**Figure 3**.⑥) (147). It has been reported that lncRNA FAL1 are taken up by surrounding HCC cells and promote HCC cell proliferation and migration by competitively binding miR-1236 in recipient cells, which in turn upregulates the expression of their target genes AFP and ZEB1 (**Figure 3**.⑦) (148). sEVs-CircFBLIM1 can promote HCC progression through the miR-338/LRP6 axis (**Figure 3**.⑧) (149). The sEVs-circ-PTGR1 downregulates miR449a-MET expression, disrupts tumor microenvironment homeostasis, and promotes HCC migration and invasion (**Figure 3**.⑨) (150). EVs complement factor H (CFH) elevates C3a and C5a levels, exacerbating inflammatory responses and tumor growth (**Figure 3**.⑩) (151).

5.2 The Role of HCC Cell-Derived EVs on Angiogenesis

HCC is typically a highly angiogenic tumor and therefore angiogenesis is closely related to the prognosis. We have known that EVs-loaded cargo is able to promote angiogenesis and increase vascular permeability. Altered vascular permeability implies altered endothelial continuity, allowing cancer cells to infiltrate and attach to the microvascular endothelial lining and form tumor metastases. For example, Lin, XJ et al. found that delivery of EVs-miR-210 to endothelial cells to target SMAD4 and STAT6 for pro-angiogenesis (**Figure 3**.⑪) (152). Besides, EVs-miR-103 inhibits the expression of VE-Cad, p120 and ZO-1 and reduces endothelial integrity to promote tumor invasion (**Figure 3**. ⑫) (153). EVs-LncRNA H19 induces the production of the pro-angiogenic cytokine (VEGF) and its receptor VEGF-r1 in HUVECs and stimulates angiogenesis (**Figure 3**. ⑬) (154). Interestingly, Y Zhou et al. found that ovarian cancer-derived EVs carry NID1 through ERK/MAPK to promote EMT, accelerate angiogenesis, and promote tumor invasion (155), but the role of NID1 in HCC is still unclear (156). In addition, HCC cell-derived EVs can promote angiogenesis in HUVECs, and the amount of HepG2-derived EVs determines the amount of angiogenesis, lumen formation. The sEVs may influence human umbilical vein lumen formation *via* the VEGF receptor and the angiogenesis-associated heat shock protein HSP70 (157).

HCC cells-derived EVs carrying proteins were found to inhibit angiogenesis by reducing VEGF through activation of AMPK signaling iynamic network microenvironment consisting of hepatocytes and their surroundings, suchn HCC (158). At the genetic level, CLEC3B-related genes are closely associated with angiogenic genes. In experiments, cells with high levels of CLEC3B formed fewer vessels than those with low levels. Likewise, in animal studies, immunohistochemical detection of tumor tissue from *in situ* tumor-implanted mice showed a significant reduction in CD31-positive and CD34-positive endothelium (EC) in CLEC3B high-isogenic grafts. Thus, high levels of CLEC3B EVs significantly reduce the expression of endothelial growth factor (EGF) in HCC, thereby reducing angiogenesis.

5.3 Inhibition of HCC Growth by EVs-Loaded Cargo of Different Cellular Origin

When certain signaling pathways are blocked by EVs-loaded cargo, the growth and distant metastasis of HCC may also be inhibited. For example, when Vps4A is overexpressed in HCC cell-derived EVs, it inhibits the PI3K-Akt pathway and thereby inhibits the metastasis of HCC (159). When normal cells secrete sEVs containing SENP3-EIF4A1, SENP3-EIF4A1 inhibits HCC cell proliferation by suppressing miR-9-5p in HCC cells and activating the expression of ZFP36 (160). In contrast, EVs-circ-0051443 promotes HCC cell apoptosis and inhibits tumor growth by competing with miR-331-3p in HCC cells and upregulating BAK1 expression (161). Interestingly, Huang, X et al. proposed that IncRNA 85 regulates the invasion of cancer cell by targeting miR-324-5p and through ceRNA mechanisms, and more importantly, miR-324-5p overexpressed can reducing migration by regulating the expression of MMPs, ETS1 and SP1 genes in HCC (162, 163). When tumor-associated fibroblasts (CAFs) secrete EVs containing miR-320a, miR-320a inhibits HCC growth by suppressing the PBX3/ERK1/2/CDK2 pathway in HCC cells (164). For example, EVs enriched in LncRNA H19 were secreted by CD90+ cancer cells to promote angiogenesis, inducing the production and secretion of the pro-angiogenic cytokine VEGF and its receptor in HUVECs (154). What's more, it has been shown that co-culture of Huh7 cells with HepG2 cells, where Huh7 secretes EVs containing miR-122, has an inhibitory effect on tumor growth, when co-cultured HepG2 cells attenuate this inhibitory effect by secreting IGF1 (165).

Alteration of original physiological functions between HCC cells through the delivery of cargo molecules in EVs. Some goods are markers to diagnose HCC from other liver diseases; Some can determine the effectiveness of HCC treatment and predict the recurrence rate of HCC; Some can be used as vehicles for delivering drugs for the treatment of HCC. In conclusion, EVs loaded with cargo play different roles in the migration of HCC, regulating the talks between HCC and cells (**Table 2**).

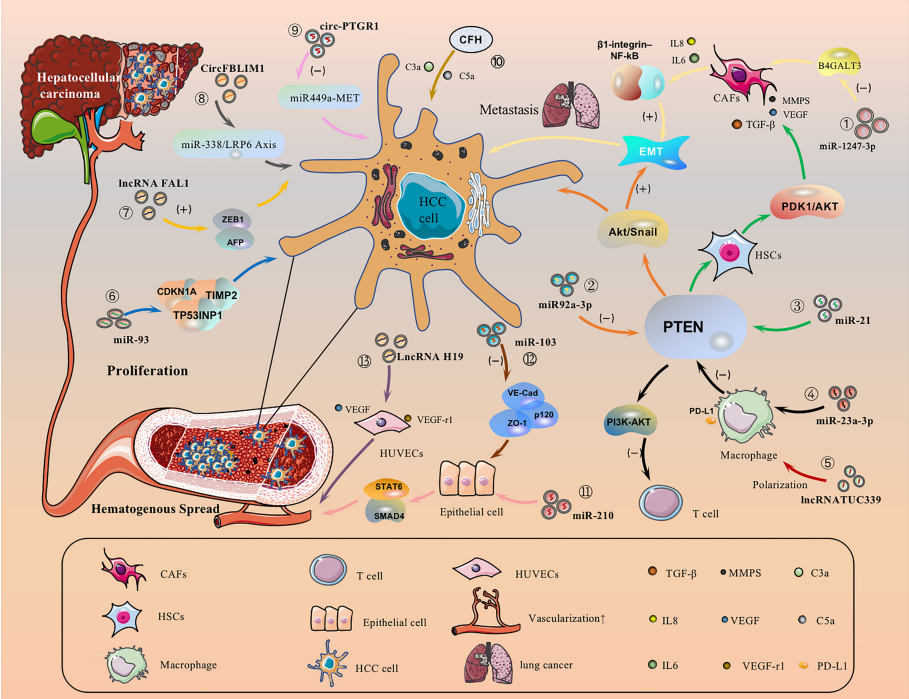

FIGURE 3 | HCC cell-derived EVs carry cargo and regulate different receptor cells.

6 REGULATION OF HCC BY DIFFERENT CELL-DERIVED EVs IN THE MICROENVIRONMENT

There is growing evidence that the dynamic network microenvironment consisting ofhepatocytes and their surroundings, such as cancer cells, immune cells, cytokines andextracellular matrix is also a key factor in tumor metastasis. Liver is rich in immune cells, which can greatly produce EVs, and has a unique immune-tolerant microenvironment, which is a huge challenge for HCC immunotherapy (170). Among them, various immunosuppressive cell subsets and signaling pathway-mediated pre-tumor immune responses play a key role in "tumor immune escape". EVs are not restricted by space and material and can interact with cancer cells anywhere in the body. EVs produced by cancer cells can also interact with nearby immune cells (171, 172). The interaction between tumor and the immune system determines the progression of the tumor at the early stage. In conclusion, HCC occurs not only because hepatocytes contain sufficient genetic mutations, but multiple interrelated factors in the hepatic microenvironment influence the progression of HCC, and the mechanistic features of these new factors have prompted the search for new therapeutic approaches to treat not only the tumor itself but also the hepatic microenvironment to prevent recurrence and treatment resistance, some of which have yet to be fully elucidated.

6.1 Mesenchymal Stem Cells-Derived EVs

MSCs are present in bone marrow, umbilical cord blood and adipose tissue and are adult stem cells with multidirectional differentiation potential (173). MSCs attenuate fibrosis by upregulating hepatocyte growth factor (HGF) (174, 175), insulin growth factor (176), and MSCs-derived EVs improve hepatocyte regeneration and modulate immune activity, demonstrating therapeutic benefits in various liver diseases (173). Meanwhile, the role of MSCs-derived EVs cannot be ignored. Experiments have shown that ADMSC (adipose-derived mesenchymal stem cells)-derived EVs promote anti-tumor responses of NKT cells, leading to early ADC increase and low-grade tumor differentiation (177). In addition, an anti-tumorigenic effect of MSC-EVs was also observed in a CCl4-induced mouse liver tumor model. After treatment with EVs, the growth of liver tumor was significantly inhibited by inhibiting oxidative stress (178). Bruno, S et al. have demonstrated that EVs in human BM-MSCs can induce HepG2 cell cycle blockers and apoptosis necrosis *in vitro*, which inhibit tumor growth in the body. However, EVs secreted by fibroblasts formed by differentiation of human derived MSCs lack antitumor effects (179). In addition, miR-122 delivered *via* AMSC-derived EVs may provide new therapeutic options for HCC (**Figure 4**. ①) (180). It remains unclear that whether MSCs-derived EVs can inhibit HCC progression by carrying cargo, and it provides a new direction for the possibility of using MSCs-derived EVs as carriers to exert anti-tumor effects.

TABLE 2 | The cargos and functions of EVs related with HCC.

Name of the Cargo in EVs	Cargo Type	Mechanism of the Cargo	Function of the Cargo	Vivo or vitro	Cell lines	Refs
miR-429	miRNA	Targeting the RBBP4/E2F1/OCT4 axis in recipient cells, promote liver T-ICs properties	Facilitate HCC	Vitro	T-ICs	(46)
miR-142-3p	miRNA	Down-regulation of RAC1	Suppressed migration of HCC	Vivo	Hepa1-6	(166)
miR-221	miRNA	Binding to the target sites in the 3'-UTR of p27/Kip1 tumor suppressor gene	Promote proliferation of HCC	Vitro	SMMC-7721	(135)
miR-25	miRNA	Attenuating p53 and enhancing FOXM1 expression	Mediate sorafenib resistance in HCC	Vitro	HepG2	(138)
miRNA let7b	miRNA	Targeting proinflammatory cytokine IL-6	Attenuates tumor inflammation	Vivo, Vitro	H22	(136, 141)
miR-34a	miRNA	miR-34a was down-expressed in HCC, promoted the translation of antiapoptotic factors	Promote the conversion of CHB to HCC	Vitro	–	(139)
CD147	protein	Induce upregulation of MMPs in fibroblasts, leading to extracellular matrix degradation	Promote tumoral invasion	Vitro	–	(136)
miR-1247-3p	miRNA	Targets B4GALT3, activate β1-integrin–NF-κB signaling, activated CAFs secrete pro-inflammatory cytokines	Promote lung migration of liver cancer	Vivo, Vitro	CSQT-2	(143)
miR-103	miRNA	Inhibiting the expression of VE-Cad, p120 and ZO-1, attenuated the endothelial junction integrity	Promote vascular permeability and metastasis	Vivo	MHCC97H	(153, 167)
miR-638	miRNA	Attenuate endothelial junction integrity	Promote vascular permeability and metastasis	Vivo	HuH-7M	(168)
miR-93	miRNA	Directly inhibiting the expression of TIMP2/TP53INP1/CDKN1A	Promote proliferation and metastasis of HCC	Vitro	SKHEP1	(147)
miR-23a-3p	miRNA	Promotes PD-L1 expression in macrophages and inhibits T-cell function through miR-23a–PTEN–AKT signaling pathway	Promote proliferation and metastasis of HCC	Vivo, Vitro	HepG2	(145)
lncRNAFAL1	lncRNA	Competitively binding to miR-1236, indirectly up-regulated the expression of AFP and ZEB1	Promote proliferation of HCC	Vitro	Huh7	(5, 148)
lncRNA 85	lncRNA	Targeted miR-324-5p and regulated its expression through a ceRNA mechanism	Promote proliferation and metastasis of HCC	Vitro	HepG2	(163)
lncRNATUC339	lncRNA	Excess lncTUC339 expression in macrophages promoted M(IL-4) polarization	Suppress the immune response to tumor cells	Vitro	HL-7702	(147)
circUHRF1	circRNA	Upregulate TIM-3 expression and suppress the production of IFN-γ and TNF-α	Inhibit NK cell function	Vivo	SMMC-7721	(169)
Vps4A	protein	PI3K/Akt pathway was inactivated by Vps4A-overexpression	Inhibit the growth and metastasis of HCC	Vivo	Hep3B	(159)
CFH	protein	Increase the production of C3a and C5a	Promote proliferation and metastasis of HCC	Vivo, Vitro	Huh7	(151)

6.2 Cancer Stem Cells-Derived EVs

Cancer Stem Cells (CSCs), with proliferative and differentiation potential, is more easily contributing to tumor recurrence (181–184). It is reported that EVs derived from CSCs can induce tumor growth, metastasis, participating in angiogenesis and maintaining the stem cell phenotypes (185–188). EVs released from CSCs containing multiple cargoes, including proteins and multiple RNA (189). EVs can make the microenvironment to change in the direction of promoting tumor occurrence and metastasis. For example, Domenis, R et al. found that CSC-derived EVs inhibits T cells through monocyte-specific secretion of IL-10 (190). In addition, fibroblasts can be converted into cancer-associated fibroblasts (CAF) through the uptake of CSC-derived EVs, promoting tumor progression and metastasis (191). It was also found that CSCs-like CD90+ hepatocytes regulate the endothelial phenotype by releasing EVs containing H19 lncRNA, significantly increase VEGF expression, and promote intercellular adhesion, induce angiogenesis, and affect the tumor microenvironment (154). What's more, Alzahrani FA et al. showed that hepatic CSCs-derived EVs were able to increase the expression of Bcl2, TGFβ1, NFκB, MMP9, VEGF, 13K, ERK and decrease the levels of Bax, p53, TIMP1 mRNA in the liver of mice, suggesting that CSCs-derived EVs promote hepatocellular carcinoma cell invasion while upregulating TGFβ1-induced EMT (**Figure 4**. ②) (192). However, it is of interest that CSCs-derived EVs and MSCs derived EVs had opposite effects on HCC growth and progression *in vivo*, and neither involved promotion or inhibition of HCC-induced oxidative stress or antioxidant activity. As can be seen, these studies have showed new insights into the treatment of HCC, and more research is needed to clarify the mechanisms involved.

6.3 Macrophages-Derived EVs

Depending on the state and functional status of macrophages after activation, they can be divided into M1 and M2 macrophages, with M1 macrophages playing a tumoricidal role and M2 macrophages promoting tumorigenesis (193). M1 macrophages are involved in the polarization of Th1 and high expression of IL-6, IL-12, TNF-α, iNOS, ROS to promote the occurrence of inflammation (194). EVs from M1 macrophages induce stronger antigen-specific cytotoxic T-cell responses in lymph nodes, enhance immune responses to cancer vaccines, and are used as effective vaccine adjuvants (195). In the TME, tumor-associated macrophage (TAM)-derived EVs significantly

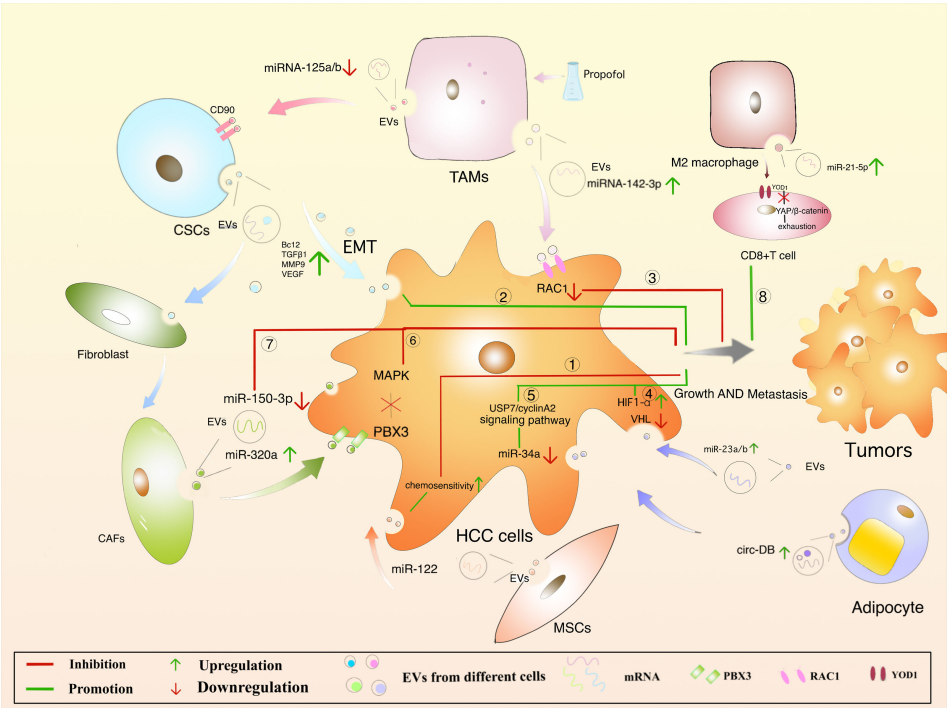

FIGURE 4 | Regulation of HCC by different cell-derived EVs.

downregulate miRNA-125a and miRNA-125b (miRNA-125a/b targets CD90, a stem cell marker for HCC) and promote the progression of HCC (196). The macrophages were treated with propofol to help secrete more EVs with miRNA-142-3p, which can be absorbed by HCC cells, and furtherly, RAC1 inhibited the migration and tumor growth in mice (**Figure 4**. ③) (166). M2 macrophages are involved in Th2 polarization and highly express IL-4, IL-10, TGF-β, CD206, CD163, CCL22, etc., while reduce the expressing of IL-12,downregulate the immune response and promote tumor progression (197). In an experiment by Jian Pu et al. in which EVs were injected into a mouse model of liver cancer, M2 macrophage-derived EVs were found to promote CD8+ T cell failure *via* the miR-21-5p/YOD1/YAP/β-catenin axis (**Figure 4**. ⑧) (198). Thus, it seems that M2 macrophages are closely associated with the malignant development of HCC (199).

6.4 Adipocytes-Derived EVs

Adipocytes mainly play a role in providing metabolic substrates for tumor cells. There is evidence that adipose-derived EVs can promote tumor growth in HCC by downregulating VHL, delivery of miR-23a/b. Studies *in vivo* have shown that increasing levels of EVs-miR-23a/b, VEGF, GLUT1 and HIF1α accelerated tumor growth and rate in high fat diet mice (**Figure 4**. ④) (200). Visceral adipocyte exocytosis induces dysregulation of the TGF-b pathway in HepG2 cells in high body fat individuals, but not in low body fat individuals (201). Zhang, H et al. suggested that EVs-circ-DB was upregulated in HCC patients with high body fat and its positively correlated USP7 was also increased (202). Mature adipocyte-derived EVs and HCC cellular effects lead to a decrease in miRNA-34a (tumor suppressor), while an increase in the USP7/Cyclin A2 signaling pathway (pro-cancer), a promotion of HCC cell growth, and a reduction in DNA damage (**Figure 4**. ⑤). Nevertheless, once circ-DB is knocked out, these effects will disappear. Furthermore, adiponectin is an abnormally abundant adipocytokine that regulates sEVs biogenesis by binding to T-cadherin and reduces cytosolic ceramide levels by releasing EVs (203, 204). sEVs are formed through the non-dependent mechanism of ESCRT, a process in which ceramide is essential and accordingly lipocalin is crucial in regulating their exocytosis. sEVs as a biological delivery vehicle for cancer treatment has been a hot research topic recently, but the role of adipocyte-derived EVs in HCC still requires further investigation.

6.5 Fibroblasts-Derived EVs

The connective tissue is rich in fibroblasts. Understanding the regulation of CAF in HCC is critical. CAFs-derived EVs are low in miR-320a, which binds to its direct downstream target PBX3 and inhibits HCC by suppressing MAPK pathway activation (**Figure 4**. ⑥) (164). The expression of CAFs-derived EVs-MiR-150-3p is reduced, which can inhibit the migration and invasion of hepatocellular carcinoma cells (**Figure 4**.⑦) (205), suggesting it may be a new therapeutic option. Meanwhile, studies have reported that miR-195 in HCC has been downgraded to VEGF, CDC42, CDK1, CDK4, CDK6, and CDC25 (206, 207). As

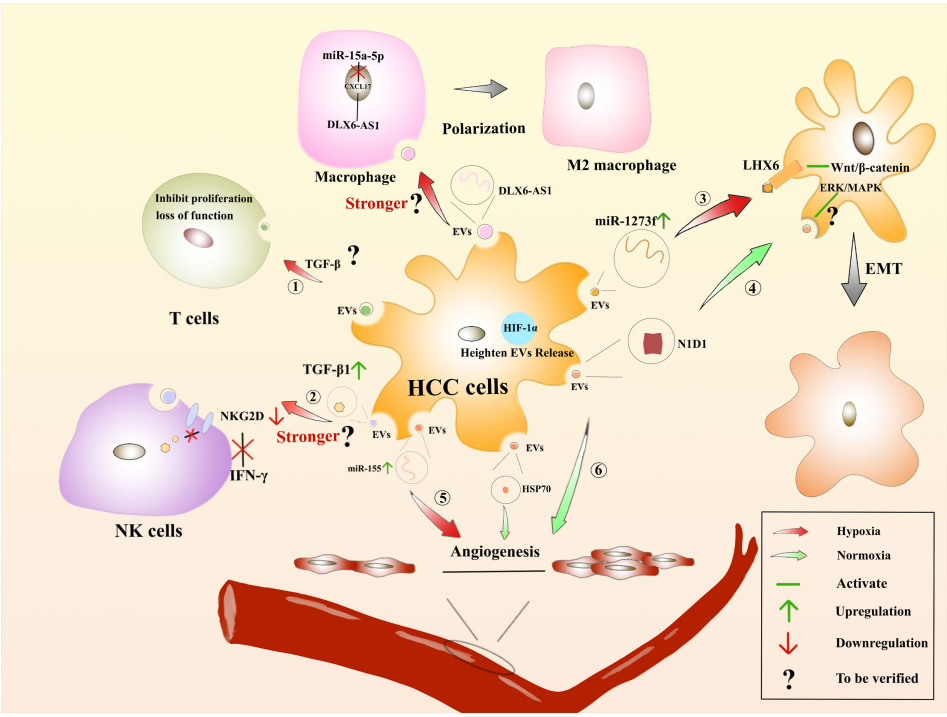

FIGURE 5 | Hypoxia-induced microenvironment affects the regulation of HCC by EVs : The role of EVs derived from HCC on immune cells in the hypoxic environment. ① Suppressing the proliferation of T cells or rendering them incompetent. ② Whether the inhibitory effect on IFN-β production by NK cells and the process of inducing macrophage polarization are enhanced remains to be verified. The role of HCC-derived EVs facilitates EMT. ③ In the hypoxic environments, miR-1273f is upregulated in HCC-derived EVs, acting on LHX6 to activate Wnt/β-catenin to promote EMT. ④ In the normoxic environment, HCC-derived EVs contain N1D1, which may activate the ERK/MAPK pathway in recipient HCC cells to promote EMT. Regulation of angiogenesis by HCC-derived EVs. ⑤ In the hypoxic environments, miR-155 is upregulated in HCC-derived EVs and promotes angiogenesis. ⑥ In the normoxic environment, HCC-derived EVs are enriched in N1D1 and HSP1, which promote angiogenesis.

described, understanding the mechanism of fibroblasts-derived EVs on HCC can help design new therapeutic approaches.

7 HYPOXIA-INDUCED MICROENVIRONMENT AFFECTS THE REGULATION OF HCC BY EVs

Many solid tumors live in the hypoxic microenvironment. Hypoxia promotes the production and release of EVs from cancer cells. Studies have showed that the number of sEVs in breast cancer cells and oral squamous carcinoma cells was significantly increased under hypoxic conditions (208). Hypoxia-inducible factor-α1 is a regulator of cells under hypoxic conditions and can facilitate the release of EVs (209). The proteins and nucleic acids of sEVs are also altered in the hypoxic environment (210). Under hypoxic conditions, miR-1273f carried by sEVs could accelerate the progression of HCC, targeting LHX6, which further inhibits HCC tumorigenesis or malignant transformation by targeting the Wnt/β-catenin signaling pathway (211). Hypoxia-generated sEVs can inhibit the expression of E-cadherin, thereby promoting EMT (212). EVs derived from HCC cells could affect angiogenic endothelial cells under the hypoxic conditions through upregulation of miR-155, thereby affecting tumor angiogenesis (213). Furthermore, EVs released from epithelial ovarian cancer (EOC) cells can express more miR-21-3p, miR-125b-5p and miR-181d-5p under the hypoxic conditions, thus facilitating M2 macrophage polarization (214). Additionally, DLX6-AS1 carried by HCC is in competition with miR-155 to regulate CXCL17. M2 macrophage polarization is induced, and migration, invasion, and EMT of HCC will be accelerated (215). Unfortunately, the authors did not investigate whether hypoxia accelerates this process. Rong, L et al. saying that hypoxia enhanced the secretion of sEVs in breast cancer cells, thereby inhibiting the proliferation of T cells (216). Moreover, hypoxia induced a significant increase in TGF-β1 content in cancer cell-derived EVs, decreased the expression of the activation receptor NKG2D, and inhibited the cytotoxicity of NK cells and also reduced the production of IFN-γ (217). Therefore, the tumor hypoxic microenvironment is closely related to tumor development, treatment and prognosis, which has become a research hotspot to find new treatments for HCC (**Figure 5**).

TABLE 3 | EVs as biomarkers for the diagnosis of HCC.

Classification		Biomarkers	Expression	Species	Type of biological fluid	AUROC	Clinical significance	Refs
m/lEvs		AnnexinV+EpCAM+ASPGR1+CD133+taMPs	↑	Human	serum	0.7439	Diagnosis of HCC/CCA from LC	(225)
		EpCAM+AnnexinV +ASGPR1+taMPs	↑	Human	serum	0.7322	Diagnosis of HCC/CCA from LC	(225)
		Total m/lEVs of peripheral blood	↑	Human	serum	0.83	Diagnosis of E-HCC from LC (TNM stage I)	(226)
sEVs	microRNA	miR-148a	↑	Human	serum	0.871 0.860	Diagnosis of HCC from NC Diagnosis of E-HCC from LC	(227)
		miR-122	↑	Human	serum	0.990 0.795	Diagnosis of HCC from NC Diagnosis of E-HCC from LC	(227)
		miR-1246	↑	Human	serum	0.825 0.761	Diagnosis of HCC from NC Diagnosis of E-HCC from LC	(227)
		miR-638	↑	Human	serum	——	Associated with tumor recurrence, As a prognostic marker	(228)
		miR-125b	↑	Human	serum	0.739	Prediction of recurrence and survival	(229)
		miR-93	↑	Human	serum	0.825	The prognosis and diagnosis of HCC	(147)
		miR-665	↑	Human, Mice	serum	——	Diagnosis and prognosis of HCC	(230)
		miR-92b	↑	Human, Rats	serum	0.702	Prediction of E-HCC relapse after LDLT	(231)
		miR-21	↑	Human	serum	——	Detection of E-HCC, Prognostic marker	(232)
		miR-718	↑	Human	serum	——	Prediction of HCC relapse after LDLT	(233)
		miR-21-5p	↑	Human	serum	0.71	Diagnosis of HCC from LC	(234)
		miR-21, miR-10b	↑	Human, Mice	serum	——	Prognostic markers of E-HCC	(235)
		miR-18a, miR221, miR-222, miR224	↑	Human	serum	——	Diagnosis of HCC from LC/CHB	(223)
		miR-101, miR106b, miR-122, miR-195	↑	Human	serum	——	Diagnosis of HCC from CHB	(223)
		miR-122, miR148a, miR-1246	↑	Human	serum	——	Diagnosis of HCC from LC	(227)
		miRNA-519d, miR-595, miR-939	↑	Human	serum	——	Diagnosis of HCC from LC	(222)
		miR-10b-5p, miR-221-3p, miR-223-3p, miR-21-5p	↑	Human	plasma	0.86	Diagnosis of HCC from CH or LC	(234)
	lncRNA	lncRNA-HEIH	↑	Human	serum	——	Diagnosis of HCV-associated HCC from CHC	(236)
		LINC02394	↑	Human	serum	0.719	Diagnosis of HCC from CHB	(237)
		LINC00635	↑	Human	serum	0.750	Diagnosis of HCC from CHB	(237)
		LINC00161	↑	Human	serum	0.794	Prediction of HCC growth and metastasis	(238)
		lncRNA-ATB	↑	Human	serum	——	The prognosis of HCC	(239)
		Lnc85	↑	Human	plasma	0.869	Diagnosis of AFP-negative HCC from healthy controls and LC	(163)
		SENP3-EIF4A1	↑	Human, Mice	plasma	0.8028	The diagnosis of HCC	(160)
	circRNA	circFBLIM1	↑	Human, Mice	serum	——	The therapeutic target of HCC	(149)
		circ-0051443	↑	Human, Mice	plasma	0.8089	The diagnosis and therapeutic target of HCC	(161)
		circRNA-100338	↑	Human, Mice	serum	——	The diagnosis and therapeutic target of HCC	(240)
		circUHRF1	↑	Human, Mice	plasma	——	The therapeutic target of HCC	(169)

(Continued)

TABLE 3 | Continued

Classification	Biomarkers	Expression	Species	Type of biological fluid	AUROC	Clinical significance	Refs
	circ-DB	↑	Human, Mice	adipocyte	——	The prognosis of HCC	(202)
proteins	LAPTM4B-35	↑	Human	serum	——	Prediction of recurrence and diagnosis of HCC	(241)
	SMAD3	↑	Human, Mice	peripheral blood	0.70	The diagnosis of HCC	(242)
	RAB5A	↑	Human	serum	——	The diagnosis and therapeutic target of HCC	(243)
	ENO1	↑	Human, Mice	serum	——	The prognosis of HCC	(244)
Other combinations	miR-122, miR-148a, AFP	↑	Human	serum	0.931	Diagnosis of HCC from LC	(227)
	SMAD3+ATP	↑	Human, Mice	peripheral blood	0.90	The diagnosis of HCC	(242)
	lncRNA-RP11-513I15.6, miR-1262/RAB11A	↑	Human	serum	——	Diagnosis of E-HCC from CHB	(245)
	miRNA-21, lncRNA-ATB	↑	Human	serum	——	The prognosis of HCC, overall survival	(239)
	ENSG00000258332.1, LINC00635, AFP	↑	Human	serum	0.894	The diagnosis and prognosis of HCC	(237)
	AFP、ENST00000248932.1, ENST00000440688.1, ENST00000457302.2	↑	Human	plasma	0.905 0.879	Predict the probability of HCC in the cancer-free groups Predict the probability of HCC in the CH groups	(246)
Total EVs	Total EV	↑	Human	serum	0.83	Detection of HCC	(225)
	AFP, GPC3, ALB, APOH, FABP1, FGB, FGG, AHSG, RBP4, TF	↑	Human	plasma	0.93	Diagnosis of E-HCC from LC	(247)
	LINC00853	↑	Human	serum	0.956	Diagnosis of E-HCC from CH、LC	(248)

8 BIOMARKERS

EVs are providing important links for intercellular information transfer (218), and specific proteins and nucleic acids in EVs are important biomarkers for clinical diagnosis of various liver diseases.At present, the clinical assessment of liver damage is mainly based on liver enzyme profiles, such as aspartate aminotransferase (AST), alanine aminotransferase (ALT) (219–221). However, these enzyme markers lack specificity for liver diseases. Traditional tumor markers such as AFP, AFP-L3 are susceptible to other liver diseases and cannot analyze HCC for etiology, which has certain limitations. Therefore, to find new specific markers for patients with liver disease is significant. Much research mentioned that the proteins and nucleic acids carried by EVs can serve as markers to predict the prognosis of patients with liver disease (222–224).

8.1 EVs-Associated Nucleic Acids as Biomarkers for HCC Diagnosis

8.1.1 miRNAs

miRNAs in serum EVs hold great potential as novel diagnostic biomarkers, and some of which have been reported worldwide (**Table 3**). Elevated levels of miRNA-21 and lncRNA-ATB expression were found to have higher specificity and sensitivity for HCC (232, 239). Patients with postoperative recurrence of HCC have significantly reduced the expression of miRNA-718, which was associated with the highly aggressive nature of HCC (233). Interestingly, Wang, Y et al. proposed that EVs-miR-122, EVs-miR-148a and EVs-miR-1246 in HCC patients serum were apparently higher than those in the LC and the NC group, and that these miRNAs combined with AFP could effectively reduce the rate of misdiagnosis (227). However, for HCC patients with low AFP expression, whether or not with hepatitis virus infection, sEVs' miRNAs are more indicative of being markers of HCC when they are expressed as miR-10b-5p+ miR-221-3p+ miR-223-3p and miR-10b-5p+ miR-221-3p+ miR-223-3p+ miR-21-5p (234). Tian X et al. indicated that an acidic environment triggers HIF-1α and HIF-2α activation and facilitates the expression of EVs-miR-21 and EVs-miR-10b, significantly promoting the progression of HCC both *in vivo* and vitro (235, 249). We also find that several miRNAs are studied at high frequency, such as miR-21 and miR-122, and the results may differ in different study contexts. Besides, we read that some serum miRNAs are biomarkers of HCC (250–256), but it is not explicitly stated that these miRNAs are associated with EVs, and their roles in the progression and recurrence of HCC need to be further explored.

TABLE 4 | EVs-associated proteins as biomarkers of liver disease.

Liver disease	Biomarkers	Types	Function	References
Non-alcoholic steatohepatitis(NASH)	ASGPR1+	Protein	A surrogate noninvasive biomarker of portal hypertension in patients with cirrhotic NASH.	(267)
	CD4+	Protein	Biomarkers of nonalcoholic fatty liver(NAFL)and CHC	(268)
Toxic acute liver injury	Apolipoprotein A-1	Protein	Tentative hepatotoxic markers during hepatic damage	(269)
	Carboxylesterase-1	Protein	Hepatotoxic markers during hepatic damage	(269)
	Carboxylesterase-3	Protein	Non-invasive indicator of drug toxicity	(270)
CCA	AnnexinV+EpCAM+ASGPR1 +CD133+ taMPs	–	A novel biomarker of HCC and CCA liquid biopsy	(225)
	MMP-7	Protein	Biomarkers for the diagnosis of CCA	(271)
Liver fibrosis	CD8+	Protein	A biomarker for liver fibrosis	(272)
	CD14+	Protein	A tamps biomarker for liver fibrosis	(273)
Alcoholic steatohepatitis (ASH)	CYP450-2E1	Cytochrome	A potential biomarker for liver injury	(274)
	CD40L	Protein	A potential biomarker for ASH	(275)
Alcoholic hepatitis	CD34+ ASGPR	Protein	Biomarkers of alcoholic hepatitis	(276)
	CK18	Protein	Biomarkers of alcoholic hepatitis	(277)

8.1.2 lncRNAs

In recent years, the potential of EVs-derived lncRNAs in the prognosis of HCC has also attracted growing research interest. lncRNAs alter lncRNA expression can contribute to the cancer phenotype by stimulating cell proliferation, angiogenesis, immune evasion, and inhibition of apoptosis. Among them, linc-VLDLR was identified as a lncRNA enriched in EVs that contributes to the cellular stress response (257). ENSG00000248932.1, ENST00000440688.1 and ENST00000457302.2 were significantly increased in HCC patients, suggesting that lncRNAs may predict tumorigenesis and can be used to dynamically monitor HCC metastases (246). The expression of lncRNA-HEIH was higher in patients with HCV (hepatitis C virus)-associated HCC than that of CHC (chronic hepatitis C) patients (236, 258). Some indicated that sEVs levels of ENSG00000258332.1 and LINC00635 in serum were significantly high and it would be more specific and sensitive when they combined with serum AFP to detect HCC (237). Huang X and Kim S et al. suggested that EVs-derived Lnc85 and LINC00853 showed high positivity in AFP-negative patients with early HCC and were significantly better than AFP, respectively, which is particularly relevant to patients with AFP-negative tumors (163, 248). The potential of EVs containing lncRNAs as biomarkers in the process of HCC diagnosis cannot be ignored, and to find more specific markers for HCC is the next research direction.

8.1.3 CircRNA

There is growing evidence that circRNA in EVs has certain advantages in terms of abundance and stability, indicating that they are promising therapeutic targets for HCC. Similar to miRNA and lncRNA, changes in circRNA expression can also affect the occurrence and progression of HCC (259). In addition, circFBLIM1 was significantly expressed in HCC serum sEVs and promoted HCC progression by affecting the miR-338/LRP6 axis (149). Similarly, Bai N et al. found that circFBLIM1 acts as ceRNA to facilitate HCC by sponging miR-346 (260). In contrast, sEVs-circ-0051443 inhibits HCC progression by regulating miR-331-3p/BAK1 (161). Moreover, Huang XY et al. indicated that HUVECs receiving the circRNA-100,338 could boost the metastatic capacity of HCC cells, which may be related to the regulation of angiogenesis (209). Furthermore, serum EVs-circrna-100, 338 in patients with radical hepatic resection HCC are persistently hyperexpressed, dedicating lung metastases and low survival (240). Ultimately, circMTO1 (261), circSETD3 (262), cSMARCA5 (263), and hsa_circ_0068669 (264) also play key roles in HCC and are potential therapeutic targets, but it remains unclear whether these circRNAs and EVs are related.

8.2 EVs-Associated Proteins as Biomarkers of Liver Disease

EVs proteins change with the environment and state of liver cells, it can be used directly or indirectly as a biomarker in different liver diseases (265, 266) to predict the progression of the corresponding liver disease (**Table 4**). CYP450-2E1 (227) and protein tyrosine phosphatase receptor (sPTPRG) isoforms associated with EVs are biomarkers of liver injury, and sPTPRG in plasma reflects the extent of liver injury (274, 278). If CD8, CD14, and connective tissue growth factor (CCN2) are highly expressed in EVs, they can be used to assess the degree of liver fibrosis (272, 273, 279). High expression of Apolipoprotein A-1 by EVs elevates liver-specific proteins such as FGB, causing toxic acute liver injury (269). Studies have shown that EVs containing Carboxylesterase-1 and Carboxylesterase-3 can be evaluated for hepatotoxicity (269, 270). JH H et al. indicated that EVs highly express AnnexinV+EpCAM+ASGPR1+CD133+ taMPs, which can be a novel biomarker for HCC and CCA liquid biopsies (225). If MMP-7 is highly expressed in EVs, it could be a marker for the differential diagnosis of CCA (271). Hepatocytes secrete EVs if ASGPR1+, which can be an alternative non-invasive biomarker of portal hypertension in NASH patients (267).

High CD4+ expression in EVs can be a biomarker to diagnosis nonalcoholic fatty liver (NASH) from chronic hepatitis C (CHC) (268). Positive CD34+ with ASGPR (heavy alcoholic hepatitis) or CK18 (alcoholic hepatitis) in EVs can be used as biomarkers (276, 277), among them, CD34 can also be used as a biomarker to determine heavy alcoholic hepatitis (276).

ENO1 upregulates the expression of integrin α6β4 and activates the FAK/Src-p38MAPK pathway (244). Gorji-Bahri G et al. suggested that RAB5A knockdown could be used as a therapeutic target to control the progression of HCC (243). Pang Y et al. saying that LAPTM4B-35 is associated with the HCC relapse, drug resistance, and it is expected to be a new diagnostic marker for HCC (241).

Many studies have shown that EVs affect the progression of various liver diseases by regulating cellular functions and activating key signaling pathways in receptor cells, obviously, which are newly discovered potential biomarkers, to open up new ways to clinically distinguish different kinds of liver disease. Unfortunately, the role of EVs in the diagnosis, prognosis determination and predictive value of liver diseases is still lacking sufficient clinical evidence. Studies on the sensitivity and specificity of these markers in liver disease have also been reported relatively rarely, and relevant applications remain to be further investigated.

9 VESICLE-LOADED DRUGS

9.1 EVs are Natural Nanocarriers

EVs are endogenous cell-derived membranous structures, natural nanocarriers with very low cytotoxicity and immunogenicity, protecting the transported RNA from disassembly and phagocytosis by ribonucleases, with inherent activity targeting and ability to cross biological barriers (30). EVs can transport a wide variety of bioactive molecules, thus altering the physiological functions of the recipient cells and reducing the accumulation of chemotherapeutic drugs in non-target organs, thereby reducing off-target toxicity. Additionally, EVs can bind to each other through various ligand receptors, especially cytokinesis (280). EVs are efficient as synthetic nanocarriers. EVs as nucleic acid and drug delivery vehicles has been extensively studied (281, 282). Notably, EVs as drug carriers need to find an efficient method as cargo loading. Different techniques such as electroporation (283), incubation (284), sonication (285), and freeze-thawing have been applied for the EVs loading (286). What's more, EVs can also be loaded with specific cargoes with endogenous mechanisms such as direct transfection or co-incubation to deliver the cargo to the cytoplasm (287, 288). However, these loading techniques may lead to some changes in the morphological characteristics and physicochemical properties of EVs, as well as aggregation of themselves or of the cargo they carry (289, 290). A more accurate understanding of the proteomic profile of EVs and the factors influencing protein composition will facilitate the development of protein-based therapeutic strategies for EVs in the future (291, 292).

9.2 Application of Drug-Carrying EVs in HCC

We review emerging strategies for targeted delivery using EVs and explore the use of them for the treatment of hepatocellular carcinoma. Treatment of H22 cells with the chemotherapeutic drug methotrexate (MTX) and irradiation with UV light, which could secrete Microparticles (MPs) when co-incubate with the remaining H22 cells, effectively kill tumor cells and reduce adverse effects, while impeding drug efflux (98). We read that RBC-EVs loaded with doxorubicin or sorafenib showed enhanced therapeutic effects in mouse models of *in situ* HCC through a macrophage-dependent mechanism compared with conventional doses of doxorubicin and sorafenib (293). More importantly, drug-loaded RBC-EVs did not show systemic toxicity, whereas conventional doses of doxorubicin and sorafenib did. The main challenges in the current clinical application of EVs are the limited yield and the susceptibility to contamination of EVs with various centrifugation methods (105, 114), which affects the purity and biological properties of EVs. In addition, although EVs are good natural carriers, how to load substances efficiently such as antitumor drugs or genes into EVs is still an urgent technical problem to be solved. Drug-carrying EVs are promising for clinical applications in the treatment of liver diseases, and careful selection of cells of origin for EVs, the creation of appropriate methods for loading the molecules they carry, overcoming low yields, etc. are current research hotspots.

10 DISCUSSION

EVs are sensory molecules for information exchange between tumor cells in the microenvironment, activating different signaling pathways and influencing the development, progression and metastasis of tumors (294–297). In recent years, EVs have become promising vehicles in liver disease for their low toxicity, high stability and preferential absorption (298). Today, the application of EVs is still in its early stages. Although there have been clinical trials choosing miRNAs for liver disease, they are still not available for clinical use (298), lacking a number of clinical trials to demonstrate the effectiveness of EVs. The mechanisms and clinical applications of EVs in liver disease need to be studied in more depth. EVs may be an effective intervention in the future, showing a new light for oncology patients. What's more, EVs can also alter the function of recipient cells and is crucial in the genesis, development and pathogenesis of HCC. Circulating EVs, as a novel signaling modality, which are involved in multiple processes including tumor development and metastatic drug resistance, are promising biomarkers for diagnosing liver disease and monitoring treatment response (46).

Notably, our current understanding of EVs is still inadequate and standard methods for isolating and tracking EVs are lacking. EVs are nearly released by all cells in the body, and many mechanisms involved in their production, transport, uptake and involvement in cancer development have not been fully explored (299), and challenges remain in the extraction, identification and processing of EVs biomarkers for analysis. In addition, the complexity of the immune response and microenvironment in the liver poses a significant challenge to the routine treatment of patients with HCC (300). Therefore, it is

important to improve isolation techniques, tracking methods, screening for tissue-specific markers of EVs or the identifying EVs of tissue-specific origin in lesions. Making full use of the different extraction techniques available and optimising them is an important next step in research. In addition, experiments *in vitro* and *in vivo* on EVs still have many limitations, so there is an urgent need to establish well-developed experimental models to further explore their properties and mechanisms of action, and to explore the potential of using this intercellular communication modality in the TME for molecular diagnosis and targeted therapy of tumors. In conclusion, current studies indicate that EVs is crucial in mediating the progression of liver disease and therefore can be thought as a potential therapy for HCC. With a more comprehensive understanding of EVs, more valuable references will be provided for the prevention, diagnosis and prognosis of HCC.

AUTHOR CONTRIBUTIONS

JW: conceptualization, methodology, writing-original draft, writing-review & editing, visualization and supervision. XW: writing-original draft, formal analysis and resources. XZ: writing-original draft, formal analysis and project administration. TS: writing-original draft, software. YL: writing-original draft, data curation. WW: writing-original draft, methodology. YH: Conceptualization and Supervision. All authors contributed to the article and approved the submitted version.

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GLOSSARY

ADC	apparent diffusion coefficient
ADMSC	adipose-derived mesenchymal stem cells
AF4	Asymmetrical flow field-flow fractionation
AHSG	alpha 2-HS glycoprotein
ALB	albumin
ALT	alanine aminotransferase
APOH	apolipoprotein H
ASGPR1	asialoglycoprotein receptor
ASH	Alcoholic steatohepatitis
AST	aspartate aminotransferase
AUROC	Area under Receiver Operating Characteristics
BCA	bicinchonic acid
BM-MSCs	Bone marrow mesenchymal stem cells
CAFs	Cancer-associated fibroblasts
CCA	Cholangiocarcinoma
CCN2	Connective tissue growth factor
ceRNA	competing endogenous RNA
CFH	Complement Factor H
CH	chronic hepatitis
CHB	chronic hepatitis B
CHC	chronic Hepatitis C
CHOP	enhancer-binding protein homologous protein
CK18	Cytokeratin-18
CLEC3B	C-Type Lectin Domain Family 3 Member B
CSCs	Cancer stem cells
CTGF	connective tissue growth factor
DCP	des-gamma-carboxy prothrombin
DDS	drug delivery system
EVs	Extracellular vesicles
EC	endothelial cells
EGF	endothelial growth factor
E-HCC	early-stage hepatocellular carcinoma
EMT	Epithelial–mesenchymal transition
ENO1	Alpha-enolase
EpCAM	epithelial cell adhesion molecule
ESCRT	endosomal sorting complex required for transport
ELISA	enzyme linked immunosorbent assay
FCS	fluorescence correlation spectroscopy
FABP1	fatty acid binding protein 1
FGB	fibrinogen beta chain
FUS	focused ultrasound
GEVs	Glioma-derived EVs
GGT	glutamyl aminotransferase
GPC3	glypican3
HCC	hepatocellular carcinoma
HCV	Hepatitis C Virus
HDL	High-density lipoprotein particles
HEMs	Adult human epidermal melanocytes
HGF	Hepatocyte growth factor
HIF-1α	Hypoxia Inducible factor 1 α
HIF-2α	Hypoxia Inducible factor 2 α
HSCs	Hepatic stellate cells
HUVECs	Human umbilical vein endothelial cells
IL	nterleukin
ILVs	intraluminal vesicles
iNOS	Inducible nitric oxide synthase
LAMP2B	lysosomal associated membrane protein 2B
LC	liver cirrhosis
LDLT	living donor liver transplantation
LG3BP	galectin-3-binding protein
LFIA	Lateral-Flow Immunochromatographic Assay
MMP	Matrix metalloproteinase
MPs	Microparticles
MSC	mesenchymal stem cells
MVBs	multivesicular bodies
m/lEVs	medium/large EVs
MDVs	Mitochondria-Derived Vesicles
MAC	membrane attack complex
NTA	nanoparticle tracking analysis
NAFL	Nonalcoholic fatty liver
NASH	non-alcoholic steatohepatitis
NC	normal control
NVs	Nanovesicles
PIGR	polymeric immunoglobulin receptor
RBP4	retinol binding protein 4
ROS	reactive oxygen species
sEVs	small EVs
SNX9	sorting nexin 9
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SAH	Severe alcoholic hepatitis
SMAD3	SMAD Family Member 3
sPTPRG	Protein tyrosine phosphatase receptor Gamma
TAMs	Tumor-associated macrophages
TF	transferrin
TGF-β	Transforming growth factor
TME	Tumor microenvironment
TNFα	Tumor necrosis factor alpha
TSG101	tumor susceptibility gene 101 protein
TRPS	tunable resistive pulse sensing
VEGF	Vascular endothelial growth factor

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REVIEW
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EV-Mediated Chemoresistance in the Tumor Microenvironment: Is NF-κB a Player?

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Drug resistance is a major impediment to patient survival and remains the primary cause of unsuccessful cancer therapy. Drug resistance occurs in many tumors and is frequently induced by chemotherapy which triggers a defensive response both in cancerous and cancer-associated cells that constitute the tumor microenvironment (TME). Cell to cell communication within the TME is often mediated by extracellular vesicles (EVs) which carry specific tumor-promoting factors able to activate survival pathways and immune escape mechanisms, thus sustaining tumor progression and therapy resistance. NF-κB has been recognized as a crucial player in this context. NF-κB activation is involved in EVs release and EVs, in turn, can trigger NF-κB pathway activation in specific contexts, based on secreting cytotype and their specific delivered cargo. In this review, we discuss the role of NF-κB/EVs interplay that sustain chemoresistance in the TME by focusing on the molecular mechanisms that underlie inflammation, EVs release, and acquired drug resistance.

Keywords: extracellular vesicles, NF-κB, drug resistance, tumor microenvironment, inflammasome

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INTRODUCTION

Cancer chemotherapy resistance is the innate and/or acquired ability of cancer cells to escape the effects of chemotherapeutics and represents a great challenge in cancer therapy to improve clinical outcomes. The development of resistance occurs in many tumors and depends partially on genetic instability, heterogeneity, speedy mutation in the tumor cell, cytogenetic changes, and intra-neoplastic diversity (1, 2). The tumor microenvironment (TME) is also considered to be a factor for resistance development in many cancers, as chemotherapy frequently triggers a defensive response not only in cancerous cells but also in cancer-associated cells within the TME (3). Activation of survival pathways and immune escape mechanisms are often mediate by extracellular vesicles (EVs), which release their cargo in recipient cells within TME. EVs are lipid-contained vesicle, which are classified based on their size, biogenesis, and release mechanism. EVs include microvesicles (MVs), exosomes (EXs) and apoptotic bodies (ABs) (4). MVs are 100 to 1000 nm in size, EXs are smaller and range in size from 30 to 150 nm, while ABs show a size ranging from 50 to 5000 nm. MVs and ABs directly bud from cytoplasmic membrane, while EXs are produced by inward budding of plasma membrane and formation of endosomes, which mature into multivesicular bodies (MVBs) and subsequently were secreted into extracellular space (5, 6). Among EVs, exosomes are the best characterized. After formation, early endosome is processed in MVBs by the

endosomal sorting complexes required for transport (ESCRT), directing by the invagination of MVBs outer membrane and packaging of biomolecules (7). EVs support intracellular communications within the TME by carrying specific tumor-promoting factors that positively regulate several pro-survival pathways including NF-κB, which plays a pivotal role in this context.

NF-κB transcription factor family consists of a group of inducible effectors which regulate a plethora of genes involved in several physiological processes such as inflammation (8), differentiation (9), survival (10), proliferation (11), and immunity (12). However, dysregulated NF-κB signaling is often found in many pathological conditions including inflammatory disorders (13), autoimmunity (14), and cancer (15). NF-κB family comprises five structurally related members, specifically, p65 (RelA), RelB, c-Rel, p50 (NF-κB1), and p52 (NF-κB2) that form homodimers and heterodimers able to specifically bind the consensus κB site 5'-GGGPuNNPyPyCC-3 (16) with different transcriptional activity (17). p50 and p52 are active forms of precursors proteins p105 and p100 respectively that, in turn, operate both as NF-κB precursors and inhibitors of NF-κB dimers (18). Activation of NF-κB pathway occurs through two major mechanisms, namely canonical (or classical) and non-canonical (or alternative) pathways (19). In absence of stimuli, NF-κB dimers are retained within the cytosol in inactive forms through their interaction with IκB (inhibitor of kappa B) proteins (20). In the canonical pathway, upon stimulation [*i.e.*, lipopolysaccharide (LPS), tumor necrosis factor α (TNF-α)], IKK (IκB kinase) (21) proteins (IKKα, IKKβ, IKKγ) assemble into multiproteic complexes and trigger NF-κB activation. Specifically, in presence of stimuli, activated IKK complex phosphorylates IκB proteins, thus inducing its ubiquitination (22) and proteasomal degradation and allowing, in turn, NF-κB dimers to translocate into the nucleus and activate its target genes. In the non-canonical pathway, induced by a subset of tumor necrosis factor receptor (TNFR) superfamily members upon stimulation by several factors such as lymphotoxin B and BAFF, the activation of the heterodimers p100/RelB is triggered by NF-κB inducing kinase (NIK) that activates IKKα complex and in turn promote the processing of p100 in p52 and the nuclear translocation of the active p52/RelB dimer.

While the role of NF-κB in promoting cancer progression and drug resistance is well-known, emerging evidence are pointing out for a crucial interplay between NF-κB and EVs that sustain TME remodeling, tumor inflammation and therapy resistance. Here, we discuss this crosstalk by focusing on the molecular mechanisms that underlie inflammation, EVs release, and acquired drug resistance.

NF-κB: A Master Regulator of Inflammation and Therapy Resistance in Cancer

NF-κB Signaling in Inflammation and Cancer

During carcinogenesis, parenchymal cells continuously interact with the surrounding environment establishing a plethora of interactions with stromal cells (*i.e.*, fibroblasts, endothelial cells, adipose cells, mesenchymal stem cells), immune cells, and extracellular matrix that constitute the TME (23). In this context, inflammation plays a key role in contributing to carcinogenesis and promoting the metastatic phenotype (24, 25). NF-κB constitutive activation is widely recognized as a hallmark of many types of tumors including hepatocellular carcinoma (26), breast cancer (27), lymphoid malignancies (28), colorectal (29) and prostate cancer (30). In addition to promoting tumor cell survival, oncogenic NF-κB signaling operates in the TME, thereby linking inflammation and cancer (15, 31). The inflammatory response is mainly induced by TNF-α, Interleukin-1β (IL-1β), and Interleukin-6 (IL-6). These cytokines usually are not overexpressed in healthy tissues but are significant upregulated in response to several pathological stimuli. Although released to protect the host, these cytokines often trigger a positive feedback mechanism promoting a chronic inflammation that, in turn, sustains, carcinogenesis and tumor progression (32). Accordingly, NF-κB activation in non-malignant tumor-associated cells has been shown to amplify the production of cytokines and other specialized effectors that promote tumor-cell proliferation, invasion and therapy resistance, while suppressing anti-tumor immune responses (33). Damage-associated molecular patterns (DAMPs), are endogenous molecules produced by dying cancer cells in response to stress and cell injury (34). After production, DAMPs are secreted through several mechanisms including extracellular vesicles (EVs) (35); these are recognized by specific pattern recognition receptors (PRRs) expressed on several cells, such as monocytes and macrophages, which activate different inflammatory pathways including NF-κB pathway. In turn, NF-κB activation causes the release of proinflammatory cytokines, such as IL-1β (36). As part of a positive feedback loop, active IL-1β binds to IL-1 receptors (IL-1R) on cancer cells and further stimulate NF-κB signaling, thus inducing the expression of pro-inflammatory cytokines TNF-α and IL-6 and sustaining NF-κB-mediated chronic inflammation (37–39). Moreover, enhanced or deregulated NF-κB activity in fibroblasts and macrophages promotes their switching to cancer-associated fibroblasts (CAFs) and tumor-associated macrophages (TAMs), respectively, thus supporting tumor progression, vascularization and tumor growth, as observed in several cancers (40–45). NF-κB signaling also plays an important role in macrophages polarization (46); M1-type macrophages have a pro-inflammatory activity and tissue damaging properties, while M2 macrophages, with their anti-inflammatory phenotype, promote cell proliferation and tissue repair. Interestingly, although NF-κB represents a key transcription factor in M1 macrophage during the early stage of tumorigenesis, it also plays a pivotal role in advanced stages where it polarizes TAMs toward the immunosuppressive and tumor-promoting M2 phenotype. Indeed, we have demonstrated that NF-κB activation, through its target gene *GADD45B*, prevents TAM polarization to M1, thus inhibiting their antitumor activity (47, 48). Furthermore, it was observed that NF-κB p50 protein suppresses M1-type polarization and supports M2 immunosuppressive phenotype (49). The role of NF-κB in CAFs and TAMs polarization represents a hallmark of inflammatory TME and has been well explained in many excellent reviews (50, 51). In summary, as discussed below, NF-κB-mediated

inflammation constitutes an important link between EVs activity and acquired drug resistance.

NF-κB-Driven Drug Resistance

In addition to promote initiation and tumor progression, NF-κB signaling fosters cancer resistance to chemotherapy. Although chemotherapy is the gold standard option for many types of cancers, multi-drug resistance (MDR) occurring in late/advanced stages significantly limits its long-term efficacy. Several authors reported that various anti-cancer drugs can activate NF-κB pathway by different mechanisms (**Figure 1**). The microtubule stabilizer paclitaxel, triggers NF-κB cascade by binding the toll-like receptor 4 (TLR4) (52). Other microtubule polymerization inhibitors, such as vinblastine and vincristine, can activate NF-κB, specifically by inducing protein kinase C-mediated phosphorylation and subsequent degradation of IκBα (53, 54). Another class of drugs able to induce NF-κB are the topoisomerases inhibitors, such as doxorubicin and SN38, that act by directly activating IKK complex (55). Once activated, NF-κB promotes chemoresistance in different ways, including the induction of anti-apoptotic genes, thus increasing resistance to drug-induced damage, and the overexpression of efflux pumps to prevent xenobiotic accumulation. In A549 human lung adenocarcinoma cells, chemotherapy-induced NF-κB activation leads to the expression of anti-apoptotic proteins like BCL-xL and BFL1 that in turn promote cancer cell survival (56). Furthermore, NF-κB induces resistance to apoptosis by upregulating the expression of inhibitors of apoptosis proteins (IAPs) (57) and suppressing the TNF-related apoptosis-inducing ligand (TRAIL) pathway (58). In addition, activation of NF-κB signaling can lead to chemoresistance by directly inducing the expression of efflux pumps proteins, such as human multidrug resistance protein 1 (MDR1), also known as P-glycoprotein 1 (P-gp), and ATP-binding cassette sub-family B member 1 (ABCB1). P-gp is an ATP-dependent transporter with a broad spectrum of activity, and it is able to efflux nonionic and amphipathic xenobiotics like anthracyclines, vinca-alkaloids and taxanes. It has been observed that NF-κB can transactivate the promoter of *MDR1* (59) and that the inhibition of this signaling results in the downregulation of *MDR1* in different types of cancers (60–62). NF-κB is also a transcriptional regulator of cyclooxygenase-2 (COX-2) (63), whose activity showed a strong correlation with P-gp expression in hepatocellular carcinoma (64) and colorectal cancer (65), and with multidrug

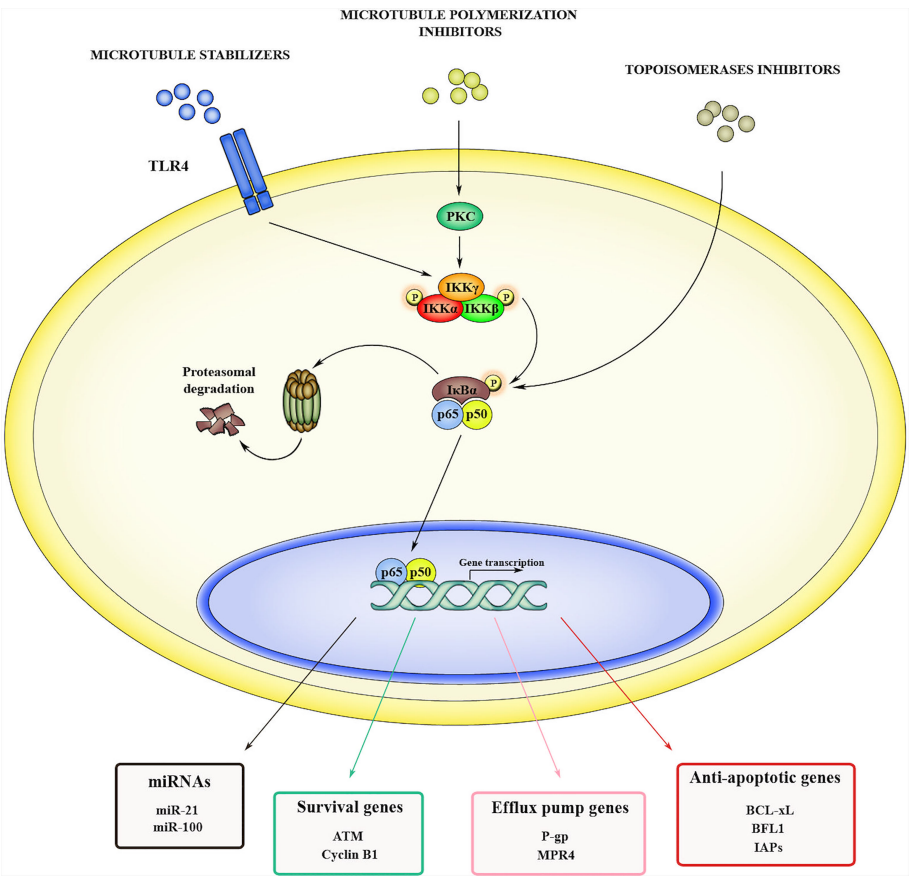

FIGURE 1 | Mechanisms of NF-κB-induced drug resistance. NF-κB can be activated in cancer cells in response to several anticancer drugs, including microtubule stabilizers (*i.e.*, paclitaxel), microtubule polymerization inhibitors (*i.e.*, vinblastine and vincristine) and topoisomerases inhibitors (*i.e.*, doxorubicin and SN38). When activated, NF-κB promotes drug resistance by inducing the transcription of genes such as miRNAs, as well as genes codifying for prosurvival factors, anti-apoptotic effectors, and efflux pumps.

resistance protein 4 (MRP4) in lung cancer (66). Another important mechanism in NF-κB-mediated drug resistance involves microRNAs (miRNAs). In this regard TRAIL cascade activation induces NF-κB with subsequent upregulation of miR-21 and miR-100, which in turn activate TNF Receptor Associated Factor 7 (TRAF7) and sustain NF-κB signaling, thus generating a positive feedback loop that suppresses TRAIL-induced apoptosis (67). NF-κB is also involved in radiotherapy resistance through the transcription of prosurvival genes that mediate resistance to ionizing radiation such as DNA-damage sensor ataxia-telangiectasia mutated (ATM) and cyclin B1 (68). In addition, overexpression of several miRNAs, such as miR-125b and miR-668, leads to NF-κB activation by targeting TNF alpha induced protein 3 (TNFAIP3/A20) and IκBα, respectively, thus promoting radioresistance in nasopharyngeal carcinoma (69) and in breast cancer (70). These findings show that NF-κB activation induces drug resistance at multiple levels and that its inhibition could represent an efficient approach to improve clinical outcome.

EVs in TME-Cancer Cell Crosstalk and Drug Resistance

EVs Roles in the Bidirectional Cell to Cell Communication Within the TME

The crosstalk between cancerous and surrounding cells in TME through EVs is essential to sustain tumor growth, change cell phenotype and induce metastatic switch (71) (**Figure 2**). The key role of EVs in TME is due to their capacity to transfer proteins, lipids, nucleic acids, and membrane receptors, thus providing different information based on the specific composition of their cargo. Exosome secretion and its intracellular trafficking involve a subset of small GTPase named Rabs which are demonstrated to be expressed in a context-dependent manner (72–74). In some cancers, Rabs overexpression is linked with tumor progression and worse clinical outcome (75). Once released, exosomes interact with target cells through specific receptors or molecules such as intercellular adhesion molecule 1 (ICAM1) and heat shock protein 70 (Hsp70), expressed on dendritic cell-derived and on mast cell-derived exosomes, respectively. While ICAM 1 is recognized by lymphocyte function-associated antigen 1 (LFA-1) (76, 77), Hsp70 interacts with low density lipoprotein receptor-related protein 1 (LRP1/CD91) on antigen presenting cells (78). Due to their crucial role in cell-cell communications, exosomes activity in TME has been associated with tumor progression and induction of metastatic phenotype (79, 80). Importantly, the bidirectional cell to cell communication between tumor and stromal cells sustains cancer progression toward advanced stages (81). Within TME, the communication between CAFs and TAMs with tumor cells is mediated by CAFs/TAMs derived exosomes. It was observed that CAF-derived exosomes (CDEs) containing intact metabolites are able to promote oncogenic transformation, by inhibiting oxidative phosphorylation and

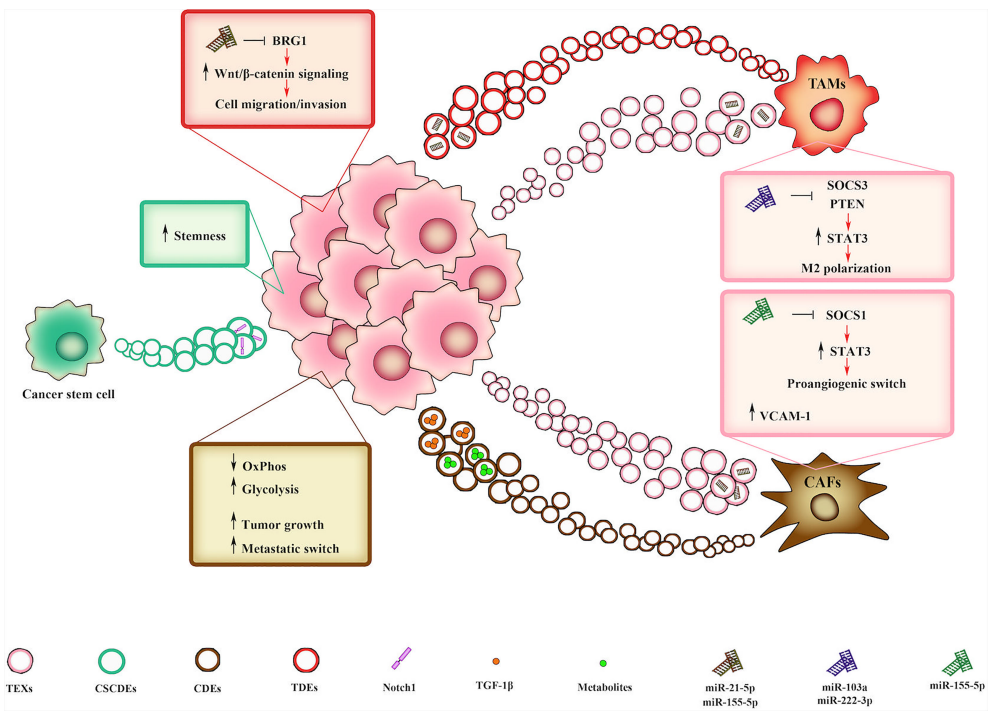

FIGURE 2 | EV-mediated interplay within the TME mediates tumor progression. CAF-derived exosomes (CDEs) promote either metabolism deregulation or metastatic switch in tumor cells by delivering intact metabolites or TGF-β, respectively. TAMs-derived exosomes (TDEs) loaded with miR-21-5p and miR-155-5p can promote cell migration and invasion by downregulating BRG1 gene, thus sustaining Wnt/β-catenin signaling. CSCs-derived EVs (CSCDEs) confer stemness traits to recipient cells by delivering notch receptor 1 (notch1) protein. In turn, tumor-derived exosomes (TEXs) induce CAF transformation and macrophage M2 polarization by delivering miR-155-5p, miR-103a and miR-222-3p, that in turn affect STAT3 signaling.

increasing glycolytic metabolism (82, 83). Moreover, CAFs induce tumor growth and metastatic phenotype switching by producing exosomes with high concentration of TGF-1β (84). Lan et al. demonstrated that TAMs-derived exosomes also mediate cell migration and invasion in colon cancer *via* miRNAs (miR-21-5p and miR-155-5p)-mediated downregulation of *BRG1* gene (85), whose decreased expression is responsible for Wnt/β-catenin signaling activation (86). On the other hand, tumor cells modulate and "re-educate" surrounding cells by secreting tumor-derived exosomes (TEXs). Xiao et al. showed that melanoma-derived EVs promote CAFs transformation by inducing VCAM-1 expression *via* extracellular signal-regulated kinase 1/2 (ERK1/2)-activation (87). Moreover, miR-155-5p in melanoma-derived exosomes triggers the proangiogenic switch of CAFs by targeting suppressor of cytokine signaling 1 (SOCS1), thus activating the janus kinase (JAK)2/signal transducer and activator of transcription (STAT)3 (JAK2/STAT3) pathway (88). As to TAMs transformation, Hsu and collaborators reported that in hypoxic lung cancer miR-103a-loaded EVs promoted M2 macrophage polarization by inhibiting phosphatase and tensin homolog (PTEN) and enhancing protein kinase B (PKB/Akt) and STAT3 activity (89). Moreover, ovarian cancer-derived exosomes induce macrophages polarization by delivering miR-222-3p which target SOCS3, thus sustaining STAT3 signaling (90). Furthermore, cancer stem cell (CSCs)-derived EVs (CSCDEs) play a significant role in TME remodeling. Sun et al. showed that glioblastoma stem cell (GSCs)-derived exosomes are able to confer stemness traits and enhance tumorigenicity in non-GSC glioma cells by delivering notch receptor 1 (notch1) protein and activating Notch signaling in recipient cells (91). Collectively, these studies underline the central roles of EVs in sustaining TME remodeling and fostering cancer progression.

EVs-Mediated Chemotherapy Resistance

In addition to promote tumor growth and progression, EVs also play a pivotal role in cancer drug resistance by taking part in several processes (*i.e.*, direct removal of drugs, incorporation of efflux pumps and miRNAs/long non-coding RNAs (lncRNAs) delivery) (**Figure 3**). Direct removal of drugs from intracellular space has been reported by Shedden et colleagues; they observed the release of doxorubicin-containing vesicles in doxorubicin-treated MCF-7 breast cancer cells and demonstrated that vesicles accumulated the drug passively. Moreover, they also founded a correlation between vesicle-shedding-associated gene expression and doxorubicin resistance in several cancer cell lines (92). Furthermore, Federici and collaborators identified cisplatin-enriched exosomes in human metastatic melanoma cells and founded that EVs-mediated drug elimination is enhanced by tumor acidic microenvironment (93). EVs are also able to incorporate efflux pumps which can actively transport drugs into intraluminal space; drug resistant cancer cells can transfer efflux pumps *via* EVs to the sensitive surrounding ones, thus conferring them resistance traits (94–97). In addition, EVs can incorporate factors that induce the expression of efflux pumps. In this regard, Ma et al. demonstrated that adriamycin-resistant breast cancer cell line MCF-7/ADM showed elevated levels of Ca^{2+}-permeable channel TRPC5, and that TRPC5 expression is essential for P-gp induction (98). The suppression of TRPC5 activity as well as of P-gp expression reduced drug resistance and tumor growth both *in vitro* and *in vivo* suggesting that inhibiting either P-gp or TRPC5 could be an attractive strategy to overcome drug resistance (98). They also founded that TRPC5-carrying EVs released from MCF-7/ADM cells can transfer chemoresistance properties to drug sensitive recipient cells (99). Accordingly, Wang et al. showed that exosomes-carrying Ca^{2+}-permeable channel TRPC5 acts as a noninvasive chemoresistance marker that could predict chemoresistance in metastatic breast cancer patients (100). EVs can also transport drug-metabolizing enzymes, such as glutathione S-transferase P1 (GSTP1), that contribute to the intracellular detoxification as observed in chemotherapy-resistant breast cancer (101). Another crucial mechanism in drug resistance involves miRNA and lncRNA. As reported by Lunavat and collaborators, miR-211–5p, which is overexpressed in response to treatment with BRAF inhibitors (Vemurafenib and Dabrafenib), induced drug resistance in melanoma cells (102). Furthermore, Mikamori et al. evidenced that long exposure to gemcitabine increased miR-155-loaded EVs secretion in pancreatic ductal adenocarcinoma, thus conferring drug resistance through inhibition of pro-apoptotic stress-induced p53 target gene tumor protein p53-inducible nuclear protein 1 (TP53INP1) (103). Again, Shen et al. showed that breast cancer cells treated with sublethal dose of chemotherapeutic drugs released miR-9-5p, miR-195-5p, and miR-203a-3p-enriched EVs, which simultaneously targeted transcription factor One Cut Homeobox 2 (ONECUT2), thus conferring stemness and resistance traits in recipient cells (104). Qu et al. founded that, in renal cell carcinoma (RCC), lncARSR (lncRNA Activated in RCC with Sunitinib Resistance)-loaded exosomes competitively bind miR-34/miR-449 and enhance AXL and c-MET receptors signaling that are responsible for Sunitinib resistance through activation of STAT3, AKT, and ERK signaling (105). In light of this finding, lncARSR represent a potential therapeutic target to overcome sunitinib resistance in RCC (105). Other two lncRNAs involved in drug resistance are lncRNA urothelial carcinoma-associated 1 (UCA1) and lncRNA prostate androgen-regulated transcript 1 (PART1); Yang et al. founded that exosomal UCA1 is associated with Cetuximab resistance in colorectal cancer and could predict clinical outcome of Cetuximab therapy (106), while Kang and collaborators demonstrated that lncRNA PART1 is able to confer resistance to Gefitinib in esophageal squamous cell carcinoma by inducing B-cell lymphoma 2 (Bcl-2) expression through inhibition of miR-129 (107).

In summary, these findings show how EVs can exploit different mechanisms to sustain the acquisition of drug resistance by cancer cells, making them a central player in tumor evolution.

LINKING NF-κB AND EVS ACTIVITY IN CANCER PROGRESSION AND THERAPY RESISTANCE

Reciprocal Regulation Between EVs and NF-κB Signaling in the TME

NF-κB is directly involved in EVs trafficking and EVs-mediated chemoresistance, and, at the same time, EVs are responsible for NF-κB activation (**Figure 4**).

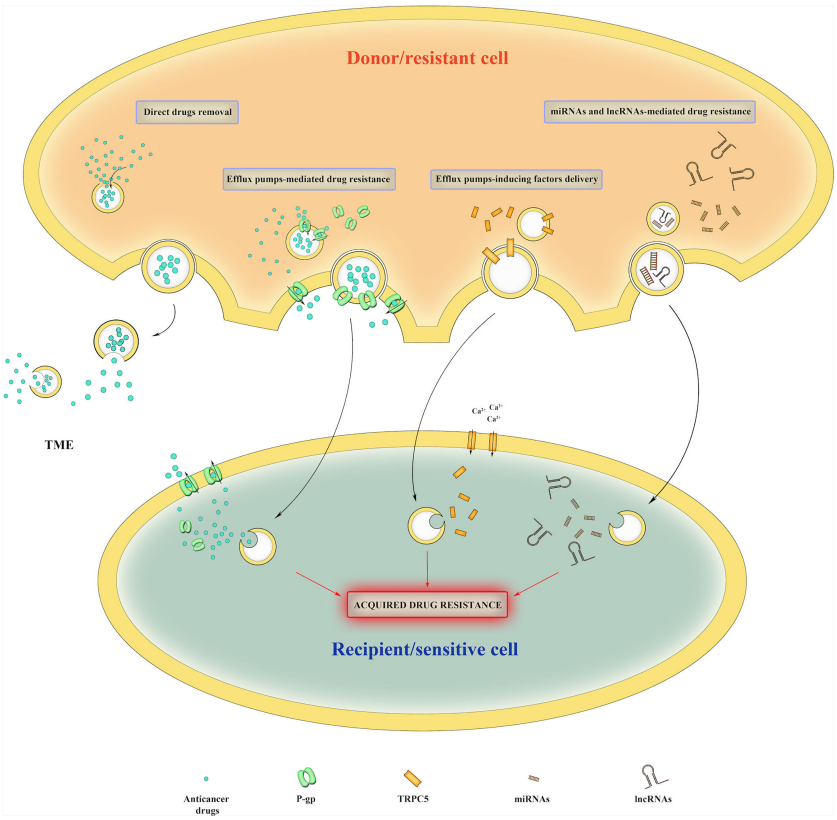

FIGURE 3 | Mechanisms of EV-mediated drug resistance. EVs can directly remove intracellular drugs from cancer cells. They can also deliver efflux pumps, efflux pumps-inducing proteins, miRNAs and lncRNAs to recipient cells, thus conferring them drug resistance traits.

Hypoxia is recognized as a hallmark of cancer (108) and promotes the immunosuppressive phenotype within the TME (109). In addition, hypoxia promotes EVs biogenesis and release in order to support intercellular communication and to compensate nutrient's deficiency and oxygen starvation in cancer cells (110–112). In response to oxygen starvation, cancer cells activate hypoxia-inducible factors (HIFs) (113), a family of transcription factors involved in adaptation to hypoxic condition through modulation of angiogenesis, metastasis, and drug resistance (114), as well as in the regulation of small Rab GTPases (*i.e.*, Rab22A) which in turn control intra- and intercellular trafficking of EVs (112). It is well-known that NF-κB, in response to inflammatory stimuli, upregulates HIF-1α and HIF-1β expression (115–117). Notably, hypoxia, per sé, can induce NF-κB, leading to an inflammatory response (118). Indeed, it has been observed that inhibition of oxygen sensors prolyl hydroxylases (PHDs) by low oxygen tension, stabilizes IKKβ with subsequent p65 nuclear accumulation and signaling transduction (119). Importantly, NF-κB is also able to directly regulate Rab proteins, as observed by Feng *et al.* in colon cancer stem cells. They identified a functional NF-κB binding site in the Rab27A promoter and observed that increased p65 levels induced Rab27A expression and enhanced EVs secretion in HT29 cells (120). In turn, Rab27A can promote tumor proliferation and chemoresistance *via* NF-κB, in bladder cancer (121). Indeed, Rab27A overexpression was associated with increased phosphorylation of p65 and increased expression of the antiapoptotic gene *Bcl-2*, and conversely, pharmacological inhibition of NF-κB by BAY 11-7082 abrogated cisplatin resistance and cancer cell survival (121). NF-κB activation also influence EVs cargo. Yang *et al.* reported an altered exosomal protein profile in NF-κB knockout mice following ischemia-reperfusion (I/R) injury in skeletal muscles, suggesting that NF-κB contributes to EVs production. Specifically, in the exosomes of NF-κB knockout mice, they observed upregulation of several proteins such as protease serine 1 and glyceraldehyde-3-phosphate dehydrogenase-like isoform 1, and downregulation of other factors including apolipoprotein B, complement component C3 prepropeptide, and immunoglobulin kappa light chain variable region (122). It has been reported that NF-κB can also drive transcription of specific miRNAs including miR-21; indeed, it was observed that miR-21-carrying exosomes released by TAMs induced cisplatin resistance in gastric cancer cells modulating PTEN/PI3K/AKT signaling pathway (123).

In accordance with the reciprocal interplay between EVs and NF-κB signaling, EVs can modulate NF-κB activity in recipient cells (**Figure 4**). Bretz et al. showed that exosomes from various

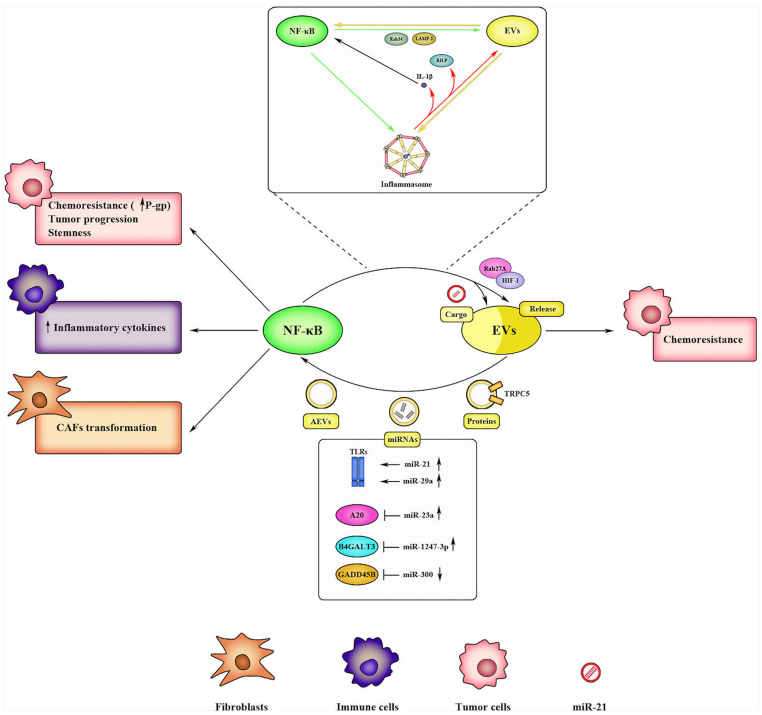

FIGURE 4 | Bidirectional communication between NF-κB and EVs within the TME. NF-κB promotes EV-mediated chemoresistance in cancer cells by regulating the expression of EV-releasing factors (*i.e.*, Rab27A, Rab34, LAMP-2, HIF-1). NF-κB also affects EV cargo by inducing miRNA transcription (*i.e.*, miR-21). Reciprocally, EVs activate NF-κB through several mechanisms, such as by acting as DAMPs or delivering specific proteins and miRNAs. Upon activation, NF-κB induces chemoresistance, progression, and stemness in tumor cells, inflammatory cytokines release in immune cells, and CAFs transformation in fibroblasts. Inflammasome often mediates the crosstalk between NF-κB and EVs. Activated inflammasome promotes EV release through specific factors (*i.e.*, RILP) and sustains NF-κB signaling through IL-1β production. In turn, NF-κB regulates inflammasome activation by promoting priming step, and reciprocally, EVs induce inflammasome signaling, thus generating a positive feedback loop.

body fluids stimulate the NF-κB-dependent production of pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-6 in monocytic cell line THP-1 *via* TLR2 and TLR4 activation (124). Gastric (125) and breast cancer (126) derived TEXs induce the expression of pro-inflammatory cytokines by macrophages *via* NF-κB, and the NF-κB activation in myeloid cells could involve TLR2 signaling (126). NF-κB pathway can be also regulated by specific EVs-delivered factors (*i.e.*, proteins, miRNAs, lncRNAs), which can directly or indirectly activate/inhibit this pathway depending on the context. As previously mentioned, exosomes-carrying Ca^{2+}-permeable channel TRPC5, responsible of P-gp-induced chemoresistance, are associated with IL-6 expression and increased phosphorylation of p65 in nasal polyps (127), suggesting a possible role of NF-κB in TRPC5-mediated drug resistance. Delivered miR-21 and miR-29a, can directly trigger NF-κB-mediated inflammatory response by binding murine TLR7 and human TLR8 (128). Commonly, miRNAs modulate NF-κB pathway in an indirect manner. Fang et al. showed that miR-1247-3p-carrying TEXs promote liver cancer stemness and chemoresistance *via* inhibition of beta-1,4-galactosyltransferase 3 (B4GALT3) and activation of β1-integrin–NF-κB pathway in fibroblasts (129). Activated CAFs further induce tumor progression by secreting pro-inflammatory cytokines, including IL-6 and IL-8 (129). Furthermore, Li and colleagues observed that miR-23a-enriched exosomes activate NF-κB pathway in macrophages by targeting its negative regulator A20 (130) while Chen and collaborators founded that exosomal miR-300 controls melanoma cell progression targeting GADD45B expression, a NF-κB-induced pro-survival factor (131). MiRNAs can also be downregulated to sustain NF-κB pathway. Wang et al. demonstrated that when the usually low expressed miR-192-5p is overexpressed in TAMs-derived exosomes, it suppresses endometrial cancer progression through NF-κB inhibition (132). With respect to lncRNAs, Li et al. founded that exosomal lncRNA FMR1-AS1 (FMR1 antisense RNA 1) is associated with CSC-like phenotype by binding TLR7 in female esophageal carcinoma (133). Moreover, expression of lncRNA BORG (BMP/OP-responsive gene) in triple-negative breast cancer (TNBC) (134) and lncRNA HOTAIR (HOX antisense intergenic RNA) (135) in colorectal cancer promotes chemoresistance *via* NF-κB. NF-κB activation can be triggered also by so-called "apoptotic exosome-like vesicles" (AEVs). Recently, Park and collaborators demonstrated that AEVs, during apoptotic process, act as DAMPs and activate NF-κB pathway, thus promoting an inflammatory response (136).

EVs and NF-κB Interplay: The Crucial Role of Inflammasome

Inflammasomes represent an important link between NF-κB and EVs activity in the TME (**Figure 4**). Inflammasomes are multimeric protein complexes that are assembled upon recognition of specific pathogen-associated molecular patterns (PAMPs) and DAMPs by PRRs (137) during innate immune response. Inflammasome consists of cytoplasmic PRRs 'sensors' such as nucleotide-binding domain and leucine-rich repeat receptors (NLRs), the 'adapter' apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC), and an 'effector' pro-caspase 1 (138). In this context, NF-κB plays a leading role; indeed, DAMPs are recognized by toll-like receptors (TLRs), a subset of membrane-bound PRRs which trigger NF-κB cascade and subsequent transcription of IL-1β (139). IL-1β is released as precursor protein (pro-IL-1β) and is processed in its active form by caspase-1 (140) through the formation of a multi-protein complex termed inflammasome. Although the existence of several NLRs has been reported, nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family pyrin domain containing 3 (NLRP3) is the best characterized inflammasome. Activated NF-κB upregulates NLRP3 (141, 142) that is necessary for the inflammasome assembly and for the pro-IL-1β processing. The process that lead to the inflammasome activation consist of two phases, priming and activation (143). In the priming step, NF-κB induce transcriptional upregulation of NLRP3 inflammasome components while the activation step led to full induction and inflammasome complex formation. In detail, NLRs act as DAMPs sensors leading to recruitment of ASC and subsequent activation of caspase-1 thus promoting processing of pro-IL-1β in active IL-1β. Importantly, it is known that inflammasome activation induce EVs secretion (144) by several mechanisms. One of those could be represented by *de novo* synthesis and production of IL-1β, which, in turn, activate NF-κB signaling and induce the expression of factors involved in EVs secretion. Gutierrez et al. demonstrated that activation of NF-κB can induce several membrane-trafficking regulators such as lysosome-associated membrane protein 2 (LAMP-2) and ras-related protein Rab34 (145). EVs secretion can also be promoted by inflammasome itself through caspase-1-dependent cleavage of the trafficking adaptor protein rab interacting lysosomal protein (RILP) (146). Furthermore, inflammasome-derived exosomes can activate NF-κB in macrophages leading to their pyroptosis *via* up-regulation of NLRP3 and pro-IL1β (147). While these findings show how inflammasome affects EVs secretion, it is worthy to note that EVs can also influence inflammasome activity, thus revealing a bidirectional crosstalk. Hence, EVs can either positively or negatively affect inflammasome activity depending on the nature of the EVs releasing cells (148). Exosomes from LPS-treated macrophages are able to activate NLRP3 inflammasome in AML-12 hepatocytes (149). Again, EVs from palmitate-treated Huh7 hepatocytes induce production of IL-1β in mouse bone marrow-derived macrophages (150) and exosomes from ARPE-19 exposed to photooxidative blue-light activate NLRP3 inflammasome *in vitro* (151). However, inflammasome activation is repressed in THP1 cells treated *in vitro* with human amniotic fluid derived EVs, as well as in cardiomyocytes isolated from a mouse model of doxorubicin-induced cardiotoxicity following treatment with embryonic stem cell-derived EVs (152, 153). Although inflammasome involvement in cancer is still debated and sometimes controversial, several findings underline its role in tumorigenesis, cancer progression and drug resistance (154). In this context, NLRP3 inflammasome activity is associated with carcinogenesis in head and neck squamous cell carcinoma (155) and with proliferation and migration in A549 lung cancer cells (156).

Further, TEX-mediated inflammasome activation in non-cancer cells within the TME was shown to exacerbate inflammatory response and sustain tumor progression. In prostate cancer, EVs released from advanced-stage tumor cells were found to activate inflammasome in non-cancerous prostate cells and induce M2 polarization in THP1 (157). Moreover, Liang et al. showed that tripartite motive containing 59 (TRIM59)-loaded EVs released from lung cancer cells promoted tumor progression *in vitro* and *in vivo*. Mechanistically, TRIM59 promoted abhydrolase domain containing 5 (ABHD5) degradation and activation of NLRP3 inflammasome in macrophages, which in turn released proinflammatory cytokines, thus sustaining cancer cell proliferation and invasion (158). Hwang et al. also demonstrated that colorectal CSCs-derived exosomes induced IL-1β expression in neutrophils *via* NF-κB, thus promoting tumorigenesis in colorectal cancer cells (159). Moreover, inhibition or deletion of inflammasome components NLRP3, ASC, or caspase-1 is protective against pancreatic ductal adenocarcinoma as it is associated with the reprogramming of innate and adaptive immunity in TME (160). Inhibition of NLRP3 inflammasome also suppresses metastatic potential of melanoma cancer cells (161). Inflammasome is also involved in drug resistance and can be activated following chemotherapeutic treatments, likely as result of NF-κB stimulation. Zhai et al. founded that NLRP1 inflammasome activation induces drug resistance in melanoma cells through release of IL-1β (162). Again, Theivanthiran et al. observed activation of NLRP3 inflammasome following treatment with anti-PD-1 checkpoint inhibitor with subsequent infiltration of granulocytic myeloid-derived suppressor cells and reduction of antitumor response (163). As reported by Feng et al. inflammasome activation is also involved in in 5-fluorouracil resistance of oral squamous cell carcinoma (164).

All together, these findings highlight an intricate interplay between NF-κB and EVs activity which influence tumor growth and drug resistance. Although further studies are needed to fully elucidate the molecular mechanisms underlying this reciprocal regulation between NF-κB and EVs, it could represent yet another way through which NF-κB orchestrate tumor behavior within the TME.

CONCLUSIONS

The bulk of evidence summarized herein shows that EVs play a key role in drug resistance through several mechanisms,

including direct load and expulsion of chemotherapeutics, as well as delivery of pro-survival, anti-apoptotic, and stemness-associated factor cargo. The pharmacological target of EV biogenesis, uptake, and transfer to suppress chemoresistance has shown promise, but most of the data obtained were generated *in vitro* (165), and further *in vivo* investigation are needed. Another potential issue is represented by the heterogeneity of EVs, as not all EV communication is pro-tumorigenic. Thus, a better understanding of the molecular mechanisms underlying EV processes will significantly contribute to develop more specific approaches for overcoming drug resistance. In this review, we discuss the complex crosstalk between NF-κB pathway and EVs, as NF-κB activation affects EVs formation and release, and EVs, in turn, can trigger NF-κB activation. Since NF-κB/EVs interplay profoundly contribute to fuel aggressive disease and desensitize cancer cells to drugs, this axis could represent a promising target. However, as seen for EVs, targeting NF-κB core pathway produces off target effects due to the lack of anti-cancer specificity, as this factor is a master regulator of several biological processes. Therefore, the identification of NF-κB targets involved in this interplay in specific cancer models could be a useful strategy to overcome NF-κB/EV-mediated resistance.

AUTHOR CONTRIBUTIONS

MDVN and DVec wrote the manuscript. IF, DVer and MP made the figures. EA DC and FZ revised the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Extracellular Vesicles and Resistance to Anticancer Drugs: A Tumor Skeleton Key for Unhinging Chemotherapies

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Although surgical procedures and clinical care allow reaching high success in fighting most tumors, cancer is still a formidable foe. Recurrence and metastatization dampen the patients' overall survival after the first diagnosis; nevertheless, the large knowledge of the molecular bases drives these aspects. Chemoresistance is tightly linked to these features and is mainly responsible for the failure of cancer eradication, leaving patients without a crucial medical strategy. Many pathways have been elucidated to trigger insensitiveness to drugs, generally associated with the promotion of tumor growth, aggressiveness, and metastatisation. The main mechanisms reported are the expression of transporter proteins, the induction or mutations of oncogenes and transcription factors, the alteration in genomic or mitochondrial DNA, the triggering of autophagy or epithelial-to-mesenchymal transition, the acquisition of a stem phenotype, and the activation of tumor microenvironment cells. *Extracellular vesicles* (EVs) can directly transfer or epigenetically induce to a target cell the molecular machinery responsible for the acquisition of resistance to drugs. In this review, we resume the main body of knowledge supporting the crucial role of EVs in the context of chemoresistance, with a particular emphasis on the mechanisms related to some of the main drugs used to fight cancer.

Keywords: chemoresistance, extracellular vesicles, metastasis, tumor recurrence, tumor microenvironment

INTRODUCTION

Extracellular vesicles (EVs) are a heterogeneous population of double membrane-enclosed lipidic structures, which are actively secreted by eukaryotic and prokaryotic cells (1, 2). EVs are recently recognized as mediators of communication due to their molecular cargo consisting of biomolecules (lipids, nucleic acids, carbohydrates, and proteins) transferable to neighboring cells (3–5). EVs can be classified into three different subtypes according to their size, biophysical properties, and biogenesis: small EVs (exosomes), medium EVs (microvesicles), and apoptotic bodies (6) (**Figure 1**). Small EVs are nano-sized vesicles smaller than 150 nm, which originate from intraluminal vesicles (ILVs) through the formation of multivesicular bodies (MVBs) (7). As a next step, these ILV-containing MVBs can either be redirected to degradation in the lysosome or fused with the plasma membrane (PM), thus leading to the release of exosomes. The three main mechanisms of ILV formation are described. The first mechanism requires the presence of

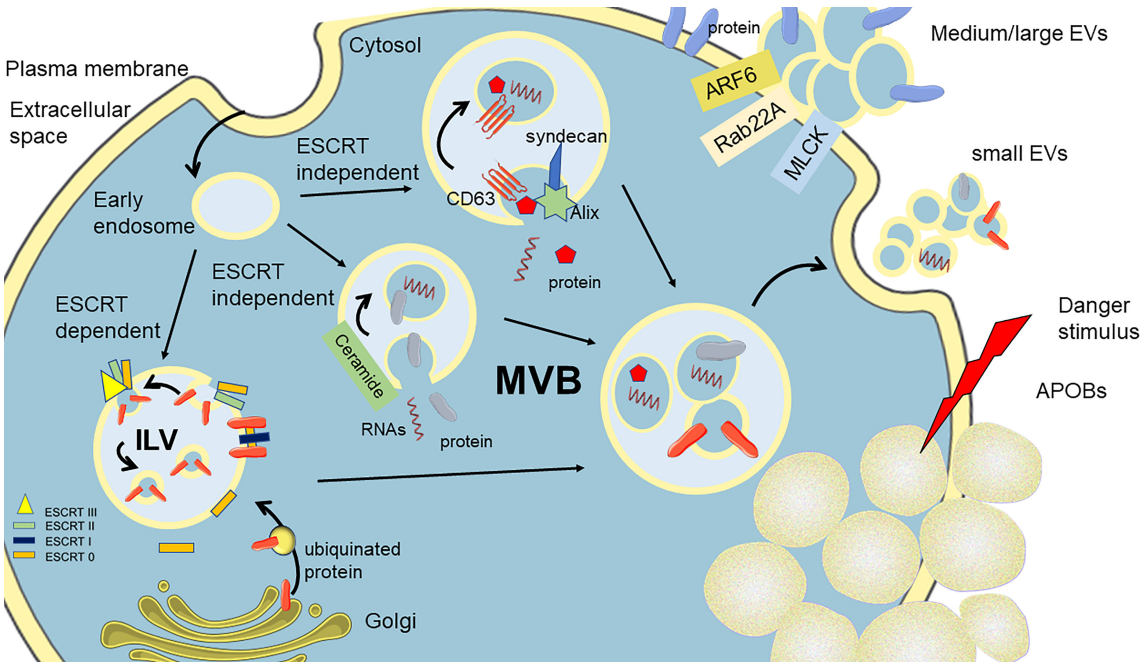

FIGURE 1 | Schematization of the biogenesis of the three classes of EVs. Small EVs arise from the shedding of MVBs fusing with the plasma membrane (PM). Three main paths are described for the formation of MVBs, entrapping in the cytoplasmatic components of ILVs. Medium and large EVs are derived for the budding of PM, pulling the constituent of PM. Apoptotic bodies (APOBs) are released after an irreversible injury to the cell, triggering apoptotic cell death and the perturbation of PM. MVBs, multivesicular bodies; ILVs, intraluminal vesicles; ESCRT, endosomal sorting complexes required for transport.

endosomal sorting complexes required for transport (ESCRT) complex members (8). These proteins have been described to select ubiquitinated proteins and segregate them into microdomains found on the endosomal membrane. ESCRT-0, ESCRT-I, and ESCRT-II are held responsible for the binding, through the tumor susceptibility gene 101 (TSG101), of specific cargoes selecting ubiquitinated proteins and segregating them into microdomains found on the endosomal membrane. Subsequently, these complexes recruit the apoptosis-linked gene 2–interacting protein X (ALIX), which aids in recruiting the ESCRT-III complex containing proteins involved in vesicle budding and the release from the plasma membrane. The second pathway, independent of ESCRT, requires only ALIX, and transmembrane proteins, such as syntenin and syndecan, which are responsible for recruiting tetraspanin CD63 (the main marker of small EVs) and other specific molecular cargoes (i.e., adhesion molecules, growth factors, and integrins) along with the interaction with proteins involved in the release from the cellular membrane (8). The third mechanism of ILV biogenesis mainly involves the participation of membrane lipid microdomains or lipid rafts. One of the main players is ceramide, generated by the neutral sphingomyelinase enzyme finally favoring the bending toward the lumen of the MVB membrane (9). Finally, MVBs can be degraded by fusion with lysosomes or can be shuttled to the membrane for the fusion and release of their cargo. On another side, medium/large EVs, also known as microvesicles, ectosomes, or microparticles, range between 50 and 1,000 nm. They are described to be released from the cell surface by blebbing from the plasma membrane. The biogenesis of EVs involves many partners, among which are small GTPases (such as ADP-ribosylation factor 6, ARF6), Ras-related proteins (Rab-22A), and phospholipases (PLD), the latter inducing a phospholipid redistribution and positioning of phosphatidylserines to the outer shell in EVs (10). The final recruitment of extracellular signal-regulated kinase (ERK) and the phosphorylation of the myosin light-chain kinase (MLCK) induce the invagination of the plasma membrane and EV release. Apoptotic bodies (APOBs) are the third subtype of EVs and vary in size, ranging from 50 to 2,000 nm in diameter, ultimately produced by the programmed cell death apoptosis (11). One of the main features of apoptotic bodies is that mechanisms for specific sorting of organelles, RNA, and DNA fragments can be detected, which are absent in other EV subtypes. APOBs are far to be inactive particles but are now shown to be lively involved in biological processes. During their biogenesis, EVs entrap different macromolecules, such as nucleic acids (DNA, mRNA, miRNAs, long non-coding RNAs), lipids, proteins (cytosolic factors, receptors, and ligands), and organelles, which are then shuttled to surroundings where they exert metabolic changes in target cells.

A physical/molecular interaction between EVs and cell membranes triggers the EV uptake. This interaction has been shown to occur *via* different routes, including a direct fusion between EVs and the plasma membrane (12), as well as EV internalization *via* clathrin-, lipid draft-, and caveolae-dependent endocytosis, macropinocytosis, and phagocytosis (13–15).

Indeed, the EV uptake is likely dependent on many factors: the EV subtype, protein, and lipidic composition of the released EVs as well as the composition of the plasma membrane of recipient cells, cell metabolic status, and extracellular space conditions (i.e., pH, oxygen tension, and extracellular matrix components). The exchange of EVs is nowadays recognized as a crucial axis in the intercellular communication, exerting autocrine, paracrine, and systemic effects. EVs orchestrate physiological regulation in all tissues. The involvement of EVs is reported in many physiological processes such as angiogenesis [i.e., *via* the shedding of EV-encapsulated angiogenetic factors such as tetraspanin8, L-selectin, vascular endothelium growth factor receptor 1 (VEGFR1), and CD147], liver function and metabolism (i.e., asialoglycoprotein receptor-, apolipoprotein E/AV- and glutathione S-transferase-enriched EVs), bone resorption (i.e., pro-osteoclastogenic RANKL-positive EVs from osteoblasts), cornea wounding (i.e., fibronectin- and thrombospondin 1-enriched EVs from corneal epithelial cells), lung cell differentiation (i.e., EV-mediated shuttling and *de novo* transcription of pulmonary epithelial cell mRNAs), muscle regeneration (i.e., shuttling of α-Klotho transcript inducing muscle rejuvenating), bowel barrier integrity (epithelial cell-derived EVs alleviate gut injury after intestinal ischemia/reperfusion by miR-23a-3p), gut microbiota (*Escherichia coli* Nissle 1917 release vesicles positively modulates the intestinal epithelial barrier through upregulation zonulin-1/-2 and claudin-14), and immunity (macrophage-derived EVs contains alarmins orchestrating immune regulation) (16–24). Similarly, tissue dysfunctions and diseases are sustained by EV exchange including but not limited to stroke, obesity, skeletal muscle atrophy, colitis, and major depressive disorder (25–32). Finally, malignant transformation and cancer progression are fueled by a massive switch of EVs in many types of tumor (33–38). Carcinogenesis is a complex transformation of a cell by which specific traits or "hallmarks" are acquired, shifting from a healthy cell to a cancer one (39). According to Hanahan and Weinbergs' theory, the mandatory hallmarks include the following: sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. All of them are reported to be triggered by EVs. EVs from gastric cancer SGC7901 cells sustain proliferation by PI3K/Akt and MAPK/ERK activation; EVs are able to directly or epigenetically reduce the cytoplasmatic levels of phosphatase and tensin homologue (PTEN) to evade the growth suppressor (40–42). Many papers reported the mechanisms of resisting cell death induced by EVs: multiple myeloma cells reduced by EVs the levels of the pro-apoptotic protein Bcl-2-like protein 11 (Bim); the antiapoptotic protein survivin is shuttled in EVs from HeLa cervical carcinoma cells irradiated with a sublethal dose of proton, and both gastric- and bladder cancer-derived EVs suppressed the apoptosis of respective cancer or suppressive immune cells *via* the upregulation of Bcl-2 and cyclin-D1 expression and the downregulation of Bax and caspase-3 (43–48). The examples of replicative immortality triggered by EV cargoes are described for the shuttling of telomerase (TERT) transcript in target cells, as well as of TP53 and β-catenin (49–51). Angiogenesis is one of the most described event regulated by EVs, due to the enrichment in the EVs of VEGF, VEGFR, MMPs, and correlated agonists (52). Finally, metastatization support by EVs is widely investigated in a large body of literature reporting the plethora of their molecular cargo involved in the process, such as amphiregulin, C-X-C chemokine receptor type 4 (CXCR4), epidermal growth factor receptor (EGFR), interferon regulatory factor (IRF)-2, miR-105 (downregulating zonula occludens-1), and many others (53).

In this review, we will discuss the involvement of tumoral EVs in the insurgence of chemoresistance.

MECHANISM OF RESISTANCE TO CHEMOTHERAPEUTICS

Chemoresistance and radioresistance remain the main complications of cancer therapy, hindering the improvement of clinical outcomes for patients suffering from cancer since they cause cancer relapse and metastasis (54–56). Multiple mechanisms of resistance to drugs are reported, and the tight interconnection and support among them are one of the main issues for overcoming this crucial tumor feature. In the next section, we will provide an overview of the main mechanism of drug resistance reported in cancers (**Figure 2**).

Transporter Proteins

The exchange across the plasma membrane is a pivotal mechanism of cellular homeostasis. The translocation of ions, lipids, amino acids, sugars, and xenobiotics occurs mainly through transporters or channels. The ATP-binding cassette (ABC) proteins and major vault protein (MVP) are the main players in these mechanisms. P-glycoprotein [P-gp, ABCB1, or multiple drug resistance 1(MDR1)] is an ATP-dependent efflux pump widely expressed in many tissues: capillary endothelial cells, intestinal epithelium, liver cells, and the renal proximal tubule (57). P-gp is one of the most powerful detoxification tools for cytotoxic drugs in cancer cells *via* direct efflux. P-gp overexpression has been observed in different kinds of hematological and solid tumors, such as leukemia, neuroblastomas, and ovarian and breast cancers, demonstrating its contribution to chemoresistance (58, 59). The ABCG2 encodes for another member of the ABC superfamily, also known as breast cancer resistance protein (BCRP) (60, 61). It has been reported that ABCG2 is an estrogen-inducible gene, associated with a higher tolerance of breast cancer cells against cytotoxic drugs (i.e., mitoxantrone) (62). Major vault protein (MVP) is a protein localized to a nuclear pore as a ribonucleoprotein with a hollow barrel-like structure responsible for gating ribosomes, hormones, and drugs (63). MVP was first discovered as a new 110 kD drug transporter in doxorubicin-resistant lung cancer cells, and it was later reported in many types of tumors (64). In triple-negative breast cancer cells MDA-MB-231, MVP has been demonstrated to be upregulated by the Notch1 intracellular domain and the

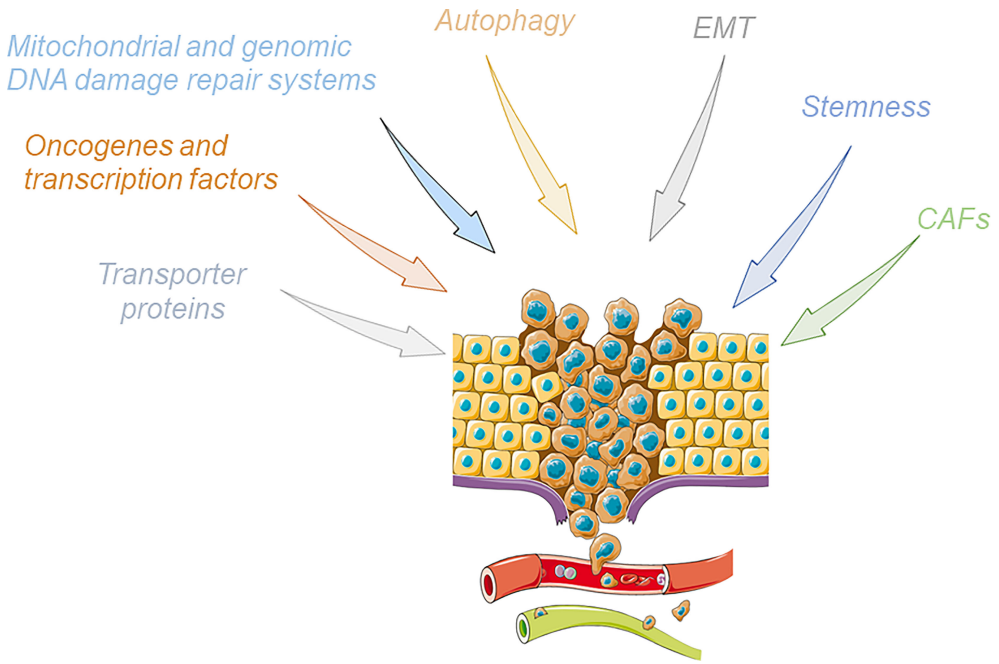

FIGURE 2 | Cartoon summarizing the main mechanisms involved in the induction of the phenomenon of tumor drug resistance. Upon the influence of these pathways, drug-resistant cancer cells acquire an enhanced invasive ability, break away from the original tumor site, and finally metastasize to other organs through the blood or lymphatic systems. This figure was drawn with the support of the bioicons website (https://bioicons.com/).

activation of the AKT pathway and promoting the epithelial-to-mesenchymal transition (EMT) and the chemoresistance of cancer cells (65).

Oncogenes and Transcription Factors

The overactivation and mutations of genes encoding for proteins involved in pivotal cellular processes (proliferation, survival, and transformation) is a frequent strategy of cancers to overcome the effect of cytotoxic drugs. The most upregulated pathways in chemoresistance are JAK/stat3, PI3K/Akt/mTOR, Src/FAK/ROS, and SOS/Grb2/Ras cascades. In turn, oncogenes can be upstream activated by receptors. For example, all the above pathways can be commonly activated by the EGFR. Accordingly, the overexpression or gain-of-function mutations of EGFR are reported in different types of aggressive and chemoresistant cancers. EGFR promotes metabolic processes critical for cancer cell proliferation both directly by phosphorylating rate-limiting enzymes or indirectly through the activation of the MYC transcription factor and of the AKT signaling cascade (66–68). A mutated p53 is another common feature of many cancers (69). The protein p53 is involved in the sensitivity of cells to DNA-damaging drugs through DNA damage-response sensors ataxia telangiectasia mutated protein (ATM) and ataxia telangiectasia and Rad3-related protein (ATR) and their downstream cell cycle regulator checkpoint kinases 1 and 2 (Chk1 and Chk2) (70, 71). Some mutated p53 forms are very stable to degradation and ubiquitination and heterodimerize with wild-type p53, working as a dominant-negative able to disrupt most or all normal p53 functions, such as apoptosis or cell cycle arrest (72–74). Many mutated p53 forms can stimulate the mammalian target of rapamycin (mTOR) and block autophagy, leading to proliferative and anti-apoptotic responses in breast and pancreatic cancers (66). On other hand, the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is another master player in dampening apoptosis induced by a variety of stimuli, including tumor necrosis factor-α (TNF-α), γ-radiation, and chemotherapeutics (75). Cancer usually expresses high levels of constitutive NF-κB activity and the exposition to cytotoxic agents increases NF-κB activity, resulting in cell growth and survival and finally resistance to the therapeutic agents. NF-κB induces the overexpression of downstream anti-apoptotic genes, such as the radiation-inducible immediate-early gene (IEX-1L), the inhibitor of apoptosis (IAP), and growth arrest and DNA damage-inducible 45 beta (Gadd45β), B-cell lymphoma-extra large (Bcl-xL), cyclin D1 and c-Myc, and many others, finally contributing to the chemoresistance. Thus, the NF-κB signaling pathway could be a potent target for improving the chemosensitivity of the tumor cells (76).

Mitochondrial and Genomic DNA Damage Repair Systems

Many types of tumors harbor somatic mutations in the mitochondrial genome (mtDNA), resulting in mitochondrial dysfunction. Many mutations in the mitochondrial genes of cancer cells overload mitochondrial activity mainly because

cancer cells shift their metabolism, requiring more glycolysis or oxidation. Proliferator-activated receptor gamma co-activator (PGC)-1α and mitochondrial transcription factor A (TFAM) are overexpressed in cisplatin-resistant ovarian cancer; similarly, PGC-1β confers the chemoresistance of lung cancer cells to cisplatin associated with mtDNA mutations (77, 78). Mitochondrial dynamics (fission and fusion) are critical for metabolic adaptations. Mitochondrial fusion with efficient ATP production was frequently observed in chemoresistant cancer cells (79). Dynamin-related protein 1 (DRP1) promotes mitochondrial fission, and its hyperexpression induces chemoresistance in lung, breast, thyroid, and colon cancers (80–84). DNA damage is the goal of many chemotherapeutic drugs, acting as alkylating (i.e., cisplatin) or antimetabolites (5-fluorouracil) to the DNA molecules. Cancer cells counteract their effects through a strengthening of the DNA repair, occurring mainly *via* nucleotide excision repair and base excision repair machinery. Excision Repair Cross-Complementation Group (ERCC) 1 is involved in the nucleotide excision repair pathway and has been reported to be associated with chemoresistance in melanoma and ovarian cancer and colorectal cancer (CRC) (85–87). Similarly, ERCC2 also supports chemoresistance in ovarian cancer (88). Reversionless 3-like (REV3L), the catalytic subunit of DNA polymerase ζ, modulates sensitiveness to 5-fluorouracil in lung and esophageal carcinoma (89, 90).

Autophagy

Cells upon nutrient starvation, hypoxia, cellular stress, or metabolic alteration initiate autophagy to degrade cellular-damaged organelles and recycle amino acids or fatty acids *via* autophagosome formation. This adaptation strategy aims to favor cell survival and proliferation and is therefore adopted by cancer cells to fight drugs. CRC tissues were reported to be characterized by a significantly higher expression of autophagy-related genes such as Beclin-1, microtubule-associated protein 1A/1B-light chain 3 (LC3), and Rictor, which levels are positively correlated with the level of MDR-1 (91). The pro-survival role of autophagy was also confirmed in breast, ovarian, esophageal, lung, prostate, glioma, bladder, renal, and pancreatic cancers (92). The crucial role of autophagy was confirmed by the use of autophagy inhibitors, such as 3-methyladenine, able to sensibilize tumor cells to drugs (93). Human leukemia cells resist doxorubicin and vincristine by secreting high mobility group box 1 (HMGB1), responsible for overexpressing LC3-II in cancer cells (94). HMGB1 overexpression also contributed to the chemoresistance of neuroblastoma cells by inducing Beclin-1-mediated autophagy (95).

Epithelial-to-Mesenchymal Transition

EMT is a complex process wherein epithelial cells depolarize, lose their cell–cell contacts, and acquire an elongated, fibroblast-like morphology. This mechanism is a means by which tumor cells increase their metastatic potential and can be triggered by extracellular signals (collagen, hyaluronic acid, and integrins), growth factors and cytokines (TGF-β, VEGF, EGF, and HGF), non-coding RNAs, or hypoxia (96). Under EMT, cancer cells enhance mobility, invasion, and resistance to apoptotic stimuli. Finally, through EMT, tumor cells acquire stemness (see next paragraph) and chemoresistance. Targeting EMT could indeed be an effective approach to obstacle chemoresistance (97). Colon cancer cells have been reported to encounter EMT and gain doxorubicin chemoresistance *via* the upregulation of TGF-β signaling (98). In hepatoma cells, gemcitabine supports EMT upon PDGF-D trigging, while oxaliplatin exposition induces EMT through BMP4/MEK1/ERK/ELK1 pathway activation (99, 100). In breast cancer cells MCF-7, EMT driven by Snail upregulation is reported to be associated with 5-fluorouracil insensitiveness (101).

Stemness

The concept of stemness in the cancer field is nowadays widely accepted, and cancer stem cells (CSCs) are the actual dogma for the basis of cancer recurrence and chemoresistance (102). The definition of CSCs is the same as the normal tissue stem cells: the ability of a small subset of cells in a tissue having the capacity for self-renewal and to reform in a host the complete tissues containing all the cellular hierarchy from whence the stem cells were derived (103). Similarly to stem cells, CSCs can be purified as a poorly or negatively stained side population (SP) by flow cytometry, so-called because of their characteristic hallmark to exclude the Hoechst from the nucleus, while other tumor cells are highly positive for the nuclear DNA staining (104). This feature is associated with a high expression of the ATP-binding cassette transporter protein ABCG2/Bcrp1 (105). The other molecular signatures of CSCs reported included Oct4, Nanog, Sox2, ALDH, CD44, CD117, CD133, Notches members, and many others (106–109). CD44, a hyaluronic acid receptor, is highly expressed by cancer stem cells and interacts with the WNT/β-catenin pathway, leading to more aggressive tumors in pre-clinical models and patients suffering from CRC (110). CD44-expressing ovarian cancer stem cells are more resistant to platinum salts and to paclitaxel (PTX) than CD44-negative cells (111). CD133-expressing ovarian cancer stem cells have been shown to have increased engraftment capacities with chemoresistance to cisplatin (112).

Cancer-Associated Fibroblasts

CAFs are vital constituents of the tumor microenvironment, a special stroma that interacts with cancer cells to promote tumorigenesis and progression. CAFs are recognized as potential targets for anti-cancer therapy since they are described to promote both cancer metastasis and chemotherapy resistance. Tumor cells depend upon the tumor stroma since it provides nutritional support and survival signals for tumor maintenance and proliferation. Upon certain stimuli, the fibroblast inside tumor stroma becomes "activated" (113). Accordingly, fibroblasts acquire different morphology and expression profiles (114). These CAFs produce growth factors that promote tumor growth, angiogenesis, and the recruitment of protumorigenic inflammatory cells. For example, CAFs specifically produce fibroblast activation protein alpha (FAP), changing different processes such as the extracellular matrix remodeling and composition as well as immune surveillance. CAFs can also affect the sensitivity of tumor cells to chemotherapy or radiotherapy (115, 116). The main mechanisms

reported for CAF-mediated chemoresistance are the release of secreted factors, the promotion of cancer stemness, the modulation of cancer metabolism, and the induction of immune escape. The pleiotropic cytokine interleukin(IL)-6 is one of the main CAF-secreted factors. In esophageal squamous cell carcinoma (ESCC), IL-6 released by CAFs increased the chemoresistance of ESCC to cisplatin by increasing the chemokine receptor CXCR7 expression in tumor cells through the STAT3/NF-κB axis (117). Similarly, CAFs can release IL-8, promoting chemoresistance to cisplatin in human gastric cancer *via* NF-κB activation and ABCB1 upregulation (118). CAFs support the chemoresistance of tumor cells by promoting stemness. In colon cancer, Lotti *et al.* showed that CAFs upon the FOLFOX protocol released IL-17 which sustains the reservoir of CD44-positive self-renewing tumor-initiating cells (119). In breast cancer, CAFs secreted soluble factors such as activin A, insulin growth factor (IGF)-1, and leukemia inhibitory factor (LIF), all of which enhanced CSC proliferation and self-renewal *via* the activation of hedgehog signaling (120). Cancer and the tumor microenvironment acquire peculiar metabolic needs switching toward aerobic glycolysis (Warburg effect) (121). In lung carcinoma, EGFR- or MET-expressing cancer cells exhibited an elevated glycolysis activity and increased production of lactate that induced CAFs to secrete large amounts of HGF through an NF-κB-dependent mechanism. Subsequently, HGF activated MET-dependent signaling and enabled cancer cells to resist tyrosine kinase inhibitors (122). The escape from immune surveillance is a pivotal pro-survival event adopted by cancer cells, and CAFs can directly promote this phenomenon. In fact, in pancreatic cancers, CAFs have been reported to actively switch polarizing macrophages toward the immunosuppressive M2 phenotype by the release of IL-8, the granulocyte-macrophage colony-stimulating factor (GM-CSF), and monocyte chemoattractant protein-1 (MCP-1) (123). In breast cancer, CAFs over-express chitinase-3-like-1 (Chi3L1), a secreted glycoprotein, involved in macrophage recruitment and M2 polarization (124). In fact, genetic *in vivo* ablation of Chi3L1 in fibroblasts reduced tumor growth and macrophage recruitment while enhancing tumor infiltration by T cells.

CHEMORESISTANCE AND EVS

A consistent body of evidence showed that EVs are an invaluable tool for tumor cells for protecting against cytotoxic agents. Generally, EVs sequester and extrude far from the tumor cells a drug, gaining resistance to chemotherapy. Shedden *et al.* measured this feature by the correlation of a "vesicle shedding index" with the sensitivity of breast cancer cells MCF7 for a range of drugs (125). Accordingly, other authors reported that the release of EVs from resistant cells is higher compared to parental sensitive cells in different cancer cell lines, such as ovarian and pancreatic cancers (126, 127). The higher vesiculation allowed to export drugs, allowing the cells to be more resistant. Furthermore, the tumor EVs can be "upgraded" with specialized molecular machinery to more efficiently load drugs inside. Cancer cells, such as MCF7, overexpress upon doxorubicin exposition the *ABC* genes (encoding for ATP-binding cassette transporters known to confer resistance to multiple drugs) (128). The protein is found not only as a membrane transporter, to extrude drugs from the cytoplasm to the extracellular space, but is also present on the surface of EVs (129). Interestingly, the orientation of the protein on EVs can be reversed (130, 131). This feature allows importing the drugs inside EVs before their release from cells and improves the resistance of cancer cells to chemotherapeutics. Moreover, EVs can dampen the effectiveness of biological drugs. In fact, EVs act as a decoy or antagonist of monoclonal antibody-based therapies.

In the next section, we will discuss the EV-based strategy adopted by cancer cells to overcome chemotherapeutic agents (**Figure 3**).

MECHANISMS OF DRUG RESISTANCE ACTIVATED BY EV MOLECULAR CARGOES

In recent years, not only the tumor progression and growth but also the response to drugs and the outcome of antitumoral therapies have been associated with the specific effects of EVs, and precise pathways favoring tumor growth and facilitating metastasis have been described (132). For example, EVs are enriched in particular families of non-coding RNAs (microRNAs (miRNAs), long non-coding RNAs (lncRNAs), and circular RNAs (circRNAs)] involved in the epigenetic regulation of gene expression (133). *MALAT1*, a long-non-coding RNA associated with tumor metastasis and invasion in lung cancer and hepatocellular carcinoma (HCC), has also been found to be enriched in EVs from cervical carcinomas and breast cancer cells (134–136). LncRNA *TUC339* is responsible for regulating the proliferation and adhesion of HCC and is shuttled in EVs (137, 138). Through the shuttling of miRNAs, tumor cells can also acquire insensitiveness toward drugs. Yoshida *et al.* described in human biopsies of patients suffering from osteosarcoma (OS) an upregulation of miR-25-3p, negatively correlated with the clinical outcome (139). The same group demonstrated that miR-25-3p silences the Dickkopf WNT signaling pathway inhibitor 3 (DKK3) gene, thus supporting *in vitro* cancer growth and resistance to different chemotherapeutics [methotrexate, cisplatin, doxorubicin, and docetaxel (DOC)]. Similar effects have resembled after direct DKK3 silencing. Finally, miR-25-3p was found in cancer cell-derived EVs. In a similar study, Pan et al. confirmed the clinical relevance of EV-mediated drug resistance in OS patients (140). The authors revealed that circulating EVs from 43 OS patients presented the overexpression of the circular RNA circRNA103801 compared to healthy subjects. This EV cargo showed a prognostic value for patients, having an inverse correlation with the overall survival. The authors further investigated that the overexpression of circRNA103801 in human OS cell line MG63 conferred resistance to cisplatin and cells released EVs enriched in the same circRNA. The uptake of these EVs from *naïve* MG63 and U2OS cells increased the resistance to cisplatin,

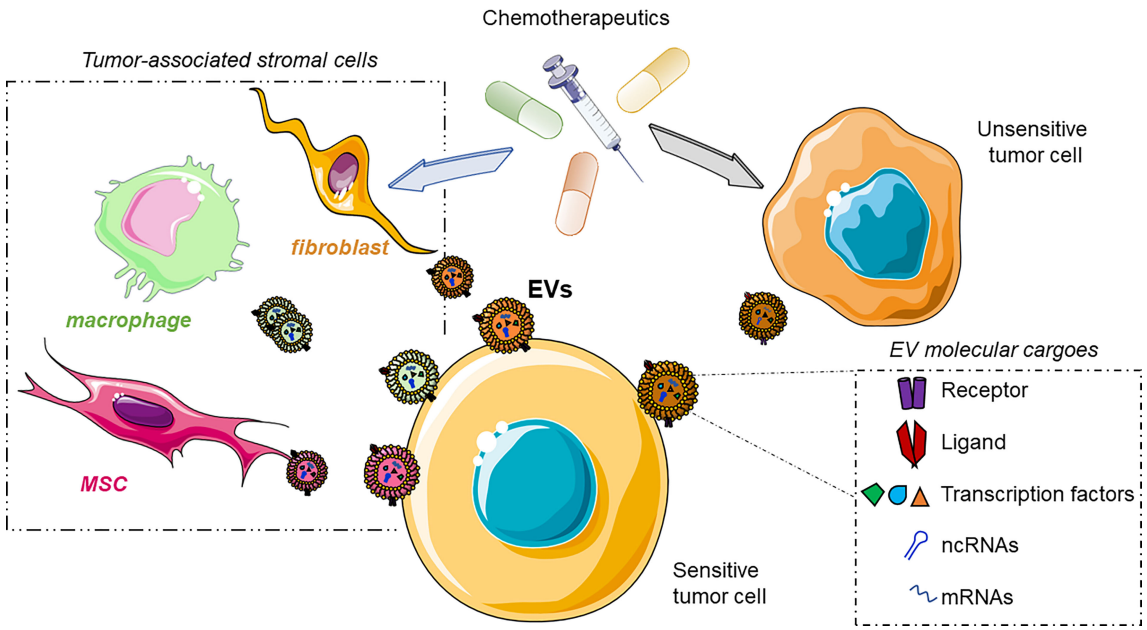

FIGURE 3 | Cartoon illustrating the involvement of EVs in the spreading of chemoresistance among the cells of the tumor microenvironment (TME). The drugs induce the modification of the gene expression of primary tumor cells or TME cells. These cells release EVs enriched in the molecular players involved in the acquisition of drug resistance in the EV-donor cells, finally taken up by the sensitive tumor cells acquiring the insensitiveness toward the chemotherapeutic. Bottom-right box: molecular effectors involved in drug resistance shuttled through the EVs. ncRNAs, non-coding RNAs; mRNAs, messenger RNAs. This figure was drawn with the support of the bioicons website.

upregulating the expression of P-gp and multidrug resistance protein 1 (MRP1). Takahashi et al. showed that HCC protects from sorafenib-induced apoptosis and cytotoxicity through the release of EVs enriched in *Linc*-ROR, *via* a TGFβ-CD133 axis (33). Exosomal miR-222 is responsible for the resistance to tamoxifen in MCF7 cells, suppressing p27 and estrogen receptor (ER) alpha expression (141). In the next paragraph, we will deeply focus on some EV-based mechanisms specifically interfering with some of the most important chemotherapeutics used for fighting cancers (**Figure 4**).

Platin (Platinum-Based Drug) Resistance by EV Molecular Cargoes

Platins are coordination complexes of platinum having a mainly alkylating activity on DNA, acting as intrastrand, interstrand, or DNA-protein crosslinks (142). An interesting study from Weinman *et al.* clarifies the correlation between EVs and drug resistance in a spontaneous canine model of OS (143). Based on the lag time between amputation and the start of adjuvant carboplatin treatment (good, disease-free interval >300 days; poor, disease-free interval <100 days), the animals have been divided into two cohorts and the protein profile of circulating EVs was run by mass spectrometry. The proteomic profile identified that tetranectin (TN) is decreased in the poor prognosis group and can be used as the most reliable biomarker. TN, a member of the C-type lectin family, shows a proteolytic activity. In the bone, TN has a crucial role in mineralization during osteogenesis and in extracellular matrix stiffness. Accordingly, the genetic loss of TN causes extracellular matrix softening and skeletal deformities (144). In a mouse model of CRC, LncH19-enriched EVs have been revealed to be promoters of oxaliplatin resistance. LncH19-EVs are released by CAFs and uptake by CRC cells SW480. Inside target cells, H19 activated the β-catenin pathway *via* acting as a competing endogenous RNA sponge for miR-141, an inhibitor of the cancer stemness. The overexpression of H19 was also confirmed in CRC patient samples at different tumor node metastasis stages (145). Lin and colleagues found that carnitine palmitoyltransferase 1A (CPT1A) was more highly expressed in colon cancer tissues than in noncancerous tissues and confirmed that CPT1A was increased by oxaliplatin stimulation in human colon cancer cell lines HCT116 and SW480. Silencing RNA could reverse the sensitivity of drug-resistant colon cancer cells to oxaliplatin (146). An elegant study showed in CRC cells the role of the antisense-RNA PGM5-AS1 in oxaliplatin resistance. Comparing tumor biopsies and perineoplastic tissues from patients, the authors found PGM5-AS1 as the second most downregulated ncRNA. In oxaliplatin-resistant SW480 cells, the downregulation of PGM5-AS1 is accompanied by the upregulation of the transcription repressor growth factor independent 1B (GFI1B). Further experiments demonstrated that GFI1B suppresses the expression of non-coding antisense RNA PGM5-AS1, which acts as a sponge for has-miR-423-5p to upregulate the expression of Nucleoside Diphosphate Kinase 1, NME1, and EVs can be involved in the intercellular exchange of the member of these pathways, contributing to resistance to

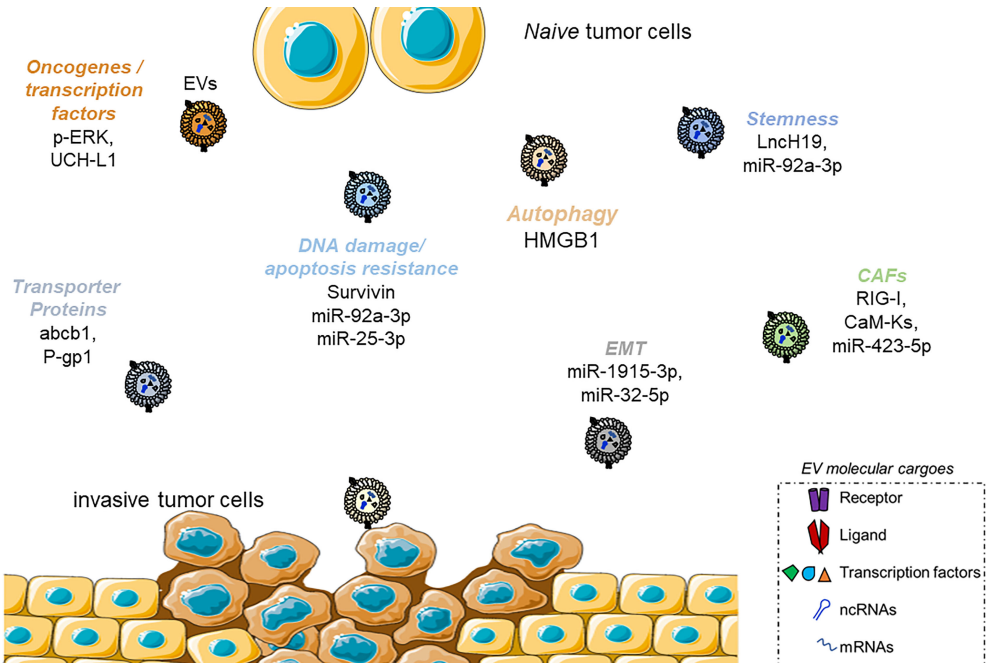

FIGURE 4 | Scheme summarizing the mechanism of drug resistance activated by EVs. Examples of molecular players for each category, as described in **Figure 2**, are reported. Only molecules shuttled by EVs are reported.

oxaliplatin (147). Another miRNA involved in CRC was reported by Xiao *et al.* They found that the exosomal delivery of miR-1915-3p can improve the chemotherapeutic efficacy of oxaliplatin in CRC cells by suppressing the expression of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) and ubiquitin carboxyl-terminal hydrolase 2 (USP2) and inducing the expression of E-cadherin (148). Another study showed the involvement of cirRNAs in the EV-based resistance to the FOLFOX regimen (oxaliplatin, 5-fluorouracil, folic acid) in CRC patients (149). A microarray profiling of exosomal circRNAs in FOLFOX-resistant HCT116 colon cancer cells identified 105 upregulated and 34 downregulated circRNAs compared to parental cells, with hsa_circ_0000338 being the most upregulated. Finally, the drug resistance can be transferred from resistant cells into sensitive cells *via* the uptake of exosomes. As reported in the previous paragraph, cancer cells can take advantage of CAF to acquire chemoresistance. Indeed, it has been shown that EVs from CAFs sustain chemo- and radio-resistance to the cisplatin of breast cancer MDA-MB-231 cells by activating retinoic acid-inducible gene I (RIG-I), the signal transducer and activator of transcription (STAT) 1, and NOTCH3 pathways (150). These studies are summarized in **Table 1**.

Antimetabolite Resistance by EV Molecular Cargoes

5-fluorouracil (5-FU) is an antimetabolite working as a thymidylate synthase inhibitor, finally depleting the cells of the pyrimidine thymidylate, a nucleotide pivotal for DNA replication (151). Although 5-FU is the first-choice drug for cancer treatment, its efficiency is limited by the acquisition of an innate or acquired resistance. Zhao *et al.* showed that circ_0000338 is upregulated in 5-FU-resistant CRC both *in vitro* and *in vivo* as well as in CRC patients (152). The authors

TABLE 1 | Resistance to platins mediated by EVs.

Drug	Cancer Type	EV Cargo	Effects	Refs
Cisplatin	Osteosarcoma patients and *in vitro*	miR-25-3p	Inhibition of DKK3	(139)
Carboplatin	Osteosarcoma, spontaneous canine tumor	Tetranectin	Unreported	(143)
Oxaliplatin	CRC, *in vivo* and *in vitro*	LncH19-EVs	Sponge for miR-141 and β-catenin activation	(145)
Oxaliplatin	CRC *in vivo*	CPT1A	Unreported	(146)
Oxaliplatin	CRC *in vitro*	PGM5-AS1	Sponge for miR-423-5p and upregulation of NME1	(147)
Oxaliplatin	CRC *in vitro*	miR-1915-3p	Suppression of PFKFB3 and USP2	(148)
Oxaliplatin (FOLFOX)	CRC patients, *in vitro*	circ_0000338	Unreported	(149)
Cisplatin	Breast cancer, *in vitro*	EVs from CAF, unreported	Activation of RIG-I, STAT1, and NOTCH3	(150)

revealed that exosomes containing circ_0000338 are delivered from resistant to sensitive cells and confirmed that circulating EVs in CRC patients were enriched in circ_0000338. Further experiments revealed miR-217 and miR-485-3p as the target miRNAs of circ_0000338. Particularly, miR-217 induces tumor suppression by targeting downstream genes such as astrocyte elevated gene-1 (AEG-1), mitogen-activated protein kinase (MAPK), and zinc finger E-box binding homeobox 1 (ZEB1). On the other part, miR-485-3p counteracts CRC development, inhibiting the targeting protein for Xklp2 (TPX2). Runbi Ji *et al.* showed that EVs from human mesenchymal stem cells (MSCs) isolated from the umbilical cord promoted the 5-FU resistance of gastric cancer both *in vitro* and *in vivo* (153). In fact, the exposition to MSC-EVs induced in gastric cancer cells HGC-27, MGC-803, and SGC-7901 the activation of calcium/calmodulin-dependent protein kinases (CaM-Ks) and Raf/MEK/ERK kinase pathways, culminating in the upregulation of MDR, multidrug resistance-associated protein (MRP) and lung resistance-related protein (LRP), and finally, insensitiveness to apoptosis induced by 5-FU. In another study, miR-92a-3p expression in the circulating EVs of CRC patients has been demonstrated to be correlated with metastasis to the liver and chemoresistance to 5-FU (154). The main players in this context were CAFs, exhibiting the upregulation of miR-92a-3p, compared to normal fibroblasts, delivered in the surrounding by EVs. Once uptaken by cancer cells, miR-92a-3p-EVs induced the stemness, EMT, metastatization, and 5-FU resistance of cancer cells both *in vitro* and *in vivo*. Finally, F-Box and WD Repeat Domain Containing 7 (FBXW7) and Modulator of Apoptosis 1 (MOAP1) were identified as the main targets of miR-92a-3p. Consistently, CRC biopsies resulted to being enriched in miR-92a-3p and depleted in FBXW7 and MOAP1. Colon cancer cells exposed to 5-FU are also able to increase angiogenesis (155). Upon exposition to 5-FU, cancer cells HCT-15 released EVs enriched in growth/differentiation factor 15 (GDF15), which binds the TGF-βIII receptor. The activation of the receptor induces the suppression of Smad signaling and the upregulation of periostin in endothelial cells, culminating in the increase of angiogenesis. In HCC, Fu and colleagues found that HCC cells Bel7402 resistant to 5-FU produce EVs enriched in miR-32-5p shuttled to sensitive parental cells, in which it induces a decrease in PTEN and the activation of the PI3K/Akt, triggering EMT, angiogenesis, and finally, chemoresistance (156). Lastly, the relevance of miR-32-5p and PTEN in human HCC samples was investigated, confirming a negative correlation between them. This evidence is summarized in **Table 2**.

Doxorubicin Resistance by EV Molecular Cargoes

Doxorubicin (DXR), also known as Adriamycin (ADR), is an anthracycline antibiotic with antineoplastic activity, isolated from the bacterium *Streptomyces peucetius* var. *caesius*, acting as intercalating base pairs in the DNA helix (157). Additionally, DXR inhibits topoisomerase II. DXR also forms oxygen free radicals, resulting in cytotoxicity secondary to the lipid peroxidation of cell membrane lipids. Primary human OS cells MG63 treated with DXR increased their expression of P-gp1. Consistently, EVs from DXR-treated MG63 presented with higher levels of both the ABCB1 transcript and P-gp-encoded protein expression, which can be transferred to untreated MG63 cells, conferring their drug resistance (158).

Breast cancer cells MCF7 treated with ADR showed a high level of UCH-L1 and phospho-ERK, involved in the overexpression of ABCB1, compared to control cells. EVs from ADR-resistant MCF7 conferred to *naïve* MCF7 cells reduced sensitivity to ADR and increased p-ERK and P-gp1 levels (159). Interestingly, circulating EVs from breast cancer patients were positive for UCH-L1 and show an inverse correlation with response to treatment. MCF-7 exposed to ADR and DOC increased both cellular and exosomal miR-222, an inhibitor of phosphatase and tensin homolog (PTEN) gene, a tumor suppressor that negatively regulates the synthesis of phosphatidylinositol trisphosphate and the Akt signaling (141). Upon the uptake of these EVs, interstitial M2 macrophages underwent activation and polarization to support cancer cells. Accordingly, miR-222 has also been found in EVs from the plasma and tissue of chemoresistant patients (141). The overexpression of glutathione-S-transferase P1 (GSTP1, a phase II-metabolizing enzyme that detoxifies chemicals by conjugating with glutathione) is a described tool for cancer cells for counteracting chemotherapeutics like ADR. Yang *et al.* found that this enzyme is present in EVs from ADR-resistant MCF7 and, accordingly, in the sera of chemoresistant patients (160). A study conducted on HCC cells reported the role of long non-coding RNA linc-VLDLR in resistance toward DXR. Linc-VLDLR promoted the expression of the PCNA and ABCG2 genes, and the EV-mediated transfer of linc-VLDLR can result in the chemoresistance of HCC (161). **Table 3** summarizes these studies.

Taxane Resistance by EV Molecular Cargoes

Taxanes are diterpenes firstly isolated from the plants of *Taxus* spp. Taxanes work as microtubule-stabilizing drugs, inhibiting

TABLE 2 | Resistance to antimetabolites mediated by EVS.

Drug	Cancer Type	EV Cargo	Effects	Refs
5-FU	CRC patients and *in vitro*	circ_0000338	Repression of miR-217 and miR-485-3p upregulation of AEG-1, MAPK, ZEB1, and TPX2	(152)
5-FU	Gastric cancer patients and *in vitro*	EVs from MSCs	Upregulation of CaM-Ks, ERK, MDR, MRP, and LRP	(153)
5-FU	CRC patients *in vitro*	miR-92a-3p from CAFS	Downregulation of FBXW7 and MOAP1	(154)
5-FU	CRC *in vitro*	GDF15	Suppression of Smad and upregulation of periostin in target endothelial cells	(155)
5-FU	HCC patients and *in vitro*	miR-32-5p	Downregulation of PTEN and activation of EMT	(156)

5-FU, 5-fluorouracile.

TABLE 3 | Resistance to doxorubicin (DXR) mediated by EVS.

Drug	Cancer Type	EV Cargo	Effects	Refs
DXR	Osteosarcoma, *in vitro*	P-gp1	Transfer of mRNA and protein of MDR in sensitive cells	(158)
DXR	Breast cancer, patients and *in vitro*	UCH-L1 and p-ERK	Increase of ABCB1	(159)
DXR	Breast cancer, patients and *in vitro*	miR-222	Suppression of PTEN in M2 macrophage	(141)
DXR	Breast cancer patients and *in vitro*	GSTP1	Enhancement of detoxification pathway	(160)
DXR	HCC *in vitro*	Linc-VLDLR	Upregulation of PCNA and ABCG2	(161)

the depolymerization of microtubules during cell division (162). PTX, DOC, and cabazitaxel are widely used for the treatment of many different cancers. Exposure to the DOC of MCF7 cells induced the expression of P-gp1. Moreover, EVs from DOC-treated MCF-7 expressed higher levels of P-gp compared to EVs from *naïve* MCF-7, and the incubation with DOC-MCF7 EVs reduced the cell apoptosis of *naïve* MCF-7 (163). In another study, breast cancer cells MDA-MB-231 exposed to PTX specifically released EVs enriched in survivin, an inhibitor of apoptosis (164). The survivin-enriched EVs exerted protective effects on drug-sensitive fibroblasts and SKBR3 cells when exposed to PTX. Shan *et al.* described the role of CAF-EVs in the taxane resistance of prostate cancer cells. CAFs released EVs enriched in miR-423-5p, which is internalized inside prostate cancer cells LNCAP, 22RV-1, and C4 suppressing GREM2 [encoding for the gremlin2 protein inhibitor of bone morphogenetic protein (BMP) family members] and increasing TGF-β, overall leading to a reduced sensitivity to taxanes (165). These results are reported in **Table 4**.

Biological Drugs by EV Molecular Cargoes

Monoclonal antibodies are the newest frontier of anticancer drugs. A deeper knowledge of cancer biology and molecular profile allows to precisely target a specific member of pivotal pathways for cancer growth. Unfortunately, tumors can find a strategy to also counteract these agents. Cetuximab is a humanized mouse monoclonal antibody against the EGFR. Zhang *et al.* showed that EVs derived from cetuximab-resistant RKO colon cancer cells induced cetuximab resistance in cetuximab-sensitive Caco-2 cells. RKO cells and RKO-EVs resulted in depleted PTEN and enriched phospho-Akt, and the EV effects were abrogated by the Akt inhibitor LY294002 (166). In another study, circulating EVs from patients suffering from CRC have been exploited as a predictive biomarker for the response to cetuximab (42). In particular, circulating EVs from metastatic and chemoresistant subjects resulted in enriched lncRNA urothelial carcinoma-associated 1 (UCA1). *In vitro* experiments revealed that exosomes from cetuximab-resistant Caco-2 cells can transmit drug UCA1 and resistance to sensitive parental cells. Epidermal growth factor receptor 2 (HER2)-positive tumors can be targetable with the monoclonal antibody trastuzumab. Disappointingly, tumor cells can neutralize trastuzumab by EV release, *via* a decoy-like system. HER2-positive breast cancer cells BT474 and SKBR3 release HER2-positive EVs able to bind trastuzumab, while EVs from triple-negative cells MDA-MD-231 do not. On this basis, SKBR3 cells treated with autologous EVs were less sensitive to the effect of trastuzumab since EVs sequester trastuzumab, reducing the efficacy of the chemotherapy against the primary tumor (167). Finally, circulating EVs from HER2-positive breast cancer patients at an early stage showed lower binding to trastuzumab compared to EVs from patients with advanced disease. Rituximab is a monoclonal antibody against CD20, a standard in the management of malignant B-cell lymphoma (168). Aung *et al.* showed that leukemic cells released CD20-enriched EVs intercepting rituximab, thus protecting cancer cells from the complement-dependent cytolysis induced by rituximab (169). Lubin and colleagues reported that neuroblastoma cells released programmed death-ligand 1 (PD-L1)-EVs that bind to PD-1 on the surfaces of cytotoxic T cells, preventing the targeting of tumor cells and finally allowing immune evasion (170). Other authors described that the response to pembrolizumab (an anti-PD-1 antibody) in patients suffering from melanoma can be reduced by EVs (171). After treatment with pembrolizumab, melanoma cells released EVs enriched in PD-L1, which suppresses the proliferation of cytotoxic T cells and facilitates the immune evasion of tumor cells, counteracting the efficacy of pembrolizumab. **Table 5** summarizes these studies.

DISCUSSION

EVs are nowadays reported to be responsible for sustaining many aspects of tumor biology. Cancer recurrence and metastatization are the main clinical challenges to offer to patients a perspective of a free-disease lifespan or at least a lifetime with a steadied cancer. Resistance to therapies is one of the causative agents of those challenges. Many mechanisms are

TABLE 4 | Resistance to taxanes mediated by EVS.

Drug	Cancer Type	EV Cargo	Effects	Refs
Docetaxel	Breast cancer, *in vitro*	P-gp	Expression of functional P-gp	(163)
Paclitaxel	Breast cancer, *in vitro*	Survivin	Inhibition of apoptosis	(164)
Taxanes	Prostate cancer, *in vitro*	miR-423-5p from CAF-EVs	Inhibition of GREM2 and increase of TGF-β in cancer cells	(165)

TABLE 5 | Resistance to biological drugs mediated by EVS.

Drug	Cancer Type	EV Cargo	Effects	Refs
Cetuximab	CRC, *in vitro*	p-Akt	Depletion of PTEN	(166)
Cetuximab	CRC patients and *in vitro*	UCA1	Unreported	(42)
Trastuzumab	Breast cancer patients and *in vitro*	HER2	Neutralizing trastuzumab	(167)
Rituximab	B-cell lymphoma, *in vitro* and patients	CD20	Neutralizing rituximab	(168, 169)
Pembrolizumab	Melanoma patients and *in vitro*	PD-L1	Neutralizing pembrolizumab	(171)

described to drive chemoresistance, and, of note, they frequently overlap, making it virtually impossible to counteract once activated. EVs are recently indicated as a further mechanism supporting chemoresistance. By the means of EVs, insensitive cancer cells can educate sensitive cognate cells, shuttling directly a functional molecular apparatus. On this basis, EVs gain clinical interest as a manageable biomarker of cancer aggressiveness and predisposition to chemoresistance, becoming a promising liquid biopsy.

Moreover, EVs also offer a new strategy to fight cancer *via* the inhibition of the release or the interaction of EVs with target cells. Unfortunately, some issues dampen the use of EVs in clinical and therapeutic management. In fact, while in basic and preclinical studies, the key involvement of EVs is incontrovertible, these results are dampened in patients and not completely reproducible, mostly comparing *in vitro* studies with human trials. A reason for that can be the use of different procedures adopted to isolate EVs since a universal consensus is still lacking on this aspect. It is nowadays reported that the specific isolating procedures can enrich protein contamination (i.e., lipoproteins or protein aggregates), and certain EV subpopulations, in turn, selecting a particular molecular cargo not strictly linked to the real biological condition, making it hard to extrapolate a comprehensive and objective interpretation.

Nevertheless, many authors are exploiting the use of natural or modified EVs as a drug delivery system. A successful and effective encapsulation of chemotherapeutics has been reported for DXR, cisplatin, and methotrexate in EVs from lung (human A549 cells), hepatocarcinoma (murine H22 cells), and breast (human MCF-7 cells) cancer cells. The efficacy has been demonstrated *in vitro* in animal models and in a clinical trial (patients suffering from stage IV lung carcinoma) (172). Paclitaxel was also loaded in EVs from human prostate cancer cells (LNCaP and PC-3 cells) (173). Murine macrophage RAW 264.7 cells were tested as a source of EVs for loading DXR targeting lung and colon (both *in vitro* and in animal models) cancers (174, 175). Similarly, EVs from RAW 264.7 packaged with DXR were effective in H22 tumor-bearing mice (144). Primary murine osteoblast-EVs have been loaded with dasatinib and successfully mitigated exacerbated osteolysis *in vivo* (18).

This is a very stimulating and active field since EVs offer many advantages in drug administration compared to the classical or liposomal formulation. The naturally occurring EV composition can confer very selective tropism to a specific tissue or cell, as well as present a higher biologic activity due to the ability to convey complex molecular machinery to the targets sustaining the required therapeutic effect. Moreover, natural EVs can be engineered for acquiring further or better properties (175).

As discussed above, several issues still curb the possibility to produce EVs for therapeutic use, mainly because the option to produce EVs under Good Manufacturing Process conditions is still lacking, although many efforts are leading in this direction. In this sense, all the key unit operation and process steps are under consideration for standardization and assessment for EV safety and de-risking, considering and not limited to the following: choice and characterization of the cell source, isolation methods, drug-loading methods (loading efficacy/cost ratio for large-scale production), the eradication of potential contaminants and impurities, best formulation, and the shelf life of final EV products. The recent case of a public safety notification on exosome products from the Food and Drug Administration for a group of patients in Nebraska, who have experienced adverse effects from the administration of improper EVs, is an exemplificative of the urgency to have regulatory monitoring about the use of EVs for human health (https://www.fda.gov/vaccines-blood-biologics/safety-availability-biologics/public-safety-notification-exosome-products). The increasing availability of new analytical techniques is predictable to provide new insights into the distinctiveness of EVs and may unlock the full potential of EVs for clinical management.

AUTHOR CONTRIBUTIONS

SP: Conceptualization, Literature Review, Visualization, Writing—Review and Editing. AV: Coordination, Writing—Original Draft. RS: Supervision, Writing—Review and Editing. AC: Conceptualization, Literature Searching and Critical Review, Writing—Original Draft. All authors have read and approved the final manuscript.

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Role of exosomal microRNAs in cancer therapy and drug resistance mechanisms: focus on hepatocellular carcinoma

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Extracellular vesicles (EVs), defined as intercellular messengers that carry their cargos between cells, are involved in several physiological and pathological processes. These small membranous vesicles are released by most cells and contain biological molecules, including nucleic acids, proteins and lipids, which can modulate signaling pathways of nearby or distant recipient cells. Exosomes, one the most characterized classes of EVs, include, among others, microRNAs (miRNAs), small non-coding RNAs able to regulate the expression of several genes at post-transcriptional level. In cancer, exosomal miRNAs have been shown to influence tumor behavior and reshape tumor microenvironment. Furthermore, their possible involvement in drug resistance mechanisms has become evident in recent years. Hepatocellular carcinoma (HCC) is the major type of liver cancer, accounting for 75-85% of all liver tumors. Although the improvement in HCC treatment approaches, low therapeutic efficacy in patients with intermediate-advanced HCC is mainly related to the development of tumor metastases, high risk of recurrence and drug resistance. Exosomes have been shown to be involved in pathogenesis and progression of HCC, as well as in drug resistance, by regulating processes such as cell proliferation, epithelial-mesenchymal transition and immune response. Herein, we summarize the current knowledge about the involvement of exosomal miRNAs in HCC therapy, highlighting their role as modulators of therapeutic response, particularly chemotherapy and immunotherapy, as well as possible therapeutic tools.

KEYWORDS

exosomes, miRNAs, hepatocellular carcinoma, therapy, drug resistance

Introduction

Liver tumor is one of the most common types of cancers (1), displaying the 7th highest age-adjusted incidence rate in the world. Hepatocellular carcinoma (HCC) is considered the most frequent liver neoplasm (1) and the third most common cause of cancer-related death worldwide (2), predominantly in Asian countries, due to late diagnosis and lack of effective surveillance programs for high-risk people. In this context, several non-invasive diagnostic biomarkers are considered, but, unfortunately, they do not reach necessary sensitivity and specificity, especially for early stage-HCC (3). On the other hand, liver biopsy is a limited procedure due to its invasiveness, and imaging methods (e.g. ultrasonography, magnetic resonance and/or computed tomography) are usually utilized for diagnosis, even though small tumors can be often missed (4, 5).

HCC initiation, development and progression are dependent both on intrinsic (e.g. gene mutations) and extrinsic (e.g. viral infections, western type diet intake, alcohol consumption) factors able to induce in liver cells the typical responses of malignant transformation, leading to apoptosis evasion, cell proliferation and survival, neovascularization (4). In particular, non-alcoholic fatty liver disease (NAFLD) is now considered the most important liver chronic disease (6) and it has been shown that, among HCC predisposing factors, not only high-fat (7), but also high-carbohydrate/western type diet can induce disease progression up to tumor formation in a NAFLD/NASH mouse model (8–10). The progression of the disease includes a passage through a cirrhotic stage in a large majority of HCC cases (up to 90%) (11). Oxidative damage (12), inflammation (13), hepatocyte compensatory regeneration (14), with consequent accumulation of gene mutations, are typical HCC features. Mutational HCC landscape includes many genes with different mutation frequency, such as *TP53* (30%), *CTNNB1/β-catenin* (26%), *ARID1A* (8%), *ARID2* (6%), *AXIN* (6%) (15). Several pathways, such TGF-β, Wnt/β-catenin, Hedgehog, Notch, EGF, HGF, VEFG, JAK/STAT, Hippo, and HIF are dysregulated and play a crucial role in HCC, leading to uncontrolled cell division and metastasis. For some of them, small promising molecules for therapeutic approaches are under investigation (16).

To date, the main approach in HCC management is radiofrequency ablation (RFA), surgical resection or liver transplantation, if feasible, and outcome of patients untreatable with resection curative methods is dependent on the response to the currently available therapies. Novel treatments, principally based on sorafenib and regorafenib, two tyrosine kinases inhibitors (TKIs) seem, however, to induce a moderate increase of HCC patients' survival (17). MicroRNAs (miRNAs) are short non-coding RNA molecules able to regulate gene expression at the post-transcriptional level (18), playing a pivotal role in high-impact disorders, including neurodegenerative (19), cardiovascular (20) diseases and cancer (21, 22). They work by fine-tuning key physiological and pathophysiological processes, such as proliferation, survival, apoptosis, invasion, angiogenesis, epithelial-mesenchymal transition (EMT), metastasis and resistance to therapeutic treatments as well (23, 24). MiRNAs dysregulated expression levels are described both in tumor tissues and serum/plasma, where they are included in macromolecular protein complexes (25–27) or encapsulated in microvesicles/exosomes (28, 29) to be protected from endogenous RNase, being so easily detectable and quantifiable. Such properties allowed the identification of miRNAs as potential diagnostic, prognostic and predictive cancer biomarkers. Furthermore, aberrant miRNA expression in cancer led to the characterization of oncomiRs and tumor-suppressor miRs, playing a role in promoting or suppressing oncogenesis, respectively. The possibility to synthesize and obtain molecules able to specifically repristinate physiological conditions (i.e. antagomiRs inhibiting oncomiRs, mimics replacing tumor suppressor miRs) made these molecules of great interest for innovative cancer therapeutic strategies (30). Furthermore, several miRNAs were described as biomarkers for therapy response and disease-free survival/clinical progression in HCC patients (31–34).

In this review, we report recent advances on exosomal miRNAs in HCC, by focusing on their involvement and role in therapeutic responses.

MiRNA biogenesis

In the canonical pathway, microRNAs are transcribed, from intergenic or intragenic genomic regions, by Polymerase II in the nucleus, thus originating long double stranded-hairpin primary transcripts (pri-miR). Subsequently, RNase III enzyme Drosha, associated to RNA binding protein (RBP) DGCR8 (DiGeorge critical region 8), cleaves pri-miRNAs to generate pre-miRNAs (60-100 nucleotides in length hairpin precursors) which are subsequently transferred to the cytoplasm by Exportin 5 through a Ran (Ras-related nuclear protein)-GTPase-dependent mechanism. There, pre-miRNAs are cleaved again by RNase enzyme Dicer, linked to the trans-activation-responsive RNA-binding protein (TRBP), to produce mature double-stranded miRNAs. Mature miRNAs associate to a member of Argonaute family (Ago1-2-3-4 paralogs in mammals) thus generating the ribonucleoprotein miRNA-induced silencing complex (miRISC). Two main mechanisms for miRNA-mediated regulation are described through the interaction between the seed region and specific partially or perfectly complementary microRNA responsive elements (MREs), mainly located at the level of target mRNA 3'-UTR, with consequent translation repression or mRNA decay by deadenylation followed by decapping, respectively (18, 35, 36).

A DICER-independent mechanism has been described as well for pre-miR-451, involved in erythropoiesis. This is due to the stem-loop structure, too short to be processed by DICER. In this case, miRNA's maturation requires direct loading into Ago2 and subsequent cleavage by its catalytic centre (37).

Exosomes

Cells can secrete different types of extracellular vesicles (EVs). This feature is conserved from bacteria up to higher organisms (38, 39), and it was originally intended to discard unwanted or unnecessary molecules (40). However, it is known that EVs are involved also in exchanging nucleic acids, lipids and proteins among cells; moreover, they play a role in favouring intercellular communication, at the level of both physiological and pathophysiological processes (41). Three main types of EVs have been described: exosomes, microvesicles and apoptotic bodies which differ based on their biogenesis and release mechanisms, content, size and role (42, 43),.

Exosomes are nano-sized biovesicles (diameter 30-150 nm) secreted by all cell types. They can be detected in most of body fluids and are delimited by a lipid bilayer membrane which protects and aids to deliver cargos to recipient cells (44). Exosome biogenesis occurs as a part of membrane-trafficking processes: cargos are insourced and distributed into early endosomes at the level of endosomal system. Subsequently, late endosomes/multivesicular bodies (MVBs), containing intraluminal vesicles (ILVs), are generated from early endosomes. ILVs can sequester lipids, proteins and other cargos from cytosolic compartments and Golgi apparatus. After, MVBs containing cargos are driven at the level of the plasma membrane, where they merge with it, so that ILVs are released, as exosomes as an outward budding (43, 45, 46) (**Figure 1**).

The MVBs formation seems to be dependent also on stimulation by growth factors (47) and can occur mainly by endosomal sorting complex required for transport (ESCRT) pathway-dependent mechanisms (48). In addition, ESCRT-independent mechanisms have been described for MVBs formation (49–51) since it was demonstrated that MVBs can still form after depletion of ESCRT components (52). ESCRT (ESCRT-0/I/II/III) and accessory (e.g. Alix, TSG101, HSC70, HSP70) proteins are contained in exosomes following ESCRT-dependent MVEs generation, irrespective of the cell types, and for this reason they are considered as exosomal markers (42, 43, 53). On the other hand, in ESCRT-independent mechanism, other molecules (e.g. tetraspanins, CD63, CD9, CD81) are commonly found in exosomes, but also detected in other types of vesicles, such as MVs (54, 55). Based on information reported by some databases, exosomes can contain more than 8,000 proteins and 190 lipids. Integrins, tetraspanins, MHC-II complex proteins are described in the exosomal membrane, whereas other (CD55, trombospondin, ALIX, lactadherin) are included into exosomes during the biogenesis (56).

Exosome can load different and tissue-specific cargos, such as proteins, nucleic acids, lipids and metabolites, depending on the type of cell from which they are produced. Following their uptake by recipient cells, *via* exosomal fusion or endocytosis, they provide autocrine, paracrine and endocrine functions, thus

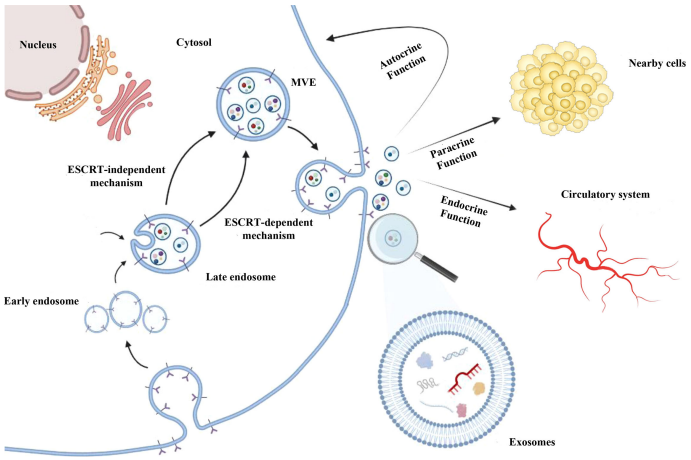

FIGURE 1
Exosomes biogenesis. After being generated by endocytosis at the level of endosomal system, exosomes are included as invagination within the multivesicular endosomes (MVEs) lumen and released as an outward budding upon the fusion of MVEs with the cell membrane. MVEs formation can occur mainly by endosomal sorting complex required for transport (ESCRT) pathway-dependent mechanism; ESCRT-independent mechanisms were also described. Exosomes can contain different cargos, such as proteins, lipids, DNA, mRNAs, lnRNAs and miRNAs. Exosomes are uptake by recipient cells thus favouring intercellular communication through their autocrine, paracrine and/or endocrine function.

favouring intercellular communications (55, 57) (**Figure 1**), participating in cell homeostasis (58), stimulating immune responses (59), promoting tissue repairing (60), cell survival (61), and modulating angiogenesis (62). For these reasons, they are considered as crucial modulators of intercellular cross-talks at the base of several high-impact diseases (63–65), including cancer.

Exosomal miRNAs in HCC

Exosomal miRNAs have been shown to regulate tumor behavior and reshape tumor microenvironment (TME). Increasing evidence indicates that exosomal miRNAs contribute to HCC pathogenesis and progression by regulating processes such as cell proliferation, metastasis and immune response (66), thus showing considerable potential as diagnostic, predictive and prognostic biomarkers as well as new therapeutic tools (67). **Table 1** shows a comprehensive overview of exosomal miRNAs and related functions in HCC development and progression, based on the most recent literature. The utility of exosomes as vectors of biological therapeutic agents, such as miRNAs, has been actively explored in HCC (67). In the following sections, we will discuss, among those reported in **Table 1** the most relevant miRNAs described as mediators of therapeutic response and/or putative treatment tools in HCC.

Exosomal miRNAs as modulators of therapeutic response

Several studies have described the ability of exosomal miRNAs to modulate HCC therapeutic response to different drugs, such as sorafenib, 5-fluorouracil (5-FU) and doxorubicin, or immunotherapy (107, 108). Experimental evidence suggests the involvement of exosomal miRNAs both in drug resistance mechanisms and in drug sensitivity improvement.

Lou et al. observed that miR-122-enriched exosomes, obtained from adipose tissue-derived mesenchymal stem cells (AMSC) after transfection with miR-122 expression plasmids, inhibited HCC cell proliferation and increased sensitivity to 5-FU and sorafenib both *in vitro* and in xenograft mouse models, by targeting and downregulating the expression of CCNG1, ADAM10 and IGF1R, genes involved in tumorigenesis and drug sensitivity in several cancer types. Thus, the authors highlighted the potential use of exosomal miR-122 to improve therapeutic response and revert drug resistance (71).

Similarly, exosomal miR-744 was able to rescue sorafenib sensitivity in resistant HepG2 cancer cells, by targeting PAX2, involved in the regulation of chemotherapy response in several cancers (88). Decreased level of miR-744 was found in HCC tissues, in exosomes from patients' sera and HCC cells resistant to sorafenib: the restoration of miR-744 expression in HepG2 cancer cells and the subsequent release of miR-744-enriched exosomes led to decreased miR-744-induced cell proliferation and resistance to therapy (88).

On the contrary, miR-32-5p was found to contribute to multidrug resistance in HCC cells (94). The authors observed that exosomes from multidrug-resistant Bel/5-FU cells were able to deliver miR-32-5p into sensitive Bel7402 cells, thus inducing angiogenesis, EMT and drug resistance through PI3K/AKT pathway activation. Increased levels of exosomal miR-32-5p were also found in sera from HCC patients, associated with poor prognosis (94).

Resistance to chemotherapy, is one of the main factors responsible for high mortality rate in HCC patients, and the identification of mechanisms underlying chemoresistance as well as the enhancement of therapeutic options is of great clinical interest. In this context, the possible role of the exosomal tumor suppressor miR-199a-3p was recently explored in two studies (78, 79).

Lou et al. showed that exosome-mediated crosstalk between adipose tissue-derived mesenchymal stem cells (AMSCs) hyper-expressing miR-199a-3p and HCC cells increased the tumor sensitivity to doxorubicin by targeting mTOR signaling pathway. *In vivo* experiments, based on AMSC-Exo-199a injection into a HCC mouse model, confirmed increased doxorubicin anti-tumor effect in HCC (78).

Likewise, Zhang et al. observed that exo-miR-199a-3p restored sensitivity to cisplatin (DDP) and decreased tumor growth in chemo-resistant HCC cells. Due to the ability of exo-miR-199a-3p to overturn DDP resistance, the authors highlighted its great potential as an alternative therapeutic option in DDP-refractory HCC (79).

In a study aimed at elucidating the possible mechanism by which hepatitis B core antigen (HBc) promotes doxorubicin resistance in HCC, Wei et al. suggested that HBc led to upregulation of exosomal miR-135a-5p inducing cell proliferation, anti-apoptotic effects, and drug resistance. VAMP2 was identified as a novel miR-135a target, and its level decrease was linked to cell proliferation, apoptosis escape and drug resistance, thus identifying the miR-135a-5p/VAMP2 axis as a key regulatory chemo-resistance mechanism in HCC (99).

Exosomal miR-451a acts as tumor suppressor miRNA and its expression is down-regulated in HCC (84). Xu et al. used human umbilical cord mesenchymal stem cells (hucMSCs) derived exosomes to treat Hep3B cells and assess paclitaxel resistance. The authors demonstrated that exosomal miR-451 slowed EMT progression and reduced proliferation, migration and resistance to paclitaxel by suppressing ADAM10 in HCC cells, thus acting as a chemosensitivity-inducing factor and promoting HCC cell apoptosis (85).

Semaan et al. reported that the use of exosomal miR-214 from human cerebral endothelial cell-derived exosomes (hCEC-

TABLE 1 Overview of exosomal miRNAs involved in HCC pathogenesis.

	miRNA	Function in HCC	Anti-cancer drug (s)	Targets/pathway	Donor cell	Recipient cell	Reference
Tumor Suppressor miRNAs	miR-9-3p	Reducing proliferation and motility		HBGF-5, ERK1/2			(68)
	miR-26a	**Suppressing cell proliferation and migration**		**CCND2, CCNE2, CDK6**	**HEK293T**	**HCC cells (HepG2)**	(69)
	miR-31 and miR-451a[1]	**Anti-cancer activity**		**CDK2, SP1, BCL2α, MDR1**	**RCC**	**HCC cells (HepG2)**	(70)
	miR-122	**Increasing sensitivity to chemotherapeutic agents/ inhibiting cell proliferation**	**5-fluorouracil (5-FU) and Sorafenib**	**ADAM10, IGF1R, CCNG1**	**AMSCs**	**HCC cells (HepG2)**	(71)
		Inhibiting growth and proliferation/increasing senescence		CAT1, FTF2B			(72)
	miR-142 and miR-223	**Inhibiting cell proliferation**		**STMN1, IGF-1R**	**TAMs**	**HCC cells (Huh7)**	(73)
	miR-142-3p	**Suppressing invasion and tumor growth**		**RAC1**	**TAMs**	**HCC cells (Hepa 1-6)**	(74)
	miR-145	Suppressing tumorigenesis and metastasis		GSK-3β/MMPs pathway			(75)
	miR-146a	Anti-cancer activity *via* immune system stimulation		M2 and T-cells			(76)
	miR-150-3p	Suppressing cancer progression		—			(77)
	miR-199a-3p	**Increasing sensitivity to anti-cancer drugs/ inhibiting invasion**	**Doxorubicin**	**mTOR pathway**	**AMSCs**	**HCC cells (Huh7,SMMC-7721, PLC/PRF/5)**	(78)
			Cisplatin (DDP)	**ATM, mTOR and DNMT3A**	**HEK293T**	**HCC cells (Huh-7, Huh-7/DDP)**	(79)
	miR-200b-3p	**Suppressing angiogenesis**		**ERG**	**HCC cells (HLE, Hep3B)**	**Endothelial cells (Huvecs)**	(80)
	miR-214	**Reducing viability and invasion in combination with anti-cancer drugs**	**Oxaliplatin and Sorafenib**	**P-gp, SF3B3**	**hCEC**	**HCC cells (HepG2, Hep3B)**	(81)
	miR-320a	**Inhibiting proliferation, migration and metastasis**		**MAPK pathway (PBX3)**	**CAFs**	**HCC cells (MHCC97H and SMMC-7721)**	(82)
	miR-335-5p	**Inhibiting proliferation and invasion**		**CDC42, CDK2, CSNK1G2, EIF2C2, EIF5, LIMaK1, NRG1, PLK2, RGS19, TCF3, THBS1, YBX1, ZMYND8**	**CAFs**	**HCC cells (MHCC97L, MHCC97H, Huh7 and HepG2)**	(83)
	miR-451a[1]	Suppressing survival and angiogenesis		LPIN1			(84)
		Suppressing drug resistance, proliferation, migration and invasion	**Paclitaxel**	**ADAM10**	**HUC-MSCs**	**HCC cells (Hep3B and SMMC-7721)**	(85)
	miR-490	**Inhibiting metastasis**		**EGFR-AKT-ERK1/2 pathway (ERGIC3)**	**MCs**	**HCC cells (HepG2, Hep3B)**	(86)

(Continued)

TABLE 1 Continued

	miRNA	Function in HCC	Anti-cancer drug (s)	Targets/pathway	Donor cell	Recipient cell	Reference
	miR-718	Decreasing tumour aggressiveness and recurrence		HOXB8			(87)
	miR-744	**Inhibiting proliferation and chemoresistance**	**Sorafenib**	**PAX2**	**HCC cells (HepG2)**	—	(88)
OncomiRs	miR-10b	Promoting proliferation and metastasis		HIF-1α, HIF-2α			(89)
	miR-21			PTEN			
	miR-21	**Promoting angiogenesis, migration and tumorigenic function**		**PDK1/Akt pathway (PTEN)**	**HCC cells**	**HSCs**	(90)
				—	**HCC cells (SK-hep-1)**	—	(91)
	miR-23a-3p	**Regulating PD-L1 expression which helps tumor cells escape from antitumor immunity**		**PTEN**	**ER-stressed HCC cells**	**Macrophages**	(92)
	miR-25-5p	Enhancing cell motility		—			(93)
	miR-32-5p	**Promoting multidrug resistance**	**5-fluorouracil (5-FU), Oxaliplatin (OXA), Gemcitabine (GEM), Sorafenib**	**PI3K/Akt pathway (PTEN)**	**Multidrug-resistant HCC cells (Bel/5-FU)**	**sorafenib-sensitive HCC cells (Bel7402)**	(94)
	miR-92a-3p	Promoting EMT and metastasis		PTEN			(95)
	miR-92b	Enhancing migration ability of cells and decreasing NK cell-mediated cytotoxicity		CD69 on NK cells			(96)
	miR-93	Increasing proliferation and invasion		TP53INP1, TIMP2, CDKN1A			(97)
	miR-103	Increasing vascular permeability and metastasis		VE-Cad, p120, Zo-1			(98)
	miR-135a-5p	**Promoting survival, proliferation and chemotherapy resistance**	**Doxorubicin**	**VAMP2**	**HCC cells (HepG2)**	—	(99)
	miR-155	**Promoting angiogenesis**		**VEGF and HIF-1α, MVD**	**HCC cells (PLC/PRF/5 and HuH7)**	**Endothelial cells (Huvecs)**	(100)
		Promoting proliferation		PTEN			(101)
	miR-210	**Promoting angiogenesis**		**SMAD4, STAT6**	**HCC cells (QGY-7703, HepG2, SK-Hep-1, Huh-7 and Hepa1-6)**	**Endothelial cells (Huvecs)**	(102)
	miR-224	Promoting proliferation and progression		glycine N-methyltransferase			(103)
	miR-655	Stimulating proliferation		MAPK/ERK pathway			(104)
	miR-1247-3p	CAFs activation and secretion of pro-inflammatory cytokines promoting cancer progression		NF-kB pathway (B4GALT3)			(105)
	miR-1273f	Stimulating proliferation and metastasis hypoxia-induced		Wnt/β-catenin pathway			(106)

AMSCs: Adipose tissue-derived mesenchymal stem cells; CAFs: Cancer-associated fibroblast; EMT: Epithelial-mesenchymal transition; hCEC:human cerebral endothelial cell; HEK293T: human embryonic kidney 293 cells; HSCs: Hepatocyte stellate cells; HUC-MSCs: Human umbilical cord mesenchymal stem cells; MCs: Mast cells; NK: Natural killer; RCC: renal carcinoma endothelial cells. TAMs: Tumor associated macrophages. [1]The same miRNA was described in references 70 and 85.
MiRNAs described in sections 4.1 and 4.2 for their potential therapeutic applications are highlighted in bold.

Exo-214) in combination with oxaliplatin or sorafenib could effectively reduce cancer cell viability and invasion of HepG2 and Hep3B cells compared to monotherapy. At the molecular level, this effect seems to be mediated by the glycoprotein P-gp and the splicing factor SF3B3 (81).

Inhibiting the release of exosomal oncomiRs, or increasing the effect of tumor suppressor miRNAs, could also play a synergic role in immunotherapy: for example, it has been shown that the inhibition of miR-23a-3p in HCC cancer cells could prevent their exosomal release and the consequent expression of PD-L1 in macrophages (92).

Similarly, it has been observed that high VEGF expression, in response to hypoxia, in TME can have immunosuppressive effect in tumors, resulting in decreased efficacy of PD-L1 and PD-1 inhibitor drugs (109, 110). Therefore, inhibition of VEGF and other hypoxia-induced factors, such as exosomal miRNAs involved in the regulation of angiogenesis in TME, could improve the efficacy of current immunotherapies (111). In this context, other studies led to hypothesize that targeting specific exosomal miRNAs, released from HCC cells and able to stimulate angiogenesis and HCC proliferation, such as miR-210 and miR-155, could therefore interfere with cellular crosstalk that promotes angiogenesis, further improving therapy (100, 102).

Exosomal miRNAs as main therapeutic tools

Exosomes naturally act as carriers of nucleic acids, proteins and lipids from donor to recipient cells. They are characterized by high biocompatibility, low immunogenicity, low toxicity and ability to cross the blood-brain barrier. These features make them promising vehicles for the delivery of chemical and biological drugs (107). In this section, we focus on exosomal miRNAs described for their potential application as main biological therapeutic agents in HCC.

Zhang et al. observed that exosomes released from cancer-associated fibroblasts (CAFs) overexpressing miR-320a were able to transfer this miRNA into HCC cells and suppressed HCC cell proliferation and metastasis both *in vitro* and *in vivo* by targeting PBX3 (82).

Through the same experimental approach, Wang et al. demonstrated that CAFs-derived miR-335-5p-enriched exosomes could inhibit HCC cell proliferation and invasion, by regulating genes including CDC42, CDK2, CSNK1G2, EIF2C2, EIF5, LIMaK1, NRG1, PLK2, RGS19, TCF3, THBS1, YBX1, and ZMYND8 (83).

Xiong et al. showed that stimulation of mast cell with hepatitis C virus E2 envelope glycoprotein (HCV-E2) resulted in miR-490 expression increase in mast cells as well as in secreted exosomes. Furthermore, the delivery of Exo-miR-490 to HCC recipient cells inhibited the ERK1/2 pathway, thereby suppressing cell migration and metastasis (86).

MiR-21 is a well characterized oncomiR, involved in cell proliferation and metastases through the inhibition of genes such as PTEN (112), PDD4, RECK, and SULF-1 (113). The ability of miR-21 to confer resistance to chemotherapy in cancer cells has also been reported (112, 114).

Zhou et al. showed the ability of HCC-derived exosomal miR-21 to convert hepatocyte stellate cells (HSCs) to CAFs, resulting in angiogenesis promotion through increased secretion of VEGF, MMP2, MMP9, bFGF and TGF-β by CAFs. Results of this study provided further insight into the crosstalk between cancer cells and their microenvironment during tumor progression, providing new information of potential clinical utility in HCC (90).

Interestingly, Liang et al. used nanoparticles loaded with small interfering RNA (siRNA) to downregulate the expression of the pro-oncogenic factor Sphk2 in HCC cells to reduce exosomal miR-21, thus decreasing tumor cell migration and exosome-mediated tumorigenic function. The anti-tumor effect of Sphk2 siRNA was also demonstrated in a xenograft mouse model resulting in reduced HCC tumor progression. Therefore, targeting exosomal oncomiR secretion could represent a new therapeutic strategy (91).

Moh-Moh-Aung et al. reported that the downregulation of exosomal miR-200b-3p in HCC cells led to the promotion of angiogenesis through endothelial ERG expression increase, thus providing new insights into possible targetable mechanisms to improve the efficacy of anti-angiogenic therapies (80).

Immune cells play a key role in tumorigenic process, therefore the collection and possible engineering of exosomes from these cell types might represent an anti-tumor strategy that requires further investigation.

It has been shown that exosomal miR-142 and miR-223, transferred from tumor associated macrophages (TAMs) to HCC cells, can suppress cancer cell proliferation through the modulation of genes involved in cell cycle regulation, such as the miR-223 target gene STMN1 (73).

Furthermore, the use of the intravenous anaesthetic propofol, induced the secretion of miR-142-3p-enriched exosomes from TAMs, and the internalization of these vesicles into HCC cells led to the inhibition of cell invasion *in vitro* and tumor growth *in vivo* through down-regulation of miR-142-3p target gene RAC1 (74).

Liang et al. described an alternative approach to deliver antioncomiRs-enriched exosomes to HCC cells. In their study, HEK293T cells were engineered to secrete exosomes actively loaded with miR-26a by electroporation. These exosomes were able to selectively target HepG2 cells, thus decreasing cancer cell migration and proliferation *in vitro* though the inhibition of key cell cycle regulators such as CCND2, CCNE2, CDK6 (69).

The same approach was described by Pomatto et al. who used renal carcinoma endothelial instead of HEK293T cells,

loaded with tumor suppressor miR-31 and miR-451a able to induce chemosensitivity (70). Overall, results of these two studies highlight the interesting possibility of using engineered exosomes as therapeutic agents.

Conclusion

MiRNAs are considered pivotal modulators of intercellular crosstalk and miRNA transfer *via* exosomes has been described as one of the possible strategies by which resistant HCC cells can share their resistance with neighbouring cells, thus hindering therapies.

In this context, based on the results principally obtained from *in vitro* and *in vivo* models, exosomes can be also considered as promising vehicles of miRNAs for therapeutic purposes, representing a great resource for the design of new treatment strategies with potential efficacy, especially in combination with chemotherapy, TKIs or immunotherapy, currently considered as a cutting-edge cancer treatment.

In addition to exosomal miRNAs directly targeting HCC cells, an interesting alternative could also be HCC tumor microenvironment targeting, in terms of CAFs, immune cells or tumor endothelial cells, on which exosomal miRNAs could induce desirable responses.

Furthermore, technological advances, focused for example on vesicles and donor cells engineering, offer an unprecedented opportunity to improve and provide novel tools for potential therapeutic applications of exosomal miRNAs in HCC and other types of cancers.

Overall, although more in-depth studies to elucidate the exact biological role and possible applicability of exosomal miRNAs in HCC treatment are required, these mediators can represent promising factors of potential therapeutic utility in HCC patients.

Author contributions

VZ, CC, and AT: original draft preparation; VZ, CC, RC, and AC: Bibliographic information; MDVN and FZ: reading and editing manuscript; EA and AT: conceptualization, review and editing manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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Cyclooxygenase-2 Upregulated by Temozolomide in Glioblastoma Cells Is Shuttled In Extracellular Vesicles Modifying Recipient Cell Phenotype

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Temozolomide (TMZ) resistance is frequent in patients with glioblastoma (GBM), a tumor characterized by a marked inflammatory microenvironment. Recently, we reported that cyclooxygenase-2 (COX-2) is upregulated in TMZ-resistant GBM cells treated with high TMZ concentrations. Moreover, COX-2 activity inhibition significantly counteracted TMZ-resistance of GBM cells. Extracellular vesicles (EV) are considered crucial mediators in orchestrating GBM drug resistance by modulating the tumor microenvironment (TME) and affecting the surrounding recipient cell phenotype and behavior. This work aimed to verify whether TMZ, at low and clinically relevant doses (5-20 μM), could induce COX-2 overexpression in GBM cells (T98G and U87MG) and explore if secreted EV shuttled COX-2 to recipient cells. The effect of COX-2 inhibitors (COXIB), Celecoxib (CXB), or NS398, alone or TMZ-combined, was also investigated. Our results indicated that TMZ at clinically relevant doses upregulated COX-2 in GBM cells. COXIB treatment significantly counteracted TMZ-induced COX-2 expression, confirming the crucial role of the COX-2/PGE2 system in TMZ-resistance. The COXIB specificity was verified on U251MG, COX-2 null GBM cells. Western blotting of GBM-EV cells showed the COX-2 presence, with the same intracellular trend, increasing in EV derived from TMZ-treated cells and decreasing in those derived from COXIB+TMZ-treated cells. We then evaluated the effect of EV secreted by TMZ-treated cells on U937 and U251MG, used as recipient cells. In human macrophage cell line U937, the internalization of EV derived by TMZ-T98G cells led to a shift versus a pro-tumor M2-like phenotype. On the other hand, EV from TMZ-T98G induced a significant decrease in TMZ sensitivity in U251MG cells. Overall, our results, in confirming the crucial role played by COX-2 in TMZ-resistance, provide the first evidence of the presence and effective functional transfer of this enzyme through EV derived from GBM cells, with multiple potential consequences at the level of TME.

Keywords: extracellular vesicles, glioblastoma, temozolomide, COX-2, COXIB, celecoxib, NS398

INTRODUCTION

Extracellular vesicles (EV), a heterogeneous population of lipid bilayer-enclosed structures released from all cell types, have been defined as critical mediators of intercellular communication by transferring functional genomic and proteomic cargo. The molecular content of EV, namely proteins, nucleic acids, and lipids, reflects the status and phenotype of the releasing cells. The EV are essential actors in the tumor microenvironment (TME), including glioblastoma (GBM), the most common and aggressive type of primary intracranial tumor in humans, representing about 81% of the malignant oncological lesions of the brain (1). Nowadays, although remarkable advances in GBM therapy have driven significant progress, chemoresistance remains the main hurdle in patient survival. Temozolomide (TMZ) is an oral DNA alkylating agent currently used as a standard first-line treatment for adult patients affected by newly diagnosed GBM. This drug exerts its antitumor activity by interfering with DNA replication. TMZ methylates DNA leading to the formation of O6-methylguanine, the most potent cell-killing lesion, which mispairs with thymine during the next cycle of DNA replication. Although TMZ can improve the overall survival of patients, the therapeutic outcomes remain unsatisfactory (2).

The ability of GBM cells to dynamically modulate the EV cargo composition in response to chemotherapy and hypoxia is becoming increasingly evident; on the other hand, how the EV cargo can affect the target cell phenotype, modifying sensitivity to chemotherapy drugs, thus promoting chemoresistance (3–5), is currently being studied.

The combined use of the chemotherapeutic agent TMZ with COX-2 inhibitors (COXIB) has been investigated as an alternative strategy to fight GBM progression by counteracting chemoresistance (6). As the proinflammatory enzyme COX-2 is recognized as a crucial mediator in GBM biology, selective COXIB were defined as an extremely promising GBM therapy increasing sensitivity to chemotherapy without other side effects (7). In this regard, recently, our group reported that the selective COX-2 inhibitor, NS398, counteracted chemoresistance to TMZ, used at high concentrations (200 μM) for 72 h, in resistant GBM cell line (T98G) abrogating TMZ-induced COX-2 upregulation and COX-2-dependent pathways involved in TMZ-resistance (8). TMZ exposure of resistant T98G, but not sensitive and COX-2 null U251MG cells, led to a significant and dose-dependent upregulation of COX-2. Moreover, NS398 enhanced the chemosensitivity to TMZ in GBM cells downregulating TMZ-induced COX-2 expression. The ability of Celecoxib (CXB), a selective COXIB approved by the Food and Drug Administration (FDA) and widely used for its anti-inflammatory, analgesic, and antipyretic actions, to suppress the growth of GBM cell lines, U373 and T98G, partly by inhibiting the NF-kB signaling pathway, has also been reported (9). A recent *in vitro* study highlighted the CXB ability combined with TMZ at a high concentration (250 μM) to reverse chemoresistance of TMZ-resistant GBM cell lines, LN229 and LN18, affecting cell proliferation and inducing apoptosis and autophagy by the inhibition of the mitochondrial metabolism and respiratory chain (10). Also, CXB has been studied in several clinical trials in combination with other drugs such as TMZ (11, 12). In general, the results from these *in vivo* studies support the potentially effective use of low-dose metronomic CXB combined with TMZ in treating GBM patients not eligible for standard treatment (13–15).

In this study, the first aim was to analyze the effect of TMZ, at clinically relevant concentrations (5-20 μM), on T98G ability to release EV when daily exposed to long-term treatment (5 days), a condition conceived to mimic the clinical and therapeutical setting, during which intratumoral TMZ concentrations between 1 and 35 μM are achieved (16). Thus, to improve the transferability of *in vitro* results to *in vivo* studies, we initially evaluated whether TMZ at clinically relevant concentrations could induce a cytotoxic effect and a significant increase of COX-2 level similar to what we previously registered with TMZ at higher concentrations in T98G cells, chosen as TMZ-resistant/COX-2 positive cell line (8). The U251MG cells were used as a negative control, being TMZ-sensitive andCOX-2 null. Also, we preliminarily verified whether COX-2 inhibition could counteract the resistance of T98G cells exposed to TMZ. COX-2 levels were evaluated in EV secreted by T98G and U87MG cells after the scheduled treatment program with drugs alone or in combination. COX-2 over-expression or exogenous PGE2 had been reported to promote the macrophage polarization to M2 phenotype in breast cancer (17). Also, the COX-2 inhibition caused the loss of M2 features and suppressed the tumor metastasis (18). Based on these findings, we have investigated the effects of EV released by T98G cells after treatments on the phenotype of macrophages used as recipient cells. Moreover, the ability of the EV released by TMZ-treated T98G cells to affect TMZ-sensitivity was evaluated in U251MG recipient cells.

MATERIALS AND METHODS

Cell Culture and Treatments

Human GBM cell lines T98G and U87MG were acquired from the European Collection of Authenticated Cell Cultures (ECACC) and U251MG cell line was acquired from Cell Lines Service (CLS). Cells were cultured using manufacturer recommendations in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) of fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (complete medium) (EuroClone, West York, UK) at 37°C in 5% CO_2 and 95% humidity and media was totally replaced every 3 days. Human monocyte cell line, U937, widely used in *in vitro* experiments as a human macrophage model (19, 20), was acquired from Cell Lines Service (CLS) and cultured in RPMI-1640 medium (EuroClone, West York, UK) supplemented with 10% (v/v) of FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (complete medium) in a 5% CO_2 humidified atmosphere at 37°C.

T98G was chosen as chemoresistant cell line for TMZ, displaying a LC50 ranging from >250 mM to 1585 mM, U87MG as chemosensitive cell line for TMZ, showing a LC50 ranged into 7 μM to 172 μM and U251MG cells as chemosensitive cell line, showing a LC50 around 50 μM (21).

From the temozolomide (TMZ, Sigma-Aldrich, Saint Louis, MO, USA) stock solution (51.5 mM in dimethyl sulfoxide, DMSO, which had no significant effect on treated cells), the concentrations of 5, 10, and 20 μM were daily added to cells, initially plated at 3.5×10^3 cells/cm^2, for 5 days. Based on previous reports (22), the Celecoxib (CXB) concentration of 8 μM was used (Sigma-Aldrich, Saint Louis, MO, USA). NS398 (N-[2-(Cyclohexyloxy)-4-nitrophenyl] methanesulfonamide) (23) (Sigma-Aldrich, Saint Louis, MO, USA), was stored as stock solutions in DMSO at −20°C according to the manufacturer instruction and diluted in cell culture medium just before use at the low concentration of 20 μM for the indicated times. In order to assess the effect of TMZ alone or in combination with CXB or NS398 on GBM cells and on the GBM-EV content, the cells were plated for all experiments in 25 cm^2 plates at 3.5×10^3 cells/cm^2, left to adhere and then daily treated or not (CNTR) with TMZ, CXB or NS398, at selected concentrations alone or in combination. The treatment with the DMSO alone (vehicle) was referred throughout the manuscript as "control" (CNTR).

To mimic the clinical condition as much as possible, the exposure to TMZ and the combined treatment schedule with CXB or NS398, were repeated daily for 5 days (referred as a "long-term exposure") (24). To analyze cell viability following treatments, the 0.04% Trypan blue (EuroClone, West York, UK) solution was used. The cells were transferred to a Bürker counting chamber and then counted by microscopy (Eclipse 50i, Nikon Corporation, Tokyo, Japan). The GBM cell lines morphology was visualized and imaged by Nikon Eclipse TS100. Where not otherwise specified, the reagents and consumables were purchased from EuroClone (EuroClone, West York, UK). All cell lines were routinely tested for mycoplasma and were negative prior to use.

Extracellular Vesicle Isolation

To isolate EV, the cells were cultured in complete medium, replacing the FCS with Hyclone 40 nm filtered serum (Thermo Scientific, Rockford, IL, USA) and supernatants collected after treatments were centrifuged at 600×g for 15 min and then at 1500×g for 30 min at 4°C to remove cells and large debris, respectively. The resulting supernatants were then centrifuged at 100,000×g for 90 min at 4°C in an Optima XPN-110 Ultracentrifuge Rotor 70Ti, Quick-Seal Ultra-Clear tubes, k_{adj} 197, brake 9 (Beckman Coulter, CA, USA). Isolated EV were resuspended in Dulbecco's phosphate-buffered saline (PBS) (EuroClone, West York, UK) according to proper dilutions, and the determination of their quantification was carried out by measuring the vesicle-associated protein levels using DC Protein Assay (Bio-Rad, Hercules, CA, USA) using BSA as standard.

Transmission Electron Microscopy

Transmission electron microscopy (TEM) was performed on EV isolated as described above. To this aim, after collection, EV, resuspended and diluted in PBS according to proper dilutions, were adsorbed onto 300-mesh carbon-coated copper grids (Electron Microscopy Sciences, Hatfield, PA, USA), fixed in 2% glutaraldehyde (Electron Microscopy Sciences) in PBS for 10 min, rinsed in Milli-Q water, and negative stained with 2% phosphotungstic acid. Grids were examined with a Philips CM 100 transmission electron microscope TEM (Philips, Eindhoven, Netherlands).

NanoSight

EV number and size were assessed by the nanoparticle tracking analysis (NTA). Using a NanoSight NS300 (NanoSight Ltd., Amesbury, UK), EV were visualized by laser light scattering. Briefly, EV-enriched pellets were resuspended in sterile PBS to generate a proper dilution and five recordings of 60 sec were performed for each sample; 1498 frames in total were examined, captured, and analyzed by applying optimized settings. Data were analyzed with the NTA software, which provided the concentration measurements (particles/ml) and size distribution profiles for the EV in solution.

Protein Extraction and Western Blotting Assay

GBM cell pellets were homogenized and lysed in ice-cold RIPA buffer (phosphate buffer saline pH 7.4) (Merck KGaA, Darmstadt, Germany) supplemented with 100 mM protease inhibitor cocktail (Sigma-Aldrich, Saint Louis, MO, USA). Protein lysates (25 μg/lane) were separated on 10% SDS–polyacrylamide gel under reducing conditions with β-mercaptoethanol 5% and electroblotted onto 0.45 μm nitrocellulose membrane sheets (Whatman-GE Healthcare Life Sciences, UK). To remove non-specific binding sites, membranes were incubated with 5% non-fat dry milk in Tris buffered saline for 1 h at room temperature and then incubated overnight at 4°C with primary antibodies: rabbit monoclonal anti-COX-2 (Cell Signaling Technology, Danvers, MA, USA; dilution 1:1000), mouse monoclonal antibody anti-MGMT (BD Biosciences, San José, CA, USA; dilution 1:500), rabbit polyclonal anti-β-catenin antibody (Cell Signaling Technology, Danvers, MA, USA; dilution 1:1000), and mouse monoclonal antibody for anti-β-actin (Bio-Rad, Hercules, CA, USA; dilution 1:1000). As secondary antibodies, peroxidase conjugated anti-rabbit and anti-mouse IgG antibodies (dilution 1:2000) were acquired from Sigma-Aldrich (Saint Louis, MO, USA).

For Western blotting on EV lysate proteins (10 μg/lane) were resolved on 10% SDS–polyacrylamide gel electrophoresis under non-reducing conditions with heating (CD63, TSG101) or without heating (CANX) and blotted to nitrocellulose membranes (Whatman-GE Healthcare Life Sciences, UK); blocking was performed for 90 min in 10% non-fat dry milk in TBS containing 0.5% Tween-20 (TBS-T) at room temperature. The blots were then incubated at 4°C overnight with primary antibodies diluted in TBS-T containing 1% non-fat dry milk: rabbit polyclonal anti-CANX antibody (Immunological Sciences, Italy; dilution 1:1000), mouse monoclonal anti-CD63 (Santa Cruz Biotechnology Inc, Dallas, TX, USA; dilution 1:400), rabbit polyclonal anti-TSG101 (Immunological Sciences, Italy; dilution 1:2000). The membranes were washed in TBS-T and incubated for 1 h at room temperature in a peroxidase-

conjugated secondary antibody diluted in TBS-T containing 1% non-fat dry milk (goat anti-mouse IgG-HRP, 1:10,000 dilution; goat anti-rabbit IgG-HRP, 1:7500 dilution; Santa Cruz Biotechnology, Inc.).

Chemiluminescent detection was performed using the ECL (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Emission was captured using the chemiluminescence documentation system ALLIANCE (UVITEC, Cambridge UK). For EV proteins, the relative protein levels were calculated based on GAPDH (OriGene, Rockville, MA, USA; dilution 1:1000) as the loading control.

Prostaglandin E_2 (PGE_2) Level Assay

The levels of secreted PGE_2 were measured in supernatants of GBM cells daily exposed or not (CNTR) for 5 days to TMZ, CXB, or NS398, as described above. The supernatants were then assayed for prostaglandin E_2 (PGE_2) levels by an enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical Company, Ann Arbor, MI, USA). Results are presented as fold increase of released PGE_2 vs. CNTR.

Evaluation of Growth of U251MG Recipient Cells

The U251MG cells were seeded at $4x10^3/cm^2$ and, once attached, were exposed to EV (30 μg/ml) derived from TMZ-treated T98G cells or untreated for 18 h to allow the internalization. Then, TMZ (10 μM) was added or not at culture media for 5 days, and cell growth was measured by Trypan blue staining as above described.

Macrophage Polarization

The differentiation of U937, an oncogenic human monocyte cell line, into cells possessing a macrophage-like phenotype (M0) was achieved by exposure to 100 ng/ml of Phorbol-12-Myristate-13-Acetate (PMA; Sigma-Aldrich, Saint Louis, MO, USA) as previously reported (20, 25). After 48 h, the PMA-treated monocytes, referred to as "macrophage-like", undergo a series of morphological and functional changes becoming adherent. The U937 were cultured in RPMI 1640 medium supplemented with 10% FBS, at a density of 1×10^4 cells/ml in 6-well culture plates. Macrophage M2 polarization was obtained by incubation with 20 ng/ml of interleukin 4 (IL-4) (Peprotech, Rocky Hill, NJ, USA) and 20 ng/ml of interleukin 13 (IL-13) (Peprotech, Rocky Hill, NJ, USA) for additional 72 h.

Extracellular Vesicles Labeling and Uptake of PKH26-Labeled T98G-EV by U937 Cells

To verify the uptake and target cell interaction of EV derived from T98G previously treated with CXB (8 μM), NS398 (20 μM), TMZ (10 μM) alone and in combination by U937 macrophagic cells, the fluorescent lipid membrane dye molecule PKH26 (PKH26 Red Fluorescent Cell Linker kit - Sigma-Aldrich, Saint Louis, MO, USA) staining was assessed according to manufacturer's instructions. Briefly, the U937 cell line was grown on coverslips in a 12-well plate (seeded at 5×10^4 cells/coverslips) and, once attached, was incubated in the presence or absence (CNTR) with EV derived from T98G treated as previously reported. For the PKH26 staining, the obtained EV were resuspended in 1 ml Diluent C. Then, 6 μL of PKH26 were added to each sample in sterile conditions. The EV suspension was mixed for 30 s with the stain solution and incubated for 5 min at room temperature. The labelling reaction was stopped by adding 2 ml of 10% BSA in sterile PBS. Labeled EV were ultracentrifuged as previously described. Negative technical control was made by adding the same volume of diluent C and PKH2 as samples. Afterward, U937 cells were incubated for 18 h at 37°C in a 95% air 5% CO_2 atmosphere, with 30 μg PKH26-labeled EV derived from T98G previously treated with COX-2 inhibitors and TMZ alone and in their combinations. The coverslips were mounted with Vectashield® Antifade Mounting Medium with DAPI (Vector Laboratories, Inc., Burlingame, CA, USA), and the effective EV internalization was observed by fluorescent microscopy (Nikon, Eclipse 50i, Tokyo, Japan). All images were acquired at 100×magnification.

EV-COX-2 Immunofluorescence Staining

The U937 and the U251MG cells, both plated at 5×10^4 cells/coverslips, were differently treated as above reported. For labeling, the coverslips were washed, fixed with 4% formaldehyde for 20 min, permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, Saint Louis, MO, USA) for 5 min, and blocked with 3% BSA (Sigma-Aldrich, Saint Louis, MO, USA) for 20 min at room temperature. Cells were incubated overnight at 4°C with rabbit monoclonal anti-human COX-2 (Cell Signaling Technology, Danvers, MA, USA; dilution 1:400), and afterward with a FITC conjugated goat anti-rabbit polyclonal IgG secondary antibody (Millipore EMD, Darmstadt, Germany; dilution 1:1000) for 1 h at room temperature and washed. Coverslips were mounted with VECTASHIELD® Antifade Mounting mounted Medium with DAPI (Vector Laboratories, Inc., Burlingame, CA, USA) and visualized as previously described.

TGF-β1 ELISA

The levels of released TGF-β1 were quantified in the U937 cell supernatants using a human TGF-β1 enzyme-linked immunosorbent assay (ELISA) kit (Sigma Aldrich, Saint Louis, MO, USA), as described in the manufacturer's instructions. Briefly, the U937 cells were plated at 1×10^5 cells/ml and treated with EV derived from T98G previously daily exposed for 5 days with CXB, NS398, TMZ as single agent or in combination. The EV treatment lasted 72 h, then the media were collected, cleared of cellular debris/dead cells by centrifugation at 1000× g for 15 min, and the TGF-β1 concentration was then determined in the medium using the ELISA kit. Results are expressed as pg/ml.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 6.01, (GraphPad Software, San Diego, CA, USA). The data were evaluated using the one-way ANOVA test followed by Tukey or Dunnett's *post hoc* test, where specified. Testing for synergistic or additive effects of combination therapy was performed according to Bliss independence analysis (26).

Experiments were independently repeated three times at least and performed in duplicate or triplicate, and the results were shown as the means ± SEM (standard error mean). P-value <0.05 was considered to indicate a statistically significant difference.

RESULTS

Effect of TMZ Alone or Combined With COXIB on GBM Cell Lines

TMZ affects the viability and proliferation of GBM cell lines when used at high concentrations (27, 28) while away from clinical practice. Thus, we firstly investigated the effect of a 5-day treatment with low clinically relevant concentrations of TMZ (5–20 μM) on the cell growth rate of T98G and U87MG. As expected, none of the TMZ tested concentrations significantly influenced the T98G cell number and no dead cell was detected at 5 days, confirming their high resistance (**Figure 1A**). The microscopy observation also supported these data. T98G cells exposed to low TMZ concentrations showed a cell density similar to control (CNTR) (**Supplementary Figure 1A**). We then evaluated the ability of TMZ to upregulate COX-2 in T98G cells treated as above described, through Western blotting (**Figure 1B**). TMZ incubation at 5 μM did not significantly affect the COX-2 expression compared to the CNTR sample, while the highest TMZ concentrations (10 and 20 μM) markedly influenced it. The levels of β-catenin and MGMT, two proteins strictly associated with COX-2 activity and strongly implicated in the GBM chemoresistance (29, 30), were also evaluated. The results showed that the expression of the β-catenin proportionally increased to TMZ concentrations being significant at 10 μM and 20 μM (**Figure 1C**). MGMT expression showed a similar trend (**Figure 1D**). Conversely, in the U87MG cell line, the daily treatment with TMZ at all concentrations reduced the cell proliferation rate evaluated at 5 days (**Figure 2A**), as confirmed by images taken by contrast-phase microscope (**Supplementary Figure 1B**). The levels of COX-2 and β-catenin were dose-dependently upregulated by TMZ exposure (**Figures 2B, C**). TMZ treatment did not induce MGMT expression in the U87MG cell line (MGMT negative) (**Figure 2D**). In line with our previous results (8), TMZ did not induce COX-2 expression, at all tested concentrations, in the TMZ-sensitive/COX-2 negative U251MG cells (31) (**Supplementary Figure 2A**), nor modulate β-catenin or induce the expression of MGMT (**Supplementary Figures 2B, C**, respectively).

To further elucidate the role of COX-2 in TMZ resistance, GBM cell lines were exposed to COXIB, alone or in combination with TMZ, after which the COX-2 expression and activity, as well as cell number, were evaluated. GBM cell lines were treated daily with CXB (8 μM), NS398 (20 μM), or TMZ (10 μM) alone or with their combinations (CXB+TMZ or NS398+TMZ) for 5 days. Representative Western blot images and the results from densitometric analysis of COX-2 levels in T98G cells are shown in **Figure 1E**. COXIB, used alone, did not influence the levels of COX-2. On the other hand, a significant increase of COX-2 was detected after treatment with 10 μM TMZ compared to CNTR. Of note, CXB or NS398 combined with TMZ significantly downregulated the TMZ-induced COX-2 (**Figure 1E**). PGE_2 levels were directly measured in supernatants of cell cultures to evaluate the COX-2 activity and verify the specificity of COXIB. TMZ-treated cells released high amounts of PGE_2, in line with the COX-2 upregulation. Both the drug combinations significantly reduced the COX-2 activity compared to TMZ alone (**Figure 1F**). A similar COX-2 expression and activity trend was observed in U87MG cells in which the TMZ-induced COX-2 upregulation was counteracted by drug combination treatments (**Figures 2E, F**). The protein was not expressed in the COX-2 null U251MG (**Supplementary Figure 2D**). Accordingly, PGE2 was undetectable in the U251MG culture media (not shown).

Next, we evaluated the effect of CXB, NS398, TMZ, and their combination on GBM cell number. After 5 days of continuous, scheduled treatment with COXIB, the T98G and U87MG cell number was decreased compared to CNTR cells (**Figures 1G**, **2G**). As previously reported, COX-2 inhibitors reduced the growth rate of GBM cell lines (8, 32). The reduced cell growth rate following CXB or NS398 treatment could be due to the inhibition of basal COX-2 activity and COX-2-dependent signaling pathways. Of note, both drug combinations, CXB +TMZ and NS398+TMZ, caused a statistically significant decrease in T98G and U87MG viable cell numbers compared to CNTR, TMZ alone, and relative COXIB alone (**Figures 1G**, **2G**). A Bliss independence test was performed to assess the nature of TMZ and COXIB interaction (synergistic or additive), suggesting that drug combination treatment had a synergistic effect on GBM cells vs. single agents.

Upregulation of COX-2 in EV Derived from GBM Cells Treated with TMZ is Counteracted by COXIB

We then investigated the effect of TMZ on the EV content of COX-2 in the T98G and U87MG cells. Firstly, the EV released from both GBM cell lines were collected and characterized according to MISEV (33) by TEM, Western blotting of specific markers, and NTA analyses. The TEM images showed T98G-EV and U87MG-EV with a round-shaped, membrane-enclosed structure (**Figures 3A, B**). Western blotting was performed to evaluate the expression of the specific EV markers, CD63 and TSG101, and to verify the absence of endoplasmic reticulum marker calnexin, indicating the EV purity without contamination of cell debris and organelles (**Figures 3C, D**). Isolated EV were then analyzed by NTA to determine the particle number and size distribution, displaying that most EV had a diameter less than 200 nm (*small vesicles*) (**Figures 3E, F**, respectively) (33). Next, T98G- and U87MG-derived EV were evaluated for the presence of COX-2 protein by Western blotting. The results evidenced that COX-2 protein was shuttled in EV of both cell lines, with levels dependent on TMZ concentrations, resulting significantly higher at 10 and 20 μM in T98G and at all tested concentrations in U87MG (**Figure 4A**). This trend was in line with COX-2 upregulation induced by TMZ in T98G (**Figure 1B**) and U87MG cells (**Figure 2B**).

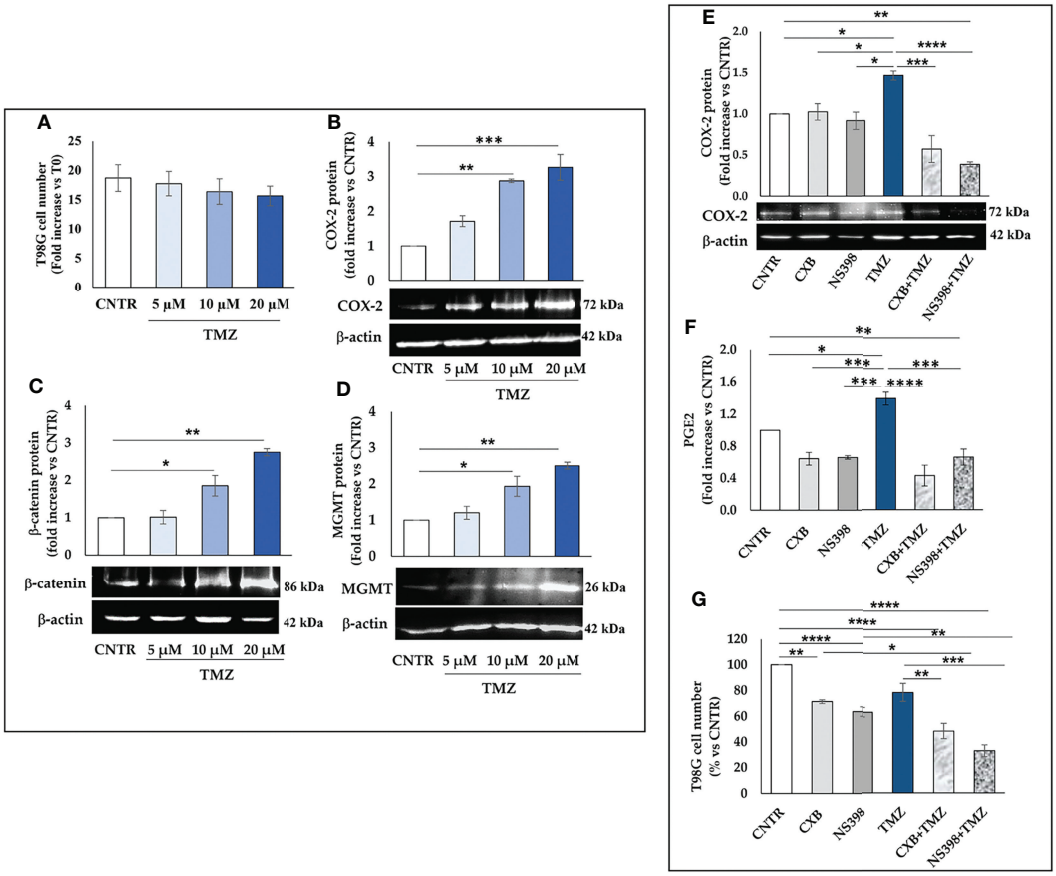

FIGURE 1 | Influence of TMZ on T98G TMZ-resistant cells. Cells were daily incubated with increasing doses of TMZ (5-20 μM) for 5 days and **(A)** cell number was detected by Trypan blue staining. All values are given as fold increase vs. initial time (T0). The results, derived from three experiments performed in duplicate, are expressed as mean ± SEM. The one-way analysis of variance (ANOVA) followed by Dunnet *post hoc* test show not significant differences. Influence of TMZ on **(B)** COX-2, **(C)** β-catenin and **(D)** MGMT levels was assessed by Western blotting in the presence of vehicle (CNTR), or TMZ, as previously described. The obtained values were normalized vs. β-actin and presented as fold increase vs. CNTR. Data are from three independent experiments and values are expressed as mean ± SEM. For comparative analysis of data, a one-way ANOVA with Dunnet *post hoc* test was used (*P<0.05; **P<0.01; *** P<0.001 vs. CNTR). Representative images of each immunoblotting are shown. **(E)** Influence of the COXIB combined with TMZ on COX-2 levels was verified by Western blotting assay in T98G cells daily incubated or not (CNTR) with Celecoxib (CXB) (8 μM), NS398 (20 μM), TMZ (10 μM) or with the co-treatments (CXB+TMZ and NS398+TMZ) for 5 days. Densitometric analysis was performed by normalizing vs. β-actin and presented as fold increase vs. CNTR. Data from three independent experiments are expressed as mean ± SEM. Representative images of each immunoblotting are shown. C+ = positive control (not treated T98G cells). **(F)** PGE2 levels (fold increase vs. CNTR) released by T98G treated as above described, were assayed by ELISA kit. Results are expressed as mean ± SEM of three experiments in duplicates. **(G)** Effect of COXIB, CXB and NS398, TMZ and drug combinations on T98G cell number was evaluated by Trypan blue staining. All values are given as fold increase vs. CNTR of two independent experiments performed in triplicate (mean ± SEM). For comparative analysis of data groups, a one-way ANOVA with Tukey *post hoc* test was used (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001).

Next, we evaluated whether COX-2 levels in EV released from T98G and U87MG exposed to low concentration of TMZ could be modulated by the concurrent treatment with CXB or NS398. The Western blot results of both cell lines showed a similar trend: the daily exposure to CXB and NS398 alone did not significantly modulate the content of COX-2 when compared to EV derived from CNTR (**Figure 4B**). On the other hand, an evident reduction of COX-2 in EV derived from T98G and U87MG treated with both drug combinations (CXB+TMZ and NS398+TMZ) was observed, suggesting that COXIB counteracted the increase of EV-COX-2 content induced by TMZ (**Figure 4B**).

Intracellular Uptake of EV-COX-2 Protein in Recipient Human Macrophage Cell Line

It is also known that EV can mediate the communication in TME promoting tumor escape (34). Herein, we investigated the hypothesis that EV from TMZ-resistant T98G treated with COXIB- and TMZ, alone or in combination, could directly influence macrophage polarization. The internalization of T98G-EV by the U937 macrophage cell line was verified by PKH26 red fluorescent staining. Non-polarized U937 macrophages (M0) were incubated for 18 h with 30 μg/ml of PKH26-labeled EV derived from T98G exposed to COXIB, TMZ, and their mixture, as previously stated. U937 cells were

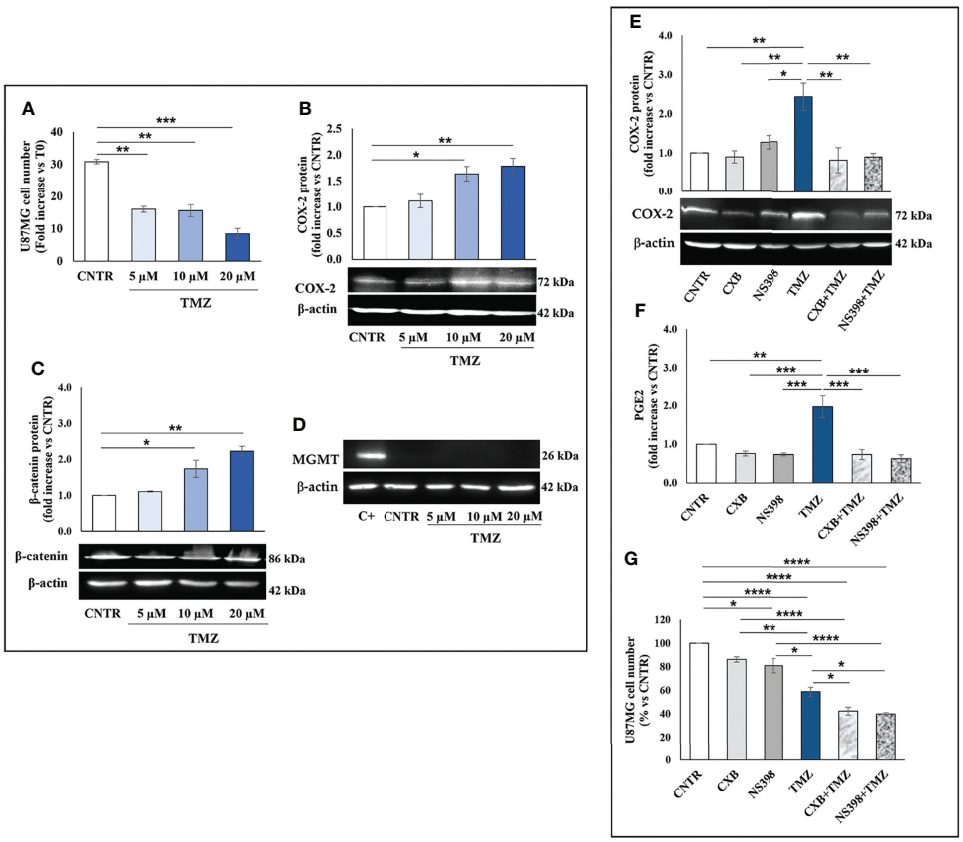

FIGURE 2 | Influence of TMZ on U87MG TMZ-sensitive cells. Cells were daily incubated with increasing doses of TMZ (5-20 μM) for 5 days and **(A)** cell number was detected by Trypan blue staining. All values are given as fold increase vs. initial time (T0). The results, derived from three experiments performed in duplicate, are expressed as mean ± SEM. The one-way analysis of variance (ANOVA) followed by Dunnet *post hoc* test show not significant differences. Effect of TMZ on **(B)** COX-2, **(C)** β-catenin and **(D)** MGMT levels was assessed by Western blotting in the presence of vehicle (CNTR), or TMZ, as previously described. The obtained values were normalized vs. β-actin and presented as fold increase vs. CNTR. Data are from three independent experiments and values are expressed as mean ± SEM. For comparative analysis of data, a one-way ANOVA with Dunnet *post hoc* test was used (*P<0.05; **P<0.01; *** P<0.001 vs. CNTR). Representative images of each immunoblotting are shown. **(E)** Influence of the COXIB combined with TMZ on COX-2 levels was verified by Western blotting assay in U87MG cells daily incubated or not (CNTR) with Celecoxib (CXB) (8 μM), NS398 (20 μM), TMZ (10 μM) or with the co-treatments (CXB+TMZ and NS398+TMZ) for 5 days. Densitometric analysis was performed by normalizing vs. β-actin and presented as fold increase vs. CNTR. Data from three independent experiments are expressed as mean ± SEM. Representative images of each immunoblotting are shown. C+ = positive control (not treated T98G). **(F)** PGE2 levels (fold increase vs. CNTR) released by U87MG treated as above described, were assayed by ELISA kit. Results are expressed as mean ± SEM of three experiments in duplicates. **(G)** Effect of COXIB, CXB and NS398, TMZ and drug combinations on U87MG cell number was evaluated by Trypan blue staining. All values are given as fold increase vs. CNTR of two independent experiments performed in triplicate (mean ± SEM). For comparative analysis of data groups, a one-way ANOVA with Tukey *post hoc* test was used (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001).

permissive to PKH26-labeled EV entry. When PKH26-labeled EV were incubated with human U937 cells, we observed an effective uptake into the cytoplasm of all recipient cells, as indicated by red fluorescence (**Figure 5**).

Therefore, COX-2 protein levels were evaluated in recipient U937 cells (M0) following exposure to EV derived from T98G treated as above described. Fluorescence images revealed the presence of COX-2 protein in macrophage (M0) appearing diffuse throughout the cytoplasm (green fluorescence). Macrophages M0 (COX-2 negative) not exposed to T98G-EV were used as a negative control. A significant COX-2 presence in U937 exposed to EV from TMZ-T98G cells was observed. Of interest, a lower COX-2 content was detected in U937 incubated with EV-CXB+TMZ and EV-NS398+TMZ compared to EV-TMZ (**Figure 6A**). The COX-2 quantification by Western blot analysis showed a significant increase in COX-2 level when U937 were exposed to EV-TMZ (**Figure 6B**). On the other hand, COX-2 levels in U937 exposed to EV-COXIB+TMZ were comparable to those of U937 exposed to EV-CNTR (**Figure 6B**).

Effect of EV from T98G Treated With COXIB in Combination With TMZ on Macrophages

GBM is sustained by a complex and highly immunosuppressive TME also responsible for therapy resistance. In this context, we finally investigated whether the EV secreted by T98G treated

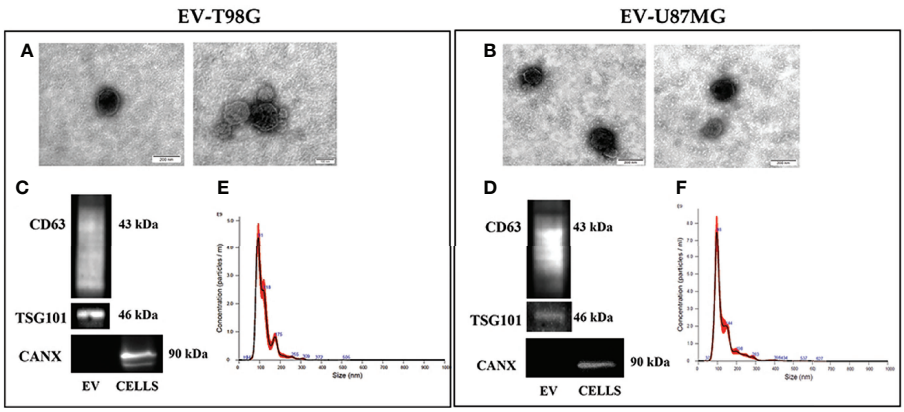

FIGURE 3 | Characterization of EV population secreted from GBM cell lines, T98G and U87MG. **(A)** and **(B)** Representative electron microscopy images showing whole and round-shaped EV derived from T98G and U87MG, respectively. **(C)** and **(D)** Western Blot analysis of specific markers CD63, TSG101 and Calnexin on EV isolated from GBM cells. **(E)** and **(F)** Representative Nanoparticle Tracking Analysis (NTA) profiles of T98G-EV and U87MG-EV. Each curve was generated from five measurements.

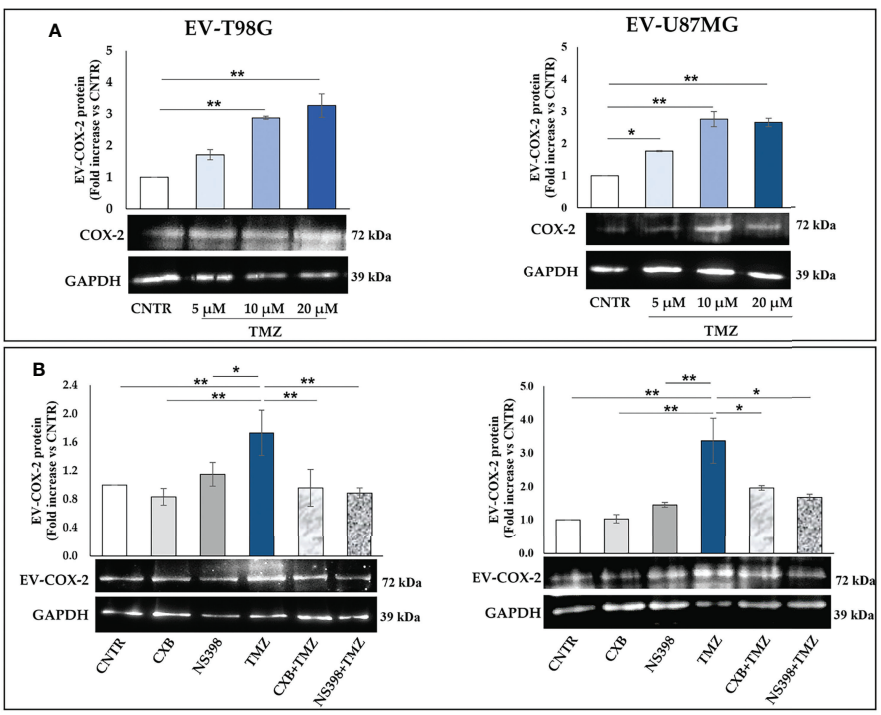

FIGURE 4 | **(A)** COX-2 protein is transferred into EV derived by T98G and U87MG treated with TMZ in a dose-dependent way. GBM cells were daily exposed to increasing concentrations of TMZ (5–20 μM) for 5 days, EV were collected from cell supernatants and COX-2 levels were analysed by immunoblotting assay. Densitometric bands were normalized vs. GAPDH. Data from three independent experiments are shown as the mean ± SEM and expressed as fold increase vs. CNTR. Images from one representative out of three independent experiments are presented. For comparative analysis of groups of data, one-way ANOVA followed by Dunnett's post hoc test was used (*P<0.05; **P<0.01 vs. CNTR). **(B)** COXIB, CXB or NS398, alone and in combination with TMZ, modulate the cargo of EV released from GBM cells. T98G and U87MG cell lines were daily exposed for 5 days to CXB (8 μM), NS398 (20 μM), TMZ (10 μM), alone or in combination and representative COX-2 and GAPDH immunoblots are shown. Following densitometric analysis, obtained values were normalized vs. GAPDH. Data are from three independent experiments, and values (mean ± SEM) are expressed as fold increase vs. CNTR. For comparative analysis of data, a one-way ANOVA with Tukey post hoc test was used (*P<0.05; **P<0.01).

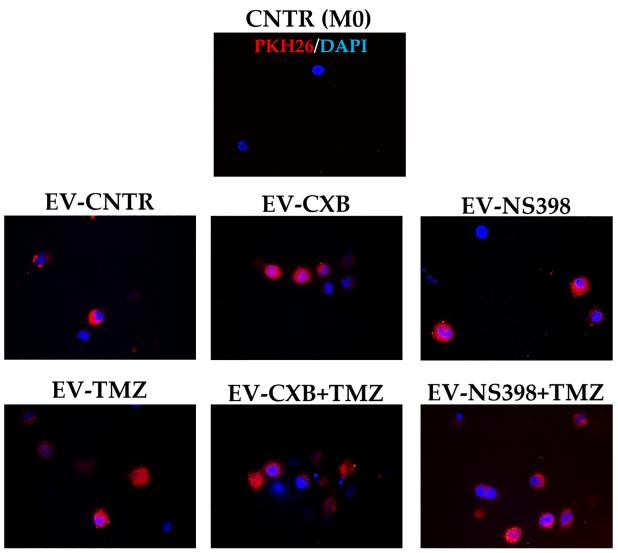

FIGURE 5 | Macrophage U937 intracellular uptake of EV derived from control (CNTR) and CXB-, NS398-, TMZ-, drugs combination-treated T98G assessed by PKH26 staining. PKH26-labeled EV-COX-2 (red) were incubated at 30 μg/ml with U937 cells for 18 h. The nuclei of U937 were counterstained with DAPI (blue). CNTR (M0) = U937 cells not exposed to EV. Representative immunofluorescence images are from one out of three independent experiments. All images were acquired at 100× magnification.

with COXIB, TMZ, and respective combinations, containing different levels of COX-2, were able to induce a phenotype modulation in the recipient U937 cells. To verify the macrophage M2 polarization after T98G-EV internalization, TGF-β1 released in the culture medium was assayed. M2 polarized macrophages, obtained as described in the materials and methods section, were used as the positive control. The extracellular levels of TGF-β1 released by U937 cells significantly increased after exposure to EV from TMZ-treated T98G (EV-TMZ) when compared to EV from CNTR ($P<0.05$) (**Figure 7**). No effect was observed when U937 cells were exposed to EV derived from T98G treated with single COXIB. In contrast, the TGF-β1 level was significantly lower in the supernatants of U937 treated with EV-COXIB+TMZ than EV-TMZ (**Figure 7**).

Effect of EV Released from TMZ-Treated T98G Cells on U251MG

To further explore the role of EV-COX-2 in the TMZ resistance, we examined the cell growth of U251MG recipient cells, exposed to EV secreted by T98G treated or not with TMZ (10 μM). The EV internalization in U251MG target cells was allowed by incubation for 18 h. Representative images of immunofluorescence staining of U251MG cells treated with EV secreted from T98G cells showed the COX-2 delivery in the recipient cells, as detectable by green fluorescence (**Figure 8A**). Of note, in U251MG exposed to EV from TMZ-T98G, the fluorescence appeared more intense than relative CNTR according to TMZ's ability to upregulate shuttled-COX-2 levels. No green fluorescence was detected in U251MG treated with or without TMZ.

As expected, TMZ treatment significantly reduced the U251MG cell growth versus CNTR ($P<0.01$) (**Figure 8B**). The uptake of EV from T98G-CNTR did not influence the cell number with respect to U251MG CNTR. Differently, the

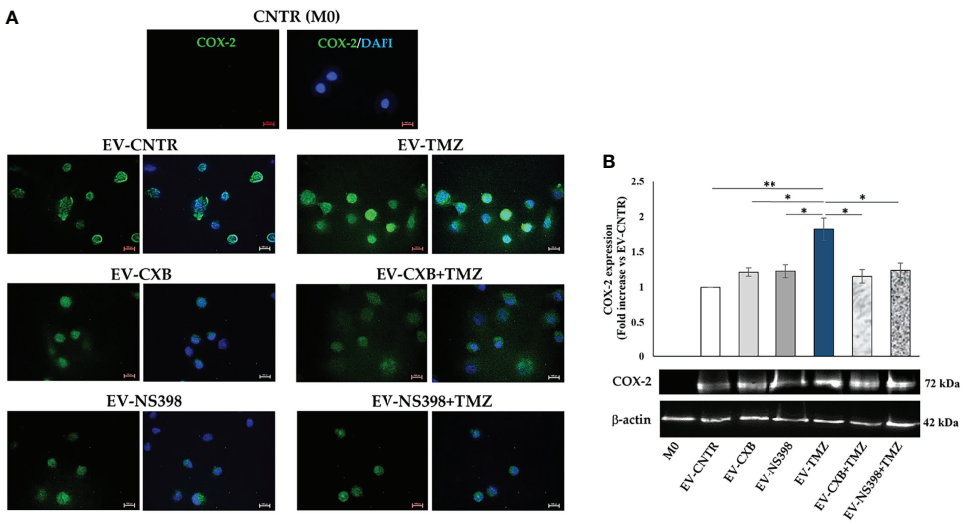

FIGURE 6 | COX-2 levels in human macrophage cells after T98G-EV internalization. U937 macrophage cells (M0) were incubated with 30 μg/ml of EV from T98G daily treated in the absence (CNTR) or presence of COX-2 inhibitors CXB (8 μM), NS398 (20 μM), TMZ (10 μM) and their combinations for 5 days. **(A)** Representative immunofluorescence images from one out of two independent experiments showed the COX-2 (green) transferred by T98G-EV in U937 cells. Nuclei were counterstained with DAPI (blue). No green fluorescent signal was detected in the CNTR (M0) sample (U937 cells not exposed to EV). All images were acquired at 100× magnification (Scale bar = 10 μm). **(B)** COX-2 levels were analyzed by immunoblotting assay. Densitometric analysis was performed by normalizing vs. β-actin and expressed as fold increase vs. EV-CNTR. Data are from two independent experiments (mean ± SEM). For comparative analysis of data, a one-way ANOVA with *post hoc* Tukey test was used (*$P<0.05$, **$P<0.01$).

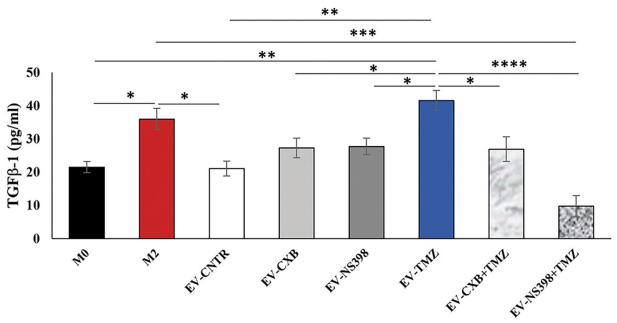

FIGURE 7 | Effect of EV from treated-T98G on U937 macrophage cell line. The macrophage U937 cells (M0) were incubated with EV derived from T98G treated in the absence (CNTR) or presence of COXIB, CXB (8 μM), NS398 (20 μM), TMZ (10 μM) and their combinations for 5 days. Levels of TGF-β1, M2-related marker, were analyzed by ELISA kit in the cell supernatants of U937 exposed to EV from T98G. Macrophage M2 polarization was obtained by incubation with 20 ng/ml of interleukin 4 (IL-4) and 20 ng/ml of interleukin 13 (IL-13) for 72 h. The results relative to three experiments in duplicate are expressed as mean ± SEM. For comparative analysis of data, a one-way ANOVA with Tukey *post hoc* test was used (*P<0.05; **P<0.01; ***P<0.001).

internalization of EV T98G-TMZ significantly increased the U251MG growth rate when compared to CNTR (P<0.05) and TMZ (P<0.001) (**Figure 8B**). Of note, when the U251MG were exposed to TMZ for an additional 72 h, the cell growth did not decrease in cells that have previously internalized the EV derived from CNTR and TMZ-treated T98G, showing a similar trend to those without TMZ treatment. Therefore, the EV derived T98G were able to strongly hinder the chemosensitivity. Representative images from microscopic observations confirmed these results (**Figure 8B**).

DISCUSSION

In this work, we show evidence that low and clinically relevant TMZ concentrations, able to upmodulate COX-2 in T98G and U87MG cells, led to a dose-dependent increase of the COX-2 levels in secreted EV. It is fair to point out that the results obtained with repeated exposure to TMZ for 5 consecutive days at clinically relevant concentrations, showing the TMZ-induced COX-2 increase as well as the increased sensitivity to TMZ in the presence of COXIB, have the same trend as those obtained in our previous work (8) where high doses of TMZ for 72 h were used on TMZ-resistant cells. Hence, the similarity between the effects of metronomic low dose application and those of single high dose protocol could slightly mitigate the criticalities raised toward *in vitro* studies in which the use of un-physiological high TMZ concentrations is questioned since it does not faithfully reproduce the clinical situation.

The ability of TMZ to induce an upregulation of COX-2 expression could be ascribed to the action exerted by the drug at the EGF/EGFR pair level leading to the NF-κB transcription factor activation able to upregulate the COX-2 expression due to the presence in the COX-2 promotor of the NF-κB response element (35). The NF-kB/COX-2 signaling is a crucial regulator of the malignant phenotype and chemoresistance in GBM (36). NF-κB signaling pathway has also been reported to influence radiotherapy tolerance of glioma cells through regulating COX-2 expression, with potential therapeutic approaches for the treatment of glioma (37).

The TMZ-induced increase of COX-2 in EV was significantly counteracted by the treatment of the cells with COXIB in combination with TMZ. The EV derived from T98G exposed to drug combination treatment were able to prevent the TGF-β1 release by recipient macrophage cells induced by EV secreted by TMZ-treated T98G, thus neutralizing the M2-polarization.

Experimental studies carried out in recent years on the EV derived from GBM have revealed numerous molecules in their cargo (38); however, to the best of our knowledge, the presence of COX-2 protein in EV derived from GBM cells has never been verified. In 2017, Kim and colleagues (39) reported the transfer of the COX-2 protein by exosomes from COX-2-positive lung cancer cell lines affecting the phenotype of monocytes THP-1, used as recipient cells. The COX-2 uptake by THP-1 determined an increased production of PGE2 and VEGF sustaining tumor growth. In our previous study, the addition of NS398 caused a functional change of EV released by GBM stem cells which, in turn, provoked a decrease in cell migration and autophagy induction in adherent U87MG and T98G, used as recipient cells (40).

The neuroinflammation and the role of inflammatory mediators, such as COX-2, are critical components in establishing an immunosuppressed microenvironment, thus fueling GBM proliferation, invasion, and maintenance of stemness features (41). COXIB enhanced the tumor-associated-macrophage-mediated anti-tumor immune responses by increasing monocyte cytokine production (42). The COX-2 role in macrophage polarization was also analyzed after NS398 treatment of bone marrow-derived macrophages that increased the secreted levels of TNFα and reduced the IL-10 secretion (42). Our present findings show that EV released by TMZ-treated T98G shuttled COX-2 and, after effective internalization by U937 macrophage cells, induced a higher level of TGF-β1, a hallmark of the transition in M2 macrophage state (43). Of interest, the EV-COX-2 made the recipient U251MG, TMZ-sensitive cells, less responsive to TMZ action, suggesting a possible role of EV derived from TMZ-resistant cells in the transfer of chemoresistance through the COX-2 delivering.

Overall, the results suggest that COX-2 shuttled by EV can modulate the fate of cells in the GBM microenvironment making them less sensitive to the alkylating drug action. **Figure 9** shows a graphical representation of the main results.

Although our data represent the first evidence of the presence and effective functional transfer of COX-2 through EV derived from GBM cells, further and more comprehensive studies are needed on the multiple consequences at the level of TME of the presence of COX-2 in EV. Likewise, it will be essential to deepen COXIB's effects on tumor cells and the TME. Although many questions remain unanswered regarding the precise molecular and cellular mechanisms involved in GBM resistance, strategies

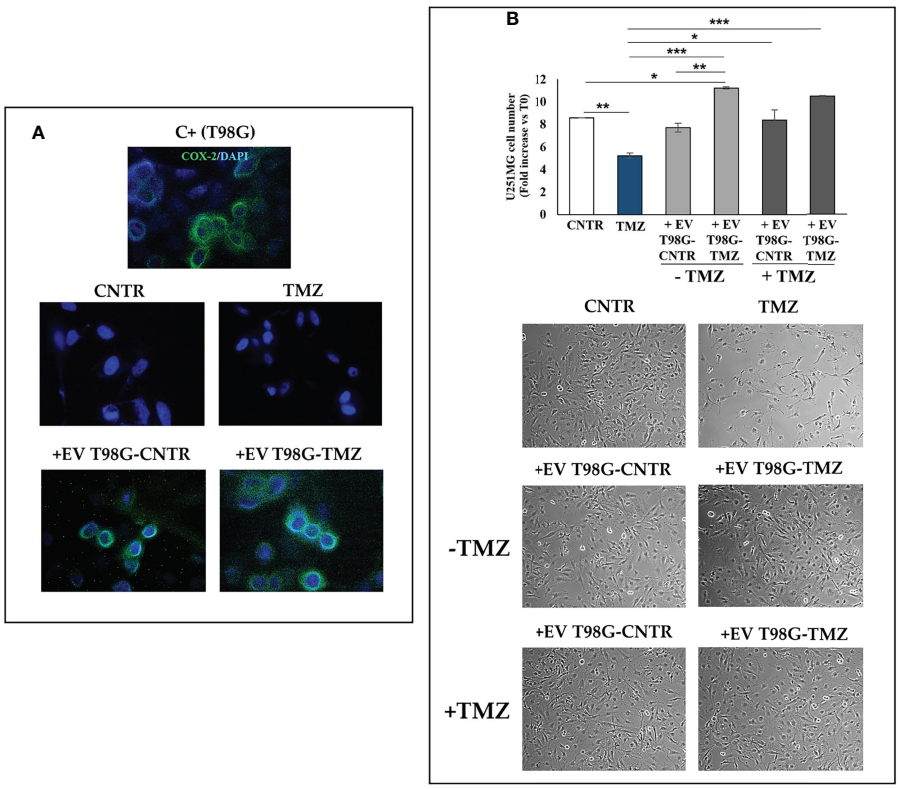

FIGURE 8 | Effect of EV released from TMZ-treated T98G cells on U251MG TMZ-sensitive cells. The U251MG cells were incubated in presence or absence (CNTR) of TMZ (10 μM) or with 30 μg/ml EV derived from T98G treated with or without TMZ (10 μM) for 5 days. **(A)** Representative immunofluorescence images of U251MG cells treated or not as above described and stained with anti-COX-2 antibody (green) from one out of two independent experiments are shown. Nuclei were counterstained with DAPI (blue). T98G cells are used as positive control (C+). All images were acquired at 100× magnification. **(B)** Cell number was recorded by Trypan blue staining and representative images were taken at 10× magnification. All values are given as fold increase vs. initial time (T0). The results, derived from three experiments performed in duplicate, are expressed as mean ± SEM. For comparative analysis of data groups, a one-way ANOVA with Tukey *post hoc* test was used (*P<0.05; **P<0.01; ***P<0.001).

to modulate the TME can offer a new perspective on the clinical approach.

We highlighted a possible scenario to counteract the TMZ-resistance using COXIB to modulate the GBM neuroinflammation and immunosuppression in the TME through EV and enhance the TMZ therapeutic effectiveness. Our data support a combined therapeutic strategy of administering COXIB and TMZ to modulate the content of local EV with the aim of improving the response to chemotherapy in GBM patients.

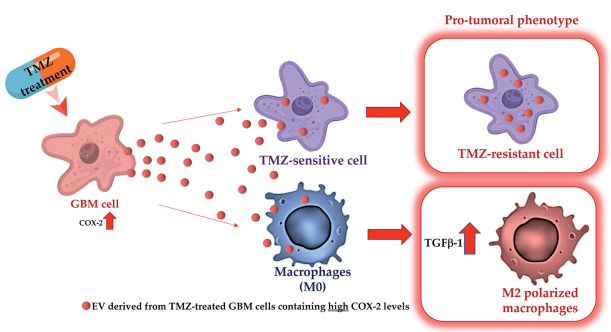

FIGURE 9 | Graphical representation of experimental design and main obtained results.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

MGC, BC, and PP contributed to conception of the study; FL, FRA, SA, and PP performed the experimental work. IG and VD performed the EV isolation and characterization. FL, FRA, IG, PP performed data analyses. PP and FL performed the statistical analysis of data, wrote the original manuscript, and produced all figures. MGC, EA, VD, BC, and PP performed the revision and editing of the manuscript and provided overall guidance for the

experiments. FL, MGC, BC, and PP provided for the founding acquisition. All authors listed contributed to final manuscript revision and agreed to the published version of the 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/fonc.2022.933746/full#supplementary-material

Supplementary Figure 1 | Effect of TMZ on GBM cell viability. Representative phase-contrast images (10× magnification) of **(A)** T98G and **(B)** U87MG in the absence (CNTR) or presence of TMZ at several concentrations for 5 days are shown.

Supplementary Figure 2 | Influence of TMZ on **(A)** COX-2, **(B)** β-catenin and **(C)** MGMT expression in U251MG TMZ-sensitive cell line. Immunoblotting assays were performed on cells incubated for 5 days in the presence or absence (CNTR) of TMZ (5-20 μM). β-actin serves as internal control. The images are representative of three independent experiments. **(D)** Influence of the COXIB combined with TMZ on COX-2 levels was verified by Western blotting assay in U251MG cells daily incubated or not (CNTR) with Celecoxib (CXB) (8 μM), NS398 (20 μM), TMZ (10 μM) or with the co-treatments (CXB+TMZ and NS398+TMZ) for 5 days. Representative images of each immunoblotting are shown. C+ = positive control (not treated T98G).

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The history of small extracellular vesicles and their implication in cancer drug resistance

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Small extracellular vesicles (EVs) in the last 20 years are demonstrated to possess promising properties as potential new drug delivery systems, biomarkers, and therapeutic targets. Moreover, EVs are described to be involved in the most important steps of tumor development and progression including drug resistance. The acquired or intrinsic capacity of cancer cells to resist chemotherapies is one of the greatest obstacles to overcome to improve the prognosis of many patients. EVs are involved in this mechanism by exporting the drugs outside the cells and transferring the drug efflux pumps and miRNAs in recipient cells, in turn inducing drug resistance. In this mini-review, the main mechanisms by which EVs are involved in drug resistance are described, giving a rapid and clear overview of the field to the readers.

KEYWORDS

extra cellular vesicles, cancer, drug resistance, therapy, oncology

Introduction

Extracellular vesicles (EVs) are small cell-released particles with a diameter ranging from 30 to 1,000 nm (1). EVs are a heterogeneous population that can differ in size, properties, and biological function and classified according to their biogenesis pathway (2). In addition, from the first attributing role, consisting in managing cellular waste, nowadays it is well recognized that EVs play a central role in cell–cell communication (3), both in physiological and in pathological conditions, and their cargoes have been distinguished in different components from proteins to miRNA, going through mRNA and lncRNA, among others (4). EVs were also employed as drug delivery systems (DDS) displaying very suitable properties for this purpose and obtaining interesting results in preclinical and clinical trials (2, 5).

The history of EVs started in the second half of the 1940s in the previous century, when in 1945 Chargaff working on blood coagulation observed small "*membrane debris*" sedimented at high-speed centrifugation of plasma supernatant (6). The following year,

his observation was reported as "a variety of minute breakdown products of blood corpuscules" (7). Twenty-one years later, Peter Wolf described in more detail Chargaff's remarks, saying that it could be a material "originated from platelets but it is distinguishable from the intact ones". This claim was confirmed by electron microscopy images that Wolf himself described as "platelet dust" (8). For almost 20 years, other electron microscopy images showed structures with a size under 1,000 nm. In particular, in 1974, Nunez et al. reported, for the first time, structures later called multivesicular bodies (MVB) (9), opening up the path in the identification of a subtype of EVs that originated from MVB, later called exosomes or small EVs (30–150 nm). The biogenesis of these structures was demonstrated to start from late endosomes, which are formed by the inward budding of MVB membranes forming intraluminal vesicles (ILVs), which fuse back with the plasmatic membrane and released by cells as small EVs [later called exosomes (10)] as described by Cliff Harding in 1983 (11). Starting from the early 1980s, many studies on EVs have increased the knowledge in this field and scientists began to deeply understand the multiple biological functions in which EVs are involved. For almost a decade, small EVs were identified as a vehicle to remove unnecessary molecules from cells, like a cellular garbage disposal (12). In the 1990s, small EVs were identified to have an immunological function (13), followed by a large number of studies highlighting that EVs were involved in intercellular communication mechanisms playing a role in physiological or biologically important processes, such as lactation, inflammation, cell proliferation, and neuronal function (14–16). Moreover, other studies showed that EVs are implicated not only in pathological processes, namely, thrombosis (17), diabetes, and atherosclerosis (18), but also in the development and progression of diseases such as liver (19) and neurodegenerative diseases (20) and, recently, in cancer (21, 22). In cancer, many processes like cell proliferation, migration, invasion, epithelial-to-mesenchymal transition, angiogenesis, lymphogenesis, immune suppression, and metastasis (23) are regulated by EVs. In the late 1990s, important studies were published about EVs. Starting with the work of Raposo et al. (13) that demonstrates that EVs derived from immune cells are capable of presenting antigens, other groups started new projects about a new vaccine approach based on EVs. The first approach on vaccines using EVs was explored by Zitvogel et al. in 1998 (24). In their work, the authors described how EVs secreted by dendritic cells loaded with tumor antigens are able to eradicate cancer cells. Based on advances in the next decades, Escudier et al. conducted a clinical trial (25). This work has been a starting point for many studies on the physiological role of EVs and their possible applications as biomarkers, and an opportunity to new therapeutic approaches. In the last few years, lines of evidence for the implication of EVs in the development of anticancer drug resistance have increased and have been extensively studied. This mini-review will focus on the role of EVs in cancer drug resistance exploring and describing the main mechanisms of action through a synthetic description of the major scientific works in the field. Also, a brief description of the most important research papers is provided in Table 1, which aims to give an impression of this field and, overall, to give the readers a rapid and clear overview of the involvement of EVs in drug resistance mechanisms.

The term EVs used in this review, independent of the term used in the article referred to, refers to a mixed population of small EVs ranging from 30 to 200 nm since the available isolation methods are not able to discriminate vesicles originated from different pathways.

EVs mediated drug resistance

The hallmarks of drug resistance are basically summarized in six points: (1) alteration of drug targets (2), activation of drug pumps, (3) detoxification mechanisms, (4) reduced susceptibility to apoptosis, (5) increased ability to repair DNA damage, and (6) altered proliferation. Also, local modifications of stroma, tumor microenvironment (TME), and local immunity could contribute to the development of resistance (66). Keeping in mind these notions, EVs are involved in cell–cell communication and cargo sharing/delivery, and these characteristics have been associated with chemo- and targeted therapies' resistance as detailed here. In the next paragraphs, the most important mechanisms by which EVs regulate drug resistance will be described.

Activation of drug-efflux pumps

Efflux pump mechanisms are physiologically important in many processes such as toxin clearance from the gastrointestinal tract, elimination of bile from the hepatocytes, effective functioning of the blood–brain and placental barrier, and the renal excretion of drugs. In drug-resistant tumors, the overexpression of these proteins (67) allow the cells to reduce the intracellular drug concentration to a sublethal dose. Many research papers described the role of EVs in the transferring of drug efflux pumps from resistant to sensitive cancer cells. Among the delivered proteins are frequently described ATP-binding cassette (ABC) family, like P-glycoprotein (P-gp, MDR1, and ABCB1), breast cancer (BC)-resistant proteins (ABCG2, BRCP, and ABCA3), and multidrug-resistant protein 1 (MRP-1) (45, 46, 68–72). The mechanism by which EVs transfer proteins among cells is commonly called EVs-mediated horizontal transfer of drug efflux pumps. BC cells were able to export doxorubicin in the extracellular medium by EVs shedding, thus reducing intracellular accumulation of the drug. Moreover, EVs mediate the transfer of functional proteins or RNAs (miRNA and mRNA) that modulate the expression and function of P-gp. The P-gp is found to be overproduced in cancer cells to remove cytotoxic drugs from cells and is

TABLE 1 EVs cargoes and drug resistance mechanisms.

miRNA						
Cell of origin	**EV content**	**Target**	**Cancer type**	**Type of resistance**	**Mechanism**	**Ref.**
MCF-7 and MDA-MB-231 DOX and PTX-resistant cells MCF7 CSCs	miR-155	TGF-β, FOXO-3a, and C/EBP-β mRNA	BC	DOX and PTX resistance	Contributing to drug resistance and promoting EMT and CSC phenotypes	(26)
MDA-MB-231 cells	miR-1246	CCNG2	BC	DOC, EPI, and GEM resistance	Promoting cell proliferation, migration, and drug resistance	(27)
BC cells resistant to TAM	miR221/222	P27 and ERα	BC	TAM resistance	Downregulation of p27 and ERα protein increasing cell proliferation	(28)
Trastuzumab-resistant BC cells	miR-567	ATG5	BC	Trastuzumab resistance	Regulating autophagy	(29)
MCF7	miR-567	ATG5	BC	Trastuzumab resistance	MiR-567 delivered by EVs revert cell resistance to trastuzumab	(30)
HL60/AR	MRP-1; miR19b, miR20a	HL60	Acute myeloid leukemia	MDR	Transferring chemoresistance through EVs from resistant to sensitive cells	(31)
MiaPaCa, Colo-357	miR-155	Unknown	Pancreatic cancer	GEM	Small EV-mediated mechanism of drug-induced acquired chemoresistance in PC cells. miR-155 induced suppression of gemcitabine-metabolizing enzyme, DCK	(32)
MCF7-Tam	miR-221/222	MCF7	BC	TAM	Vesicles containing miR-221/222 act as signaling molecules in cell–cell communication for tamoxifen resistance	(33)
786-0 Sor res, ACHN sor res.	miR-31-5p	786-0 Sor sens, ACHN sor sens	Advanced renal cell carcinoma	Sorafenib	EVs shuttled miR-31-5p can transfer resistance information from sorafenib-resistant to sensitive cells by directly targeting MLH1	(34)
SYO-1, HS-SYII, 1273/99 and YaFuS-resistant cells	microRNA-761	SYO-1, HS-SYII, 1273/99, and YaFuS	Synovial sarcoma	Pazopanib	EV miR-761 delivering affects chemosensitivity of synovial sarcoma cells to Pazopanib by targeting TRIP6, LMNA, and SIRT6	(35)
TMZ-resistant GBM cells	miR-1238	GBM-sensitive cells	Glioblastoma	Temozolomide	MiR-1238 levels are higher in TMZ-resistant GBM cells	(36)

(Continued)

TABLE 1 Continued

miRNA

Cell of origin	EV content	Target	Cancer type	Type of resistance	Mechanism	Ref.
					and their small EVs than in sensitive cells. Higher levels of miR-1238 are found in the sera of GBM patients than in healthy people. The loss of miR-1238 may sensitize resistant GBM cells by directly targeting the CAV1/EGFR pathway	

Proteins

Cell of origin	EV content	Target	Cancer type	Type of resistance	Mechanism	Ref.
ADM-resistant MCF-7 cells	UCH-L1, P-gp	MAPK/ERK	BC	ADM resistance	Overexpression of UCH-L1 enhanced multidrug resistance in BC	(37)
Peripheral blood Evs from BC patients	TRPC5	P-gp	BC	Anthracycline/ taxane-based chemotherapy	EVs stimulate the production of P-gp in the recipient cells by $Ca2^{+}$- and NFATc3-mediated mechanisms	(38)
EVs derived by PTX treated MDA-MB-231 cells	Survivin	N/A	BC	PTX resistance	Promoting cell survival and drug resistance	(39)
DOC-resistant variant of MCF-7	P-gp	Stimulating drug efflux	BC	DOC resistance	Drug resistance is transferred as well as P-gp from drug-resistant to sensitive BC cells	(40)
HER2-positive BC cells	TGFβ1 and PD-L1	Unknown	BC	Trastuzumab resistance	Neuromedin U induces the escape of immune response in HER2-positive BC cells by increasing the expression of TGFβ1 and PDL1	(41)
HER2 positive SKBR-3 and BT474 cells	HER2	Unknown	BC	Trastuzumab resistance	Inhibition of Trastuzumab activity *in vitro*	(42)
Basal-like BC cells	PD-1	Unknown	BC	Immunosuppression	ESCRT-related protein ALIX regulates EGFR activity and PD-L1 surface presentation in BC cells	(43)
Mesenchymal stem cells	TGFβ, C1q and semaphorins	PDL-1 overexpression	BC	Immunosuppression	Inducing differentiation of monocytic myeloid-derived suppressor	(44)

(Continued)

TABLE 1 Continued

Proteins						
Cell of origin	**EV content**	**Target**	**Cancer type**	**Type of resistance**	**Mechanism**	**Ref.**
					cells into highly immunosuppressive M2-polarized macrophages at tumor beds	
Su-DHL-4, Balm-3, OCI-Ly1	CD20	Unknown	B-cell lymphoma	Rituximab resistance	EVs protect target cells from rituximab action through the expression of CD20	(45)
DU145RD and 22Rv1RD	MDR-1/P-gp	Unknown	Prostate cancer	DOC	Small EVs expelled from DU145 and 22Rv1 docetaxel-resistant variants (DU145RD and 22Rv1RD) conferred docetaxel resistance to DU145, 22Rv1 and LNCaP cells	(46)
MCF7 ADM res	P-gp/TrpC5	HME cells	BC	ADM	MCF-7/ADM cell-derived MVs transferred both P-gp and TrpC5 to HMECs, and TrpC5-containing MVs modulated the expression of P-gp in HMECs *via* the translocation of the transcription factor NFATc3	(47)
MG-63DXR30	MDR-1 mRNA/P-gp	MG-63	Osteosarcoma	DOX resistance	Multidrug-resistant osteosarcoma cells are able to spread their ability to resist to the effects of doxorubicin treatment on sensitive cells by transferring small EVs carrying MDR-1 mRNA and its product P-glycoprotein.	(48)
KBv200	ABCB1	KB	Epidermoid carcinoma	MDR	Chemotherapeutic agents can increase Rab8B-mediated release of EVs containing ABCB1 from drug-resistant cells to sensitive recipient cells; acquire a rapid but unsustainable resistance to evade the cytotoxicity of chemotherapeutic agents.	(49)

(Continued)

TABLE 1 Continued

Proteins

Cell of origin	EV content	Target	Cancer type	Type of resistance	Mechanism	Ref.
OSCC cell lines	ATP1A1, ATP1B3	Unknown	Oral squamous cell carcinoma	CPT resistance	OSCC-derived EVs may regulate cisplatin resistance through a cellular efflux system	(50)
RKO/R	p-STAT3, GSTP1p	Unknown	CRC	5-FU resistance	p-STAT3-containing small EVs contribute to acquired 5-FU resistance in CRC.	(51)
SGC-7901/VCR	CLIC1	SG7901	Gastric cancer	Vincristine	Small EVs transferring CLIC1 could induce the development of resistance to vincristine *in vitro*	(52)
BC cells under hypoxic conditions	TGFβ and IL10	Unknown	BC	Immunosuppression	Suppress T-cell proliferation *via* TGFβ	(53)
Acute lymphoblastic leukemia cell line MDR	P-gp	Unknown	Acute lymphoblastic leukemia	MDR	Purified EVs transfer functional P-gp from resistant cancer cells to drug-sensitive cells *in vitro*	(54)

LncRNAs

Cell of origin	EV content	Target	Cancer type	Type of resistance	Mechanism	Ref.
DOX-resistant breast cancer cell lines. MCF7 and MDA-MB-231	Lnc RNA-H19	Unknown	BC	DOX resistance	Inhibition of apoptosis and enhancing of cell proliferation and drug resistance	(55)
ER-positive BC cells	LncRNA-UCA1	Cleaved Caspase 3	BC	TAM resistance	Caspase 3 intracellular levels are decreased impairing TAM-induced apoptosis	(56)
HER2-positive BC cells	LncRNA-SNHG14	Bcl2/BAX signaling pathway	BC	Trastuzumab resistance	LncRNA-SNHG14 may induce resistance to trastuzumab through inhibition of Bcl2/Bax apoptotic pathway.	(57)
Eca109 MDR cells	linc-VLDLR	Eca 109	Esophageal cancer	MDR	Linc-VLDLR EVs, secreted by the drug-resistant esophageal carcinoma cells, could cause the acquired drug-resistance phenotype of target cells by regulating	(58)

(Continued)

TABLE 1 Continued

LncRNAs

Cell of origin	EV content	Target	Cancer type	Type of resistance	Mechanism	Ref.
					the expression of ABCG2	
Sunitinib-resistant renal cancer cells	LncARSR	Endothelial cells	Renal cancer	Sunitinib	LncARSR is identified as a mediator of sunitinib resistance in renal cell carcinoma by acting as a competing endogenous RNA for miR-34 and miR-449, and show that small EV-mediated transmission of lncARSR can confer resistance to sensitive cells	(59)

Other cargoes

Cell of origin	EV content	Target	Cancer type	Type of resistance	Mechanism	Ref.
Cervical cancer cells	ceRNA of miR-34b	Unknown	Cervical cancer	CPT resistance	EVs carrying HNF1A-AS1 as a ceRNA of miR-34b to promote the expression of TUFT1 and the drug resistance of CC cells	(60)
Mouse mammary tumor TS/A cells	Unknown	Unknown	BC	Immunosuppression	Inhibition of NK cell tumor toxicity stimulated by IL-2	(61)
Metastatic BC cells	Unknown	Unknown	BC	Immunosuppression	Blocking T-cell proliferation and NK cell cytotoxicity	(62)
DOX-resistant MCF-7 cells	DOX	N/A	BC	DOX resistance	DOX accumulation in shed vesicles	(63)
TAM- and metformin-resistant MCF-7 cells	N/A	N/A	BC	TAM and metformin resistance	ERα decreased activity. Activation of AKT and AP-1, NF-kB, and SNAIL1	(64)
Patients with mBC resistant to hormonal therapy	mtDNA	N/A	BC	Endocrine therapy resistance	Promoting ER-independent oxidative phosphorylation	(65)

ADM, adriamycin; BC, breast cancer; CPT, cisplatin; CRC, colorectal cancer; DOC, docetaxel; DOX, doxorubicina; EPI, epirubicina; GEM, gemcitabine; MDR, multidrug resistance; PTX, paclitaxel; TAM, tamoxifene; N/A, not applicable.

demonstrated to cover a pivotal role in drug resistance together with TRPC (transient receptor potential channel) proteins (73).

EVs could also transfer drug metabolizing enzymes to inactivate drugs. Yang et al. described that the expression of GSTP1 (glutathione S-transferase P1), an enzyme belonging to phase II drug-metabolizing proteins, was higher in doxorubicin-resistant cells and in their EVs, which are capable of transferring the GSTP1 enzyme to sensitive cells (74). Accordingly, a high level of GSTP1 in circulating EVs may be an indication of a drug-resistant profile and could be used as a drug resistance predictive marker (74) as already demonstrated for the expression of GSTP1 on tumor cells (75, 76).

Cell viability could even be enhanced by EVs' transferring of pro-survival factors like cell surface receptors, miRNAs, and cellular proteins. These cargoes could improve cell viability by decreasing apoptosis and activating proliferative signals (77–81).

Intercellular communication between the microenvironment and tumor cells

As described in the *Introduction*, EVs are involved in cell–cell communication. This mechanism could play a role in the bidirectional crosstalk between tumoral and stromal cells also regarding drug resistance mechanisms. EVs derived from cancer-associated fibroblasts (CAFs) are described to be involved in drug resistance in different types of tumor. In colorectal cancer, EVs derived from CAFs are able to induce chemoresistance to 5-FU and oxaliplatin both *in vitro* and on patient-derived mouse xenografts (82). CAF-EVs are able to promote stemness and resistance in CRC cells *in vitro* and *in vivo* also by transferring the lncRNA H19 (83), H19 is an activator of β-catenin pathway. Previous studies demonstrated that the β-catenin pathway is involved in tumor progression and drug resistance (84–86). Interestingly, CAFs are naturally resistant to gemcitabine and their EVs transfer the gemcitabine chemoresistance phenotype in pancreatic ductal adenocarcinoma (PDAC) by delivering the SNAIL mRNA that increase SNAIL protein expression promoting proliferation and drug resistance (87). A recent work highlighted that CAF-EVs are involved in oxaliplatin resistance in CRC by transferring the CCAL (colorectal cancer-associated lncRNA) and activating the β-catenin pathway (88). CCAL interacts with mRNA-stabilizing protein HuR (human antigen R) increasing β-catenin mRNA and protein levels. Another work described the effect of stromal EVs in multiple myeloma cells inducing resistance to bortezomib, which could be linked to the activation of JNK, p38, p53, and Akt pathways (89). The release of EVs from mesenchymal stem cells carrying miR-222/miR-223 is linked to drug resistance in BC cells (90). ZEB1 mRNA encapsulated in EVs derived from mesenchymal transformed lung cells can transfer gemcitabine and cisplatin chemoresistance and the mesenchymal phenotypes to epithelial NSCLC cell line (91).

RNA (miRNA, lncRNA, and mRNA)-mediated drug resistance

Micro RNAs are small noncoding RNAs of 13–29 nucleotides involved in gene regulation and different biological and pathological processes, including the formation and development of tumors and drug resistance. In the last years, miRNAs are one of the most studied cargoes of EVs. As described, drug resistance mechanisms are heterogeneous and complex, and most of them are also regulated by miRNAs (92). miRNAs could promote drug resistance through the activation of metabolizing enzymes, in turn favoring drug inactivation or the expression of drug efflux pumps. The transfer of miRNA-365 by tumor-associated macrophage (TAM)-derived EVs to pancreatic ductal cells is described to induce resistance to gemcitabine in pancreatic adenocarcinoma by upregulating the triphosphate-nucleotide pool in cancer cells and inducing the cytidine deaminase enzyme that is able to inactivate gemcitabine (93). As mentioned, EV miRNAs could regulate the expression of ABC transporters that are involved in the efflux of intracellular drugs. It is described that, in ovarian cancer (OC) cells, there is an inverse correlation between the expression of Caveolin 1 (Cav1) and ABCB1, and this proportion is supposed to be driven by Cav1 (94, 95). Kanlikilicer et al. demonstrated that Cav1 levels in macrophages when co-cultured with OC cells are selectively dysregulated by the release of miR-1246 *via* EVs by OC cells. miR-1246 secreted in EVs inhibits the expression of Cav1 and upregulates ABCB1 expression to induce tumor-promoting phenotype and drug resistance *in vitro* and *in vivo* (96). As described for the transport of drug efflux pumps, even miRNAs could display a double-action behavior in the occurrence and development of drug resistance. Some miRNAs could have a positive effect on drug resistance, enhancing drug sensitivity in cancer cells. An analysis conducted by Liu et al. showed that EVs containing miR-128-3p were able to downregulate the expression of the MDR5 protein thus enhancing oxaliplatin sensitivity in resistant colorectal cancer cells (97). Another way to inhibit drug resistance is the regulation of glycolysis. Cancer metastasis, invasion, and drug resistance are also dependent on the anabolic profile of tumor cells that promotes the decrease in extracellular pH leading to the reduction of cytotoxic T-cell function in the TME acquiring strong survival advantages (98, 99). The GLUT protein family is involved in the intracellular uptake of glucose (100) and the regulation of glycolysis could be a strategy to contrast drug resistance (101). GLUT1 is demonstrated to be overexpressed in several tumors (102, 103), and its activation is associated with the regulation of mTOR. A decreased expression of mir-100-5p is described to be involved in drug resistance in many tumors. mTOR is the target gene of miR-100-5p that decreases its expression, enhancing drug sensitivity in cancer cells (104).

mRNA-mediated EVs are another player in the resistance process. Cao et al. demonstrated that EVs containing the DNMT1 mRNA (DNA methyltranferase 1) induce the overexpression of this enzyme in the recipient cells, playing an important role in the cisplatin resistance mediated by EVs in the xenograft model (105). In this research work, the underlying mechanism is not investigated, but it could be speculated that the dysregulation of Wnt and PI3K/AKT/mTOR signaling pathways, caused by an altered methylation status in a variety of genes, was described to be associated with resistance to standard treatments in many types of cancer (106). It was also demonstrated that BC cells resistant to doxorubicin possess an increased level of mRNA coding for a

detoxifying enzyme (GSTP1) and EVs derived from those cells are capable of transferring the mRNA to sensitive cells and inducing resistance (74). *In vitro* and *in vivo* experiments demonstrated that normal astrocytes can protect glioma cells from apoptosis induced by Temozolomide (TMZ) through the transfer of the mRNA of O-6-methylguaninene-DNA methyltransferase (MGMT) by EVs (107). EVs transfer of Zinc finger E-box homeobox 1 (ZEB1), a transcription factor involved in the epithelial-to-mesenchymal transition (EMT) process, induces the mesenchymal phenotype and drug resistance in recipient lung cancer cells (91, 108). In particular, this work described how EVs derived from mesenchymal oncogenically transformed lung cells can transfer chemoresistance and the mesenchymal phenotype to recipient cells.

LncRNA delivered by EVs often serves as competing endogenous RNA (ceRNA) to help miRNA in their drug resistance regulatory mechanisms (109). LncRNAs have been identified to be involved in cancer drug resistance by affecting the expression of drug metabolizing enzymes (110). Two studies described that EVs transferring lncRNA linc-ROR (111) and linc-VLDRLR (112) induced sorafenib and doxorubicin resistance in HepG2 cells (hepatocellular carcinoma) by activating the TGF-β pathway (111) and increasing the expression of ABCG2 (112). LncRNA urothelial carcinoma-associated 1 (UCA1) in NSCLC is associated with the modulation of a gefitinib-resistant phenotype by decreasing the expression of miR-143 and consequently increasing the expression of its target FOS-like 2 (113). LncRNA SBF2-AS1 is identified to be ceRNA of miR-151 and is involved in the mechanism of DNA repair that is one of the leading mechanisms of resistance to TMZ in neurological cancers (114). In glioblastoma patients, the presence of EVs lncRNA SBF-AS1 in the serum was found to be associated with TMZ resistance (115). LncRNA could also act by regulating some RNA-binding proteins as demonstrated for AFAP1-AS1 associated with shorter time survival of HER-2-positive BC patients linked to trastuzumab resistance. AFAP1-AS1 is responsible for trastuzumab resistance by upregulating HER-2 expression through the binding of the RNA binding protein AU-binding factor 1 (116).

EVs and possible applications as biomarkers of tumor therapy resistance

EVs can be isolated from various types of body fluids including blood, urine, and saliva. It is demonstrated that in the cancer patient population, the amount of EVs present in the blood is more than double compared to healthy individuals (117), suggesting that they could be new biomarker candidates (118). A correlation between serum EVs containing miR-146-5p could predict the efficacy of cisplatin in NSCLC patients in advanced stages and utilized for the real-time monitoring of drug resistance manifestation (119). Xiao et al. described that EVs derived from the serum of drug-resistant CRC patients (5-FU resistance) are enriched in TAG72 (tumor-associated glycoprotein 72) (120, 121). In BC preclinical models, it is demonstrated that the cargoes of EVs are influenced by the stress induced from drugs and could be correlated to the transfer of resistance in metastatic sites mediated by the Pg-P protein (40) or by miR-423-5p (122). Leukemia-derived EVs are described to induce IL-8 release in bone marrow stromal cells, thus protecting the cells from the effects induced by chemotherapy (123). A high level of IL8 promotes the expression of Pg-P and is required for the expression of the MDR profile in BC (124) and, in renal cancer, is described to be associated with sunitinib resistance (125). According to the described implication of EVs in drug resistance, it could be useful to set up methods for rapid isolation and characterization of tumor-derived EVs to improve the personalization of the therapies and to predict the drug response of the patient. Moreover, targeted drugs against tumor-derived EVs should be studied to reduce their non-beneficial effects as described in the next paragraph.

Targeting EVs to reduce cancer chemoresistance

Considering the importance of EVs in the regulation of chemoresistance, a few drugs were utilized to inhibit their production, release, or action.

It is described that drug-resistant cancer cells could produce an increased number of EVs than their drug-sensitive counterpart, thus contributing to the spread of resistance (45, 126–128). Some studies reported that, in drug-resistant cells, there is a direct association between the presence of drug resistance mediators and molecules involved in the production of EVs. For instance, Annexin A3 is a protein involved in OC platinum resistance and is also demonstrated to have a role in the EVs' production (129, 130). In the last years, an increasing number of studies investigated the possibility to inhibit the release of EVs from cancer cells. GW4869 is an inhibitor of the neutral sphingomyelinase (131) and is able to sensitize cisplatin-resistant OC cells by reducing EVs trafficking (105). Moreover, rhamnose-emodin is a molecule that is described to reduce the secretion of EVs from doxorubicin-resistant BC cells, thus reducing the expression of EVs miRNAs involved in chemoresistance (132). Therapeutic targeted antibodies against cell surface receptors may be neutralized by EVs interaction. Aung et al.'s research group described how Rituximab (anti-CD20) is quenched by EVs expressing the target protein. The authors also demonstrate that by blocking EVs biogenesis with

indomethacin, the therapeutic benefits of the therapy were restored (45). In another work, indomethacin is used to block EVs secretion in order to increase the amount of cytoplasmatic doxorubicin, its accumulation in the nucleus, and cytotoxicity (133). In a CRC model, it was demonstrated that the interaction mediated by EVs between cancer stem cells and fibroblasts promoted the resistance to 5-FU and oxaliplatin and can be reverted by blocking the release of EVs (82). Xie et al. developed functionalized silica mesoporous nanoparticles (NPs) able to selectively bind $EGFR^+$-EVs derived from NSCLC through aptamer recognition. NPs, after binding to EVs in the bloodstream, are delivered to the liver and excreted in the intestinal tract to be removed from the organism. It was demonstrated that by employing this system, the *in vivo* cancer metastatic overgrowth could be reduced (134).

Conclusions

The discovery of new cancer therapies is a stimulating topic that is investigated by a lot of researchers all over the world. The need for new therapeutic approaches is required because of the interpatient variability in terms of drug response, and also the development of drug resistance represents a very hard hurdle to overcome. Drug resistance appears in almost all types of cancer, and the underlying mechanisms are not yet clearly understood. In the last few years, the wide implication of EVs in drug resistance has been investigated, and in this manuscript, the major implications in this process are described and summarized. Although many described experiments are limited to preclinical and often to an *in vitro* stage, it is necessary to deeply investigate the roles of EVs in cancer drug resistance for many important aspects.

First of all, the involvement of EVs in drug resistance and their profiling could be exploited in the clinical approach to define new hallmarks of prognosis of drug response avoiding invasive procedures. On the other hand, as already explained, clarifying the role of EVs on drug resistance could stimulate the development of new anti-cancer strategies based on EVs targeting to revert drug resistance. Most importantly, cancer-released EVs should be deeply characterized, and their peculiar properties should be investigated. In this way, the development of new targeted strategies able to discriminate tumor-derived EVs could be set up. Moreover, the employment of artificial EVs could be considered in order to revert drug sensitivity (135, 136). EVs have already been described to possess very suitable properties as DDS to be loaded with different cargoes (drugs, miRNA, and proteins) displaying high biocompatibility, and the capacity to target cells is 10 times higher compared to liposomes of the same size (137–140). EVs could also represent a new DDS against neurological malignancies due to their ability to cross the blood–brain barrier (141, 142). Due to the possibility to produce engineered EVs *in vitro*, they could be developed to target different types of malignancies. There is also the possibility of studying artificial EVs for therapeutic employment with the advantage of producing standardized EVs with a defined content to facilitate the transition into a clinical application.

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SP wrote and revised the text. FR and VC conceptualized the work and edit the final version. All authors contributed to the article and approved the submitted version.

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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.

Publisher’s note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.948843/full#supplementary-material

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Effects of osteoblast-derived extracellular vesicles on aggressiveness, redox status and mitochondrial bioenergetics of MNNG/HOS osteosarcoma cells

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Osteosarcoma is the most common primary bone malignancy. The crosstalk between osteosarcoma and the surrounding tumour microenvironment (TME) drives key events that lead to metastasization, one of the main obstacles for definitive cure of most malignancies. Extracellular vesicles (EVs), lipid bilayer nanoparticles used by cells for intercellular communication, are emerging as critical biological mediators that permit the interplay between neoplasms and the tumour microenvironment, modulating re-wiring of energy metabolism and redox homeostatic processes. We previously showed that EVs derived from the human osteosarcoma cells influence bone cells, including osteoblasts. We here investigated whether the opposite could also be true, studying how osteoblast-derived EVs (OB-EVs) could alter tumour phenotype, mitochondrial energy metabolism, redox status and oxidative damage in MNNG/HOS osteosarcoma cells.These were treated with EVs obtained from mouse primary osteoblasts, and the following endpoints were investigated: i) cell viability and proliferation; ii) apoptosis; iii) migration and invasive capacity; iv) stemness features; v) mitochondrial function and energy metabolism; vi) redox status, antioxidant capacity and oxidative molecular damage. OB-EVs decreased MNNG/HOS metabolic activity and viability, which however was not accompanied by impaired proliferation nor by increased apoptosis, with respect to control. In addition, OB-EV-treated cells exhibited a significant reduction of motility and *in vitro* invasion as compared to untreated cells. Although the antioxidant N-acetyl-L-cysteine reverted the cytotoxic effect of OB-EVs, no evidence of oxidative stress was observed in treated cells. However, the redox balance of glutathione was significantly shifted towards a pro-oxidant state, even though the major antioxidant enzymatic protection did not respond to the pro-oxidant challenge. We did not find strong evidence of mitochondrial involvement or major energy metabolic switches induced by OB-EVs, but a trend of reduction in seahorse assay basal respiration was observed, suggesting that OB-EVs could represent a mild metabolic challenge for osteosarcoma cells. In summary, our findings suggest that OB-

EVs could serve as important means through which TME and osteosarcoma core cross-communicate. For the first time, we proved that OB-EVs reduced osteosarcoma cells' aggressiveness and viability through redox-dependent signalling pathways, even though mitochondrial dynamics and energy metabolism did not appear as processes critically needed to respond to OB-EVs.

KEYWORDS

osteosarcoma, extracellular vesicles, redox status, bioenergetics, tumour microenvironment, apoptosis, mitochondria, cell communication

Introduction

Osteosarcoma is the most common primary bone tumour, especially in childhood and adolescence, representing 2% of all neoplasias in children up to 14 years of age, and 3% in those aged 14 to 19 (1, 2). The consequences of osteosarcoma are devastating, ranging from limb amputation (20% of operable osteosarcomas) to death because of lung metastases (3), not to mention the physical and psychological consequences on both children and their families. Osteosarcoma derives from a malignant transformation of mesenchymal cells and is characterised by deposition of an osteoid-like matrix, with varying degrees of mineralization that can be studied by x-ray (4). Drug resistance is a serious issue in osteosarcoma, owing to its high genetic plasticity, which eventually leads to lung metastasization, inevitably ending with the death of the patient (5–7). Treatment of osteosarcoma often relies on reactive oxygen species (ROS)-generating compounds, and an important role in osteosarcoma chemoresistance has been recently acknowledged for improved protection against redox imbalance and ROS over-production (8, 9). Cancer cells are known to show a strong antioxidant machinery that serves to counteract high ROS basal levels, which are linked to mitochondrial metabolic reprogramming and to ROS-mediated signalling promoting proliferation (10). In this context, some authors have reported a critical role for mitochondrial dysfunction in the cytotoxic effect elicited by redox-active compounds that are potentially relevant for clinical treatment of osteosarcoma (11), and others have demonstrated that chemoresistant osteosarcoma cells exhibit lower mitochondrial activity with respect to non resistant cells (12). Today, it is widely accepted that cancer cell phenotype is influenced by the tumour microenvironment (TME), which represents a complex ecosystem that includes tumour cells, immune cells and stromal cells. In particular, the interactions among the components of the TME control the tumour's fate, along with its aggressiveness and resistance against therapies (13). Extracellular vesicles (EVs), that consist of lipid bilayer nano/microparticles secreted by all cell types, are emerging as a powerful means of communication between osteosarcoma and the TME. EVs contain a plethora of bioactive macromolecules, including miRNAs, membrane and luminal proteins, lncRNAs, circRNAs, tRNAs, many of which exhibit target specificity, being uptaken by specific cell types mainly depending on their membrane proteins (14, 15). The importance of EVs in cancer is starting to be well recognised, thanks to pioneering works from the late 2010s (16–19), yet their importance in primary bone cancers has not been elucidated in detail. Some of us previously demonstrated that EVs derived from the human osteosarcoma cell line MNNG/HOS were able to influence bone cells (20), especially osteoblasts. However, whether osteoblasts are also able to influence osteosarcoma phenotype through EVs still needs to be fully elucidated. In this work, we aimed at contributing to this field, focusing our attention on the effects induced by OB-EVs in MNNG/HOS cells, in terms of tumour phenotype, energy metabolism, redox status and oxidative damage.

Materials and methods

Materials

Dulbecco's modified Minimum Essential Medium (DMEM), Dulbecco's PBS (DPBS), Foetal Bovine Serum (FBS), penicillin, streptomycin and trypsin were supplied by GIBCO (Uxbridge, UK). All sterile plasticware was from Falcon Becton-Dickinson (Cowley, Oxford, UK) or Costar (Cambridge, MA, USA). The Matrigel Matrix (cat. no. 354262) was purchased from Corning (NY, USA), the EdU (5- ethynyl-2'-deoxyuridine)-HTS Kit 488 (cat. no. BCK-HTS488) was provided by Base Click GmBH (Munich, Germany). Trypan blue 0.4% (cat. no. 15250061), Mitotracker green FM (cat. no. M7514) and RevertAid First Strand cDNA Synthesis (cat. no. K1622) were from Thermo Scientific (Waltham, MA, USA). The glutathione assay kit (cat. no. 703002) and the TBARS Assay Kit (cat. no. 10009055) were

supplied by the Cayman Chemical Company (Ann Arbor, MI, USA). The human MMPs array C1 kit (cat. no. AAH-MMP-1-8) was supplied from RayBiotech (Peachtree Corners, GE, USA), Bradford assay (cat. no. A6932,0500) was from Panreac Applichem (Darmstadt, Germany). All other reagents, including 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) bromide reduction assay, staurosporine (cat. no. 569396), N-acetyl-L-cysteine (L-NAC; cat. A7250), etomoxir (cat. E1905), oligomycin A (cat. 75351), 2,4-dinitrophenol (2,4-DNP; cat. D198501), rotenone (cat. R8875), antimycin A (cat. A8674), NADPH (cat. N7505) and GSSG (cat. G4376) were supplied by Merck Life Science (Milan, Italy). Dithiothreitol (DTT; cat. D1532) was supplied by Invitrogen (Waltham, MA, USA). The L.D.H. (LDH-P) DGKC method-based kit (cat. K2011) was supplied by Biolabo (Maizy, France). Antibodies used in this work are summarised in Supplementary Table 1.

Animal ethical approval

All procedures involving animals and their care was conducted in conformity with national and international laws and policies (European Economic Community Council Directive 86/609, OJ L 358, 1, December 12, 1987; Italian Legislative Decree no. 26, Gazzetta Ufficiale della Repubblica Italiana no. 61, March 4th, 2014; guide for the Care and Use of Laboratory Animals, National Institute of Health, Publication no. 85-23, 1985) and the Animal Research: Reporting of *in Vivo* Experiments (ARRIVE) guidelines. Animal procedures received Institutional approval by the Italian Ministry of Health (approval no. 622/2021-PR).

Cell cultures

The human osteosarcoma cell line MNNG/HOS (RRID : CVCL_0439) was obtained from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK) and grown at 37 °C, 5 % CO2 in DMEM supplemented with 10% FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine.

Osteoblast primary cultures

Calvariae from 7-day-old CD1 mice were explanted, cleaned free of soft tissues and digested three times with 1 mg/ml *Clostridium histolyticum* type IV collagenase and 0.25% trypsin, for 15, 30 and 45 min, respectively, at 37°C with gentle agitation. Cells from the second and third digestions were plated following centrifugation at 300 x *g* for 7 min and grown at 37°C, 5% CO2 in DMEM plus 10% FBS. At confluence, cells were trypsinised and plated according to the experimental protocol. The purity of the culture was evaluated by the transcriptional expression of the osteoblast biomarkers Alkaline Phosphatase (ALP), Runt-related transcription factor 2 (*Runx2*), type I collagen and osteocalcin and by the histochemical evaluation of ALP activity.

Extracellular vesicles isolation

Osteoblast derived EVs (OB-EVs) were isolated according to Ucci et al. (20) and Loftus et al. (21). Briefly, upon reaching 80% confluence, mouse primary osteoblasts were washed in DPBS and starved in serum-free DMEM to prevent contamination from FBS-EVs. After 24 h, the conditioned medium (CM) was collected and sequentially centrifuged at 300 x *g*, 4°C for 5 min to remove dead cells and at 5,000 x *g*, 4°C for 25 min to remove membrane debris. The supernatant was collected and transferred to a Beckman L7-65 ultracentrifuge in a Beckman SW41-Ti or SW28 rotor and centrifuged at 100,000 x *g*, at 9°C for 70 min. Supernatant was discarded, while the pellet, containing EVs, was resuspended in DMEM for cell treatments. To quantify OB-EVs, they were subjected to nanoparticle tracking analysis (see Supplementary Figure 1A) as well as to protein extraction, the latter giving a yield of 4.9 ± 1.3 µg/12 ml CM. Freshly-isolated EVs were used for all the subsequent experiments.

Nanoparticle tracking analysis

EVs were isolated from osteoblast CM (12 ml collected from one 175cm^2 flask, cell density = 3.5x10^4 cells/cm^2) and resuspended in 100µl of nanofiltered DPBS. EVs were then diluted 1:100 and used for nanoparticle tracking analysis using a nanosight NS300 NTA apparatus. Flow and camera gain were adjusted following the manufacturer's instructions based on the NS300 quality control parameters. Five camera acquisitions of 60 seconds each were analysed for every biological replicate.

Transmission electron microscopy

EVs were isolated from osteoblast CM (12 ml collected from one 175cm^2 flask, cell density = 3.5x10^4 cells/cm^2) and resuspended in 2% PFA. Five µl of EVs were then put onto Formvar-coated grids and allowed to adsorb for 20 min in a dry environment. Grids were washed in PBS and fixed in 1% glutaraldehyde for 5 min. Samples were washed in distilled water and contrasted with 4% uranyl-oxalate solution for 5 min. Grids were air-dried for 10 min and observed under a Philips CM 30 TEM, 80 kV.

EV internalisation assay

Extracellular vesicles derived from osteoblast CM (12 ml collected from one 175 cm^2 flask, cell density= 3.5x10^4 cells/cm^2)

were incubated at 37°C with the membrane-permeant green fluorescent dye 5-chloromethylfluoresceindiacetate (CMFDA) for 30 min followed by 5 min at 37°C with the red fluorescent membrane-labelling dye PKH26 (Sigma–Aldrich; #MINI26-1KT). Then, the EVs were washed in PBS and ultracentrifuged at 100,000 x *g*, at 4°C for 70 min. Finally, EVs were resuspended in PBS for the treatment. Target cells were incubated with stained or unstained EVs for 48h before microscopic assessment of internalisation. Nuclei were counterstained with DAPI.

MTT assay

Metabolic activity of MNNG/HOS cells was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) bromide reduction assay (22). Briefly, cells (9,000/cm^2) were plated in 96-well plates, starved O/N in serum-free DMEM and then treated with OB-EVs (isolated from 12 ml of CM collected from one 175-cm^2 flask to treat 9 wells). MTT was dissolved in DPBS at 5 mg/ml concentration, and added at 1:6 (v/v) ratio directly into the cell supernatant. Three hours later, medium was removed and DMSO was added to dissolve the precipitated formazan salts arising from the reaction. Plates were shaken at 160 rpm on an orbital shaker for 10 min, then absorbance at 595 nm was recorded and plotted as X-fold to the absorbance at t_0.

Viable cells counting

Viable cells counting was carried out through the Trypan blue exclusion test (23). MNNG/HOS cells were plated in 175-cm^2 flasks and treated with DMEM (control), OB-EVs (isolated from 36 ml of CM collected from three 175-cm^2 flask, target cell surface:isolation surface ratio=1:3) or OB-EVs + 5 mM L-NAC. Twenty-four hours later, cells were detached using trypsin-EDTA and centrifuged at 400 x *g*. Pellets were resuspended in DPBS and a 20 µl aliquot was mixed with the same volume of 0.4% Trypan blue. Cells were incubated for 2 min at RT, then 10 µl of the cell suspension were transferred to an hemocytometer for viable cell counting. Cell viability was then expressed as % live cells/total cells.

EdU (5- ethynyl-2′-deoxyuridine) cell proliferation assay

EdU cell proliferation assay was performed on MNNG/HOS cells pretreated with OB-EVs, by using the EdU (24) HTS Kit 488. Briefly, cells were plated onto cell culture dishes (90 mm Ø) and, upon reaching 80% of confluence, were starved O/N in serum-free DMEM and treated with OB-EVs isolated from 12 ml of CM collected from one 175-cm^2 flask (target cell surface: isolation surface ratio=1:3) or with DMEM, as control. After 24 h, cells were detached and seeded in 96-well plates at a density of 9,000 cells/cm^2), grown in standard conditions for 43 h and incubated for 5 hours with 5 µM EdU (48h total culture time). Cells were then washed in DPBS and fixed in 4% paraformaldehyde (PFA) for 10 min. EdU incorporation was detected using the EdU HTS Kit: cells were permeabilised with 0.5% Triton X-100 in PBS, incubated with the click assay cocktail for 30 min at RT, protected from light, and then washed twice in 1X rinse solution. Nuclei were counterstained with DAPI. Cell proliferation was assessed under a fluorescence microscope and expressed as the percentage of EdU-positive cells.

Wound healing assay

MNNG/HOS cells were cultured in cell culture dishes (90 mm Ø) until confluence reached 80%. Then, cells were starved O/N and pre-treated for 24 hours with OB-EVs (previously isolated from 12 ml of CM collected from one 175-cm^2 flask, target cell surface:isolation surface ratio=1:3) or with DMEM (control). Then, MNNG/HOS cells were detached and plated on 24 well-plates in DMEM+10% FBS. When confluence reached 100%, cell monolayers were scratched with a sterile tip to create a cross-shape wound (25). Pictures were taken at time 0 and 6 hours later under a phase-contrast microscope. Cell motility was evaluated by calculating the percentage of wound-healed area using NIH ImageJ software (RRID : SCR_003070).

In vitro invasion assay

MNNG/HOS cells were plated in cell culture dishes (90 mm Ø) and, upon reaching 80% of confluence, they were washed twice in DPBS, starved overnight in serum-free DMEM and treated with OB-EVs (previously isolated from 12 ml of CM collected from one 175-cm^2 flask, target cell surface:isolation surface ratio=1:3) or with DMEM, as control. After 24 h, MNNG/HOS cells were washed in DPBS detached by trypsin-EDTA, incubated for 30 min at 37°C in 5% CO_2, and centrifuged at 300 x *g* for 5 min at RT. Each pellet was then resuspended in DPBS, centrifuged at 300 g for 5 min at RT and resuspended in serum-free DMEM. Then, 8 x 10^5 cells were seeded in the upper compartment of each transwell, onto an 8.0 µm membrane pre-coated with Matrigel (26), while 0.8 ml of FBS were added to the lower compartment as chemoattractant. After 8 h, the cells that remained in the upper compartment were carefully removed using cotton swabs, while those migrated towards the lower compartment and remained in the matrigel were fixed with cold methanol, washed in PBS and stained with hematoxylin/eosin. Number of invaded cells per field was evaluated in seven fields/transwell.

Oncosphere formation assays

MNNG/HOS cells were plated at a density of 4,000 cells/ml in oncosphere medium on poly-hydroxyethyl-methacrylate-coated plates (27). Oncosphere medium composition was as follows: DMEM/F12 with 1% penicillin/streptomycin, 20 nM progesterone, 100 µM putrescine, 1% insulin- transferrin-selenium A, 10 ng/ml basic fibroblast growth factor, 10 ng/ml epithelial growth factor with OB-EVs or DMEM as control (27). Growth factors and OB-EVs (EVs isolated from 12 ml of CM collected from one 175-cm^2 flask and then added at 3.85cm^2 isolation surface/ml of medium ratio) of EVs were refreshed twice a week. After 7 and 10 days, pictures of the whole plates were taken with an inverted phase contrast microscope equipped with a CMOS sensor camera, and images were analysed by imageJ to evaluate oncosphere number and area.

Western blotting

MNNG/HOS cells were plated in cell culture dishes (90 mm Ø) and, upon reaching 80% of confluence, they were washed twice in DPBS, starved overnight in serum-free DMEM and treated with OB-EVs (previously isolated from 12 ml of CM collected from one 175-cm^2 flask, target cell surface:isolation surface ratio=1:3) or with DMEM, as control. After 24 h of treatment, cells were lysed in RIPA buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitors. Mouse primary osteoblasts and EVs were also subjected to protein extraction as described above. Proteins (8 µg for osteoblasts and OB-EVs and 30µg for MNNG/HOS cells) were resolved by 10-12% SDS-PAGE and transferred to nitrocellulose membranes. Blots were incubated 1 hour in 5% nonfat dry milk in TBS-T, probed with the primary antibody (Supplementary Table 1) in 1% milk O/N at 4°C, washed and incubated with the appropriate HorseRadish Peroxidase (HRP)-conjugated secondary antibody (Supplementary Table 1) for 1 h at RT. After washing, protein bands were revealed by Enhanced ChemiLuminescence (ECL) and acquired using a Chemidoc XRS+ imaging system. The analysis of band intensities was performed using the NIH ImageJ tool (RRID : SCR_003070), and β-Actin or α-Tubulin-normalised data were shown as fold *vs* DMEM (control).

Matrix metalloproteinases protein array

MNNG/HOS cells were plated in cell culture dishes (90 mm Ø) and, upon reaching 80% of confluence, they were washed twice in DPBS, starved O/N in serum-free DMEM and treated with OB-EVs (previously isolated from 12 ml of CM collected from one 175-cm^2 flask, target cell surface: isolation surface ratio=1:3) or with DMEM, as control. After 48 h, CM was collected, centrifuged at 300 x *g*, 4°C for 5 min, and subjected to the Human MMPs array C1 kit (RayBiotech, cat. no. AAH-MMP-1-8), following the manufacturer's instructions. One ml of CM from MNNG/HOS untreated or treated with OB-EVs was incubated with each membrane, which includes 10 capture antibodies printed in duplicate. Membranes were washed and incubated with the Chemiluminescence Detection Buffer mix for the detection of the positive spots. For data analysis, the intensity of each spot was determined by densitometry using NIH ImageJ, and the average background subtracted. The intensity of positive control spots were used to normalise the signal intensity of the protein of interest. Data was shown as fold *vs* control (DMEM).

Incucyte-based caspases 3/7 assay

MNNG/HOS cells were plated at a density of 9,000 cells/cm^2 in 96-well plates and starved O/N. Then, cells were stained with a caspase 3/7-sensitive probe (Sartorius, cat. no. 4440) that crosses cell membranes but is only fluorescent in cells with active caspases 3/7, along with OB-EVs (isolated from 12 ml of CM collected from one 175-cm^2 flask and then added to target cells at a target cells surface:isolation surface ratio=1:3), DMEM (control) or 5 µM staurosporine (positive control). After 30 min of incubation at RT, cells were transferred into a Sartorius Incucyte S3 live cells imager and captured (10X magnification) with a capture time interval of 2 h, across 48 h overall, using both phase contrast and green fluorescence channels. Data were analysed by Incucyte's in-bundle software (rel. 2019B), and the metric used was the number of green positive objects (casp3/7^+ cells)/confluence area. DMEM-normalised results were given including datasets from 4 independent experiments, with at least 6 technical replicates each.

Incucyte-based Mitotracker assay

MNNG/HOS were plated at a density of 9,000 cells/cm^2 in 96 well plates and starved O/N. Cells were then stained with MitoTracker green FM (Thermo Fisher, cat#M7514) following the manufacturer's instructions and incubated at 37°C, 5% CO_2 for 30 min. The staining solution was removed and cells were treated with OB-EVs (isolated from 12 ml of CM collected from one 175-cm^2 flask and then added to target cells at a target cells surface:isolation surface ratio=1:3) or DMEM (control). Then, cells were transferred into a Sartorius Incucyte S3 live cells imager and images were captured (10X magnification) with a 2-hour time interval, across 48 hours

overall, using both phase contrast and green fluorescence channels. Results were analysed using Incucyte's in-bundle software (rel. 2019B), and the metric reported was the t_0-normalised green fluorescent area/total cell area ratio. Datasets derived from 5 independent experiments, with at least 6 technical replicates.

Glutathione assay

Cells were treated as described in the Viable cells counting section, then total (tGSH) and oxidised glutathione (GSSG) levels were measured using the method described by Baker and co-workers (28). GSSG was measured by first derivatizing GSH with 2-vinylpyridine (cat. no. 132292, Sigma–Aldrich), as recommended by the manufacturer. Briefly, DMEM-, OB-EV- and OB-EV+NAC-treated cells were homogenised in the MES buffer provided by the manufacturer (1.5 x 10^7 cells/ml) and centrifuged at 10,000 x *g* for 15 min, at 4°C. Supernatants were immediately deproteinized with 5% (w/v) metaphosphoric acid (cat. no. 239275, Sigma–Aldrich), and centrifuged at 4,000 x *g* for 5 min. Protein-free supernatants (50 µl) and Assay Cocktail (150 µl) were mixed in a 96-well microplate, and the colour development was followed in a Victor3 microplate reader (PerkinElmer Inc., Waltham, MA, USA) at 405 nm for 30 min with 5-min time intervals. Calibration curves were obtained from pure GSSG- and GSH-containing reactions (range: 0-8 mM GSSG, 0-16 mM tGSH). All samples were blinded-processed in technical triplicates. Results were from 8 independent experiments.

Thiobarbituric acid-reactive substances assay

Cells were treated as described in the Viable cells counting section, and then subjected to TBARS measurement, a well-established method used to detect lipid peroxidative damage (29, 30). Briefly, DMEM-, OB-EV- and OB-EV+NAC-treated cells were extracted in PBS (6 x 10^7 cells/ml), by using three thaw/freeze cycles in liquid nitrogen. Samples were centrifuged at 16,000 x *g* for 30 min at 4°C. Supernatants (37.5 µl) were mixed with sodium dodecyl sulphate (SDS) (37.5 µl) and 1.5 ml of Colour Reagent, in triplicate, as suggested by the manufacturer. Then, reaction mixtures were incubated for 1 hour in boiling water and centrifuged at 1,600 x *g* for 10 min at 4°C. Supernatants were kept at RT for 5 min until clarified, and read at 532 nm in a Lambda 25 spectrophotometer (PerkinElmer Inc., Waltham, MA, USA). A linear calibration curve was obtained from pure malondialdehyde (MDA)-containing reactions (range: 0-50 mM). All samples were blinded-processed in technical replicates. Results were from 5 independent experiments.

Catalase enzymatic activity assay

Cells were treated as described in the Viable cells counting section, harvested and lysed (2 x 10^7 cells/ml) in a 100 mM phosphate buffer (pH 7), containing 0.1% (v/v) Triton X-100. Cell suspensions were lysed through N_2-freezing and thawing (three cycles). Then, pellets were homogenised using 1.5 ml tube pestles for 2 min in ice. Samples were centrifuged at 16,000 x *g* for 30 min at 4 °C and the resulting supernatants were used both for Bradford assay to evaluate total protein concentration, using BSA as the standard, and for the assessment of the enzymatic activity of CAT (EC 1.11.1.6). The enzymatic activity of CAT was assayed by recording the disappearance of 10 mM hydrogen peroxide at 240 nm and 25 °C, as described by Aebi (31), using a Lambda25 spectrophotometer (PerkinElmer Inc., Waltham, MA, USA). One unit was defined as 1 µmol of hydrogen peroxide consumed/min. Three independent experiments were carried out with at least three technical replicates.

Glutathione reductase enzymatic activity assay

Cells were treated as described in the Viable cells counting section, harvested and lysed (3 x 10^7 cells/ml) in a 100 mM phosphate buffer (pH 7) supplemented with 2 mM EDTA and 3 mM DTT. Cell suspensions were lysed through N_2-freezing and thawing (three cycles). Then, pellets were homogenised using 1.5 ml tube pestles for 2 min in ice. Samples were centrifuged at 16,000 x *g* for 30 min at 4 °C. The resulting supernatants were used for Bradford assay to evaluate total protein concentration, using BSA as the standard, and for the assessment of the enzymatic activity of GR (EC 1.6.4.27). The enzymatic activity of GR was assayed according to Di Ilio and colleagues (32), starting the reaction by adding GSSG (1.14 mM final concentration) and following the disappearance of 190 µM NADPH at 340 nm and 25 °C with a Lambda25 spectrophotometer (PerkinElmer Inc.). One unit was defined as 1 µmol of NADPH consumed/min. Five independent experiments were carried out with at least three technical replicates.

Lactate dehydrogenase enzymatic activity assay

Cells were treated as described in the Viable cells counting section. Twenty-four hours later, conditioned media were collected and used for the assessment of the LDH enzymatic activity. The quantitative determination of LDH activity was assayed in duplicate using the L.D.H. (LDH-P) DGKC method-based kit by following the LDH-dependent decrease of NADH absorbance at 340 nm associated with reduction of pyruvate, as

recommended by the manufacturer. Results from two independent experiments were analysed using a Kenza One analyzer (Biolabo), equipped with Kenza One v2.04 software.

Seahorse-based assays

For bioenergetic profiling, 5,000 MNNG/HOS cells were seeded onto wells of Seahorse 96-well plates coated with 0.1% Collagen Type I. After 24 h cells were starved for further 24h and treated or not with OB-EVs (isolated from 12 ml of CM collected from one 175-cm^2 flask and then added to target cells at a target cells surface:isolation surface ratio=1:3). After 24h cells were analysed by XF-96 Extracellular Flux Analyzer (Agilent Seahorse). ATP production rate was measured in DMEM XF Assay Medium (#103680-100, Agilent Seahorse) containing 1 mM pyruvate, 2 mM glutamine, and 10 mM glucose, following injection of 1.5 µM oligomycin A, 0.5 µM rotenone, and 0.5 µM antimycin A. Mitochondrial oxygen consumption rates (OCR) and glycolytic Extra-Cellular Acidification Rates (ECAR) were measured and then transformed into mitochondrial (mito-ATP) and glycolytic (glyco-ATP) ATP production rates, using validated algorithms provided in the Seahorse Agilent software. Mitostress test was conducted, and OCR was measured in the same media as above following injection of 1.5 µM oligomycin A, 75 µM 2,4-Dinitrophenol, 0.5 µM rotenone, and 0.5 µM antimycin A. Spare respiratory capacity (SRC) was calculated in accordance to the manufacturer's specifications as the difference between maximal respiration (i.e., the maximum rate measurement recorded after 2,4-DNP injection after subtracting the non-mitochondrial respiration rate) and basal respiration (i.e., the last measurement recorded prior to oligomycin A injection after subtracting the non-mitochondrial respiration rate). Seahorse values were normalised by cell number well by well.

Real time RT-PCR

Total RNA from osteoblast-derived EVs was extracted using TRIzol reagent, then RNA (1.5 µg) was reverse transcribed *via* a M-MLV reverse transcriptase-based first strand cDNA synthesis, as recommended by the suppliers. cDNA was subjected to real time PCR, using the Fast Advanced Master Mix (ThermoFisher Scientific, cat. 4444557), together with TaqMan® Gene Expression Assays with IDs Mm00439154_m1 (*Mus musculus* glutathione reductase) and Mm00802658_m1 (*Mus musculus* glutamate-cysteine ligase catalytic subunit). Reactions (in triplicates) were set up in Primo® FrameStar® 96-well PCR plates (Euroclone, cat. ECPCR0770C), which were sealed with MicroAmp™ optical adhesive films (Applied Biosystems, cat. 4360954). The thermal profile of the Applied Biosystems VIIA7 was set as recommended by the manufacturer for absence/presence assays: 2 min at 50°C, 20 sec at 95°C, then 40 cycles with 1 sec at 95°C and 20 sec at 60°C, along with a final post-read stage of 30 sec at 60°C.

Statistics

Results were expressed as means ± SEM. To compare curves in longitudinal studies, Graphpad Prism (RRID : SCR_002798, version 7.0) was used to run curve fitting tests and evaluate whether one curve could fit both the datasets compared. Shapiro-Wilk normality tests were performed to assess whether to use parametric or non-parametric tests. In experiments with more than 2 independent experimental groups, one-way ANOVA (parametric) or Kruskal-Wallis (non-parametric) was used to calculate statistical significance of differences. Unpaired Student's t-test (parametric) or Mann-Whitney (non-parametric) tests were used when comparing 2 groups only. Dunn's test was used when comparing non-normally distributed multiple groups with experimental pairing. Statistics used were specified in each figure legend. Differences were considered statistically significant when p was < 0.05.

Results

Characterisation of osteoblast-derived EVs

We first characterised the EVs isolated from mouse primary osteoblast conditioned media. EV size was confirmed by NanoSight, which also allowed to determine the EV concentration per preparation (Supplementary Figure 1A). The typical positive EV biomarkers, CD81, CD63 and Annexin II (*Anxa2*), were enriched in the EV protein lysates versus the source cells (Supplementary Figure 1B), while the mitochondrial protein SOD2, a possible negative marker for EVs, was barely detectable in EVs and well expressed in the cell protein lysate (Supplementary Figure 1B). The vesicular nature of the isolated particles was confirmed by TEM, which also showed the EV membrane integrity (Supplementary Figure 1C). Altogether, these results fulfil the requirements described in the MISEV2018 guidelines (33) to define a vesicular fraction as EVs.

We next investigated whether OB-EVs were actively internalised by MNNG/HOS cells. To this aim, OB-EVs were double-labelled with the intra-vesicular green-fluorescent dye, CMFDA, and with the red-fluorescent membrane dye, PKH26. MNNG/HOS cells were then incubated with these EVs for 48 hours. Fluorescence microscopy demonstrated that PKH26 and CMFDA fluorescence was detectable in osteosarcoma cells, suggesting internalisation of OB-EVs by the recipient cells

(Figure 1A, left panel). Negative controls treated with unstained EVs confirmed specificity of the signal (Figure 1A, right panel).

OB-EVs reduced MNNG/HOS cell viability without activating apoptosis or affecting cell proliferation

To assess whether OB-EVs could affect basic cellular activities, the first endpoints analysed were cell viability, death and proliferation. Interestingly, the MTT assay, which measures cell metabolic function, revealed a diminished activity of mitochondrial dehydrogenases in OB-EVs-treated MNNG/HOS cells, after 24 and 48 hours of treatment, as compared to control (Figure 1B). In order to investigate whether the reduction in MTT was accompanied by a reduced cell viability, we also performed a Trypan blue exclusion test, and found that OB-EVs reduced the percentage of live MNNG/HOS cells (Figure 1C). This result was confirmed by the increased extracellular levels of lactate dehydrogenase, a marker used to assess cell death (34), found in the medium of osteosarcoma cells treated with OB-EVs (+11.2% vs DMEM). Interestingly, the co-administration of the antioxidant N-acetyl-7L-cysteine (NAC) was able to revert the cytotoxic effect observed in OB-EV-treated cells (Figure 1C). To discriminate between necrotic and apoptotic death, we also performed an Incucyte-based time-course assay for detection of caspase 3/7 positive cells, and found that the % of apoptotic cells was not statistically different between OB-EV-treated and control cells (Figure 1D). As expected, osteosarcoma cells that were treated with 5 μM staurosporine (positive control) for the whole duration of the experiment showed a statistically significant increase in apoptosis (Figure 1D). Since P53 serves as a critical mediator of several types of apoptosis (35), we assessed its protein expression by western blotting, finding that it was similar in OB-EV-treated and in control cells (Figure 1E). Finally, we performed an EdU incorporation assay to evaluate whether proliferative rate was affected by the treatment with OB-EVs, and observed no statistically significant difference when comparing treated and control cells (Figure 1F).

OB-EVs shifted glutathione redox status towards oxidation, without altering lipid peroxidation profile and major ROS-scavenging protection

As reported above, the antioxidant and cysteine-donor N-acetyl-L-cysteine was able to revert the cell-damaging effects of OB-EVs. Therefore, we measured the redox balance of glutathione, which is an indicator of the antioxidant buffering system within the cell (36), along with the peroxidative damage in all the experimental conditions. Intriguingly, we observed a decreased tGSH/GSSG ratio in OB-EVs-treated MNNG/HOS, as compared to control cells (Figure 2A). Interestingly, such a redox perturbing effect was reverted by the co-administration of OB-EVs+NAC (Figure 2A). Of note, no OB-EV-dependent effect was observed on the most important enzyme for recycling oxidized glutathione (i.e., glutathione reductase). In fact, the specific activity of GR in OB-EV-treated cells was unchanged, as compared to non-treated cells (Figure 2B). Similarly, no change of GR specific activity was detected in cells treated with both OB-EVs and NAC (Figure 2B). Surprisingly, the level of lipid peroxidative damage was unchanged by OB-EVs, as shown by the unaltered levels of TBARS (Figure 2C). Since catalase (CAT) represents one of the crucial antioxidant enzymes that mitigates cellular oxidative stress, we investigated CAT activity by spectrophotometric enzymatic assay, finding that it was unchanged by OB-EVs (Figure 2D), and the same was true for the protein levels of CAT itself and of superoxide dismutase 2 (SOD2), another major mitochondrial antioxidant enzyme (Figure 2E). OB-EVs were also analysed with regard to their content in terms of mRNAs relevant to glutathione homeostasis. Our real time RT-PCR analyses revealed that two transcripts that are crucial to glutathione metabolism were undoubtedly present within the OB-EVs. In particular, our presence-absence TaqMan-based assays detected both the glutathione reductase mRNA and the glutamate-cysteine ligase catalytic (*Gclc*) subunit transcript (Supplementary Figure 1D).

OB-EVs did not alter the extension of mitochondrial network, nor did they affect mitochondrial metabolism and dynamics in MNNG/HOS

Since a pro-oxidant perturbation of the redox milieu may be linked to a pro-oxidative switch towards mitochondrial metabolism, we investigated more in detail whether and how mitochondria and cellular energy metabolism could respond to OB-EVs. *Via* a Incucyte-based assay and normalising the Mitotracker-positive area to total cell area, we found that mitochondrial network decreased in OB-EVs-treated osteosarcoma cells less rapidly than in control cells (Figure 3A), even though a direct comparison between the mitochondrial area in cells treated with OB-EVs for 24 hours and the corresponding time point in control cells did not reveal any statistically significant difference (Figure 3A). Intriguingly, this was not accompanied by a change in the ratio between glycolytic *versus* oxidative ATP produced by MNNG/HOS, as demonstrated by the Seahorse ATP rate assay (Figure 3B). However, mitostress assays showed a strong trend of reduction in basal respiration (BR, $p=0.051$), with non-significant reductions in maximal respiratory capacity (MRC) and spare respiratory capacity (SRC, Figure 3C) following OB-EVs treatment. Moreover, the expression level of two important

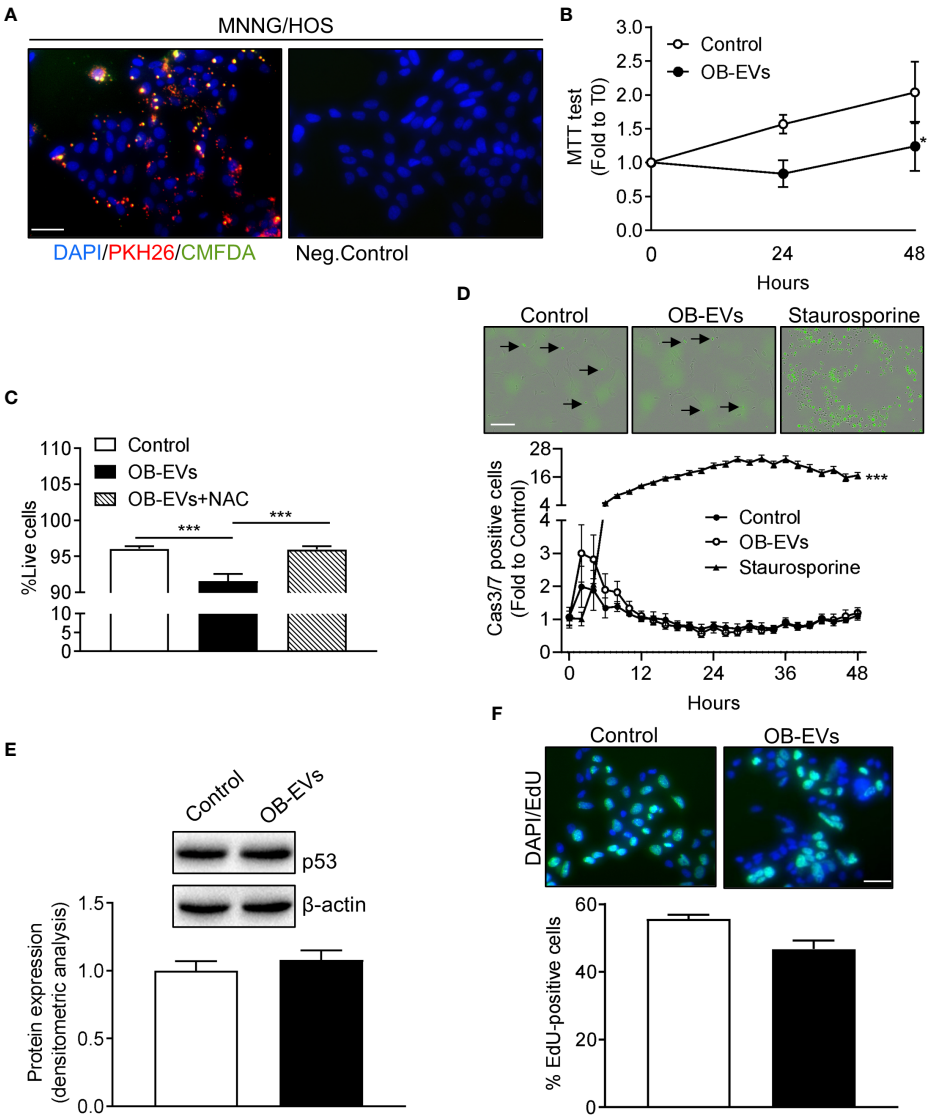

FIGURE 1
Effects of OB-EVs viability, apoptosis and proliferation of MNNG/HOS cells. **(A)** Left panel: representative fluorescence micrographs of MNNG/HOS cells incubated with osteoblast-derived EVs (OB-EVs) previously labelled with CMFDA (green cytoplasmic dye) and PKH26 (red lipophilic dye) for 48 hours. Right panel: representative image of the negative control performed on MNNG/HOS cells incubated with unlabeled OB-EVs. Cells were also stained with the nuclear dye DAPI. Data are representative of 3 independent preparations (scale bar=50 µm). **(B–E)** MNNG/HOS cells were plated and starved in serum-free DMEM O/N the next day. Then, cells were treated with DMEM (control), OB-EVs or OB-EVs + 5 mM N-acetyl-L-cysteine (NAC), for the time duration specified in the figures, or for 24 h when not specified. Cells were subjected to **(B)** MTT assay to assess cell viability/metabolic activity; **(C)** Trypan blue dye-exclusion test to evaluate the percentage of live cells; **(D)** Caspase 3/7 (Casp3/7) Incucyte-based assay for apoptosis (bar=200 µm, black arrows: apoptotic cells); **(E)** Western blotting to assess p53 protein expression/β-actin; **(F)** EdU-based assay to evaluate proliferation (scale bar=50 µm). **(B, E, F)** N=3, **(C)** N=8, **(D)** N=4. **(B, D)** Curve fitting test; **(C)** One-way ANOVA; **(E, F)** Unpaired Student's *t*-test. *p<0.05; ***p<0.001. Data are presented as mean ± SEM.

regulators of mitochondrial dynamics (namely, mitofusins 1 and 2) were not significantly affected by the treatment with OB-EVs (Figure 3D). Similar results were obtained when we measured the expression level of the master regulator of mitochondrial biogenesis, the peroxisome proliferator co-activator 1 alpha (PGC1α) (Figure 3D).

OB-EVs reduced MNNG/HOS motility and invasion

A key feature of aggressive cancer cells is the ability to migrate linearly and invade basal membranes. To check if OB-EVs affected these characteristics, we pre-treated MNNG/

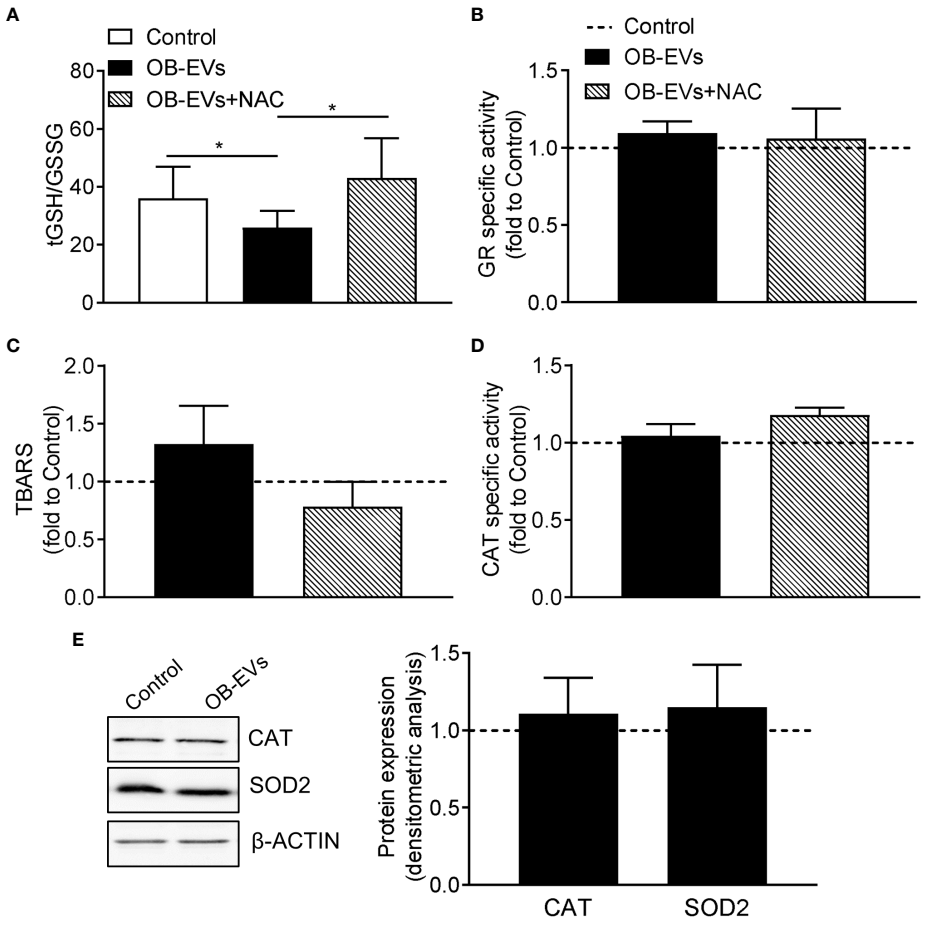

FIGURE 2
Effects of OB-EVs on redox status, lipid peroxidative damage and antioxidant protection of MNNG/HOS cells. **(A–E)** MNNG/HOS cells were plated and starved in serum-free DMEM O/N the next day. Then, cells were treated with DMEM (control), OB-EVs or OB-EVs + 5 mM N-acetyl-L-cysteine (NAC), for 24 h Cells were subjected to **(A)** quantification of total (tGSH)/oxidised (GSSG) ratio; **(B)** assessment of glutathione reductase (GR) specific activity; **(C)** assessment of TBARS to detect lipid peroxidation; **(D)** evaluation of catalase (CAT) specific activity; **(E)** β-actin-normalised immunoblot-based assessment of CAT and superoxide dismutase 2 (SOD2) protein levels. Representative images of Western blot were reported. **(A)** N=8, **(B, C, E)** N=5, **(D)** N=3. **(A)** Dunn's test, **(B, C, D)** one-way ANOVA, **(E)** one-sample *t*-test. *p<0.05. Data are presented as mean ± SEM.

HOS with EVs for 24 hours, then we assessed scratch wound healing and invasion assays to evaluate *in vitro* tumour cells motility and invasiveness, respectively. Interestingly, both functions were reduced by OB-EVs treatment (Figures 4A, B). However, metalloproteinases (MMPs) arrays ran on conditioned media of MNNG/HOS cells treated with OB-EVs showed no difference *versus* untreated MNNG/HOS (Figure 4C).

OB-EVs did not affect MNNG/HOS primary oncospheres formation

Another important characteristic of aggressive cancer cells is their *in vitro* stemness, which is an indication of how many tumour-initiating cells are present in the population of cancer cells. We assessed this by primary oncosphere formation assay (Figure 5A), but we found no differences in their number/seeded cells (Figure 5B) or total area (Figure 5C) of the spheroids in cells treated with OB-EVs *vs* control cells.

Discussion

Osteosarcoma is still a largely understudied neoplasia. The chances of survival for patients dramatically drop when metastases, preferentially to the lungs, develop (3) and therapy resistance occurs (6). Such disease progression is often achieved through the cross-regulation between osteosarcoma cells and bone cells in the TME, and EVs are

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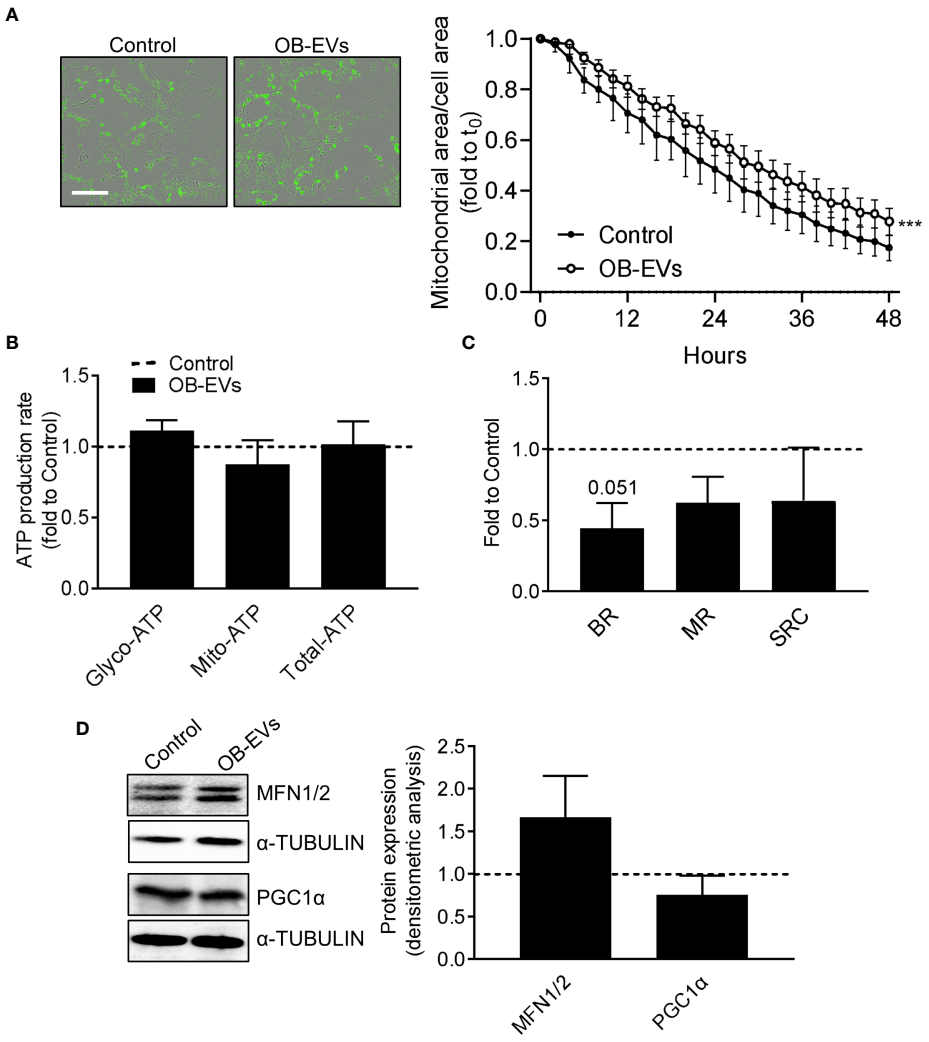

FIGURE 3
Effects of OB-EVs on mitochondrial function and dynamics of MNNG/HOS cells. MNNG/HOS cells were plated and starved in serum-free DMEM O/N and treated with DMEM (control) or OB-EVs for the time duration specified in the figures, or for 24h when not specified. Then, cells were subjected to **(A)** Mitotracker-based assessment of mitochondrial area/total cell area (scale bar=200 μm); **(B)** SeaHorse-based analysis of glycolytic/mitochondrial/total ATP production rate; **(C)** Mitostress assay to evaluate maximal respiration; **(D)** α-tubulin-normalised immunoblot-based assessment of protein expression of mitofusin 1/2 (MFN1/2) and PGC1α. Representative images of Western blots were reported. **(A)** N=5, **(B–D)** N=3. **(A)** Curve fitting test, **(B, C)** paired *t*-test. ***p<0.001. Data are presented as mean ± SEM.

emerging as key players in this regulatory interplay. Indeed, in a previous work, we demonstrated that MNNG/HOS-derived EVs influence osteoblasts, by reducing their differentiation and increasing their release of pro-inflammatory cytokines (20). Here, we wondered if also osteoblasts could signal back to osteosarcoma cells through EVs. Therefore, as a first basic approach, we aimed at evaluating whether OB-EVs could affect proliferation dynamics and viability in MNNG/HOS cells. As shown by the EdU-based approach, the proliferation rate of MNNG/HOS cells was unchanged upon treatment with OB-EVs. Conversely, we found that OB-EVs reduced the viability of MNNG/HOS cells, and this was observed using two different methods (i.e. Trypan blue-exclusion test and MTT assay). In order to verify whether such a reduction in cell viability was caused by increased apoptotic death, we measured caspase 3/7-dependent cell death by time-course fluorescence microscopy, and found no evidence of any change in casp3/7-dependent apoptotic rate in osteosarcoma cells treated with OB-EVs. Consistently, P53 protein levels were not affected by the treatment with EVs. This would suggest that the exposure to OB-EVs could induce necrotic death or even non-classical apoptosis in MNNG/HOS cells. In fact, despite the fact that it is generally acknowledged that members of the caspase family of proteases play a pivotal role

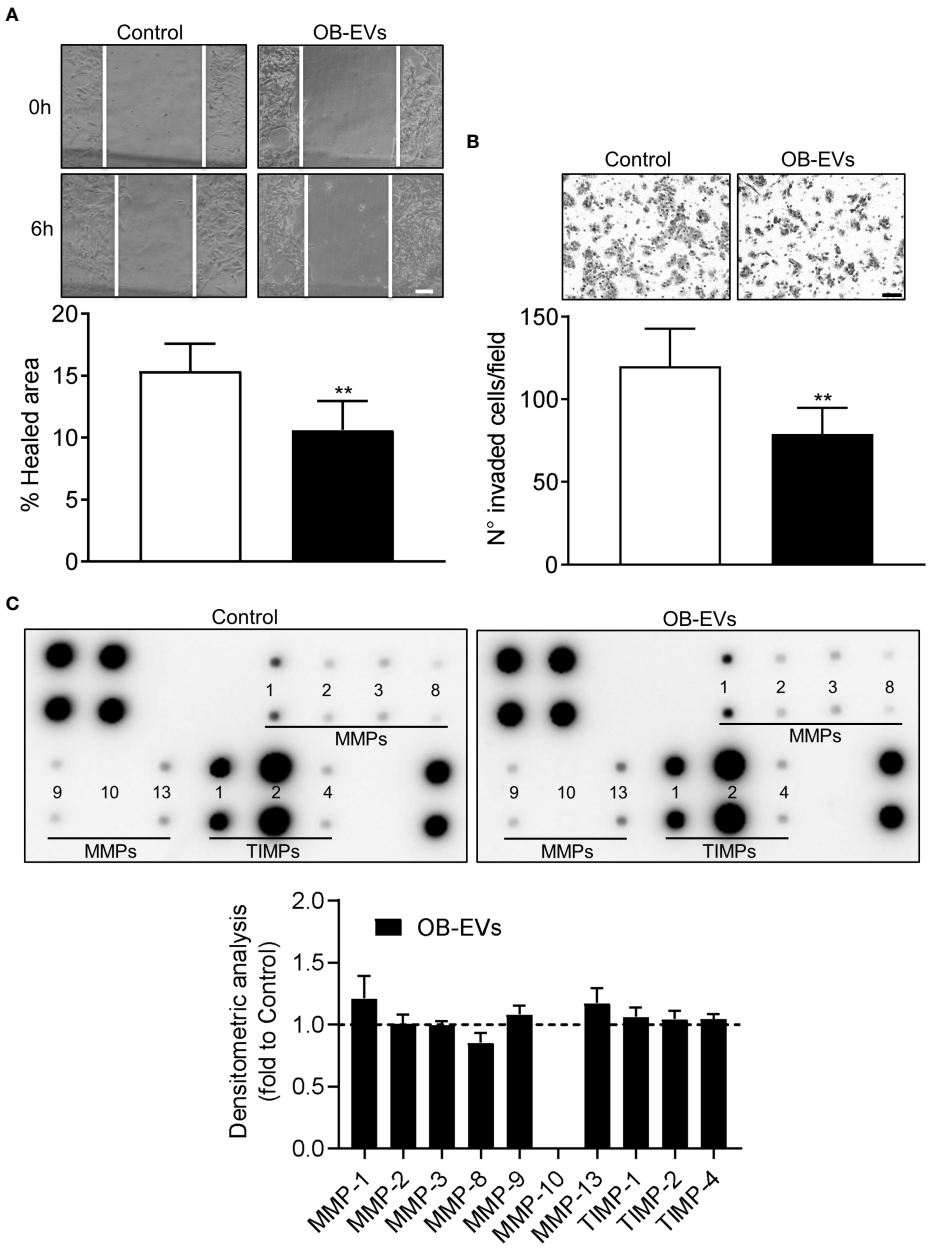

FIGURE 4
Effects of OB-EVs on motility and invasiveness of MNNG/HOS cells. MNNG/HOS cells were plated and starved in serum-free DMEM O/N the next day. Then, cells were treated with DMEM (control) or OB-EVs for 24 hours, **(A)** plated in 24 well plates to perform a scratch assay (scale bar=100 μm) or **(B)** seeded on the upper basket of a matrigel-coated transwell to perform an invasion assay over FBS (scale bar=100 μm). **(C)** Conditioned media (CM) were collected from DMEM- or OB-EVs-treated MNNG/HOS after 24 hours of treatment and subjected to metalloproteinase (MMPs) antibody array. Representative images of membranes incubated with CM from control or OB-EV-treated MNNG/HOS cells, and evaluation of secreted proteins (MMP-1, -2, -3, -8, -9, -10, -13, TIMP-1, -2, -4), as determined by densitometric analysis of spots of interest that were normalised for the positive control spots and shown as fold to control. **(A)** N=6, **(B)** N=5, **(C)** N=3. Paired Student's *t*-test. **p<0.01. Data are presented as mean ± SEM.

in the execution of apoptotic cell death (37), the existence of caspase-independent forms of programmed cell death (PCD) has been proved in the recent past (38–40). This intriguing result deserves further investigation to clarify the exact pathway activated by the exposure of osteosarcoma cells to OB-EVs, with particular focus on the redox-responsive non-classical forms of PCD, such as necroptosis, which has been recently proposed as an inducible pathway to activate an

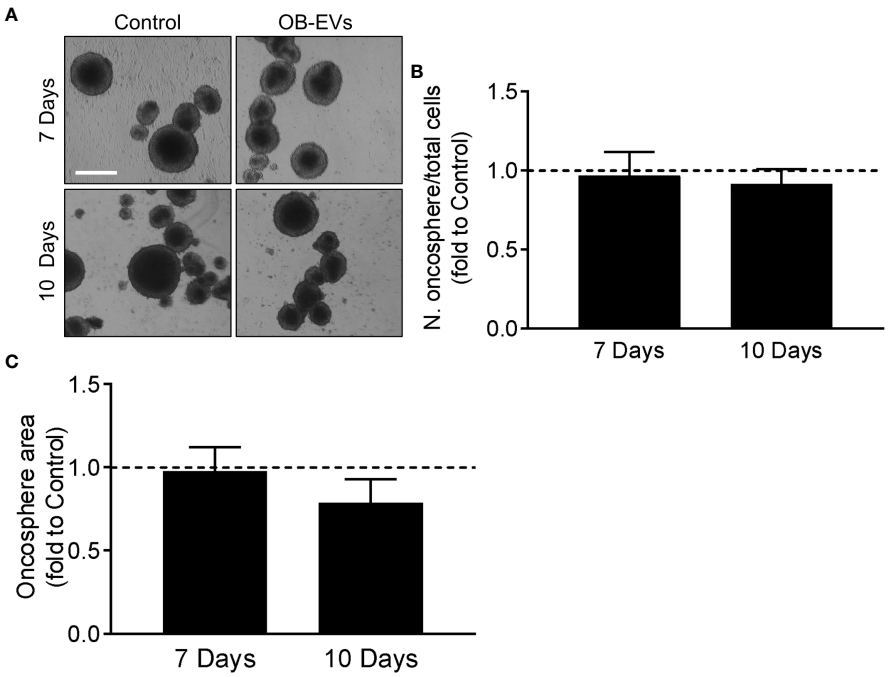

FIGURE 5
Effects of OB-EVs on stemness phenotype of MNNG/HOS cells. **(A–C)** MNNG/HOS cells were detached, reduced into a single-cell suspension and plated at a density of 2,000 cells/ml in oncosphere medium on poly-hydroxyethyl-methacrylate-coated plates and in a specifically-formulated medium to favour the formation of oncospheres, marker of stemness phenotype. Cells were then treated with DMEM (control) or OB-EVs twice a week. After 7 and 10 days, **(A)** pictures of the oncospheres were taken (scale bar=200 μm) to analyse their **(B)** number/plated cells ratio and their **(C)** total area. One sample *t*-test, N=4. Data are presented as mean ± SEM.

ordered form of cell death in osteosarcoma cells and osteosarcoma stem cells (41, 42).

The next step was to investigate two key features that often predict aggressiveness and tumorigenicity of cancer cells, such as migration and invasion capacities. With regard to this aspect, we describe here that OB-EVs reduced cellular motility and invasion capacity, even though no changes in the expression of the major matrix-degrading enzymes were detected. Taken together, these observations corroborate the idea that OB-EVs diminished the ability of osteosarcoma cells to invade the surrounding tissues, but this was probably not achieved by decreasing cellular capacity to digest matrix and basal membranes, but rather by reducing their overall ability to move. However, we cannot rule out the possibility that OB-EVs impaired some form of MMP-independent invasion process. Indeed, some authors have described that inhibitors of the proteolytic activity of MMPs were not able to prevent invasion of immortalised mouse epithelial cells through a sparse three-dimensional collagen matrix (43). Further experiments will attempt to clarify this aspect more in detail.

It is well-established that a subgroup of cancer cells, including osteosarcoma, is able to self-renew and initiate tumours *in vivo*. These are commonly termed cancer stem cells (CSCs), or tumour-initiating cells (TICs) (44, 45). CSCs have also been reported to present lower levels of ROS and a different redox balance compared to their non-stem counterparts (46), and we therefore deemed it interesting to evaluate whether OB-EVs would change the stem-like phenotype of osteosarcoma cells. To this end, we used the oncosphere formation assay, since the ability to form spheroids is directly associated with the stem cell-like phenotype in osteosarcoma (44, 47). However, our analysis showed that no differences were caused by treatment with OB-EVs, and control cells were able to form a similar number of similarly-sized spheroids.

The results presented so far suggested that OB-EVs elicited a clear cytotoxic effect in MMNG/HOS cells, significantly reducing the major traits of their malignant behaviour. In this context, redox homeostasis is increasingly considered as an essential resilience trait that helps cancer cells to preserve viability and growth capacity, along with resistance to external stressors (48–50). In particular, reactive oxygen species (ROS) serve as signalling entities in cells, regulating key cellular processes such as proliferation, survival, and adaptive response to external stimuli, as broadly reviewed by Sies and Jones (51). This is not only true in physiological conditions, but also in hyperproliferation-based diseases, such as in tumours and cancers (52). On this basis, we wanted to investigate whether

and how OB-EVs could cause biochemical and molecular alterations in the balance of the redox milieu of osteosarcoma cells. Here we show that a 24-hour treatment with OB-EVs significantly decreased the total/oxidised glutathione. Glutathione is a small endogenous thiol-containing tripeptide that in its reduced form (GSH) fuels direct and indirect ROS-scavenging antioxidant reactions within cells (53). Upon being used as a reducing molecule, GSH is promptly converted to glutathione disulfide (GSSG), therefore a decrease in the total/oxidised glutathione in a biological system indicates either that an over-production of pro-oxidants is occurring or that an impaired biosynthesis/recycling of glutathione is taking place (54, 55). In order to exclude that the recycling pathway was involved in such OB-EV-induced impairment, the specific activity of glutathione reductase (GR), the most important enzyme for recycling GSSG within cells (56) was assayed, and we found evidence that the enzymatic function of GR in OB-EV-treated cells was unaltered, nor was it changed by treatment with both OB-EVs and NAC, thus pointing out to the *de novo* formation of GSH as the redox-active biosynthetic pathway possibly involved in the pro-oxidant effect of OB-EVs on human osteosarcoma cells. This working hypothesis will be verified in the next future, by more specific experiments focused on evaluating the expression of the rate-limiting enzyme responsible for GSH synthesis (i.e., glutamate cysteine ligase). However, it should be also noted that cancer cells divert glucose utilisation to the pentose phosphate pathway (PPP) to support cell survival and growth by generating building blocks for nucleic acid synthesis, along with the NADPH needed for fatty acid synthesis and cell survival under stress conditions (57). Therefore, the possibility that the treatment with OB-EVs could alter PPP activity, thus down-regulating the synthesis of NADPH in osteosarcoma cells, will certainly be considered in our next work. Importantly, we also demonstrated that EVs isolated from osteoblasts included both the glutathione reductase and the *Gclc* subunit transcripts, thus further confirming that the glutathione redox homeostasis of the cells interacting with the vesicles might be altered.

More in general, the shift of the glutathione redox equilibrium towards a more oxidised state might indicate the occurrence of an oxidative stress condition (54, 55). On this basis, we hypothesised that OB-EVs may elicit a pro-oxidant effect on osteosarcoma cells. The fact that OB-EVs served as a ROS-dependent stimulus on MMNG/HOS cells was confirmed by the observation that the co-incubation with the ROS-scavenging compound and cysteine-donor N-acetyl-L-cysteine (NAC) was able to revert completely the pro-oxidant effect of OB-EVs in terms of glutathione redox balance. Interestingly, NAC also inhibited the cytotoxic effect of OB-EVs, thus strongly suggesting that the cell death induced by OB-EVs was dependent on the ROS-promoting effect of extracellular vesicles.

In order to further investigate more in detail how osteosarcoma cells were challenged by OB-EVs *via* ROS-dependent mechanisms, we measured the specific activity of catalase, which is known to play a critical role in first line defence against oxidative stress within cells (55, 58, 59). Our experiments revealed that the treatment with OB-EVs did not affect the specific activity of catalase, nor did the treatment change CAT protein expression level. Similar results were obtained when the protein level of another important antioxidant enzyme (namely, the superoxide dismutase 2) was studied. SOD2 and CAT are inducible enzymes whose up-regulation upon oxidative stress represents a critical step in the adaptive response of cells to pro-oxidant stimuli, at mitochondrial and peroxisomal level, respectively (47, 60–63). Taking into account the pivotal role played by mitochondria and peroxisomes in the regulation of the redox metabolism in the eukaryotic cell (64), the findings we present here suggest either that the stress level induced by OB-EVs may have cytotoxic effect before any adaptive response in osteosarcoma cells could initiate, or that the OB-EV-dependent cytotoxicity could rely more on a mild imbalance of redox homeostasis rather than on severe oxidative stress condition. In order to settle this, we measured the levels of TBARS, which is a well-established marker of lipid peroxidative molecular damage (65). Our experiments did not reveal any OB-EV-induced change in the TBARS levels. This suggests that the treatment with OB-EVs could activate a redox-dependent cell death pathway in osteosarcoma cells without initiating a state of full-blown ROS-based molecular damage. This seems to be confirmed by the evidence that mammalian catalase, which intervenes in the antioxidant defence when the level of oxidative stress within cells is severe (66), was unaffected by the treatment with OB-EVs. On this basis, our results suggest that OB-EVs may act as a cytotoxic stressor for osteosarcoma cells more through a modest imbalance of redox homeostasis rather than *via* promoting an oxidative stress condition. However, it cannot be ruled out that assessments at different time points might reveal a pattern of tentative antioxidant response, at least at the enzymatic level. Nor can we rule out the possibility that OB-EVs could cause oxidative-dependent damage to macromolecules other than lipids. Such hypotheses will be clarified by more targeted experimental approaches in the future.

Mitochondrial redox-active metabolism and ROS-promoting enzymatic reactions, along with ATP generation, seems to serve as a crucial determinant for malignant phenotype and anticancer drug-resistance (48, 67, 68). Moreover, even though many cancers exhibit increased rates of glycolytic catabolism, increasing proofs support the idea that mitochondria remain a crucial source of ATP, and serve as key organelles that integrate a plethora of metabolic and signalling pathways in tumours and malignancies (69). On this basis, we wanted to verify whether OB-EVs could also alter the function and the extension of the mitochondrial network in osteosarcoma cells. Our Mitotracker-based investigation revealed that cell growth determined a decline in the extension of the

mitochondrial compartment in both OB-EVs-treated and control osteosarcoma cells, and this was most likely due to the growth of the cytoplasm that accompanied cell growth. However, in OB-EVs-treated cells the decline of the mitochondrial network over the timeframe of 48 hours was less rapid. This could be due to the inhibiting effect of OB-EVs on cell growth, or a compensatory mechanism that counteracts a possible detrimental effect on mitochondria. Most importantly, it should be noted that the difference between OB-EVs-treated and control at 24h, *i.e.* the time at which all other assays were performed, was not statistically significant. Although OB-EVs did not change the glycolytic/OxPhos ATP ratio, MitoStress experiments revealed an almost significant trend (p=0.051) of reduction in basal respiration, and a reduction in maximal respiration and spare respiratory capacity in 3 over 4 experiments analysed in OB-EVs-treated cells. Considering the significance of such parameters (70), this result might suggest that OB-EV-treated osteosarcoma cells could have a lower threshold below which the basal ATP demands cannot be satisfied. This may be important, as the exposure to OB-EVs could weaken osteosarcoma cells towards further exogenous stressors, with clear repercussions on oncology research and clinical treatments.

Reprogramming of mitochondrial activity is thought to underlie chemoresistance and metastatic behaviour (69). In addition, mitochondrial fusion and biogenesis, which participate to the so-called mitochondrial dynamics, dictate mitochondrial morphology and function (71–73), influence the redox homeostasis and antioxidant defence of cancer cells, along with their apoptotic response to OS-promoting chemotherapeutics (74). On this basis, we investigated whether the expression of the major controllers of mitochondrial fusion and biogenesis was affected by OB-EVs, however no evidence was found for such an effect, as shown by the unaltered protein levels of MFN1/2 and PGC1α. MFN1/2 and PGC1α are key players belonging to the fine-tuned molecular pathway that govern mitochondrial dynamics (75). In this context, our results clearly suggested that the redox-dependent cytotoxic effect elicited by OB-EVs on osteosarcoma cells was not associated with any major re-organisation of the mitochondrial compartment. This finding was substantially confirmed by the IncuCyte-based analysis of the extension of the mitochondrial network. In fact, we observed that a 24-h treatment with OB-EVs did not significantly change the area of the mitochondrial network. As expected, our investigation revealed by fluorescence microscopy that the Mitotracker-positive area decreased in a time-dependent fashion. This was most likely due to cell growth over the time. However, our observations revealed that in OB-EV-treated osteosarcoma cells the Mitotracker-positive area decreased over time less rapidly than in control cells. This could be due to the cytotoxic action of OB-EVs that inhibited the growth of the confluence phase area in treated cells.

In conclusion, our findings suggest that osteoblast-derived extracellular vesicles could be an important means of cross-communication between the TME and the osteosarcoma core. For the first time to the best of our knowledge, we proved that OB-EVs reduced osteosarcoma cells' aggressiveness and viability through redox-dependent signalling pathways. However, we did not find strong evidence of the involvement of changes in mitochondrial dynamics or major energy metabolic switch in the phenotypic change induced by osteoblast-derived extracellular vesicles in osteosarcoma cells. It should be noted that OB-EVs were isolated from osteoblasts that are not being co-cultured with osteosarcoma cells, therefore representing a model of "healthy" osteoblasts, whereas we demonstrated (20) that MNNG/HOS-EVs cause devastating effects on them, thus changing their phenotype. Hence, we could speculate that keeping osteoblasts ``healthier" by preventing the effect of HOS-EVs on them, would result in reduced osteosarcoma aggressiveness. Although this has not been observed in osteoblasts, similar effects are exerted by mesenchymal stem cells-derived EVs (MSC-EVs), which are closely related to osteoblast and can differentiate into them (76). In fact, MSC-EVs reduce osteosarcoma aggressiveness when harvested from healthy MSCs (77, 78), and conversely, they promote osteosarcoma aggressiveness when co-cultures between the two cell types are established (78, 79).

Should the pathophysiological relevance of this phenomenon be confirmed *in vivo*, osteoblasts could be exploited as endogenous anti-cancer weapons, as proposed for leukaemias by other researchers (80).

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by Ministero della Salute italiano (Italian Ministry of Health).

Author contributions

MP: Methodology, investigation, data curation, visualisation, software, writing - original draft, writing - review & editing. AU: Methodology, investigation, data curation, visualisation, writing-review & editing. CP: Methodology, investigation, data curation. LG: Methodology, investigation, data curation. IF: Methodology, investigation, data curation. DC: Conceptualisation, methodology, investigation, data curation, resources. FZ: Conceptualisation, data curation, writing - review & editing. AC: Conceptualisation, data curation, methodology, review & editing. NR: Conceptualization,

supervision, funding acquisition, methodology, investigation, writing - original draft, writing - review & editing. SF: Conceptualization, supervision, funding acquisition, methodology, investigation, writing - original draft, writing - review & editing. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.983254/full#supplementary-material

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The role of extracellular vesicles and interleukin-8 in regulating and mediating neutrophil-dependent cancer drug resistance

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Tumor drug resistance is a multifactorial and heterogenous condition that poses a serious burden in clinical oncology. Given the increasing incidence of resistant tumors, further understanding of the mechanisms that make tumor cells able to escape anticancer drug effects is pivotal for developing new effective treatments. Neutrophils constitute a considerable proportion of tumor infiltrated immune cells, and studies have linked elevated neutrophil counts with poor prognosis. Tumor-associated neutrophils (TANs) can acquire in fact immunoregulatory capabilities, thus regulating tumor progression and resistance, or response to therapy. In this review, we will describe TANs' actions in the tumor microenvironment, with emphasis on the analysis of the role of interleukin-8 (IL-8) and extracellular vesicles (EVs) as crucial modulators and mediators of TANs biology and function in tumors. We will then discuss the main mechanisms through which TANs can induce drug resistance, finally reporting emerging therapeutic approaches that target these mechanisms and can thus be potentially used to reduce or overcome neutrophil-mediated tumor drug resistance.

KEYWORDS

drug resistance, neutrophil, interleukin-8, extracellular vesicles, Tumor-associated neutrophils, NETosis

1 Introduction

During the past decades, huge progress has been made in the field of cancer genetics, immunology and pathology for the identification of new markers and methods for diagnosis and treatments (1, 2). Despite these achievements, resistance to classical chemotherapeutic agents or to novel drugs is one of the major causes of therapy failure and death in cancer, still representing a crucial limiting factor in the treatment of cancer patients (3).

The mechanisms through which cancer cells get resistant or acquire resistance to drug therapies are numerous, and sometimes tumors can be resistant to multiple therapies and display, simultaneously or subsequently, different mechanisms of drug resistance. In this context, it has been proposed that the mechanisms of drug resistance in tumors can be both active (cell-autonomous) or adaptive (non-cell-autonomous): the firsts depend on cancer intracellular responses, which include, for example, genetic or epigenetic alterations that promote cell survival (4–7), while the seconds result from tumor interactions with the surrounding tumor microenvironment (TME) (8, 9) that is shaped to favor tumor growth, expansion and drug resistance.

Together with myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs), tumor-associated neutrophils (TANs) represent the most abundant population (10, 11) of immune cells infiltrated in the TME, and many studies so far have highlighted the link between elevated TAN counts and increased risk of metastasis, drug resistance and poor prognosis (12–17). In TME, neutrophils can acquire immunoregulatory capabilities, facilitating tumor progression (18) and drug resistance through a number of different mechanisms. In this context, interleukin-8 (IL-8, aka CXCL-8), a member of the CXC chemokine family that is highly produced by neoplastic cells (19), is an important chemoattractant and activator for neutrophils and is a key mediator of their biology, behavior and actions inside the tumor. On the other hand, increasing evidence is highlighting the crucial role of extracellular vesicles (EVs) in both the mediation and regulation of neutrophils' response within the TME. EVs, produced both by tumor cells and by TANs, or by other immune or stromal cells, function in fact as intercellular mediators of the communication within the TME and beyond, and can ultimately promote neutrophil-mediated tumor drug resistance (20).

In this review, we will describe the role of IL-8 and EVs in the regulation and mediation of neutrophil biology and function in the TME, promoting pro-tumoral functions of these cells, ultimately leading to neutrophil-mediated tumor drug resistance through the production of neutrophil extracellular traps (NETs) and the secretion of neutrophil-derived EVs and other factors. Finally, we will discuss emerging therapies that, targeting IL-8, EVs and neutrophil functions, could be considered as potential therapeutic tools to reduce or overcome neutrophil-mediated tumor drug resistance.

2 Neutrophils in cancer

Neutrophils are the most abundant leukocytes in the circulation, representing around 70% of all white blood cells (21). Produced in the bone marrow (BM) through the granulopoiesis (*i.e.*, progressive maturation) of hematopoietic progenitors, neutrophils are then released into the blood stream, ready to respond to a plethora of stimuli released by inflamed tissues (22). In tumors, several chemotactic and inflammatory factors, as well as EVs, are released from tumoral and non-tumoral cells and can attract mature neutrophils, which thus migrate from blood stream and infiltrate into the TME (23–25) (Figure 1).

Neutrophil recruitment into the tumor site from circulation is a multi-step process that involves several factors, but seems to be mainly regulated by two G protein-coupled receptors (GPCRs): CXCR4 and CXCR2 (26). CXCR4 is a neutrophil homing marker in the bone marrow, while CXCR2 activation by its ligands (i.e., CXCL-1, CXCL-2, CXCL-3, CXCL-5, CXCL-6, CXCL-7 and CXCL-8) induces the release of neutrophils into circulation and their recruitment into the TME (27–29). Indeed, the diverse cell types in the TME (*e.g.*, tumor cells, immune cells, fibroblasts) release large quantities of CXCR2 ligands, forming a chemotactic gradient that attracts the neutrophils from the bloodstream (29). Among the chemokines that can influence neutrophil functions, IL-8 is the master regulator of neutrophil biology and one of the most characterized chemokine in cancer as it has been found overexpressed in several tumors (30–43). Once into the TME, neutrophils turn into TANs, a plastic and dynamic population that can rapidly switch between two forms: N1 TANs with anti-tumoral functions, and N2 TANs, with pro-tumoral effects (44–46). N1 TANs are mature and short-living cells, which exert their highly cytotoxic and immune-stimulating activities by producing reactive oxygen species (ROS) and other cytotoxic substances, and by recruiting and activating other immune cells (17). On the other hand, N2 TANs are immature and long-living cells, which can produce and release cytokines, chemokines and other factors to favor pro-angiogenic, pro-metastatic and immune-suppressive activities (47). TANs polarization towards one of the two sub-populations is crucially regulated by multiple TME factors including, among others, cytokines and chemokines, such as IL-8, and also EVs, released by tumor, stromal and immune cells (17, 48, 49). In addition, TANs can also regulate cancer progression through NETosis, a process by which neutrophils extrude a sort of web-like structures called NETs (50–52). NETs are formed by DNA fibers decorated with cytotoxic enzymes, such as neutrophil elastase (NE), myeloperoxidase (MPO) and matrix metalloproteinases-9 (MMP-9) and are released by activated neutrophils into the extracellular space as mechanism of defense against pathogen micro-organisms (53). In tumors, NETs have been identified as factors that can

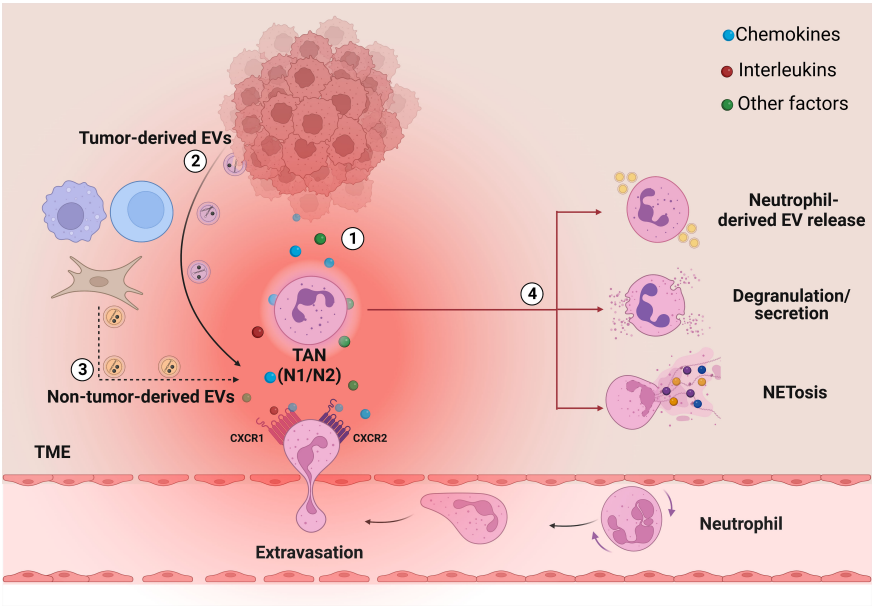

FIGURE 1
The role of secreted factors and EVs in neutrophil recruitment and activation into the TME. In TME, cancer cells can regulate neutrophil biology through the secretion of several factors, among which (1) interleukins and chemokines, such as IL-8, and (2) tumor-derived EVs. These factors regulate the recruitment of the neutrophils from the bloodstream to the tumor and the generation of TANs, which in turn promote cancer progression, metastasis and drug resistance through different cell mechanisms: release of neutrophil-derived EVs, degranulation/secretion and NETosis (4). Together with cancer cells, also other cell types in the TME can release vesicles, which are also potentially able to act on neutrophils (3, dashed arrows). Image created with biorender.com.

significantly contribute to carcinogenesis and metastasis (11, 54) in several ways, as by inducing the degradation of the extracellular matrix which promotes the extravasation of cancer cells (50), trapping circulating tumor cells (CTCs) (55, 56) or deactivating thrombospondin-1 (TSP-1), a potent inhibitor of angiogenesis and tumor progression (57, 58).

Regulated by different factors and acting through several mechanisms, neutrophils thus play a key role in hijacking the immune system response against the tumor, ultimately promoting cancer progression and tumor drug resistance (59, 60).

3 IL-8 and EVs crucially regulate and mediate the biology and functions of TANs in the TME

3.1 IL-8 and TANs

Tumor cells produce several factors, such as cytokines, chemokines, lipids, and growth factors that, not only increase their growth and survival in an autocrine manner (61), but also increase the number of circulating neutrophils by stimulating granulopoiesis in the bone marrow and promote their recruitment to TME in a paracrine manner (62–64) (Figure 1). In particular, among other factors, IL-8 has demonstrated to be crucial in tumor progression (19, 65–67), since it was found to be overexpressed in several tumors, where induces angiogenesis and is involved in the maintenance of cancer stem cells (CSCs) (68, 69). Also, a direct correlation between IL-8 and poor prognosis has been reported (70–73). IL-8 exists as a monomer or dimer and exerts its activity by binding its two receptors: CXCR1 and CXCR2 (74). It is a well-known chemoattractant able to recruit leukocytes and in particular neutrophils, which express a substantial number of IL-8 receptors on their surface (75, 76). During carcinogenesis, the IL-8 released by neoplastic cells promotes the activation of both the phosphatidylinositol-3-kinase (PI3K) and the mitogen-activated protein kinase (MAPK) signal pathways *via* CXCR2, thus leading to cell migration and survival (77–79). In addition, IL-8 mediates the formation of NETs, through the binding to CXCR1 and CXCR2 (18, 80) (Figure 1). These mechanisms help to dampen the anti-tumor immune responses and cause disfunctions of cytotoxic immune cells, thus crucially contributing to tumor growth and progression (81).

3.2 Extracellular vesicles and TANs

In addition to IL-8, EVs are other factors that are crucially involved in the regulation and mediation of TANs' pro-tumoral functions in the TME (82). EVs are heterogenous lipid bilayer

structures secreted by cells that can carry a plethora of cargoes, including lipids, proteins and nucleic acids (83–86). In the past they were divided in three subtypes (microvesicles (MVs), exosomes and apoptotic bodies) depending on their biogenesis, release pathways, size, content and functions (84, 87, 88). However, since it is not easy to clearly determine EVs biogenesis pathway, the last MISEV guidelines (MISEV 2018) suggest to classify EV subtype referring to 1) physical characteristics of EVs, such as size or density; 2) biochemical composition; or 3) descriptions of conditions or cell of origin (89). One of the main functions of EVs is to facilitate the exchanges of cellular components, acting as an intercellular communication system in both physiological and pathological conditions (88, 90, 91). The EV-mediated intercellular communication is achieved in two manners: by delivering cargoes that are within the vesicles in the target cells (92, 93), or by using EVs surface markers without requiring vesicle internalization (94). In tumors, EVs are important components of the TME and promote the crosstalk between cancer and cancer-associated cells (e.g. fibroblasts, endothelial and immune cells), creating a favorable niche that supports and nourishes the tumor, promoting its growth and progression, and also regulating tumor drug resistance.

Tumor-derived EVs are nanoscale membrane vesicles (95) that contain tumor-specific functional biomolecules both in their lumen, such as cytokines, growth factors, proteases and enzymes, as well as on their surface, including receptors/ligands, adherent molecules, or tetraspanins (96, 97). Tumor-derived EVs work in both autocrine and paracrine way to favor local invasion of tumor cells and spreading of metastasis and to induce the reprogramming of recipient cells (82, 98–101). They can also promote immune-modulation by attenuating the cytotoxic activity of T and NK cells, prompting the recruitment of regulatory B cells and Tregs and inducing the differentiation of M2 macrophages and N2 immune-suppressive sub-population of tumor-associated macrophages (TAMs) and TANs, respectively (102, 103), thereby creating a pro-tumorigenesis environment for tumor progression (104–106).

Among innate immune cells, neutrophils may be especially prone to stimulation from tumor-derived EVs (107); for example, they can promote TAN polarization into the anti-inflammatory N2 tumorigenic subtype (Figure 1). Although the underlying mechanisms remain poorly understood, Zhang and colleagues have recently started analyzing tumor-derived EVs induced N2 neutrophil polarization in gastric cancer, demonstrating that gastric cancer-derived EVs can induce the expression of programmed death-ligand 1 (PD-L1) on neutrophils, which in turn polarizes their differentiation through the N2 phenotype and suppresses T cell-mediated immunity (108, 109). On the other hand, tumor-derived EVs from murine colorectal CSCs have been shown to prolong bone marrow-derived neutrophil life-span through the activation of the NF-κB signaling, which in turn induces the expression of interleukin-1β (IL-1β) in neutrophils, thus promoting their pro-tumoral phenotype (110). Besides inducing N2 polarization, tumor-derived EVs can also modulate other properties of neutrophils' biology. Tumor-derived EVs from a metastatic human melanoma cell line (MV3), for example, have been shown to induce neutrophil chemotaxis through the CXCR2/PI3K-Akt axis and to promote the formation of NETs (103) (Figure 1), which play a crucial role in inducing cancer-associated thrombosis (111–113) and tumor drug resistance (114). Similar results have been obtained in a mouse model of breast cancer, where 4T1-derived exosomes induced NETs formation in neutrophils derived from G-CSF-treated mice and accelerated venous thrombus formation in tumor-free neutrophilic mice (115). Also the EVs released from a human cell line of breast carcinoma (MDA-EVs) induced neutrophil activation (*i.e.*, increased chemotaxis and secretion of IL-8 and MMP-9), N2-like phenotype and increase of ROS production, which were followed by augmented NETosis (116). Finally, a recent report also showed that exosomes can transfer mutant KRAS from DKO-1 colorectal cancer cells to neutrophils, resulting in increased IL-8 production, neutrophil recruitment and NETs formation, ultimately promoting tumor growth and metastasis. Interestingly, these effects were abolished by an anti-IL-8 treatment (117).

Although tumor-derived EVs represent the majority of vesicles secreted in the TME, studies have shown that EVs can be released also by other cells within the TME, such as cancer-associated stromal cells (CASCs), including fibroblasts, immune cells, endothelial cells and neurons (118) (Figure 1). These EVs can influence many aspects of tumor biology, but their direct role in the regulation of neutrophil biology has not been fully addressed yet. For example, cancer associated fibroblasts (CAFs) can secrete EVs which act on cancer cells to enhance their metastatic potential by delivering bioactive molecules, such as extracellular matrix proteins and remodeling enzymes (118). Ji et al. demonstrated that primary colorectal cancer cells can secrete integrin beta-like 1 (ITGBL1)-bearing EVs which enter the circulation, reach distant organs, and activate fibroblasts *via* the TNFAIP3-mediated NF-κB signaling (119). In addition to fibroblasts, also immune cells can release EVs within the TME ultimately exerting anti-cancer effects as for natural killer (NK) cells, or pro-cancer effects in the case of regulatory T cells (Tregs) (120). NK cell-derived EVs are released by resting and activated NK cells and both can exert cytotoxic activity on activated but not resting immune cells (121), but also exhibit immune-modulatory activity by stimulating other immune cells *via* paracrine action or through the circulatory system (122).

Further studies are needed to better understand if this subset of non-tumor-derived EVs may have a direct role in the regulation of neutrophil biology in the context of tumor progression.

4 TAN-mediated tumor drug resistance

The involvement of neutrophils in tumor drug resistance is determined by the interplay of several factors. Among others, IL-8 and EVs are key modulators of neutrophil biology and

functions within the TME. They act on neutrophils to promote tumor drug resistance which is exerted through different mechanisms, such as release of neutrophil-derived EVs, secretion of specific molecules/factors and NETosis (Figure 1). These mechanisms can act in a concerted way to promote tumor drug resistance by reducing the availability or stability of administered therapeutics, inducing ROS production or alterations of DNA damage repair pathways, and modulating antitumor immunity (123).

4.1 Tumor drug resistance promoted by neutrophil-derived EVs

Like tumor cells, neutrophils can also produce and release EVs in response to intracellular metabolic changes and/or extracellular environmental stress. As reviewed by Rubenich and colleagues, the genetic and molecular composition of neutrophil-derived EVs reflects that of the mother cell and varies depending on the existing physiological or pathological conditions (20, 124). Depending on the context, neutrophils polarize into inflammatory N1 or regenerative N2 subtypes, which are thought to be able to release two different kinds of EVs: the N1-derived and the N2-derived EVs, respectively (124).

During cancer progression, the role of neutrophil-derived EVs seems to be important for the prediction of disease outcome, although the underlying mechanisms are still unclear (125). Even if few, the available evidences on neutrophil-derived EVs isolated from tumoral contexts seem to mainly suggest a role for these vesicles in mediating cancer progression and drug resistance. On the other hand, EVs produced by neutrophils from healthy donors may possess a tumor suppressive activity both *in vitro* and *in vivo* (126). As recently demonstrated in fact, EVs from healthy neutrophils contain cytotoxin proteins that are able to activate the caspases signaling pathway and then promote tumor cell apoptosis (126) (Figure 2).

The role of neutrophil-derived EVs in drug resistance has been demonstrated by a recent work from Butin-Israeli and colleagues (127). Using samples from inflammatory bowel disease (IBD) patients, who are more prone to develop colitis-associated colorectal cancer and have an important neutrophil infiltrate in the intestinal mucosa, they demonstrated that neutrophil-derived EVs containing miR-23a and miR-155 inhibited Homologous Recombination (HR) repair by targeting the main HR regulators RAD51 while promoting non-homologous DNA end joining (NHEJ), ultimately leading to the formation of highly mutagenic DNA Double-Strand Breaks (DSBs) (127). This switch from HR to NHEJ may result in the acquisition of drug resistance in tumors (128–131) as observed in colorectal cancer, in which neutrophil-mediated NHEJ induced resistance to a lethal dose of topo-isomerase I inhibitor Camptothecin (CMPT) as tumor cells effectively resolved CMPT-induced DSBs and entered normally into cell cycle (132) (Figure 2). Other evidence for the role of neutrophil-derived EVs in cancer, suggest that they can act either as an onco-suppressor (133–136) or as an onco-promoter (137–139) in a context-dependent manner. For example, neutrophil-derived EVs containing miR-223, a miRNA essential for the development of cells of the myeloid lineage and the mobilization of neutrophils from the bone marrow (140–142), have been described to be able to both sustain and inhibit tumor growth (135, 137–139, 143). In both acute myeloid leukemia and breast cancer for instance, E2F1-dependent downregulation of EVs-transported miR-223 is associated with tumor aggressiveness and poor prognosis (135, 143). Of note, a clear role of neutrophil-derived EVs carrying miR-223 in drug resistance still remains unknown (Figure 2).

Interestingly, in addition to regulate tumor progression and drug resistance, neutrophil-derived EV have recently also been engineered to efficiently deliver anti-cancer drugs at the tumor site (126), thus not only demonstrating the intricate complexity of the processes regulating neutrophil-derived EVs content and secretion but also showing the therapeutic potential of these vesicles.

4.2 Tumor drug resistance promoted by TAN-released factors and NETosis

Attracted to the tumor site and regulated by the action of IL-8, EVs and other chemotactic factors, TANs can interfere with different antitumoral treatments not only by releasing EVs but also by secreting specific factors as well as by undergoing NETosis. During degranulation and NETosis, TANs can for example increase the secretion of matrix metalloproteinases (MMPs), such as MMP-2 and MMP-9, thus counteracting the effects of anti-angiogenic therapies. MMP-9, the production of which is also directly induced by IL-8 through CXCR2 receptor (144), can in fact cleave matrix-bound isoforms of VEGF-A into soluble fragments that are able to elicit VEGFR2 receptor activation and induce angiogenesis with a higher potential than uncleaved protein (145, 146) (Figure 3). In addition, TANs can directly secrete the pro-angiogenic cytokine IL-17 (147) or induce the activation of cathepsin B/NLRP3 inflammasome followed by IL-1β overproduction, with consequent increase of IL-17 secretion (148, 149) (Figure 3).

Besides secreting factors in the TME, TANs can mediate drug resistance also through the formation of NETs or through the activities of several NET-associated components (Figure 3). In agreement with this, increased levels of cell free cell free DNA (cfDNA), which is at least in part derived from NETs, predict limited response to chemo- and immune-therapy in several tumors (150–152). NET components, including NE, MMP-9, Cathepsin G (CG), the carcinoembryonic antigen cell adhesion molecule 1 (CEACAM1), and other factors, have been shown to promote resistance to chemotherapy through different

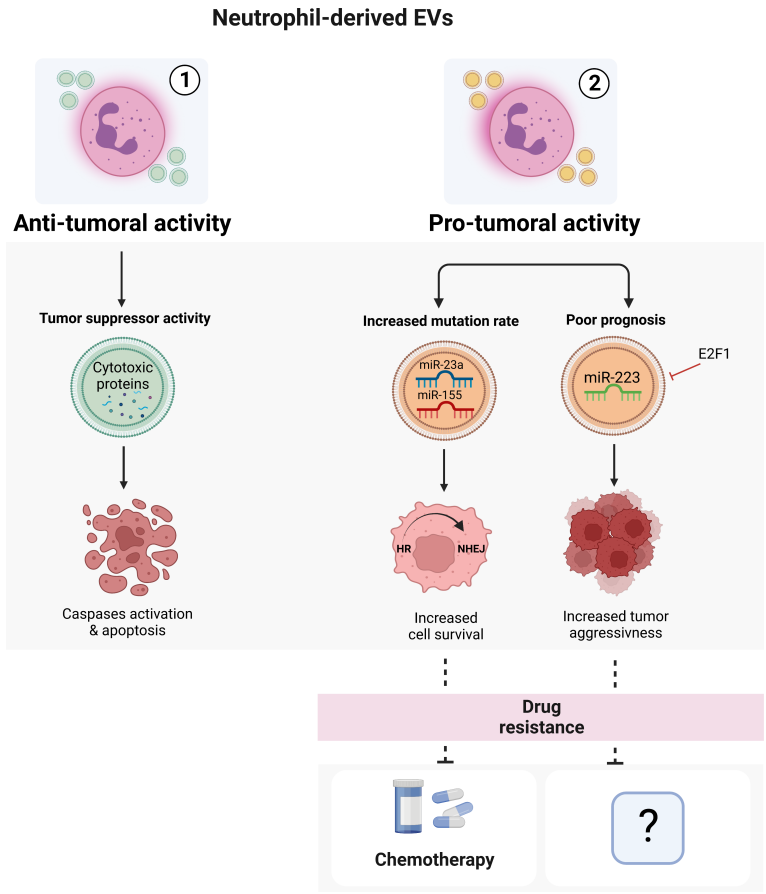

FIGURE 2
Neutrophil-derived EVs. Neutrophil-derived EVs can exert both anti-tumoral (1) or pro-tumoral (2) activities in a context-dependent manner. The EVs isolated from healthy neutrophils can induce apoptosis of cancer cells through the activation of caspases pathway. On the other hand, tumoral neutrophil-derived EVs seem to promote cancer spreading, progression and drug resistance. Image created with biorender.com.

mechanisms (55, 153–156). Preclinical studies suggest that NE can promote malignancy and resistance to chemo- and immune-therapy by inducing cell epithelial-mesenchymal transition (EMT) (153, 154, 157). Evidence emerged to support the infiltration of neutrophils into TME as a driver of EMT through NE activity (158–160). On the other hand, MMP-9 and CG, associated with NETs, mediate the degradation and remodeling of the extracellular matrix and, as discussed above, promote angiogenesis, so that their presence has been associated with tumor progression and poor response to chemotherapy (155, 161). Finally, CEACAM1 protein, that decorates NETs and facilitates NET-dependent pro-metastatic interactions by improving neoplastic cells adhesion and migration, is potentially involved also in mediating cancer response to therapy (156) (Figure 3).

Increased NETosis promotes tumor resistance also to radiation-therapy (RT) (162). In a syngeneic bladder cancer model, RT increased NET deposition and, notably, when NETosis was inhibited by DNase I or neutrophil elastase inhibitor, the overall radiation response improved. Consistently with these data, NETs have been also observed in bladder tumors of patients who did not respond to RT and had persistent post-RT relapse (163, 164) (Figure 3). In addition, tumor-associated NETs can also support metastatic cells to evade immune response by creating a physical shield from cytotoxic immune cells, such as cytotoxic CD8+ T and natural killer cells (NKs), thus preventing interactions between tumor and effector immune cells (165, 166) (Figure 3). In line with this, NETs formation has also been shown to mediate the resistance to checkpoint blockade, thus reducing responses to immunotherapy (18, 167–169). NETs can also have a role in detoxifying tetracycline drugs, such as doxorubicin (Figure 3), and degradation of NETs through DNase treatment restored chemosensitivity in animal models, demonstrating a functional role for NETs in chemo-resistance (166). Although this finding has yet to be corroborated in other tumors, this emerging evidence is notable since it raises NETs as therapeutic targets for the improvement of chemotherapy response.

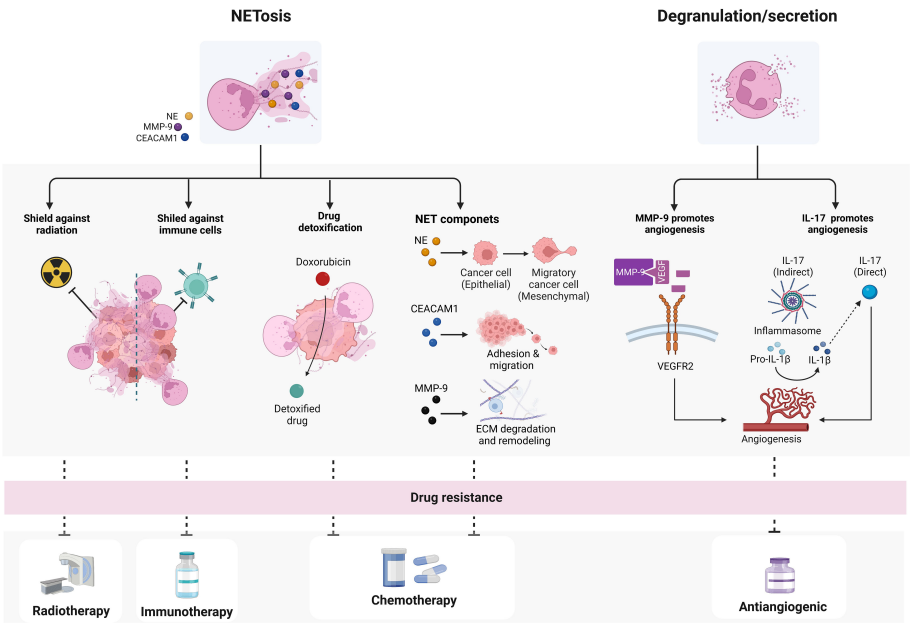

FIGURE 3
Mechanisms by which TANs may confer drugs resistance. TANs can promote drug resistance through two main mechanisms: NETosis and degranulation/secretion. NETs or NETs components can mediate resistance to immune- radio- and chemotherapy, while neutrophil-secreted factors have been shown to mainly influence angiogenesis and interfere with angiogenic therapies. biorender.com.

Pharmacological NETosis inhibition has been shown to synergize with immunotherapies, such as anti-PD-1 and anti-CTLA-4 mAbs (18, 170), possibly by favoring cytotoxic effector T cell response against cancer cells following checkpoint inhibition. As further confirmation of the role of NETosis in immunotherapy resistance, it has been demonstrated that hPMNs recruited by IL-17 in pancreatic ductal adenocarcinoma undergo NETosis, and when NETosis is abrogated, the tumor acquires an immunotherapy-sensitive phenotype (171).

In conclusion, TANs and related regulatory factors and mediators (*i.e.*, IL-8, EVs, and other secreted factors) represent potential targets for novel therapeutic approaches aiming to target cancer cells and reduce drug resistance.

5 Therapeutic strategies to inhibit neutrophils in cancer progression and cancer drug resistance

5.1 Investigational drugs

5.1.1 Targeting CXCR1/2 and neutrophils

With the aim to overcome the deleterious effects of neutrophils in cancer, the IL-8 and CXCR1/CXCR2 inhibition could reduce neutrophils migration to the tumor, thus avoiding NETs formation and eventually preventing drug resistance. In this section, we briefly report an overview of investigational drugs targeting IL-8 and its receptors CXCR1/CXCR2, and discuss their therapeutic potential in the field of cancer resistance (Table 1).

HuMax-IL8, also known as BMS-986253, is a fully human monoclonal antibody inhibitor of the IL-8 pathway. Humax-IL-8 was shown to block tumor progression (172), immune escape, EMT and MDSCs recruitment (173) in humans, thus pushing further new investigations in cancer resistance (172). HuMax-IL8 was developed for the treatment of patients with advanced solid tumors in combination with nivolumab, an anti-PD-1 monoclonal antibody immune check point inhibitor (NCT02536469), and it is currently under clinical evaluation for the treatment in several other tumors, including advanced solid tumors (NCT03400332), non-small cell lung cancer (NSCLC) (NCT04123379), advanced melanoma and metastatic renal cell carcinoma (NCT04050462), pancreatic cancer (NCT02451982), and head and neck squamous cell carcinoma (NCT04848116). In addition, HuMax IL8 is currently in phase 1b/2 trial in combination with nivolumab for treatment of men with hormone-sensitive prostate cancer (NCT03689699).

Navarixin is a CXCR1/CXCR2 receptor antagonist that impairs neutrophils recruitment (174), and that was shown to repress tumor cells metastasis and angiogenesis in preclinical models (175, 176). The molecule was shown to suppress CXCR2 signaling by decreasing MAPK/AKT pathway phosphorylation, resulting in sensitization of colorectal cancer cells to oxaliplatin

TABLE 1 Summary of the main CXCL8-CXCR1/2 inhibitors for cancer therapy.

Drug	Therapeutic combination	Indication	Trial phase/ Study type	Recruitment status	NCT number
	Nivolumab (anti PD-1)	Advanced solid tumor	Phase 1	Completed	NCT02536469
	Cabiralizumab (anti CSF1R)	Head and neck squamous cell carcinoma	Phase 2	Recruiting	NCT04848116
Humax IL8	Nivolumab (anti PD-1)	Prostate cancer	Phase 1	Recruiting	NCT03689699
	Nivolumab (anti PD-1)	Adenocarcinoma of the prostate	Phase 2	Recruiting	NCT03689699
	Nivolumab (anti PD-1)	Pancreatic cancer	Phase 2	Recruiting	NCT02451982
Navarixin	Pembrolizumab (anti PD-1)	Metastatic solid tumor	Phase 2	Completed	NCT03473925
	Durvalumab (anti PD-L1)	Metastatic pancreatic ductal carcinoma	Phase 1/2	Completed	NCT02583477
AZD5069	Durvalumab (anti PD-L1)	Advanced solid tumor and squamous cell carcinoma of head and neck	Phase 1/2	Active, not recruiting	NCT02499328
	Monotherapy	Myelodysplastic syndrome	Phase1	Recruiting	NCT04245397
SX-682	Pembrolizumab (anti PD-L1)	Metastatic melanoma	Phase 1	Recruiting	NCT03161431
	Nivolumab (anti PD-1)	Metastatic colorectal cancer	Phase 1/2	Recruiting	NCT04599140
	Nivolumab (anti PD-1)	Metastatic pancreatic ductal adenocarcinoma	Phase 1	Recruiting	NCT04477343
Reparixin	Monotherapy	Fatigue	Phase 2	Not yet recruiting	NCT05212701
		Locally advance or metastatic breast cancer			
	Paclitaxel (antineoplastic agent)	Metastatic breast cancer	Phase 1	Completed	NCT02001974
	Paclitaxel (antineoplastic agent)	Metastatic breast cancer	Phase 2	Completed	NCT02370238
	Monotherapy	Breast cancer	Phase 2	Terminated	NCT01861054
RP-72	Monotherapy or combination with gemcitabine	Pancreatic cancer	Phase 1	Recruiting	NCT04338763

treatment (177). Navarixin was assessed for its efficacy and safety in combination with pembrolizumab, an anti-PD-1 monoclonal antibody, in a phase 2 clinical trial of three types of solid tumors: programmed death-ligand 1 (PD-L1) positive refractory non-small cell lung cancer (NSCLC), castration resistant prostate cancer (CRPC) or microsatellite stable (MSS) colorectal cancer (CRC) (NCT03473925).

AZD5069 is a reversible CXCR2 antagonist that was shown to inhibit IL-8 or GRO-α-induced cytosolic calcium increase, CD11b surface expression, adhesion and chemotaxis in neutrophils (178, 179). The molecule was developed as part of combination therapies with durvalumab, an anti PD-L1 monoclonal antibody, in cancer indications including metastatic squamous cell carcinoma of the head and neck (SCCHN) (NCT02499328), and pancreatic ductal adenocarcinoma (NCT02583477).

SX-682 is a CXCR1/CXCR2 antagonist with potential anticancer activities. It exhibited significant activity in solid tumor models, where it reversed chemoresistance and extended overall survival. In syngeneic and genetically engineered mouse (GEM) melanoma models, it potently synergized with anti-PD1 therapy inducing complete remissions (180). In addition, it enhanced both PD-1 immune check point blockade, reduced MDSCs in the TME, and increased natural killer (NK) and T cells infiltration into the tumor site in animal models of head and neck tumor (181). The molecule is currently under active development as monotherapy

TABLE 2 Summary of the main anti-EV agents in cancer and cancer drug resistance in preclinical models.

Drug	Antitumor therapy	Mechanism of targeted or cancer therapy resistance	*In vitro* model	Reference
Heparin	cisplatin	EV uptake inhibitor	Ovarian cancer	Samuel P et al., 2018 (189)
Amiloride	cisplatin	EV uptake inhibitor	Ovarian cancer	Samuel P et al., 2018 (189)
Dynasore	cisplatin	EV uptake inhibitor	Ovarian cancer	Samuel P et al., 2018 (189)
GW4869	cisplatin	EV inhibitor	Ovarian cancer	Cao Y et al., 2017 (190)
		EV inhibitor	Melanoma	Matsumoto A et al., 2017 (191)
		EV inhibitor	Prostate cancer	Panigrahi GK et al., 2018 (192)
Indomethacin	doxorubicin/pixantrone	EV inhibitor	Lymphoma	Koch R et al., 2016 (193)

or in combination with anti PD-1 molecules for the treatment of myelodysplastic syndrome (MDS) (NCT04245397), melanoma (NCT03161431), metastatic colon adenocarcinoma or colorectal carcinoma (NCT04599140) and metastatic pancreatic adenocarcinoma (NCT04477343).

Reparixin is an antagonist of IL-8 that binds CXCR1 and CXCR2 receptors to prevent neutrophil chemotaxis, thus avoiding graft tissue damage in organ transplantation and cancer, including breast cancer (182, 183). The combination of reparixin with antineoplastic agent docetaxel reduced the tumor size in a model of human breast cancer cell lines and breast cancer patient-derived xenografts (184) demonstrating that reparixin is able to reduce *in vivo* the tumor-initiating ability of breast cancer cells by affecting the CSC population; in fact, in tumor-bearing mice treated with reparixin alone or in combination with chemotherapy, the CSCs proportion was far lower than in tumor from mice receiving chemotherapy alone. Additional preclinical evidence highlighted the antitumor and antistemness activity of reparixin in epithelial thyroid cancer (185) and pancreatic cancer (186). Several clinical trials were conducted to assess the efficacy of reparixin in combination with taxanes or in monotherapy in metastatic breast cancer (NCT02001974, NCT02371238, NCT0161054). A new phase 2 clinical trial (NCT05212701) has started to evaluate the efficacy of reparixin in the treatment of oncological fatigue in locally or advanced metastatic breast cancer, a highly disabling condition, very common in cancer patients.

Danirixin is a CXCR2 antagonist originally developed for the potential oral treatment of chronic pulmonary disease (COPD). The molecule is able to strongly reduce the CD11b upregulation mediated by IL-8 or GRO-α agonists in healthy donor neutrophils, thus making the molecule a potential therapeutic agent for diseases characterized by neutrophil hyperactivation (187). In addition, Danirixin was found to block migration, invasion and EMT events mediated by TAMs and IL-8 in a preclinical *in vitro* model of breast cancer (188).

RP-72 is a 72 amino-acid recombinant protein that blocks the activation of IL-8-mediated signaling transduction pathways by decreasing proliferation of susceptible pancreatic cancer cells. The protein is under a Phase 1 clinical trial development for the potential intravenous treatment of metastatic pancreatic cancer in monotherapy or in combination with antiangiogenic gemcitabine (NCT04338763).

5.1.2 Targeting EVs

Targeting EVs in cancer progression could also represent a good strategy to counteract tumor drug resistance (Table 2). In this context, promising results were obtained in an *in vitro* model of ovarian cancer, in which the treatment with heparin, amiloride and dynasore inhibited EV release after treatment with cisplatin (189) known as mechanism responsible for cancer resistance to the therapy. Similar results were obtained in another model of ovarian cancer, in which the phospholipase inhibitor GW4869 was shown to inhibit the exosomal DNA methyltransferase 1 (DNMT1)-mediated cisplatin resistance in cells, and to increase apoptosis (190). These findings suggest that the combination of cisplatin with EV inhibitors can potentially overcome the drug resistance. In a melanoma model, the same GW4869 inhibited exosome secretion that caused the induction of tumor cell proliferation and apoptosis (191). A similar effect was observed in a model of prostate cancer where treatment with GW4869 effectively reduced cancer cell viability associated to exosome secretion (192). In aggressive B-cell lymphomas, suppression of exosomal drug resistance with indometacin increased efficacy of doxorubicin therapy (193). Finally, in a tumor mice model the treatment with dimethyl amiloride (DMA), known to reduce exosome release into the bloodstream, given in combination with the chemotherapeutic drug cyclophosphamide, halted the tumor growth by 50% or more, if compared to the untreated controls (192).

Thus, new interest is arising for the development of EV/exosome pathway inhibitors. The combined use of IL-8 biological activity inhibitors that modulate the hyperactivation of neutrophils could represent a new strategy to mitigate cancer drug resistance induced by EVs release. A first example of such approach is represented by the combined blockade of IL-8 and IL-6 in osteosarcoma. Starting from data showing that osteosarcoma tumor-secreted EVs can induce a pro-metastatic phenotype by strongly inducing IL-6 production in mesenchymal stem cells (MSCs), it has been demonstrated that EVs from aggressive cancer cell lines can induce MSCs to express inflammatory cytokines and chemokines, among which IL-8 was the most upregulated one, and that this was due to tumor EV-associated non-coding RNAs. The blockade of IL-8 signaling with ladarixin (an allosteric inhibitor of CXCR1 and CXCR2) and, even more strikingly, its combination with tocilizumab (an anti-IL-6 receptor antibody) reduced lung metastasis formation in a xenograft mouse model of osteosarcoma and, notably, prevented the occurrence of MSC-induced tumor resistance to antimetastatic drugs (abstract submitted to the ASCO 2022 meeting).

6 Conclusions

TANs play a key role in tumor drug resistance, and their activities in this context are regulated and mediated by different factors. Among these, EVs and IL-8, produced either by tumoral cells or by neutrophils themselves, crucially function to both control and mediate the pro-tumoral functions of neutrophils in the TME. The role of both EVs and IL-8 is crucial for neutrophil-mediated tumor drug resistance, which is mainly due to the induction of

NETs formation and the secretion of pro-tumoral factors, including neutrophil-derived EVs. Growing evidence has highlighted the close association between high levels of IL-8, EVs production, NETosis, and limited therapeutic response in a variety of malignancies, thus paving the way to investigations on the therapeutic potential of combination treatments either of IL-8 activity blockers, or anti-EVs drugs, or NETosis inhibitors with standard antitumoral therapies, to reduce or counteract tumor drug resistance (162, 193).

In conclusion, IL-8 and EVs represent key potential targets for the development of novel therapeutic options aimed to target neutrophil-mediated tumor drug resistance.

Author contributions

MZ, AR, FR, and MSM performed data collection (literature reviewing) and prepared the original draft of the manuscript. RN revised and wrote the final version of the manuscript. MA and MCC revised the manuscript for critically important intellectual content. PGA conceptualized the study and revised and wrote the final version of the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors are employees of Dompé farmaceutici S.p.A.

Publisher's note

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