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Genetically engineered cell-derived nanovesicles for cancer immunotherapy

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The emergence of immunotherapy has marked a new epoch in cancer treatment, presenting substantial clinical benefits. Extracellular vesicles (EVs), as natural nanocarriers, can deliver biologically active agents in cancer therapy with their inherent biocompatibility and negligible immunogenicity. However, natural EVs have limitations such as inadequate targeting capability, low loading efficacy, and unpredictable side effects. Through progress in genetic engineering, EVs have been modified for enhanced delivery of immunomodulatory agents and antigen presentation with specific cancer targeting ability, deepening the role of EVs in cancer immunotherapy. This review briefly describes typical EV sources, isolation methods, and adjustable targeting of EVs. Furthermore, this review highlights the genetic engineering strategies developed for delivering immunomodulatory agents and antigen presentation in EV-based systems. The prospects and challenges of genetically engineered EVs as cancer immunotherapy in clinical translation are also discussed.

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1. Introduction

Naturally derived nanomaterials like cell-based nanovesicles are gaining dramatic attention owing to their favorable biocompatibility, diminished immunogenicity, extended blood circulation time, and appropriate nano-scale dimensions, positioning them as attractive candidates for drug delivery. Extracellular vesicles (EVs), which encompass different types of membrane-enclosed particles secreted by cells into the extracellular milieu, play an important role in cell-to-cell communication.[1,2] EVs can be classified into three major subclasses based on their size: small EVs (sEVs with <200 nm), large EVs (lEVs with 200–1000 nm), and apoptotic bodies

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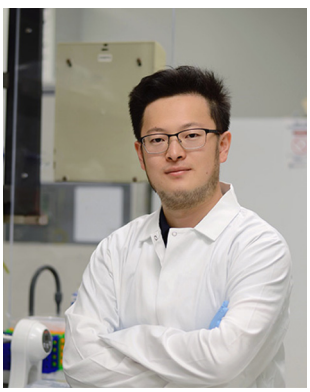

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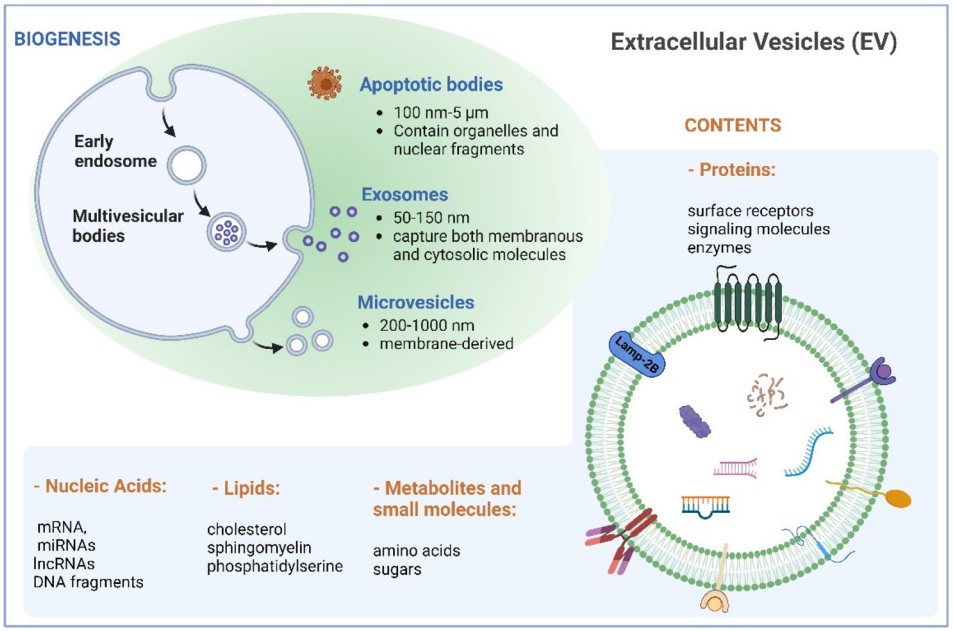

Fig. 1 Biogenesis and characteristics of extracellular vesicles. Created with BioRender.

(ABs with 100 nm–5 μm).[3] Large EVs, known as microvesicles, are generated through direct outward budding of the plasma membrane. Apoptotic bodies are produced by cells undergoing apoptosis. Exosomes, a subset of small EVs, exhibit sizes between 50 and 150 nm. Their biogenesis commences with inward budding of the plasma membrane, followed by the formation of intraluminal vesicles during endosomal processing and release into the extracellular space (Fig. 1). As such, exosomes encapsulate both membranous and cytosolic molecules, making them captivating subjects in diverse biomedical domains in recent decades.

Cancer immunotherapy, aiming to improve the ability of the immune system to recognize and eliminate tumors, represents one of the most rapidly advancing domains in clinical medicine. Steady development of cancer immunotherapy involves leveraging various elements of the immune system to treat tumors, including antibodies, cells, and proteins.[3,4] Notable advancements have been made through approaches such as adoptive cell therapy based on the injection of tumor-activated T cells and checkpoint blockade inhibitors, which prevent the deactivation of cytotoxic T cells by blocking specific inhibitory receptor interactions. These strategies have significantly improved the long-term survival rates of patients with aggressive cancers.[5,6]

With recent advancements introducing EVs as promising candidates for various therapeutic and diagnostic applications, their utilization in cancer immunotherapy has garnered advantages. In comparison with conventional cell-based immunotherapy that requires meticulous and time-sensitive preparation, EV-based therapy is cell-free and thereby offers a more practical approach by exhibiting enhanced stability for extended storage without compromising efficacy.[7–9] In addition, EVs outperform synthetic nanocarriers like liposomes or polymeric nanoparticles in terms of lower toxicity to healthy cells and environmentally friendly isolation, as they do not require organic solvents and various chemical materials for isolation.[10] EVs originating from a specific source inherently carry biologically active molecules such as nucleic acids, proteins, and glycans, establishing their nature as connate carriers. Moreover, they can be modified *via* different methods to deliver various functional cargoes and target specific signaling pathways. However, although EVs have shown promise as versatile therapeutics, the optimal cargo composition and loading strategies for maximizing their efficacy in cancer immunotherapy remain to be determined. Additionally, strategies to enhance the targeting abilities of EVs while minimizing off-target effects need further investigation. Different aspects of EV-based systems for therapeutic applications have been discussed in many recent reviews.[11–13] In this review, we specifically focus on and discuss recent advances in developing genetic engineering strategies for boosting EV-mediated cancer immunotherapy. Our discussions will help to identify key knowledge gaps in more effectively engineering EVs *via* genetic engineering as well as provide useful perspectives in introducing new functionalities to EVs as a powerful tool for cell-free cancer immunotherapy.

2. Extracellular vesicles for cancer therapy

2.1 Cell sources

2.1.1 Mesenchymal stem cells (MSCs). Mesenchymal stem cells (MSCs) represent a type of multipotent stem cells that

exist in a wide range of tissues. MSC-derived EVs not only can transfer bioactive molecules for immunomodulation, such as cytokines, growth factors, and miRNA, but also can regulate the activity of immune cells. Additionally, EVs from MSCs intrinsically exhibit tumor homing abilities, akin to their recruitment to sites of inflammation, making them candidates for anti-tumor therapy.[14] Nevertheless, accumulating evidence indicates that MSC-derived EVs can be a double-edged sword, either inhibiting or promoting tumor progression in different situations depending on their source (adipose tissue, bone marrow, and peripheral blood) and the route of administration (intramuscular, intravenous, and subcutaneous injection).[15]

Several studies have demonstrated that miRNAs encapsulated in native EVs derived from MSCs, such as miR-7-5p, miR-146b, miR-142-3p, and miR-let-7c, are capable of suppressing the development and metastasis of cancer.[16–18] For example, a recent study[19] by Wang *et al.* elucidated that miR-30b-5p shuttled by bone marrow MSC-derived exosomes could promote cell apoptosis and prevent tumorigenesis in non-small cell lung cancer (NSCLC) *via* the EZH2/PI3K/AKT axis. Conversely, a number of studies indicated the tumor-promoting effect of MSC-derived EVs,[20–23] likely due to the enriched presence of tumor-supportive miRNAs (*e.g.*, miRNA-21 and 34a) and proteins (*e.g.*, PDGFR-β, TIMP-1, and TIMP-2).[24] Ren *et al.*[20] reported that EVs isolated from human bone marrow-derived MSCs significantly increased tumor growth, intra-tumoral angiogenesis as well as macrophage M2 polarization *via* miR-21-5p delivery. In another study, Biswas *et al.*[21] showed that exosomes secreted by tumor-educated MSCs induced differentiation of monocytic myeloid-derived suppressor cells (MDSCs) into highly immunosuppressive M2 macrophages at tumor sites and thereby promoted breast cancer progression. Apart from regulating macrophage phenotypes, MSC-derived EVs have also been demonstrated to play a role in suppressing the proliferation and function of other immune cells, including natural killer (NK) cells, dendritic cells (DCs), T cells and B cells.[25,26] In some cases, they could likely induce apoptosis in activated immune cells, leading to inhibited immune responses.

Apart from utilizing the intrinsic payload, MSC-derived EVs have been used as a carrier for anti-tumor immunotherapy. Deng *et al.*[27] recently demonstrated the potential of EV-mediated delivery of CD38 siRNA to overcome the resistance of tumors to PD-1/PD-L1 inhibitors by promoting macrophage repolarization to the M1 phenotype.

2.1.2 Cancer Cells. Apart from immune cells, extensive efforts have also been made to explore the potential of EVs derived from tumor cells in delivering cancer-specific antigens as part of cancer immunotherapy strategies. However, it should be noted that tumor-derived exosomes (TEXs) also contain immune inhibitory molecules, such as Fas ligand (Fas-L), transforming growth factor beta (TGF-β) and prostaglandin E2 (PGE2), which can inhibit differentiation or decrease the cytotoxicity of proinflammatory cells (*e.g.*, NK cells, CD8 T cells, DCs),[28,29] meanwhile promoting the proliferation of anti-inflammatory cells (*e.g.*, Tregs and MDSCs). Therefore, they often would be strategically engineered to circumvent their immunosuppressive properties as well as enhance immune activities.[30–34] For instance, Rossowska *et al.*[34] genetically engineered MC38 colon carcinoma (CRC) cells for over-expression of interleukin 12 (IL-12) and shRNA targeting TGF-β1, followed by EV collection from engineered cells, which induced tumor inhibition as opposed to TEXs from unmodified MC38 cells. In another study, TEXs were painted with the functional domain of high mobility group nucleosome-binding protein 1 (HMGN1) to boost the activity of DCs.[30] In mouse models with large tumor burdens, most notably large poorly immunogenic orthotopic hepatocellular carcinoma (HCC), the engineered TEXs promoted DC activation, increased homing to lymphoid tissues, elicited long-lasting antitumor immunity, and contributed to an augmented memory T cell response.

2.1.3 Dendritic Cells. DCs are professional antigen-presenting cells (APCs) for the activation of cytotoxic T lymphocytes.[35] Consistently, EVs derived from DCs, both exosomes[36] and large EVs,[37] have been shown to bear abundant signature proteins from their producer cells, such as major histocompatibility complex (MHC) and co-stimulatory molecules, allowing the activation of T cells. Considered as the most prospective EV-based therapeutic in cancer immunotherapy, DC-derived EVs have been studied for decades and some have been progressed to clinical trials. Yet, only modest immune response can be elicited by the individual use of DC-derived EVs in part due to the lack of sufficient antigen presented on them. To enhance their anti-tumor immunity, some stimuli were administered or adjuvants like poly (I:C) and CpG DNA were co-delivered.[38] Mastumoto *et al.*[39] reported that small EVs secreted from ovalbumin-activated DCs could stimulate macrophages and DCs *in vitro* and *in vivo* through toll-like receptor 4 signaling and exhibit strong *in vivo* antitumor effects. A phase II clinical trial has confirmed the antitumor effect of mature DC-derived exosomes when used as a maintenance immunotherapy after chemotherapy in patients with advanced NSCLC (NCT01159288).[40] This similar strategy was also used to pulse DCs with an HCC antigen, α-fetoprotein, to produce exosome-based vaccines.[41] Exosomes collected from antigen-modified DCs demonstrated their potential as a cell-free alternative for DC vaccines by eliciting antigen-specific immune response in ectopic or orthotopic HCC mice. Instead of using a specific antigen, in a recent report by Xu *et al.*,[42] the authors used antigens derived from a whole tumor cell and the CpG adjuvant to activate DCs, endowing the EVs with significantly upregulated levels of co-stimulatory molecules (CD80 and CD86) and the MHC–peptide complex. The EVs were further equipped with the targeting ability by overexpressing aCD19 scFv and the immune checkpoint blocking capability by PD-L1 siRNA treatment. The multifunctional DC-EVs showed impressive ability to accumulate in huCD19-expressing solid tumors following intravenous injection and effectively reshaped the immunosuppressive tumor microenvironment.

2.1.4 Macrophages. Tumor-associated macrophages (TAMs) are the most abundant immune cells in tumor tissues.

Thus, TAM-derived EVs are appealing candidates for immunomodulation and cancer immunotherapy.[43,44] M1 macrophages are the classically activated phenotypes that exert tumor-inhibiting abilities, while M2 macrophages are the alternatively activated counterparts that promote tumor progression and immunosuppression. In analogous to their parental cells, EVs derived from M1 macrophages have demonstrated that they not only possess tumor-homing ability,[45] but also can repolarize M2 macrophages to the M1 phenotype.[43,46] A main cause of TAM-associated immunosuppression involves signal regulatory protein alpha (SIRPα) on macrophages that ligates with CD47, a "don't eat me" signal overexpressed on tumor cell surface. Accordingly, blocking the CD47-SIRPα signaling becomes an efficient strategy to re-educate TAMs to boost cancer immunotherapy. Based on this concept, Nie *et al.* prepared exosomes derived from M1 macrophages, followed by conjugation with the antibodies of CD47 and SIRPα through pH-sensitive linkers.[47] The modified M1 exosomes efficiently accumulated in tumor sites due to the targeting ability of aCD47 and then released the antibodies upon the cleavage of the pH-sensitive bond in the acidic tumor microenvironment. The synergism of M1 exosome and antibodies resulted in efficient reprogramming of pro-tumoral M2 to anti-tumoral M1 macrophages and potent anti-tumor efficacy with minor side effects.

2.1.5 T cells. T cells play a vital role in active anti-tumor immunity by recognizing and responding to antigens followed by orchestrating a targeted immune response. There are a variety of T cells, each exerting a unique immune function. EVs derived from T cells have shown great potential for cancer immunotherapy. Supporting information has been provided in some reports regarding cytotoxic CD8 T cell-derived EVs, showing that the EVs could mediate depletion of mesenchymal tumor stromal cells and cancer-associated fibroblasts.[48] Consequently, they lead to vigorous attenuation of tumor invasion and metastasis. EVs derived from other T cell subsets, such as helper CD4 T cells[49] and activated CD3 T cells,[50] have also demonstrated their therapeutic potential in cancer immunotherapy. One study by Fu *et al.* indicated that exosomes produced from CAR-T cells carried a high level of cytotoxic molecules and expressed a low level of PD1. These properties made them safer than the respective CAR-T therapy because they have a lower risk to induce cytokine release syndrome.[51] Besides, modifying the EVs or loading anti-cancer drugs could further enhance their anti-cancer efficacy.[52] For instance, Jung *et al.* developed IL-2-tethered exosomes from engineered Jurkat T cells to augment the immune responses of CD8 T cells.[52] This surface engineering of exosomes resulted in significantly modified levels of miRNAs that are crucial in activating CD8 T cells, thereby amplifying their antitumor immune effects.

2.1.6 Natural killer cells. As an important component of the innate immune system, natural killer (NK) cells can destroy harmful cells, just like cytotoxic T cells, but do not require activation by an antigen. Several studies have demonstrated that EVs secreted from NK cells contain typical NK markers (*e.g.* CD56) and cytotoxic proteins (*e.g.* perforin, granzyme and FasL), thereby capable of directly inducing tumor cell lysis or inhibiting tumor cell proliferation.[53–55] Additionally, they can enhance the anti-tumor activity of other immune cells, such as cytotoxic T cells, through intercellular signaling cytokines and chemokines such as interferon (IFN)-γ, tumor necrosis factor (TNF), interleukin (IL)-6, and granulocyte-macrophage colony-stimulating factor (GM-CSF).[56] Interestingly, it has been found that NK cell-derived EVs could re-activate the cytotoxic function of defective NK cells in the tumor microenvironment (TME), indicating their potential in anti-tumor treatments.[54] Besides their intrinsic capabilities, NK cell-derived EVs can be engineered for other purposes, such as cytokine priming (*e.g.* IL-12, IL-15)[57,58] and genetic engineering.[59,60] More information on NK cell-derived EVs can be found in another recently published elegant review.[55]

2.1.7 Other sources. Other immune cells, such as neutrophils,[61] monocytes,[62] and B cells,[63] can also serve as sources of EVs for cancer immunotherapeutic applications. Beyond eukaryotes, bacteria and archaea are able to release extracellular vesicles as well. Outer membrane vesicles (OMVs), secreted by Gram-negative bacteria, have garnered considerable attention because they can activate a variety of toll-like receptor (TLR) signaling pathways due to their inherited package of a large number of immunogenic components.[43,64,65]

In addition to employing naturally secreted EVs, parental cells or modified EVs post-isolation can be engineered to endow EVs with specific characteristics or cargoes to enhance their immunotherapeutic potential. Genetic engineering represents one versatile and effective strategy with bare limitations in cell sources. The HEK 293T cell line has been a popular candidate for its great transfectability to be a universal donor of EVs with customized properties. Of note, another source receiving great attention is red blood cells (RBCs) as they are readily available from the blood bank, thereby allowing large-scale EV production; additionally, they are enucleated cells devoid of DNA, causing no safety concern of horizontal gene transfer.[66] Le and coworkers has developed a strategy to generate scalable amounts of RBC-derived EVs for the delivery of therapeutic RNA drugs such as antisense oligonucleotides (ASOs),[67] siRNA,[68] and Cas9 messenger RNA[66] for cancer therapy with low cytotoxicity. The discussed cell sources and their characteristics are briefly summarized in Fig. 2.

2.2 Isolation approaches

Although EVs have many appealing merits for cancer immunotherapy, a standardized isolation and purification procedure with high efficiency and simplicity remains one of the major challenges hindering their clinical translation.

The most common approach to isolate EVs involves ultracentrifugation (UC) to process cell culture supernatants containing released EVs.[69–71] With a series of centrifugation steps at progressively higher *g*-force, cell debris, large particles and some other unwanted particles can be removed, and EV pellets can be obtained. Disadvantages of this method include incomplete isolation, possible protein aggregates and damage with

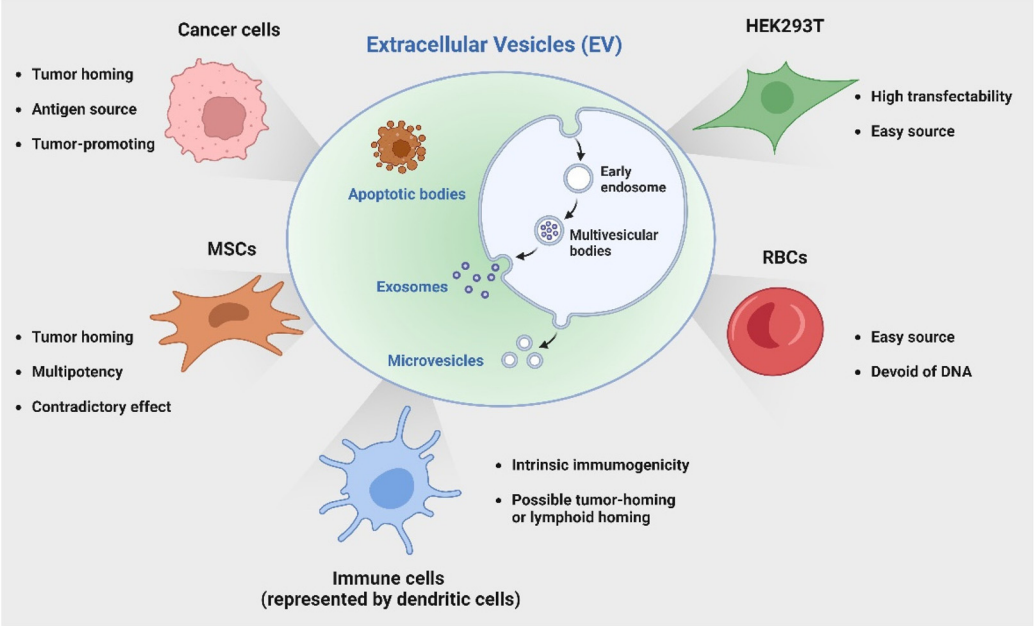

Fig. 2 Various cell sources for the production of EVs for cancer immunotherapy. Created with BioRender.

the ultra-high shear stress.[72] Another established method, density-gradient centrifugation (DGC), based on ultracentrifugation utilizes density gradients induced by sucrose or iodixanol to separate components of different buoyant densities, thereby enabling efficient purification of EVs from protein aggregates,[33,67] whereas this method requires a long processing time and expensive equipment, limiting its broad employment for scale-up applications.

Size exclusion chromatography (SEC), also known as spatial or molecular exclusion chromatography, uses a column with porous beads to allow small EVs to enter and entrap in the pores.[73] This method is frequently used in combination with ultracentrifugation. It gives rise to higher yields and more intact physiological conditions of EVs. Similarly, ultrafiltration by using membrane filters with defined molecular weight cutoffs to concentrate and isolate EVs based on their size is an easy-to-use method but may cause EV deformation upon filtration. The tangential flow filtration (TFF) method utilizes a cutoff filter similar to ultrafiltration but is specifically designed to permit the discharge of waste in a direction orthogonal to the sample flow.[74] This design prevents the formation of a filter cake and yields relatively high-purity EVs while continuously removing smaller molecules and contaminants.

Besides physiochemical properties, biological markers on the surface of EVs have been taken into consideration to develop isolation and purification methods. Immunoaffinity capture is a method that allows the selective capture of EVs expressing specific markers by antibodies that are generally prefixed to a particular carrier, such as magnetic particles or chromatography matrices. Currently, the most commonly used carrier is immunomagnetic beads. Clayton *et al.*[75] have isolated EVs derived from APCs using magnetic beads coated with antibodies specific for MHC class II. Similarly based on immunoaffinity, a strategy using microfluidic devices with antibody-coated chip surfaces for EV isolation has been described in recent years.[76–78] Kanwar *et al.* developed a platform called ExoChip to specifically capture CD63-specific exosomes from serum in an hour.[77] With narrow channels interconnected with circular chambers, an extended retention time of exosomes in the channels led to more efficient capture. Moreover, the device could simultaneously quantify the isolated exosomes. However, EVs captured by specific antibodies can only induce a very small yield since they cannot be easily eluted as intact vesicles.

2.3 Specific target

It is widely acknowledged that EVs can inherit targeting ability from their parental cells, exemplified by the tumor-homing ability of M1 macrophage-derived EVs[45] and the lymphoid homing capability of EVs derived from the mature DCs.[79] Despite this, a comparative study has revealed that systemic administration of EVs derived from most cell lines induces preferential accumulation in the liver, lungs, spleen and gastrointestinal tract.[80] To confer the targeting capabilities of EVs to other tissues or specific cell types, introducing specific targeting moieties to the EV surface is necessary. It has been validated that 80% of the proteins in exosomes are highly conserved among different cell types, some of which are used as the biomarkers of exosomes, including lysosome-associated membrane glycoprotein 2b (Lamp2b), TSG101, heat shock proteins, and the tetraspanins CD63, CD9, and CD81. Inspired by this concept, a well-studied strategy to endow EVs with targeting properties is to fuse targeting moieties with EV membrane proteins. This approach was used by Alvarez-Ervit *et al.*, who

engineered DCs to express a fusion protein of Lamp2b and brain-specific rabies viral glycoprotein (RVG), a peptide that binds the acetylcholine receptor.[81] EVs isolated from these cells demonstrated increased accumulation in the brains of mice and successfully delivered the siRNA of interest, manifesting the potential of EVs as targeted drug delivery systems. The same approach was used by Tian *et al.* to achieve targeted delivery of doxorubicin to integrin-positive breast cancer cells *in vitro* and *in vivo* by exosomes that overexpress the αγ integrin-specific RGD peptide fused to Lamp2b.[82] When using such a strategy to introduce target moieties, the choice of EV sorting domains should be considered, *i.e.*, the surface protein that is known to be enriched on EVs, according to the cell source. For instance, in HEK 293T cells, fusing moieties to the tetraspanin CD9, CD63 or CD81 would result in a significantly higher expression on EVs as compared to fusing to Lamp2b or the C1C2 domain of lactadherin.[83] Apart from utilizing exosomal surface proteins, foreign membrane proteins can be employed for the conjugation of targeting moieties. For example, Wang *et al.* designed a plasmid encoding fusion proteins of VSV-G fusogen and anti-GPC3 scFv, which can efficiently bind HCC cells.[84] After lentiviral transduction, the obtained vesicles presented anti-GPC3 on their surface and showed significantly higher accumulation in HepG2 cells both *in vitro* and *in vivo*, thus facilitating anti-cancer targeted drug delivery.

In the context of cancer immunotherapy, exosomes have been utilized for antibody display to activate T cells. A method of crosslinking T cells and cancer cells has been described by co-displaying anti-CD3 and anti-EGFR single-chain variable fragments (scFvs) on a construct of the transmembrane domain of human platelet-derived growth factor receptor (PDGFR), commercialized as pDisplay.[85] The transfected cells expressing the fusion construct produced EVs containing both antibodies against CD3 and EGFR. In such a way, the engineered EVs could redirect and activate cytotoxic T cells against triple-negative breast cancer (TNBC) cells, eliciting potent anti-tumor immunity both *in vitro* and *in vivo*. Likewise, in a more recent study, Stranford *et al.* utilized the pDisplay system to passively load high-affinity T cell-targeting domains on the EV surface, enabling the targeted delivery of biomolecules to T cells.[86] Specifically, the engineered EVs carrying substantially expressed anti-CD2 scFvs showed a 100-fold increase in binding efficiency for CD2+ Jurkat T cells than the non-targeted EVs (Fig. 3).

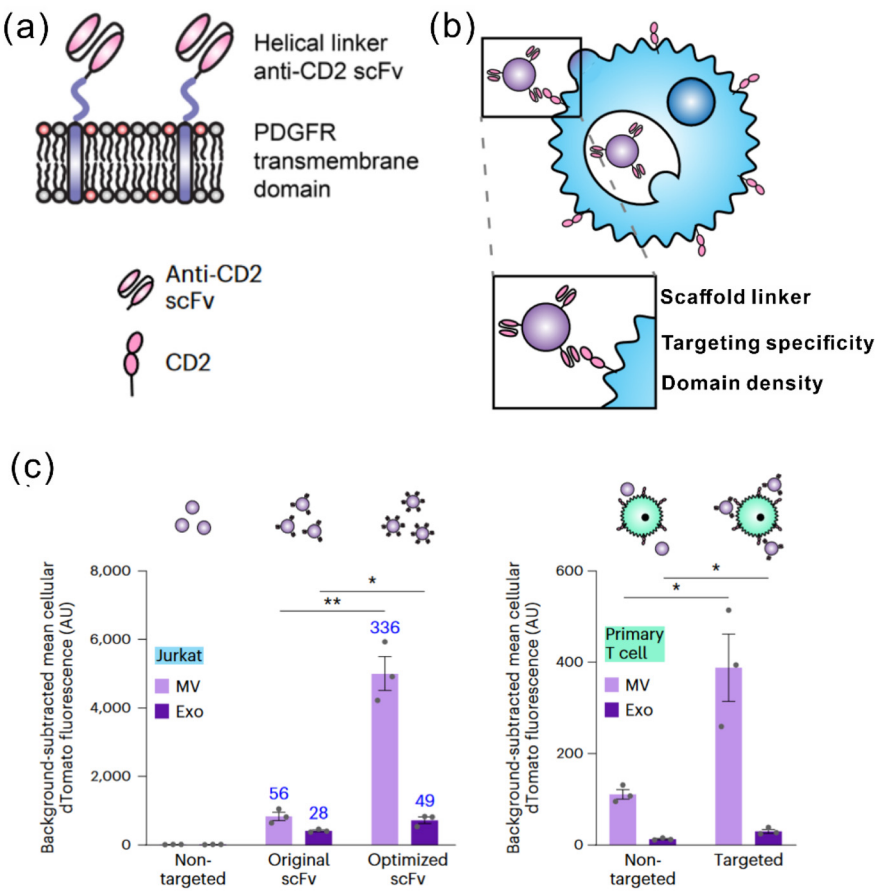

Fig. 3 A representative study employing pDisplay to achieve the targeting ability of EVs to T cells. (a) Illustration of the structures of the PDGFR transmembrane domain for anti-CD2 scFv display. (b) Illustration of EVs achieving targeting to T cells through antibody–receptor interactions. (c) Targeting EVs can efficiently bind to both Jurkat T cells and primary human CD4+ T cells. Adapted from ref. 86 with permission from Springer Nature, copyright 2023.

A more straightforward strategy to achieve the targeting ability of EVs involves an anchor peptide of CD63. Gao *et al.* reported a particular peptide, CP05, that shows a strong affinity towards the second extravesicular loop of CD63.[87] Direct painting of exosomes with different moieties conjugated to CP05 redirected systemic distribution of exosomes to specific tissues. As a proof of concept, loading of a phosphorodiamidate morpholino oligomer on exosomes *via* CP05 at extremely low doses achieved elevated dystrophin expression in muscle with functional improvement without any detectable toxicity. This strategy provides a new avenue for exosome engineering, effective conjugation of targeting moieties,[88] cargo delivery[33] and surface modification[30] with great simplicity, opening a new avenue for exosome engineering.

2.4 Biomimetic nanovesicles

Despite substantial advancements in the field of naturally-derived EVs, their relatively low yield remains a substantial impediment to widespread applications. Accordingly, various strategies have been developed to address the technical issues. Among them, biomimetic nanovesicles, also known as cell-derived nanovesicles (CNVs), have emerged as a promising alternative to EVs. CNVs are typically generated through mechanical extrusion of donor cells using nanosized filters or microchannels within microfluidic devices, resulting in a substantially higher yield.[46,84,89–91] Notably, CNVs closely resemble EVs in terms of size and protein components. Studies have revealed that CNVs share more than 70% of the same membrane proteins found in naturally occurring EVs and their batch-to-batch variation can be well-controlled and minimized to 10%.[90] In subsequent sections of this review, CNVs will be discussed within the broader context of EVs.

A study by Jang *et al.* reported a 100-fold increase in the yield of CNVs compared to that of exosomes released from the same number of cells.[89] This method relies on the serial extrusion of cells through filters with increasingly smaller pore sizes to generate CNVs. To validate the therapeutic efficacy, CNVs produced from macrophages were loaded with doxorubicin to treat immunocompetent CT26 mouse tumors. Encouragingly, CNVs showed similar anti-tumor effects to exosomes harvested from the same cells and caused minimal systemic side effects even with a 20-fold higher dose administration of the free drug. Employing this technique, Yang *et al.* generated CNVs from non-tumorigenic epithelial MCF-10A cells for siRNA delivery.[91] The yield of the nanovesicles reached around 150-fold higher that of exosomes from the same cell source. Several exosomal biomarkers, including CD63, CD9, CD81and TSG101, were detected on the surface of both vesicle types, but at higher levels on secreted exosomes as anticipated. With a siRNA against cyclin-dependent kinase 4 (CDK4) loaded *via* electroporation, CNVs could significantly downregulate the CDK4 expression *in vitro* and *in vivo*, validating their potential as effective EV substitutes for drug delivery systems.

A recent study by Wang *et al.* further equipped CNVs with efficient cytosolic delivery and target specificity by genetically engineering the producer cells, *i.e.*, HEK 293, to drive the co-expression of a viral fusogen, vesicular stomatitis virus G protein (VSV-G), and a cancer cell targeting moiety GPC3.[84] The all-in-one CNV platform demonstrated effective and selective binding to tumor cells, fulfilling endosomal escape and cytosolic delivery of different therapeutics, such as siRNA, gelonin, and paclitaxel. Ultimately, drug-loaded CNVs resulted in an enhanced tumor treatment efficacy compared to the free drug as well as nanovesicles without targeting moieties or fusogens.

3. Genetically engineered EVs for cancer immunotherapy

Genetic engineering has undergone a revolutionary advancement in its fundamental technology, positioning it as a versatile tool across a broad spectrum of biomedical applications in recent decades. To broaden and improve the functionalities of EVs, genetic engineering has been substantially employed for EV modification. In addition to the previously discussed targeting capabilities, EVs have been genetically engineered to modulate immunity within the context of cancer immunotherapy (Fig. 4). This review delves into two primary engineering directions: immunomodulatory drug delivery and antigen presentation.

3.1 Drug delivery

One hallmark of EVs is that they are naturally derived, endowing them with high biocompatibility. Moreover, the lipid bilayer membrane can be a natural barrier to prevent therapeutic cargoes from possible degradation by serum protease.[92] Therefore, EVs have become increasingly appealing as delivery carriers when compared to synthetic carriers. Exosomes, in particular, have been considered a natural alternative to liposomes as delivery systems because of their low immunogenicity, evasion from phagocytosis by monocytes and macrophages, and enhanced retention in circulation.[93] Here, we provide an overview of two different loading strategies. One is based on modulating the producer cells, commonly by genetic engineering approaches, to favor the endogenous loading of cargoes of interest during EV biogenesis. Alternatively, the cargoes can be loaded in EVs after their isolation by physical or chemical approaches. Examples of these loading techniques are shown in Table 1.

3.1.1 Exogenous loading. Performing modification after EV isolation to load cargoes is referred to as exogenous loading in our discussions. A well-utilized method is electroporation, which creates transient pores in the EV membranes under an electrical field to allow the diffusion of cargo molecules from an external solution into the inner vesicle lumen. This strategy can be highly efficient for the entry of small molecules, such as miRNA, siRNA and ASO. Mendt *et al.* isolated EVs from MSCs and exogenously loaded siRNA or short hairpin RNA against the oncogene KrasG12D by electroporation to treat pancreatic cancer.[70,94] After intraperitoneal injection, pro-

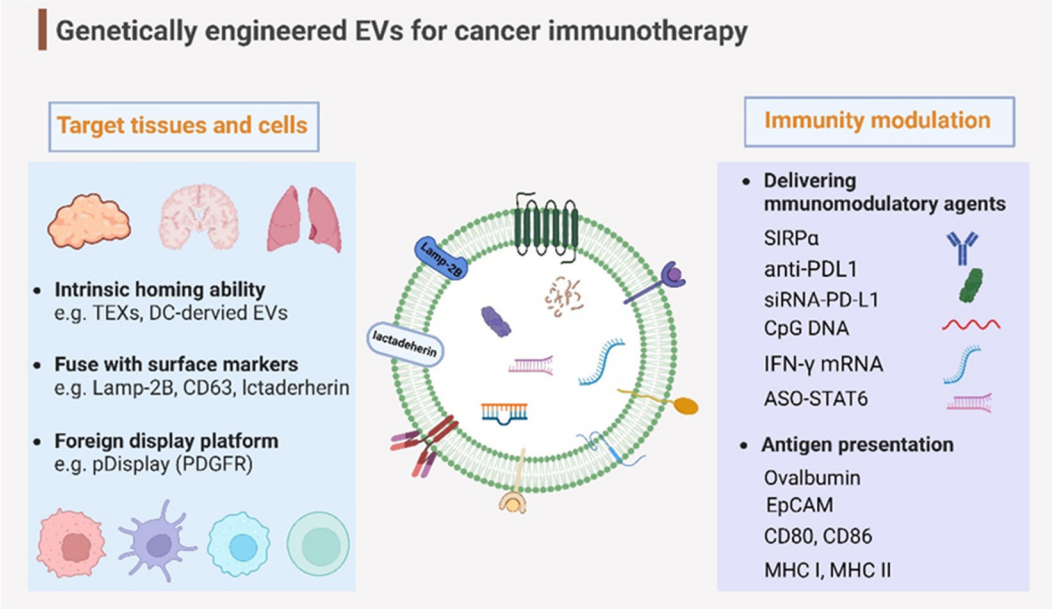

Fig. 4 Genetic engineering endows EVs with targeting capabilities to specific tissues and organs and immunity modulation for cancer immunotherapy. Created with BioRender.

found tumor regression and prolonged mouse survival were achieved in both orthotopic xenograft and genetic mouse models. Moreover, the siRNA-loaded EVs demonstrated significantly higher therapeutic efficacy when compared with liposomes carrying similar levels of siRNA, which is possibly attributed to the presence of CD47 on the surface of EVs that extended the circulation and retention of administered EVs. With these encouraging results, they are currently undergoing a phase I clinical trial (NCT03608631).[70] Aiming to block the interaction between PD-L1 and PD-1 with RNA interference, Liu *et al.* electroporated anti-PD-L1 siRNA (siPD-L1) into the EVs derived from M1 macrophages.[95] Direct release of siPD-L1 into the cytoplasm was achieved with the presence of a viral fusion protein VSV-G on the EV surface, triggering robust gene silencing and leading to the elevation of CD8+ T cell population.

Despite the common use, a major concern of the electroporation method is the generation of siRNA nanoaggregates, potentially interfering therapeutic efficacy.[67,96] Of note, some commercial non-viral transfection reagents showed higher loading efficiency than electroporation for the loading of siRNA into RBCEVs.[67] Additionally, electroporation shows a limitation in loading large cargoes, such as mRNA and plasmid DNA. An early example was the delivery of CRISPR/Cas9 gene editing tool by RBC-derived EVs.[66] Specifically, electroporation resulted in a loading efficiency of 18% for the Cas9 mRNA, and the observed gene knockout efficiency was only ~10% when the EVs were tested to deliver the DNA plasmids of Cas9 and GFP gRNA to 293T-EGFP cells.

Some studies demonstrated the feasibility of simply incubating the EVs with the cargo of interest to allow surface encapsulation. This method is generally used for hydrophobic drugs for their ability to interact with and cross the lipid bilayer EV membrane.[97] However, genetic cargoes, including RNA and DNA, are hydrophilic and thus are difficult to load passively into EVs by this method. To enhance the loading efficiency, the EV membrane can be genetically modified with some functional moieties as binding sites (anchor). For example, the streptavidin plasmid was transferred to the cells to produce SAC-EVs, which can specifically bind to biotinylated molecules to enable efficient loading of immunostimulatory CpG DNA.[31] Dooley *et al.* generated exosomes overexpressing a surface glycoprotein, prostaglandin F2 receptor negative regulator (PTGFRN), *via* virally transfecting HEK 293 cells.[98] PTGFRN+ exosomes allowed efficient tethering of a variety of cargoes, including cytokines, antibody fragments, RNA binding proteins, vaccine antigens and Cas9 (Fig. 5a). A subsequent study utilized the PTGFRN^{+} exosome platform for efficient exogenous loading of ASOs by co-incubation for 30 minutes.[71] Compared with free ASOs, enhanced delivery mediated by the PTGFRN+ exosomes has been achieved to macrophages, monocytes, and MDSCs in the liver, peripheral blood, bone marrow, and tumor microenvironment (TME). As a proof of concept, ASO-STAT6 was delivered by the PTGFRN+ exosomes, showing effective reprogramming of immunosuppressive M2 macrophages to proinflammatory M1 macrophages both *in vitro* and *in vivo*. In syngeneic models of CRC and HCC, the exosome-mediated ASO-STAT6 monotherapy resulted in >90% tumor growth inhibition and 50–80% complete elimination. This study provides one of the first descriptions of an engineered exosome therapeutic candidate delivering an ASO targeting a transcription factor that controls the macrophage phenotype for cancer immunotherapy.

Table 1 Genetically engineered EVs as carriers of immunomodulatory agents

EV type	Loading strategy	Loading method	Isolation method	Donor cell	Genetic vector	Immunomodulatory agents	Cancer model	Ref.
Exosome	Exogenous	Electroporation	UC	BM-MSC	Lentiviral	CD38 siRNA	Hepa1-6 xenograft	27
Exosome	Exogenous	Streptavidin–biotin	UC	B16BL6	Synthetic ODN	CpG DNA	B16BL6 xenograft	31
Exosome	Exogenous	Electroporation	DGC	PANC-02	Synthetic siRNA	CCL22 siRNA	PANC-02 orthotopic model	33
Exosome	Exogenous	Transfection	DGC	RBC	Synthetic siRNA	ImmRNA 3p10la9	4T1 and CA1a xenograft	67
Exosome	Exogenous	Electroporation	UC	MSC	Synthetic siRNA	siRNA-KrasG12d	Panc-1 and KPC689 orthotopic models	70
Exosome	Exogenous	Scaffold protein binding	UC	HEK 293T	Synthetic ASO	ASO-STAT6	CT26 xenograft	71
CNV	Endogenous	Viral transduction and lipid transfection	UC, DGC, and extrusion	DCs	Adenoviral and mammalian expression plasmid	GFP/OVA, M27-M30-TRP2, and anti-PD1	B16F10, MC-38, and LLC xenograft	79
Exosome	Exogenous and endogenous	Electroporation and transfection	UC	M1 macrophage	Mammalian expression plasmid	PD-L1 siRNA and VSV-G	CT26 xenograft	95
CNV	Endogenous	Viral transduction	UC and extrusion	HEK 293T	Lentiviral	aPD-L1	LLC xenograft	100
Exosome	Exogenous and endogenous	FCR–IGG binding and nanosecond pulse electroporation	UC	MEF and HEK 293T	Mammalian expression plasmid	Anti-CD71, anti-PDL1 and IFN-γ mRNA	GL261 and SB28 GBM	106
Exosome	Endogenous	Lipid transfection	UC	HEK 293T	Mammalian expression plasmid	IL-3 and BCR-ABL siRNA	LAMA84 or K562R CML xenograft	108
Exosome	Endogenous	Viral transduction	UC	NPCC	Lentiviral	miR-142-5p	5-8FR NPCC	103
Exosome	Endogenous	TACE	UC and TFF	MEF and hBMSC	Synthetic mRNA/siRNA	TP53 mRNA and $siKras^{g12d}$	Orthotopic PANC-1 and patient-derived xenograft	109
Exosome	Endogenous	CNP	UC and DGC	MEF and DCs	Synthesized mRNA	PTEN mRNA	U87 and GL261 GBM xenograft	105
Exosome	Exogenous	Electroporation	UC	NK-92MI	—	PLK1 siRNA and PD-L1 siRNA	CT26 and hepg2 xenograft	59
Exosome	Endogenous	Transduction	UC	NK-92	Lentiviral	CAR-Her2	HER2+ breast tumor brain metastasis	60

Abbreviations: CNV (cell-derived nanovesicle), TACE (Transwell®-based asymmetric cell electroporation), CNP (cellular nanoporation), UC (ultracentrifugation), DGC (density gradient centrifugation), SEC (size exclusion chromatography), TFF (tangential flow filtration), MSC (mesenchymal stem cell), BM-MSC (bone marrow-derived MSC), RBC (red blood cell), DCs (dendritic cells), NPCC (nasopharyngeal carcinoma cell), MEF (mouse embryonic fibroblasts), CML (chronic myeloid leukemia), GBM (glioblastoma multiforme), TNBC (triple-negative breast cancer).

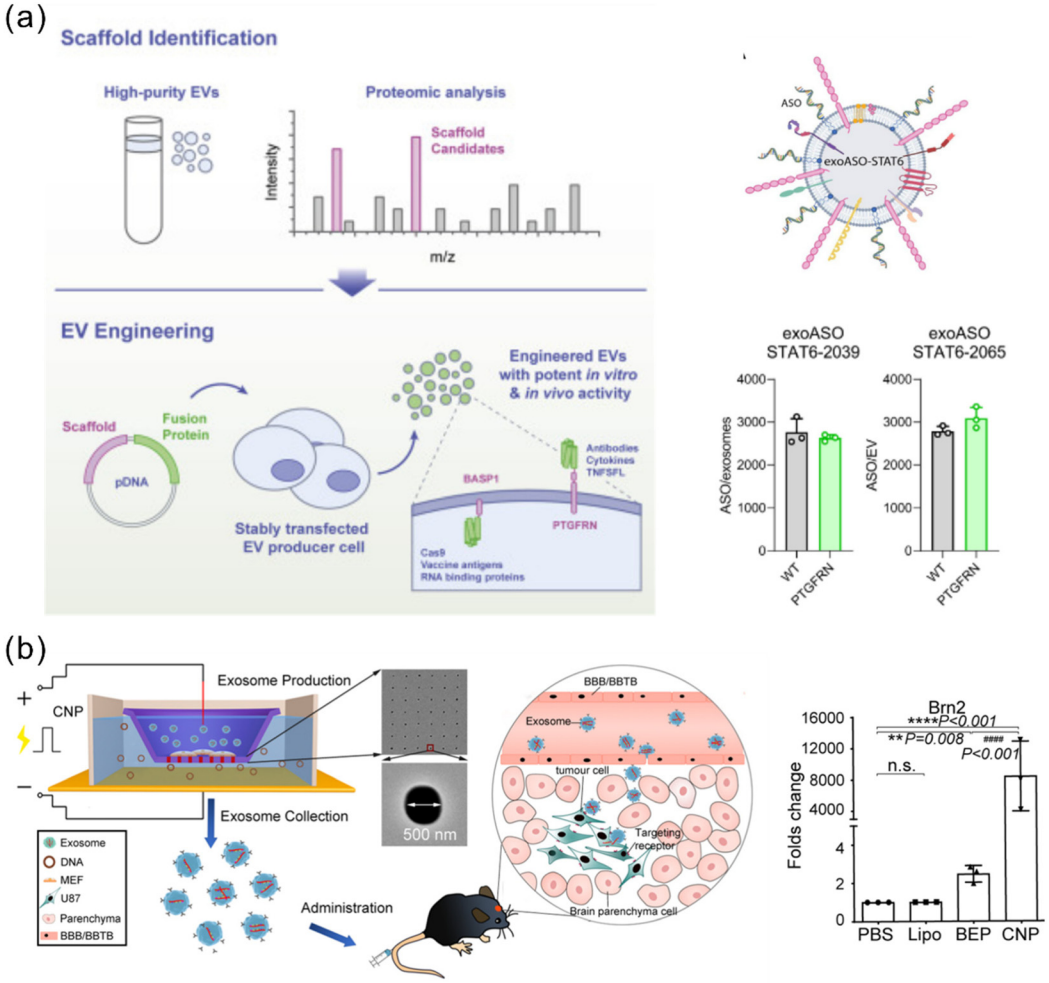

Fig. 5 Representative approaches of exogenous loading and endogenous loading for genetically engineered EVs. (a) Overexpressed PTGFRN+ on the exosome surface serves as a scaffold protein and achieves efficient loading of ASO-STAT6 with >2000 copies per exosome. Adapted from ref. 71 and 98 with permission from American Association for the Advancement of Science (copyright 2022) and from Cell Press (copyright 2021), respectively. (b) A CNP strategy achieved a high efficiency of large nucleic acid mRNA loading with a 100-fold higher level than the conventional electroporation method. Adapted from ref. 105 with permission from Springer Nature, copyright 2020.

3.1.2 Endogenous loading. Since EVs naturally bear nucleic acids from their parental cells, preconditioning cells with transfection or transduction-based approaches offer feasibility to endogenous loading of cargoes on EVs. Koh *et al.* provided an example of using exosomes as antagonists for the competitive inhibition of tumor immune evasion signals.[99] Exosomes carrying SIRPα were generated by transfecting HEK 293T cells with a plasmid encoding a high-affinity variant of SIRPα, which led to disrupted CD47–SIRPα interaction between cancer cells and TAMs, thereby facilitating prime effective anti-tumor T cell response. Based on the similar principle of immune checkpoint blockade, Li *et al.* generated a stable HEK 293T cell line expressing anti-PD-L1 scFv to produce aPD-L1 nanovesicles for remodeling the anti-inflammatory TME into a pro-inflammatory state.[100] Further loading glutamine antagonists with the aPD-L1 nanovesicles *via* electroporation facilitated the upregulation of oxidative metabolism in effector T cells to promote their differentiation into a highly activated phenotype, ultimately enhancing the efficacy of CAR-T therapy against solid tumors in immunocompetent mouse models.

Apart from proteins, RNA cargoes can also be loaded into EVs in an endogenous manner. Both small RNAs and mRNAs encapsulated in exosomes can be transferred to another cell and be functional in the new location.[101] Ohno *et al.* introduced miRNA let-7a into exosomes by transfecting HEK 293T cells using a lipofection method, which induced tumor inhibition in breast cancer mouse models.[102] Zhu *et al.* achieved endogenous loading of microRNA 142-5p within TEXs for gene silencing of epidermal growth factor (EGF) and hepatocyte growth factor (HGF), thereby accelerating the radiosensitivity of nasopharyngeal carcinoma cells.[103]

Aiming at mRNA loading, Wang *et al.* developed an approach for selective recruitment of mRNA within microvesicles by taking advantage of the binding ability of the transactivator of transcription (Tat) protein to the stem loop-containing

trans-activating response (TAR) element RNA.[104] Specifically, two expression constructs were transferred into HEK 293 cells: one being a short Tat peptide fused to the C-terminus of arrestin domain containing protein 1 (ARRDC1) and the second being TAR fused to the 5′ end of a cargo mRNA. After binding occurred, the Tat-TAR system selectively packaged TAR-labeled mRNAs into the ARRDC1-mediated microvesicles (ARMMs) during EV biogenesis. A large macromolecular CRISPR/Cas9 complex was also successfully packaged into ARMMs *via* the interaction of ARRDC1 with the WW domains in WW-Cas9 fusion proteins. The cargo-loaded ARMMs demonstrated effective translation of mRNAs into functional proteins or CRISPR/Cas9-mediated gene silencing in recipient cells.

As previously mentioned, conventional electroporation can effectively insert small nucleic acids into exosomes but has poor performance in delivering large mRNA molecules. To address this problem, Yang *et al.* designed a cellular nanoporation (CNP) device to shuttle nucleic acid cargoes from the buffer into the cells attached on the chip surface under transient electrical pulses.[105] This method allowed the production of large quantities of exosomes and efficient loading of miRNA with a higher concentration into the exosomes when compared with the conventional electroporation-based approach. More importantly, CNP achieved a high efficiency of large nucleic acid mRNA loading with a 100-fold higher level than bulk electroporation (Fig. 5b). Similarly, a recent report by Dong *et al.* developed a modified electroporation platform for higher yields of EVs (up to a 45-fold increase relative to the control group) and efficient mRNA loading.[106] It is achieved by using nano- and millisecond pulse stimulation combined with a microfluidic setup. It is demonstrated that the level of mRNAs complementary to the plasmid DNA that had been encapsulated within the EVs was 10^3-fold higher than that in the control group. As a proof of concept, a mouse embryonic fibroblast line that stably expressed CD64 was engineered by the platform to produce IFN-γ mRNA loading EVs, which enabled the immobilization of anti-CD71 and anti-PD-L1 for preferential targeting to glioblastoma cells. Treatment with the immune sEVs induced increased infiltration of effector T cells, upregulation of MHC-I on GBM cells, and re-polarization of suppressive myeloid cells, and ultimately elicited potent antitumor efficacy in preclinical GBM models, including models that are intrinsically resistant to immunotherapy. Of note, endogenous loading strategies with an expression cassette for a therapeutic gene may lead to parallel enrichment of mRNA and protein in the extracellular vesicles generated by those cells,[107] making it difficult to distinguish the newly translated protein from the mRNA in recipient cells.

A recent study has developed a strategy, termed GEMINI (genetically encoded multifunctional integrated nanovesicles), for achieving Cas9 delivery to T cells. EVs from Cas9 and sgRNA-expressing cells showed nuclease function.[86] With the equipment of anti-CD2 scFvs for T cell targeting and VSV-G display for increased EV uptake, GEMINI validated its effectiveness in delivering recombinant Cas9 RNP by the complexing of Cas9 and single-guide RNA to cut the gene encoding C–X–C chemokine co-receptor type 4 in primary human CD4+ T cells. Although not in a cancer model, this work demonstrated the large therapeutic cargo delivery of EVs to defined cell populations, as well as providing integrated tools with elaborate designs for targeting, cargo loading and vesicle fusion, which can be widely applicable for a range of applications in cancer immunotherapy.

Together, these studies shed light on the great potential of EVs as carriers for therapeutic drugs to modulate immune activity *in vitro* and *in vivo* through a variety of approaches. Still, the selection of the most suitable method for clinical application remains to be determined. Beyond developing optimal EV-based delivery systems, thorough considerations should encompass the identification of the optimal payload and the evaluation of desired on-target effects, unwanted off-target effects and potential effects on non-target cells.

3.2 Antigen presentation

To increase the responsiveness to current cancer immunotherapy, researchers have made efforts to increase antigen presentation to induce activation of T cells. The ability to express membrane proteins is a unique feature of EVs as compared to synthetic carriers, providing new opportunities for developing antitumor vaccines. Compared to the classical vaccine administration containing soluble antigens, antigens presented by EVs showed a more potent antigen-specific antitumor immune response, highlighting the potential of EVs for vaccine development.

Tumor-derived EVs, especially TEXs, naturally carry tumor-associated antigens, thereby holding the potential as cancer vaccines. Yet, the application of TEXs alone cannot produce satisfying antitumor immunity *in vivo* due to TEX-induced immunosuppression and limited immunogenicity. Modification to TEXs is necessary to enhance specific antigenicity. For example, Li *et al.* generated leukemia cell-derived exosomes (LEXs) harboring overexpressed CD80 and CD86 through lentiviral transduction, which promoted CD4+ T cell proliferation and Th1 cytokine secretion more efficiently than unmodified LEXs.[110] Another study utilizing murine melanoma B16BL6 as producer cells developed a strategy to enhance the antigen capacity of TEXs with endogenous loading of immunostimulatory CpG DNA *via* streptavidin–biotin interactions.[31] These CpG DNA-modified exosomes efficiently and simultaneously delivered tumor antigens together with the adjuvant to the same APC, inducing more potent antigen-specific immune response when compared with the simple co-administration of exosomes and CpG DNA.

The ability of DC-derived EVs to sustain similar immunostimulatory properties of their parent cells makes them among the highly auspicious candidates for anticancer vaccine development. A study has illustrated stronger *in vivo* immunostimulatory effects associated with smaller exosomes derived from DCs as compared with larger microvesicles, which is possibly due to the higher payload loading within exosomes.[111] Liu *et al.* developed a strategy to deliver an antigen to lymph nodes

(LNs) for activation of broad-spectrum T-cell responses using exosomes derived from DCs virally transduced with a plasmid encoding ovalbumin.[79] A variety of functional molecules were assessed on the surface of the vesicles, including some co-stimulatory molecules that are involved in the process of antigen presentation (CD80, CD86, CD40 and so on) and various chemokine receptors that can enhance the migration to LN (CCR2, CCR5, CCR7). The engineered exosomes showed efficient accumulation in the peripheral LNs within 12 h and elicited robust $CD8^+$ T-cell responses *in vivo*. Another interesting study for the development of cell-free DC-EV vaccines investigated the possibility of exploiting transcription-induced chimeric RNAs (chiRNAs) as neoantigens.[112] Transcription-induced chiRNAs consist of fused mRNA transcripts originating from two or more unrelated genes. This study used chiRNAs encoding for astrotactin 2, an integral membrane protein involved in neural development, and pregnancy-associated plasma protein-A, a secreted metalloproteinase that cleaves the insulin-like growth factor binding proteins. The chiRNAs were introduced into DC-EVs by lentiviral transduction, forming an anticancer vaccine termed DEX_{A-P}, which demonstrated antigen cross-presentation capability evidenced by high levels of MHC-I, MHC-II, ICAM, CD80, and CD86. Moreover, DEX_{A-P} vaccination resulted in potent anti-tumor immunity and prolonged survival in a syngeneic immunocompetent esophageal cancer model, which is a difficult-to-treat 'cold' solid tumor.

Macrophage is another APC type and as well has been engineered to enhance antigen presentation with their EVs. Wang *et al.* recently described a strategy to equip M1 macrophage-derived vesicles with antigens by incorporating tumor cell nuclei, generating chimeric exosomes (aMT-exos).[113] Tumor antigens, represented by ovalbumin, as well as various immune activation indicators represented by CD86, MHC I, and MHC II were observed in the aMT-exos. As a result, they showed potent efficacy in suppressing tumor growth and prolonging survival in multiple murine tumor models including lymphoma, TNBC, and melanoma. In terms of the underlying mechanisms, aMT-exos could enter LNs and activate prime T cells in both the classical antigen presen-

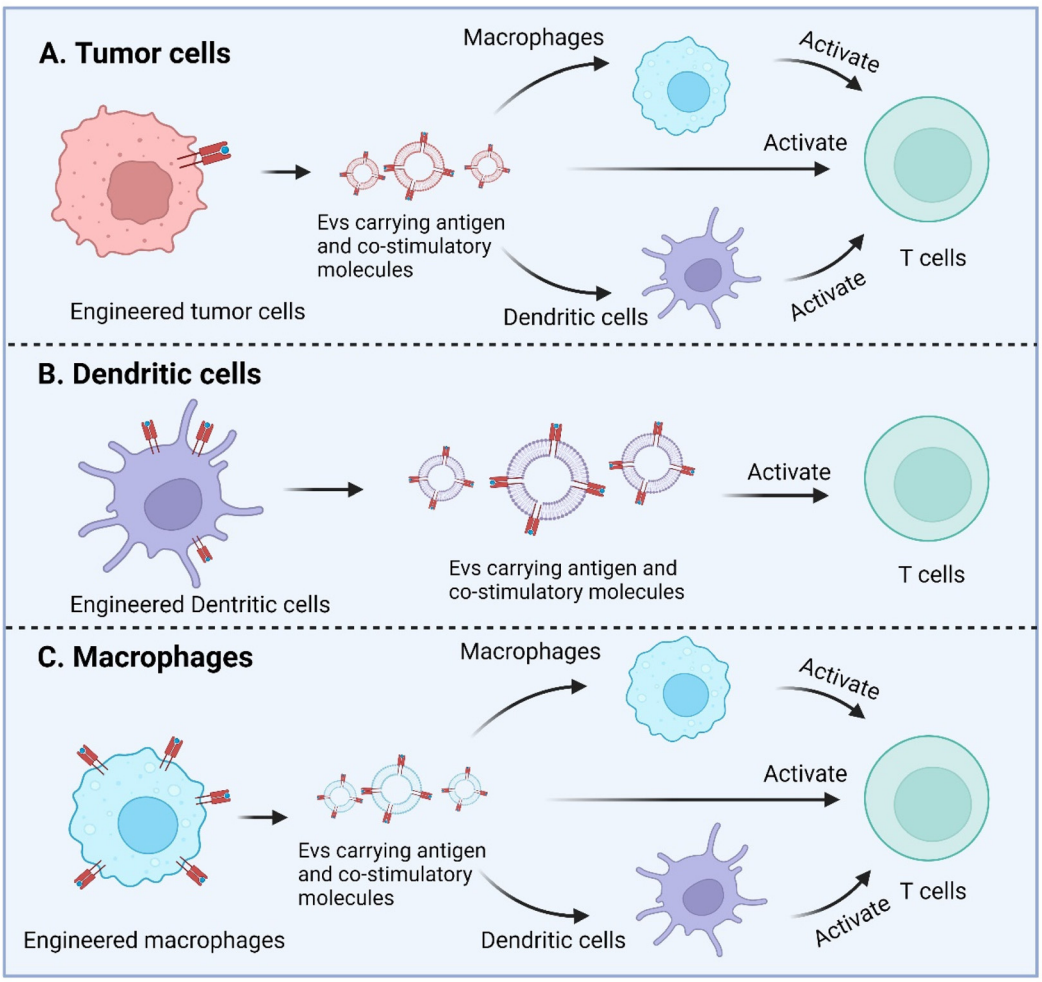

Fig. 6 Enhanced antigen presentation by genetically engineered EVs derived from tumor cells, dendritic cells, and macrophages. Created with BioRender.

tation-induced immunostimulatory manner (*i.e.* uptake by DCs or macrophages followed by downstream T cell activation) and a unique "direct exosome interaction" manner (*i.e.* direct activation of T cells with encapsulated factors). Engineered TEXs potentially can exert their functions in similar manners. For DC-derived EVs, they are more likely to only have the direct manner to interact with T cells, similar to their parental cells (Fig. 6).

Another strategy for enhanced antigen presentation to elicit immune response involves membrane engineering of tumor cells with foreign antigens. This may be achieved by exploiting the fusion ability of fusogenic liposomes or viruses. Inspired by this concept, Yang *et al.* developed a fusogenic exosome platform, mVSVG-Exo, by genetically introducing a viral fusion protein, VSV-G, to the surface of exosomes, thereby enabling transfer of active membrane proteins into the target cell surface.[114] Subsequently, the platform was utilized to achieve xenogenization of tumor cells to either enhance the antigenicity or generate danger signals for increased recognition by the immune system.[115] mVSVG-Exo mediated DC maturation by stimulating TLR4 signaling and induced cross-presentation of tumor antigens to CD8+ T cells. When combined with anti-PD-L1, the combinational treatment induced a specific and long-lasting systemic adaptive immune response that can eradicate the primary tumor and inhibit recurrence (Fig. 7). Imparting exosomes with fusion capabilities additionally facilitate delivery, in particular cytosolic delivery, of therapeutic agents. Relevant reports in this regard have been mentioned in previous sections.[84,86,95]

These studies open new avenues for engineering EVs for antigen-specific vaccines in cancer immunotherapy. Although it is yet far from the clinical translation of engineered EV-based vaccines, it is possible that an 'all-in-one' approach for generating therapeutically active EVs with antigen presentation, cytosolic delivery and minimal toxicity may be realized. The implementation of such a comprehensive approach holds promise for potential tangible clinical benefits in the future.

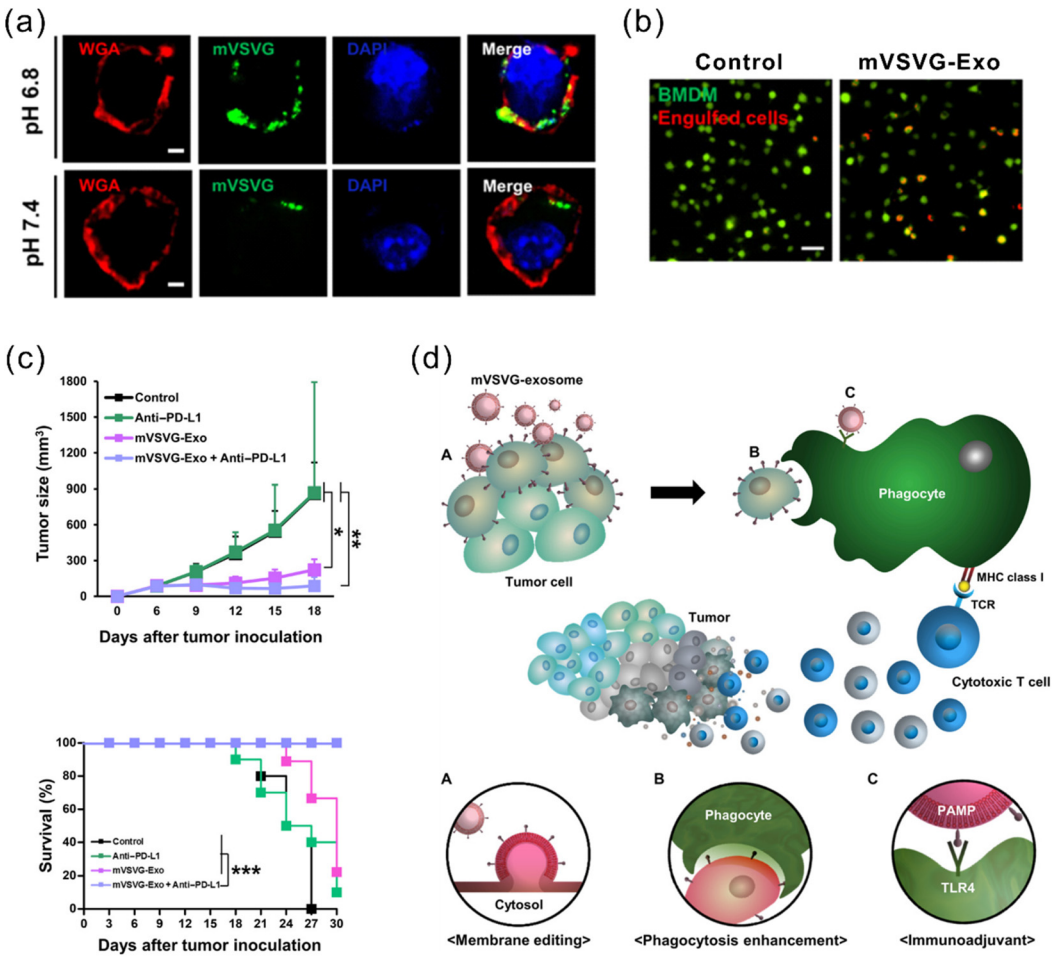

Fig. 7 A fusogenic exosome platform, mVSVG-Exo, developed for the antigen xenogenization of tumor cells to enhance anti-tumor immunity. (a) mVSVG-Exo (green) fuses with cancer cell membranes (red) at pH 6.8. (b) Cancer cells fused with mVSVG-Exo can be readily engulfed by macrophages. (c) Combined treatment with mVSVG-Exo plus anti-PD-L1 effectively induces antitumor immunity evidenced by significantly inhibited tumor growth and increased survival rates. (d) Schematic illustration of the mVSVG-Exo-mediated tumor xenogenization strategy. Adapted from ref. 115 with permission from American Association for the Advancement of Science, copyright 2020.

4. Conclusion and outlook

To sum up, the prospects of EV-based strategies in cancer immunotherapy are multifaceted. Firstly, being cell-free entities, EVs show enhanced stability and allow extended storage, which is particularly favorable for clinical applications. Secondly, their intrinsic biocompatibility, low immunogenicity, inherited surface protein presentation and encapsulation of bioactive agents make them outperformed synthetic nanocarriers. Besides, EVs can be tailored using various techniques, like genetic engineering, for targeted and efficient delivery of therapeutic agents, enhancing the precision of cancer treatment.

Despite these promising aspects, unleashing the full potential of EV-based drug delivery in cancer immunotherapy as well as their clinical translation face several challenges.[116] Owing to their inherent complexity, size heterogeneity, and batch-to-batch variations encountered both during biological processes inside the cells and during the manufacturing process, the product heterogeneity of EVs is higher than those of purely synthetic systems, potentially affecting the effectiveness of treatment. Therefore, standardization of the isolation, purification, and characterization methods for EVs is crucial for ensuring reproducibility. Of note is that large-scale production with cost-effectiveness for the clinical setting is in demand. While we mentioned in this review that some studies developed novel strategies for significantly increased yields of EVs, the required equipment or condition could be too complicated and costly for clinical-level production. To move the field forward, these pressing questions emerging from the initial stages need to be addressed.

From the translational standpoint, ensuring the safety profiles and efficacy reliability of EV-based therapy necessitates a comprehensive understanding of underlying mechanisms and potential consequences. Several pivotal questions emerge: to what extent do the functions of EV-based carriers rely on their cellular origin? What are the implications of EVs' intrinsic cargo –nucleic acids, proteins and various factors, which will be co-delivered with the EV-based delivery systems to the target cells and may exert physiological effects? How to selectively isolate functional relevant EVs while discriminating against those deemed "useless" or "trash" EVs given the potential heterogenicity in cargo content among EVs? How do EVs dynamically interact with the complex TME? What happens to EV-based carriers once administered *in vivo* in terms of biodistribution, clearance, and persistence? Addressing these questions is essential for harnessing their merits in cancer immunotherapy and accelerating the clinical translation of EV-based delivery platforms. From the genetic engineering perspective, the specific focus in this manuscript, important questions include: what safety concerns arise from genetically engineered EVs? How can we overcome limitations in loading large therapeutic payloads (*e.g.*, mRNA) without compromising EV stability? How do different encapsulation strategies influence the release of genetic cargo from EVs? How to achieve efficient endosomal escape to allow the genetic cargo to reach their intracellular target?

While understanding the essentials of EV biology remains pivotal, strategic combinations with other therapeutic modalities hold immense promise. Given the context of the volatility and complexity of tumor immune systems, achieving significant efficacy with individual therapy is challenging. Combining EV-based immunotherapy with other treatments, such as cell therapy and bio-scaffold-based strategies, holds the potential to boost the anti-tumor efficacy. Efforts can be directed towards optimizing synergistic effects and dosing schedules.

Moreover, if multi-disciplinary technologies can be combined as much as possible, such as optics, physics, clinical science, and chemistry, it would likely help to overcome existing barriers as well as expand the potential of EV-based therapy. The recent decades have witnessed rapid advancements in all these technologies. It is expected that the convergence of multidisciplinary research and strategic combinations will shape the clinical impact of EVs revolutionizing cancer immunotherapy and advancing precision medicine.

Abbreviations

APCs	Antigen-presenting cells
ASOs	Antisense oligonucleotides
CNVs	Cell-derived nanovesicles
CNP	Cellular nanoporation
CML	Chronic myeloid leukemia
CRC	Colorectal cancer
DCs	Dendritic cells
DGC	Density gradient centrifugation
EVs	Extracellular vesicles
GBM	Glioblastoma multiforme
HCC	Hepatocellular carcinoma
HEK293	Human embryonic kidney cells
IFN-γ	Interferon gamma
LNs	Lymph nodes
Lamp2b	Lysosome-associated membrane glycoprotein 2b
MHC	Major histocompatibility complex
MSCs	Mesenchymal stem cells
mRNA	Messenger RNA
miRNA	MicroRNA
MEF	Mouse embryonic fibroblasts
MDSCs	Myeloid-derived suppressor cells
NPCC	Nasopharyngeal carcinoma cells
NK	Natural killer
NSCLC	Non-small cell lung cancer
PC	Pancreatic cancer
PDAC	Pancreatic ductal adenocarcinoma
PDGFR-β	Platelet-derived growth factor receptor beta
PD-1	Programmed cell death 1
PD-L1	Programmed cell death ligand 1
RVG	Rabies viral glycoprotein
RBCs	Red blood cells
SIRPα	Signal regulatory protein α
scFvs	Single-chain variable fragments

SEC	Size exclusion chromatography
siRNA	Small interference RNA
TFF	Tangential flow filtration
TIMP-1	Tissue inhibitor of metalloproteinases-1
TLR	Toll-like receptor
TACE	Transwell®-based asymmetric cell electroporation
TNBC	Triple-negative breast cancer
TME	Tumor microenvironment
TAMs	Tumor-associated macrophages
TEXs	Tumor-derived exosomes
UC	Ultracentrifugation
VSV-G	Vesicular stomatitis virus G protein

Conflicts of interest

The authors declare no conflict of interests.

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