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Extracellular vesicles as next generation immunotherapeutics

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ABSTRACT

Extracellular vesicles (EVs) function as a mode of intercellular communication and molecular transfer to elicit diverse biological/functional response. Accumulating evidence has highlighted that EVs from immune, tumour, stromal cells and even bacteria and parasites mediate the communication of various immune cell types to dynamically regulate host immune response. EVs have an innate capacity to evade recognition, transport and transfer functional components to target cells, with subsequent removal by the immune system, where the immunological activities of EVs impact immunoregulation including modulation of antigen presentation and cross-dressing, immune activation, immune suppression, and immune surveillance, impacting the tumour immune microenvironment. In this review, we outline the recent progress of EVs in immunorecognition and therapeutic intervention in cancer, including vaccine and targeted drug delivery and summarise their utility towards clinical translation. We highlight the strategies where EVs (natural and engineered) are being employed as a therapeutic approach for immunogenicity, tumoricidal function, and vaccine development, termed immuno-EVs. With seminal studies providing significant progress in the sequential development of engineered EVs as therapeutic anti-tumour platforms, we now require direct assessment to tune and improve the efficacy of resulting immune responses - essential in their translation into the clinic. We believe such a review could strengthen our understanding of the progress in EV immunobiology and facilitate advances in engineering EVs for the development of novel EV-based immunotherapeutics as a platform for cancer treatment.

Abbreviations: ACE2, angiotensin-converting enzyme 2; APCs, antigen-presenting cells; pMHC, antigenic peptide /MHC complex; ASOs, antisense oligonucleotides; Ba-sEVs, bacterial derived sEVs; BiTE, bispecific T cell-engager; BmDCs, bone marrow derived DCs; CPC, cardiac progenitor cells; cDCs, conventional dendritic cells; CTLs, cytotoxic T lymphocytes; DAMP, damage-associated molecular pattern; DCs, dendritic cells; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; ESCRT, endosomal sorting complexes required for transport; EVs, extracellular vesicles; HIPP, Human Immunopeptidome Project; iPSCs, induced pluripotent stem cells; lEVs, large EVs; M1-/M2-sEVs, M1/M2-polarized macrophage derived sEVs; pMHC-II, MHC class II-peptide complex; MoDCs, monocyte-derived dendritic cells; MVBs, multivesicular bodies; MDSCs, myeloid-derived suppressor cells; OMV, outer membrane vesicles; PAMPs, pathogen-associated molecular patterns; PRRs, pattern recognition receptors; PBMCs, peripheral blood mononuclear cells; sEVs, small EVs; SC-sEVs, stem cell derived sEVs; STING, stimulator of interferon genes; SMAPs, supramolecular attack particles; sEVs SMART, synthetic multivalent antibodies retargeted; TMPs, T cell microvilli particles; T-sEVs, T cell sEVs; TRAIL, TNF-related apoptosis-inducing ligand; TLR, Toll-like receptor; Tu-sEVs, tumour cell derived sEVs; tSV, trans-synaptic vesicles; TAMs, tumour associated macrophages; TAA, tumour associated antigen.

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1. The diversity of extracellular vesicles

Extracellular vesicles (EVs) are key signalling players in intercellular crosstalk [1,2]. EVs are released by all cellular organisms, including plants, fungi, and bacteria [3–6], and contain and display diverse cargo of proteins, lipids, and nucleic acids (including DNA, mRNA, non-coding RNAs) which can transfer between cells to elicit functional response [7,8]. This interaction may involve not only the release and delivery of EV cargo but also cell surface interactions and target-cell modulation [9,10], including the regulation of innate and adaptive immune responses [11–14]. Functionally, EVs are involved in the processes during homoeostasis such as metabolism [15], neuronal function [16], as well as cell development and differentiation [17,18], cell proliferation, inflammation [19], embryo implantation and endometrial function [20–22], and tissue repair and regeneration [23–25]. EV-mediated crosstalk may occur unidirectionally or bidirectionally between cells or via systemic communication, during which EVs traffic to various tissues and organs [26,27].

As heterogeneously sized (~30–2000 + nm) particles, EVs include small EVs (sEVs, including exosomes), microvesicles (MVs) (or large EVs (lEVs), microparticles and ectosomes), shed midbody remnants (sMBrs), migrasomes and apoptotic bodies [10,28–34], based predominantly on their biophysical properties (size and density), differing biochemical composition, and surface charge [35] (Table 1). The nature and relative abundance of EV cargo is selectively determined during EV biogenesis [10,36,37], and vary according to subtype and state/type of the producing cell [28,38–41] as well as source tissue [15]. Indeed, oncoproteins have been shown to modulate the content of released sEVs, altering vesicle number and size and their molecular cargo [42]. sEVs (including exosomes) range in size from ~30–150 nm in diameter and density 1.08–1.14 g/ml and are derived from intraluminal vesicles (ILVs) during endosomal maturation (where late endosomes/multivesicular bodies (MVBs) release ILVs following MVB fusion with the plasma membrane); lEVs (including microvesicles, 150–1000 nm, density: 1.08–1.14 g/ml) are generated by direct budding from the cell membrane; sMBr (200–600 nm, density: 1.22–1.30 g/ml) are generated by cell abscission and cytokinesis [32,43]; migrasomes (500 nm-3 μm) are released by adherent cells during cell migration [34] and apoptotic bodies (1–5 μm) are released during the terminal stage of apoptosis (Table 1). In the context of cell signalling, sEVs, due to their small size, protective lipid bilayer, and surface receptors, can mediate paracrine and longer range, interorgan systemic crosstalk [15,26,32,38,44–46], while larger sized EVs function predominantly in local intercellular communication [10,28,47].

By transferring bioactive molecules, sEVs alter the composition and function of recipient cells; in particular, cancer cell sEVs have been shown to transfer oncogenic traits from aggressive to indolent cancer cells or to normal/stromal cells through the delivery of oncogenic cargo [60–62], including oncoproteins and mutated proteins that attenuate tumour-suppressive factors, enhance tumorigenesis, and enable tumour formation and dissemination [31,32,63,64–67]. Cancer sEVs also support tumour progression by facilitating neo/angiogenesis, modulating the immune system, promoting coagulation and thus increasing adhesion of circulating tumour cells [68], and remodelling of the local immune landscape [69] and tumour cell microenvironments (including localised primary and distant metastatic sites) [8,61,70,71]. Tumorigenesis is intimately related to the dysregulation of immune cell subsets and chronic inflammation, with cancer sEVs involved in suppression of innate immune responses through mobilization of myeloid-derived suppressor cells (MDSCs) [72], activation of tumour-associated macrophages (TAMs) [72–75], and neutrophils [76,77]. Moreover, cancer sEVs can cause immune cell dysfunction by exposing various ligands to suppress/limit adaptive immune responses through antigen-presenting cells (APCs) and cytotoxic T lymphocytes (CTLs) (blocking T cell activation, proliferation, and enhancing T cell apoptosis), and promote cancer-related inflammation and immune escape [78–91] (reviewed

Table 1
Subtypes of extracellular vesicles/particles including various biophysical and biochemical properties. Biogenesis/release mechanisms and marker compositions may differ between cell types.

	Extracellular vesicles			Extracellular particles		Other types of extracellular vesicles			
Subtype	**Small EVs (sEVs)** [29,40,46,48-51] (incl. exosomes)	**Large EVs (lEVs)** [29,36,52,53] (incl. ectosomes, microvesicles)	**Shed midbody remnants (sMBRs)** [32]	**Exomeres** [54]	**Supermeres** [55]	**Exophers** [56]	**Large oncosomes** [57]	**Migrasomes** [34,58]	**Apoptotic bodies** [59]
Biogenesis	Endosomal pathway	Plasma membrane budding	Cytokinesis (cytokinetic abscission)	Unknown	Unknown	Plasma membrane budding	Plasma membrane budding	Plasma membrane budding	Plasma membrane budding
Markers *(enrichment)*	CD63, CD81, SDCBP, ESCRT complex proteins: ALIX/PDCD6IP and TSG101	ANXA1, RPS7, ARF6	MKLP1, RACGAP1	HSP90-b, ENO1, GANAB	TGFBI, HSPA13, ENO1, ENO2	Phosphatidylserine, LC3, Tom20	CK18, GOT1	TSPAN4, ITGA5	Phosphatidylserine
Particle diameter range	30-200 nm	100-1000 nm	200-600 nm	< 50 nm	< 50 nm	≥ 1 μm	≥ 1 μm	≥ 1 μm	50-5000 nm
Buoyant density	1.08-1.14 g/ml	1.08-1.14 g/ml	1.22-1.30 g/ml						

[92]). Further, sEVs can elicit an innate immune response to enhance immune surveillance, such as inducing monocyte-mediated cancer cell clearance at the pre-metastatic niche and suppression of metastatic development [78]. As such, sEVs have gained immense pre-clinical and clinical attention in the context of antigen cross-presentation, cancer immune surveillance and tumour escape [93,94].

In this Review we critically summarise the diverse roles of sEVs in immunoregulation, including antigen presentation, cross-dressing, activation and suppression of the immune response, and expression of cell surface opsonins and complement factors as a means of immune surveillance and immunorecognition in cancer (Fig. 1). This review builds on seminal reviews and perspectives in the field of understanding EVs in the context of immune regulation and cancer [13,92,95–100]. This review highlights current and emerging therapeutic strategies in

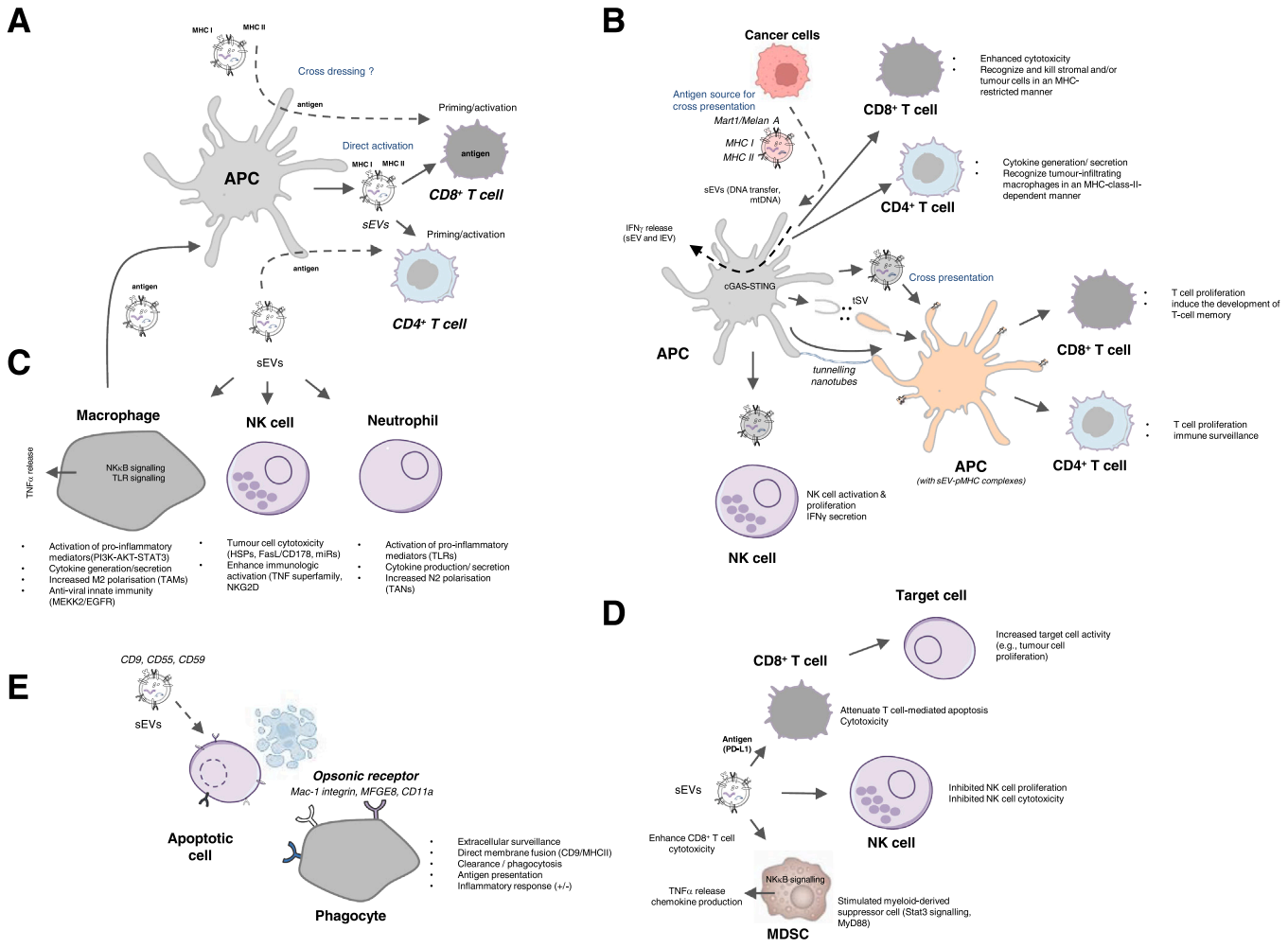

Fig. 1. Regulation of immune responses by small extracellular vesicles. (A) APC-derived sEVs can directly modulate the antigen-specific response of $CD8^+$ and $CD4^+$ T cells (direct presentation) and activation of NK cells. DC-derived sEVs (DC-sEVs), when used as potential vaccines, have been demonstrated to transfer functional pMHC (class I- and class II) to host DCs to prime $CD8^+$ and $CD4^+$ T cells, respectively. These sEVs also exhibit comparable efficacy as mature DCs to stimulate antigen-specific T cell activation in vivo; sEVs are vehicles for pMHCs, along with co-stimulatory molecules expressed by recipient DCs, to stimulate T cells. sEVs from APCs loaded with specific peptides and/ or antigens are also capable of inducing an immune response, including NKG2D ligand, IL-15/IL-15Rα-mediated activation of NK cells. Molecular regulators in sEVs (MICA/B, miR-186) have been shown to contribute to cytotoxicity of NK cells and establishment of immune escape mechanisms. **(B)** Tumour-derived sEVs (Tu-sEVs) internalised by or fused with antigen-presenting cells (APCs) have been shown to prime $CD8^+$ and $CD4^+$ T cells. These sEVs express tumour antigens (e.g., as MHC class I- and class II-peptide complexes and as luminal and membrane-surface-bound antigens). Tu-sEVs also contain/display receptor ligands and adhesion molecules (e.g., LFA1, MFGE8, TIM1/4, tetraspanins) participating either directly or indirectly in APC binding. Tu-sEVs can become antigen source for cross-presentation to modulate $CD8^+$ and $CD4^+$ T cell activation. **(C)** sEVs have been demonstrated to have specific immune cell stimulatory roles across macrophages, NK cells, and neutrophils, including activation of pro-inflammatory mediators (TNF-α, RANTES, TLRs), cytokine/chemokine generation/secretion (CCL2), and enhancing immune activation through HSP70/HSP72; activate NF-κB signalling through TLR2. Tu-sEVs transfer EGFR to macrophages to impact innate antiviral immunity via MEKK2. In pathogen infection, sEVs from infected macrophages are capable of inducing early-stage systematic inflammatory responses with cytokine storm. Moreover. macrophages can present antigens to $CD4^+$ T cells via DCs, ultimately triggering $CD4^+$ T cell activation. **(D)** sEVs suppress immune responses through different mechanisms including attenuation of T cell-mediated killing (sEVs: CD95L, TRAIL or galectin 9, promoting T cell apoptosis, PD-L1-bearing sEVs inhibit $CD8^+$ T cell cytotoxicity), NK cell cytotoxicity (blocking NKG2D-dependent cytotoxicity of NK cells and $CD8^+$ T cells), and activation of myeloid-derived suppressor cells (MDSCs) (tumour-derived sEVs contain PGE2, TGF-β, HSP72; sEVs containing MyD88 promote IL-6, TNF-α, and chemokine CCL2 production, induction of MDSCs and tumour metastasis). These mechanisms facilitate immune escape, and tumour invasion and metastasis. **(E)** sEVs can transfer complement factors to regulate/enhance extracellular surveillance, apoptotic cell phagocytosis/clearance, antigen presentation, and immune-inflammatory response. CD55/59 on sEVs from immature DCs can attenuate the inflammatory response by opsonising apoptotic cells (through opsonic receptors, e.g., iC3b) for phagocytosis. Mac-1 integrin complex in sEVs from neutrophilic granulocytes has been shown dependent on antibacterial capacity and environmental factors selectively directing sEVs with specific cell surface receptors. Further, targeting of circulating sEVs to DCs has been shown mediated by MFG-E8/lactadherin, CD9, CD11a, CD54, CD81, and phosphatidylserine on sEVs, and CD11a and CD54 on DCs.

engineering EVs as an effective means to prime or promote adaptive and innate immune responses [101–103], and technologies advancing next generation immunotherapeutics, including cancer vaccines [104] and anti-cancer therapy [105].

2. Small extracellular vesicles and their roles in antigen presentation and T cell activation

2.1. sEVs directly mediate T cell activation and cancer immunosurveillance

The engagement of T cell receptor with its specific antigenic peptide /MHC complex (pMHC) from APCs leads to T cell activation and is the keystone of the adaptive immune response, triggering T cell-mediated effector functions, including T cell proliferation, cytokine secretion and cytotoxicity [106]. sEVs can mediate T cell activation by their composition (and transfer) of functional pMHCs and other active molecules which play an important functional role in cancer immunosurveillance [80,107–110] (Fig. 1A-B). sEVs can mediate T cell activation when pMHCs on sEVs are directly engaged by antigen-specific T cells, leading to their subsequent activation [14]. sEVs have also been shown in vivo to transfer pMHCs to DCs leading to priming of $CD8^+$ and $CD4^+$ T cells [111–113]. Moreover, DC-derived sEVs (DC-sEVs) can induce T cell activation in the absence of antigen presenting cells (APCs) as they carry pMHCs and co-stimulatory molecules that include sEVs isolated from viral infected monocyte-derived DCs (MoDCs) in vitro [114].

sEV-mediated T cell activation is a selective process [75,115,116]. sEVs from ovalbumin/$OVA_{257–264}$ peptide- or OVA protein-pulsed mature bone marrow derived DCs (BmDCs) stimulated specific $CD8^+$ T cell hybridomas more efficiently than their immature counterparts, likely due to higher abundance of co-stimulatory molecules on the mature BmDC-sEVs [88]. Segura et al., [86] highlighted that DC-sEV proteome composition (from immature and mature DCs) can influence their T cell priming capacity. DC-sEVs from knock-out (KO) mice lacking MHC class II-peptide complex (pMHC-II) and co-stimulatory molecule ICAM-1 were unable to directly prime naïve T cells [86], suggesting that sEVs from DCs can differentially modulate their T cell priming abilities [86].

Although DCs are considered primary professional APCs, macrophages and B cells can also perform as APCs [117]; epithelial cells at mucosal surfaces (under pathological conditions) can also act as non-professional APCs [118]. Raposo and colleagues demonstrated that B cell-derived sEVs that bear pMHC-II could activate human and mouse antigen-specific $CD4^+$ T cell clones [80]. The presentation of these complexes to T cells suggests that sEVs can modulate the adaptive immune response. Using a mAb capable of tracking B cell surface pMHC-II after antigen pulse, Muntasell and colleagues showed that activated B cells released sEVs that express pMHC-II, which directly stimulated primed but not naïve antigen-specific $CD4^+$ T cells [85]. This study further highlighted that interaction of antigen-loaded B cells with specific $CD4^+$ T cells could be mediated by sEVs from these donor B cells, and that recycled pMHC-II complexes could directly activate $CD4^+$ T cells [85]. Such findings indicate that sEVs from (activated) B cells can act as a modulator of continuous immune response or maintain antigen-specific memory T cells.

EVs consist of variety of subtypes, which differ based on their biogenesis and biophysical/chemical characteristics, including their size, origin/biogenesis, molecular composition and function (Table 1). Various immune cell types secrete distinct types of EVs with different sizes, subcellular origin, protein composition (including surface profile), and immune cell function. The relative abundance of EVs from DCs (namely sEVs and lEVs) to the total EV population, is influenced by their maturation state [40,116,119]. Tkach et al., [116] showed that differential immune activities in vitro could be obtained from different EV subtypes from DCs (based on selected changes in luminal cargo and surface membrane composition of each subtype), and that these activities could be modulated by treating the secreting DCs with a maturation-inducing stimulus. Additionally, this study highlighted immature DCs release lEVs (DC-lEVs) to promote secretion from primary $CD4^+$ T lymphocytes of cytokines (IL-4/−5/−13), while sEVs (containing pMHC-II) promoted IFNγ secretion [116]. Here, such functional differences were, in part, shown by EV surface-exposed T cell-binding proteins CD40, DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin) (abundant only on small/large EVs) and CD80 (present on all EVs) [116]. Other studies support such differential immune cell function of sized-based subtypes of EVs. Macrophages infected with *mycobacterium tuberculosis (M. tuberculosis)* in synergy with ATP secreted EVs (sEVs and lEVs) which carry pMHC-II and triggered *M. tuberculosis*-specific T hybridoma cells; however, only lEVs stimulated naïve P25 T cells in contrast to smaller sized sEVs [120]. Such reports support the need to comprehensively decipher the protein composition and functional diversity of EVs and their subtypes, impacted by (reviewed [1,38]). Deciphering the precise mechanisms and requirements of EV (and their subtypes in) transfer between immune cells, as well as other key variables including how the stage or route of infection as well as on which antigen is being evaluated and its distribution inside the infected host cell, will be key for developing novel approaches for EV cancer vaccines that promote antigen presentation and trigger T cell activation [121].

Other cell surface and integral membrane proteins on DC-sEVs are important in mediating associated cell recognition, adhesion, antigen uptake, and recipient cell function [70,122–126]. In addition to target cell surface interaction, sEVs can also bind complement factors and complement regulatory proteins [9,127], as well as cytokines [128]. Mouse BmDC-sEVs harbouring functional membrane bound IL-15Rα (NKG2D ligand) were shown in vivo to promote IL-15Rα- and NKG2D-dependent NK cell activation and proliferation, resulting in tumour suppression [129]. Human MoDC-sEVs also carry functional NKG2D ligands and can promote the proliferation and activation of NK cells [129], suggesting their potential utility for tumour vaccination.

2.2. APCs and cross-dressing – EV-dependent approaches for intercellular antigen transfer

APCs present antigenic peptides (pMHCs) to T cells. sEVs from various cell types have been shown to play crucial roles in carrying and presenting functional pMHCs to modulate antigen-specific $CD8^+$ T cell response [90] through cross-presentation of antigen (Fig. 1A-B). Here, APCs acquire and process antigens carried by sEVs and present their peptides to $CD8^+$ T cells [130]. Moreover, cross-presentation can occur whereby antigenic pMHCs are together transferred onto DCs and then efficiently presented to T cells (termed cross-dressing) [131]. Although cross-dressing refers exclusively to the acquisition of such antigenic pMHC, other membrane-associated molecules can also be transferred in the process [87,132]. Indeed, sEVs from mouse tumour cells induced $CD8^+$ T cell activation via cross-presentation, which led to tumour rejection in vivo [107]. When loaded onto human MoDC-sEVs transferred shared tumour antigens, allowing specific human CTLs activation in vitro, while for mouse DCs stimulated with tumour derived sEVs (Tu-sEVs), induced $CD8^+$ T cell-dependent cross-presentation among different, poorly immunogenic mouse tumours in vivo [107]. Andre et al., demonstrated MoDCs pulsed with sEVs from melanoma ascites could induce Mart1/Melan A-specific, HLA-A2-restricted $CD8^+$ T cell response *ex vivo* [108]. Likewise, bulk lymphocytes from melanoma patients stimulated with MoDCs loaded with ascites-derived sEVs (A-sEVs) expanded into tumour specific CTLs. This suggests that such A-sEVs could be a suitable tumour antigen source for EV-based anti--cancer vaccines [108].

Mallegol et al., [133] demonstrated in vitro that sEVs from HLA-DR4-expressing intestinal epithelial cells (T84) (pulsed with human serum albumin peptide $HSA_{64–76}$) could not directly activate

HLA-DR4-restricted $HSA_{64–76}$-specific $CD4^+$ T cell hybridoma. However, the same T cell hybridoma could be activated by human MoDCs loaded with the same sEVs, more efficiently than MoDCs loaded with soluble antigen. Xu and colleagues revealed macrophage-derived sEVs that contain apoptotic/necrotic cell-associated antigens have a low $CD4^+$ T cell response, while their T cell response is enhanced when localised with DCs [134]. Théry and colleagues highlighted sEVs from DCs carrying functional H-Y pMHC (pMHC-II) could be transferred onto other DCs [87]. Here, such sEVs could prime naïve male antigen H-Y specific $CD4^+$ T cells in vitro and in vivo and further stimulate H-Y specific $CD4^+$ T cells in the presence of pMHC-II-deficient DCs lacking the presentation of H-Y antigen in vitro [87]. Due to the complexity and heterogenicity of DC subpopulations, traditionally DCs have been divided into two discrete subtypes, which comprised conventional (both migratory and lymphoid-resident DCs) and non-conventional DCs (MoDCs and plasmacytoid DCs) [135]. More recently, plasmacytoid DCs sEVs (pDC-sEVs) mediated conventional DCs (cDCs) to cross-prime OVA antigen specific $CD8^+$ T cells by transferring antigen in an anti-Siglec H-OVA targeted pDCs vaccination model [136]. These findings indicate that sEVs contribute to the transfer of intact pMHCs through cross-dressing, therefore potentially serving as an effective means of targeted transfer of peptide antigens. Interestingly, using the H-2 K^b and SIINFEKL-specific OT-I TCR-transgenic T cells, Wakim and Bevan showed that donor DC-sEVs alone promoted T cell proliferation, but did not transfer pMHC-I to recipient DCs to achieve cross-dressing after viral infection [130]. Clearly, with these seemingly opposing data, the context of EV-mediated cross-dressing of DCs requires further investigation. Intriguingly, a recent study by Marcoux et al., showed that lEVs from activated platelets that contained functional proteasome could directly process OVA antigen to induce OVA-specific $CD8^+$ T cell proliferation in the absence of DCs (in vitro) [137], suggesting that platelet EVs contribute to adaptive immunity through antigen cross-presentation. Biodistribution tracking analysis of murine platelet EVs revealed their localisation to target lymphoid organs (spleen and lymph nodes) and the bone marrow [137], highlighting a potential signalling mechanism where such circulating EVs have access to immune cells through the lymphatic system [138], a tissue location that is inaccessible to platelets.

Depending on the context or the activation state of the donor DCs, it has also been proposed that sEVs from DCs can induce immunogenicity or tolerance. DC-sEVs are implicated in alloantigen spreading between host DCs after transplantation [84], suggesting that EVs exchange of peptide or pMHC between cells can increase the number of cells presenting an antigen. In transplantation, direct allorecognition is triggered by migration of donor DCs from the graft to recipient lymphoid tissues to directly activate alloreactive T cells, while indirect allorecognition is initiated by recipient DCs presenting donor allogeneic antigens as a mechanism for transplant rejection [139,140]. Recently, allo-presentation was shown mediated by recipient or donor DC-sEVs, referred to as a semi-direct pathway of allorecognition [140]. Using Thy1.1 congenic, TCR transgenic 1H3.1 $CD4^+$ T cells specific for the IA^b-$IE\alpha_{52–68}$ complex, graft-infiltrating DC-sEVs were shown to facilitate transport of alloantigen $IE\alpha_{52–68}$ to recipient splenic DCs in WT mice, but not in MHC-II KO mice, to then initiate indirect allorecognition [84]. Such findings propose that sEVs from recipient DCs may amplify allorecognition during organ transplantation [84,87]. Indeed, donor DC-sEVs can efficiently transfer donor pMHC to recipient cDCs to then activate allogeneic T cells in a murine heart transplant model [141], highlighting transfer of donor DC-sEVs in the generation of allograft-rejecting immune response. Interestingly, it was even shown that donor-sEVs (sEVs secreted from donor-T cells, -DCs, or -B cells) rather than donor leukocytes, trigger allogeneic T cell response in skin, heart and islet allotransplantation models [142]. Contrastingly, DC-sEVs also can induce immune tolerance [140]. In a heart allograft rejection rat model, where administration of an optimal dose of donor DC-sEVs prior to transplantation prolonged heart allograft survival, accompanied by the delay in acute allograft rejection and elevation in anti-donor MHC-II humoral response [143]. Further, a combination of an optimal dose of donor immature DC-sEVs and donor antigen specific Treg cells (20 μg/treatment before, during and after transplantation; versus 1/10/40/80 μg) enhanced graft tolerance of a liver allograft model, resulting in a significant extension in survival [144].

pMHCs from sEVs have been associated with cross-dressing APCs to activate alloreactive T cells [84] but yet to be confirmed as a mechanism of EVs-mediated T cell cross-priming to non MHC-derived antigens [130,145]. We now need further information deciphering the precise mechanism and requirements of sEVs transfer between different DCs based on their location (i.e., conventional and non-conventional DCs [146]), dependence of cross-dressing based on EV-dependent and -independent mechanisms, the release of DC-sEVs from periphery and/or secondary lymphoid tissues [84], as well as detailed characterization of these sEVs, for their capacity to promote antigen presentation and T cell activation.

Thus, sEVs through their specific surface membrane and luminal composition dependent on cell origin, can regulate antigen presentation through intercellular antigen transfer and promote the activation of T cells for cancer immunosurveillance.

3. Small extracellular vesicles: selectively tuning immune responses

3.1. A focus on T cell-derived sEVs

sEVs derived from T cells (T-sEVs) have been shown to have a role in immune regulation. Like other immune cells, T cells release sEVs constitutively, with their release enhanced by stimuli such as TCR triggering or T cell activation [112,147–149]. Wu et al. have shown that CTLs-release sEVs that enhance activation of CTLs stimulated by low-affinity peptides [150]. Further, activated $CD3^+$ T cell-released sEVs were shown to be involved in activation and proliferation of resting autologous T cells [149]. Together with IL-2, these activated $CD3^+$ T-sEVs induced a relative increase of $CD8^+$ T cells and a distinct cytokine network, highlighting their potential in immune activation and modulation. While established that T cells release sEVs, T cell activation may impact differential release of subpopulations of sEVs. Indeed, distinct sEV populations (based on differences in their buoyant density) derived from $CD4^+$ T cells have been shown to differentially increase T cell activation dependent on level of co-stimulation [112]. In terms of T-sEVs function, sEVs from OVA-specific $CD8^+$ T cells have been observed to be recruited by DCs through LFA-1-ICAM-1 interaction, leading to down-regulation of OVA MHC-peptide presentation and apoptosis of OVA-loaded DCs, which resulted in decreased OVA-specific $CD8^+$ CTL responses in murine cancer models [151]. The role of $CD4^+$ T-sEVs in B cell responses has been highlighted to directly enhance the humoral immune response in vivo when challenged with hepatitis B surface antibody [152]. $CD4^+$ T-sEVs resulted in an antigen-dependent increase in HBsAb in sera following treatment in vivo, while in vitro promoted B cell activation, proliferation, and antibody production. Interestingly, antigen-specific $CD4^+$ T cell sEVs showed a pronounced biological effect compared to sEVs derived from non-specific $CD4^+$ T cells, with CD40L shown as a significant regulator in such sEV-mediated B cell response [152]. Such findings may highlight potential for activated T cells in promoting lower-affinity and resting T cells, and broader implications in selective activation by different sEVs of T cells in cancer immunotherapy, and as immunomodulators to promote antigen-specific humoral immune responses in vivo.

T cells may employ sEVs as a shuttle strategy to selectively remove their suppressive tRNA fragments (tRFs, a novel class of small ncRNAs produced through enzymatic cleavage of tRNAs [153]) in order to sustain their activation. Chiou et al. [115] showed that sEVs from activated T cells contain specific tRFs, which, if remaining within donor cells, inhibit their activation and cytokine production. Further, some 5'-tRFs and tRNA-derived stress-induced RNAs (tiRNAs) have been found in

sEVs and are upregulated during stress responses [154] and in cancer cells suggesting that circulating tRFs exist in a stable form and could act as novel forms of signalling molecules. Whether tRNAs/tRFs in EVs have intercellular function remains to be established.

Upon activation by interactions with antigen-bearing DCs, activated T cells can transmit sEVs that contain genomic and mitochondrial DNA (mtDNA) exposed on the EV membrane back to the presenting DCs. Such interactions can further enhance antiviral responses via the cyclic GMP-AMP synthase (cGAS)/ stimulator of interferon genes (STING) cytosolic DNA-sensing pathway (in DCs) [148] and activate caspase-dependent apoptotic mechanisms (in tumour cells) [150]. Moreover, this reciprocal signalling from activated T cells to their antigen-presenting DCs in vitro induces IRF3-dependent interferon-regulated genes and antiviral signalling factors Guanylate binding protein 5 (Gbp5) and Gbp6 [148]. Such findings suggest a feedback mechanism by which T cells enhance the activity of antigen presenting cells, promoting them to respond more efficiently to subsequent infections by the same or similar pathogen (Fig. 1**B**). As the changes in the DCs induced by T-sEVs occur in a specific antigen-dependent manner, the enhanced innate antiviral immune response triggered by such DCs responds only to specific stimuli.

The immune synapse (IS) is a highly selective interface for communication between immune cells or an immune cell and a target cell, facilitating selective transfer of specific activation signals, namely pMHC complexes, co-stimulation/repression molecules and cytokines, bidirectionally between APCs and T cells [155] (Fig. 1**B**). IS aligns processes and mechanisms for molecular information exchange at a highly organized contact area and supports the polarized transfer of genetic material by the shuttling of sEVs from the T cell to the APC [156]. More recently, specific activated T cells have been shown to mediate unique regulatory and compositional functions through release of small trans-synaptic vesicles (tSV) [155]. The inclusion of itinerant enzymes such as CD38, CD39, and CD73 in tSVs, although limited, might exert a feed-forward regulatory effect as shown for tumour EVs [157]. Other proteinaceous supramolecular attack particles (SMAPs) released from cytotoxic T cells have been shown of similar size to sEVs; they lack a phospholipid membrane while their core-shell structure enables transfer of bioactive cargo [158]. Further, telomere transfer by sEVs has been demonstrated between APCs (B cells, DCs and macrophages) and T cells (naïve and central memory T cells) independent of telomerase action [159]. Such 'telomere' sEVs retained the Rad51 recombination factor that enabled telomere fusion with T cell chromosome ends to protect from senescence before clonal division and confer long-lasting immune protection (from H1N1 influenza infection). Lanna and colleagues further isolated these telomere sEVs from blood and showed that following their transfer to T cells, they induced long-term immunological memory from both humans and mice, providing a different form of decentralized immunity [159]. Currently, challenges with understanding differences in exchange of supramolecular effectors across IS remain, including comparison of sEVs and tSVs derived from different immune cells (including DCs [40,159]).

3.2. Killer sEVs – NK cell-derived (NK-sEVs) cytotoxic regulators on tumour cells

NK cells are innate immune effector cells that play an important role in cancer immunosurveillance [160], with potent antitumour and anti-metastatic activity [161,162]. sEVs from NK cells deliver a cargo of cytotoxic proteins, including perforin, granzymes, granulysin, FasL/CD178, TNF-related apoptosis-inducing ligand (TRAIL/CD253) and small antimicrobial peptides [163–165] and nucleic acid cargo [166,167]. These effector molecules facilitate tumour cell apoptosis, including breast cancer, melanoma, and hematologic malignancies [165,168]. NK-sEVs display functions in immune surveillance and homeostasis, including cytotoxic activity against several tumour cell types and activated, but not resting, immune cells [164]. Indeed, plasma-derived sEVs from healthy donors express NK cell surface markers, such as $CD56^+$, contain cytolytic proteins, such as perforin, and show cytolysis of activated (phytohaemagglutinin) peripheral blood mononuclear cells (PBMCs) and antitumour activity [165]. Further, NK-sEVs include nucleic acid cargo, shown to contribute to cytotoxicity of NK cells and prevention of immune escape mechanisms. Indeed, tumour suppressor miR-186 was described in NK-sEVs, with its expression downregulated in high-risk neuroblastoma [166]. Ectopic delivery of miR-186 (using nanoparticle carrier) to neuroblastoma cells and NK cells inhibited tumorigenic potential through MYCN (miR-186 target regulator) to prevent TGFβ1-dependent inhibition of NK cells in vitro and in vivo. Such inhibition of neuroblastoma tumorigenesis highlights the therapeutic utility of NK-sEVs linked with signalling regulation of immune escape mechanisms in cancer. Development of EV-based mimetics using NK cell membrane extrusion has led to anti-tumour effects on xenograft glioblastoma mouse model through induction of apoptosis of cancer cells by both the extrinsic and intrinsic pathways, suggesting NK-EVs and engineered EV-like nanovesicles could be vehicles for targeted therapy [169]. Importantly, such EV mimetics combine the characteristics of donor cells and sEVs [7,170].

3.3. Macrophages, monocytes and neutrophils – patrolling and impacting immune surveillance

Macrophages and monocytes release EVs that induce both pro- and anti-inflammatory effects (reviewed [99,171]), for example, mediate release of the lipopolysaccharide (LPS) co-receptor CD14 leading to decreased CD14 on macrophage cell surface and subsequent reduced response to LPS [172]. Moreover, macrophage-derived sEVs carry enzymes that synthesize leukotriene B4 (LTB4) and LTC4 [173]. Active LTA4 hydrolase and LTC4 synthase were identified also in sEVs from human plasma, indicating that sEVs can contribute to inflammation by participating in leukotriene biosynthesis [173]. Macrophage sEVs have also been shown to deliver antigen to DCs where addition of hyaluronic acid (HA), 3-(diethylamino) propylamine, monophosphoryl lipid A, and MUC1, a tumour associated antigen (TAA), trigger sEV uptake by DCs and the release of TAA in the endocytic compartment for enhanced antigen presentation and T cell activation [174]. EVs from macrophages express CD47, a surface molecule known as the "don't eat me" signal to escape immunological surveillance, primarily by circulating monocytes [175,176]. Interleukin-1β (IL-1β), which is a cytokine that lacks the leader sequence needed for secretion by the classical pathway, is released within sEVs following endosomal sorting from macrophages [177]. Such a sorting/release mechanism is used by macrophages containing IL-1β, caspase-1, and other inflammasome components. In further intercellular crosstalk between immune cell types. Moreover, sEVs from bone marrow derived macrophages infected by *mycobacterium* were shown in a Rab27a-dependent manner to present antigen to $CD4^+$ T cells for activation, suggesting macrophage-sEVs are important in acquiring adaptive immunity against *tuberculosis* [178]. Indeed, reduction in sEVs (in Rab27a-knock-out mice) led to decrease in antigen trafficking (Ag85A) to sEVs, in part, explaining the reduced antigen presentation capacity of macrophages from these mice.

Likewise, the release of EVs from monocytes exposed to IFN-α, LPS, or in combination, could enhance production of proinflammatory cytokines by unexposed monocytes, primarily through activation of TLR4/NF-kB signalling [179]. Monocyte-sEVs have been shown to potentiate mesenchymal stem cell differentiation during the transition from injury and inflammation in bone regeneration, through elevating osteogenic factors RUNX2 and BMP-2 [180]. Further, neutrophils have been shown to release sEVs containing AnxA1, which induces macrophage phenotype switching toward a repair phenotype in atherosclerosis [181]. Human neutrophils cocultured with macrophages in vitro following TNF-α stimulation were shown to release lEVs exposing phosphatidylserine and AnxA1 and induce TGF-β production from macrophages [182]. Neutrophil-derived lEVs have also been shown to induce a protective role in arthritis [183]. Mice deficient in a lipid scramblase

(TMEM16F), a protein important in translocation of cell membrane and required for microvesiculation to generate lEVs (reviewed [184]), exhibited exacerbated cartilage damage when subjected to inflammatory arthritis [183]. Mechanistic in vitro/*vivo* studies highlighted a model where EV-associated AnxA1 interacts with its receptor FPR2/ALX to increase TGF-β production by chondrocytes and ultimately lead to reduced cartilage damage caused by inflammatory arthritis [183]. Recently, different types of vesicles/EVs from neutrophils have been shown to selectively impact the function of immune cells, suggesting that neutrophils may selectively integrate different vesicles/EVs based on their immune environment [185]. These findings are supported by sEV generation in polarised/non-polarised epithelial cells, where sEV heterogeneity exists based on different mechanism of endosomal trafficking/ILV generation [48]. Moreover, neutrophil-derived trails (NDTRs) generated from neutrophils migrating towards inflammation foci [186] can induce polarization of macrophages toward a proinflammatory phenotype, while neutrophil-sEVs can mediate their polarization toward an anti-inflammatory phenotype [185,186].

Distinct mechanisms in the formation of sEVs, specifically for activated neutrophils, have highlighted their impact on EV composition and function. In contrast to canonical $CD63^+$ MVBs, a distinct population of sEVs have been shown derived from the nuclear envelope membrane of neutrophils [49]. Here, lipid-rich microdomains (regulated by nSMase1) were linked with nuclear envelope curvature and bud formation to generate non-conventional ceramide-rich MVBs, with derived sEVs comprising $CD63^-$ LBR^+ and enzymes, 5-lipoxygenase (5-LO), 5-LO activating protein (FLAP) and leukotriene A_4 hydrolase. Like the existence of ESCRT-dependent and -independent mechanisms for the loading of select cargo in sEVs/exosomes [156,187–197], which suggests the existence of specialized mechanisms to control the selective sorting of cargo into these vesicles [198], there is also a diversity in MVBs. MVBs are highly heterogeneous according to their origination, maturation state or route. This presence of heterogeneous populations of MVBs could thereby impact the localisation and composition of released MVB-containing vesicles – from nuclear envelope and cytosolic vesicles to the endosomal system and plasma membrane (reviewed [38,199]).

3.4. Cancer cell-derived sEVs modulating the immune response

Given that cancer cells can influence the biology of local immune cells, such as NK cells and TAMs, they can establish immune escape mechanisms that inhibit or avoid the innate and adaptive antitumoral immune responses. Cancer-derived sEVs have been shown to suppress innate immune responses through mobilizing MDSCs [200], and modulating/activating TAMs [201,202] and neutrophils [76] (Fig. 1**C**). It has been reported that sEVs from TAMs could promote tumour metastasis [203] as well as chemoresistance [204,205]. Reciprocally, sEV-miR-21 from bladder cancer T24 cells could polarize THP-1 cell--derived macrophages into the M2 phenotype by activating PI3K-AKT-STAT3 signalling pathway [202]. Interestingly, this same pathway was also shown to be required for suppressing tumour cell apoptosis mediated by miR-21 transferred via sEVs from macrophages to gastric cancer cells, where enhanced activation of PI3K/AKT signalling was also met with down-regulation of PTEN [205]. Similarly, macrophages could suppress tumour immune evasion by internalising Tu-EVs and abrogating their tumour-promoting interactions with B cells [206].

Tumour sEVs (from mouse (B16) and human (A375) melanoma) have also been shown to induce patrolling $Ly6C^{low}$ monocyte expansion at the metastatic niche and TRAIL-dependent killing of melanoma cells by macrophages [78]. This immunosuppression was regulated by patrolling monocytes and NK cells, responsible for diminished metastasis through pigment epithelium-derived factor (PEDF), a potent anti-angiogenic and anti-cancer regulator. Patrolling or non-classical monocytes have also been shown to scavenge and phagocytose Tu-sEVs [207]. Hanna et al., demonstrated a cancer surveillance role of patrolling monocytes, in part through the regulation of NK cell recruitment and activity, in preventing tumour metastasis to lung in multiple mouse metastatic models [208]. Such studies may represent a novel anti-metastatic therapy by selectively targeting and tuning patrolling monocyte activity [209]. In the context of promoting metastatic growth, epithelial cells have been shown to initiate neutrophil recruitment and lung metastatic niche formation when primed with Tu-sEVs from primary tumour through TLR3 [210]. It was observed that TLR3-deficient mice displayed reduced lung metastasis as sEVs could activate TLR3 in lung epithelial cells and induce chemokine secretion (in lung) to promote neutrophil recruitment and tumour metastasis. Similarly, lin28B has been highlighted as a regulator to enable neutrophil recruitment and conversion, promoting lung metastasis of breast cancer by forming an immune-suppressive pre-metastatic niche [211]. Importantly, this study highlighted sEVs from primary (mouse models: 4 TO7, MMTV-PyMT [luminal subtype], MMTV-Neu model [$HER2^+$ subtype]) breast cancer as a critical regulator of lin28B-induced immune suppression. In this tumour environment neutrophils could be tuned by sEVs to switch to tumour promoting roles and drive metastasis. Further, Tu-sEVs have been shown to mediate delivery of EGFR and HER-2 to monocytes and promote monocyte survival prior to the formation of TAMs [212]. Tu-sEVs (from different human cancer cell models; lung adenocarcinoma A549 cells, hepatocellular carcinoma HepG2 cells, breast carcinoma MCF-7 cells) could also promote the differentiation of monocytes into MDSCs, which inhibited DC maturation [212]. Such findings provide insight into intercellular interactions between tumour and immune cells (inflammatory regulation) and monocyte survival prior to differentiation into sufficient macrophages in tumours. Such tumorigenic sEVs also can impair the differentiation of peripheral blood monocytes to functional MoDCs, instead transforming them into MDSCs [213]. This mechanism of tumour-immune system interaction is important in understanding cell-specific regulators of the tumour microenvironment.

Although released sEVs from cancer cells contain abundant DNA, the function of such sEV-DNA remains unclear (Fig. 1**B**). Cytoplasmic dsDNA is a damage-associated molecular pattern (DAMP) that can stimulate type I IFNs and other cytokines by cGAS/STING signalling [214]. In addition, mtDNA can be horizontally transferred via tumour sEVs to other cancer stem-like cells, promoting oxidative phosphorylation and resistance to hormonal therapy in breast cancer [215]. This EV-mediated mtDNA transfer may also occur in dormant or metabolically quiescent tumor cells, leading to an exit from dormancy. Recently, sEVs were shown to also carry DNA to recapitulate genomic aberrations [216]; for example, sEV-trafficked ssDNA from prostate cancer and patient plasma could amplify primary tumour oncogenes (i.e., MYC). In the setting of metastatic cancer, higher levels of sEVs/exosomal dsDNA have been found in aggressive melanoma compared with the levels found in nonmetastatic or low-metastatic melanoma [217].

The composition of sEVs from tumour cells is further impacted under certain extrinsic conditions, such as during radiation treatment or chemotherapy. Following antitumour chemotherapy, DNA levels in Tu-sEVs significantly increased, and sEV-mediated activation of DCs through cGAS-STING signalling in vitro and in vivo [218]. These findings suggest that a STING-dependent pathway could drive antitumour immunity by responding to Tu-sEV derived DNA. Interestingly, combinations of radiotherapy and immunotherapy induced a tumoricidal effect [219,220]. In mice with breast cancer, immunostimulatory DNA secretion was induced following treatment with topotecan or by irradiation, triggering an antitumour response through maturing DCs and activating $CD8^+$ T cells [221].

Other kinds of oncogenic cell stress, including DNA damage, autophagy, and endoplasmic reticulum (ER) stress, can modify EV composition and particle yield, generating damage-associated molecular patterns (DAMPs) that modulate the immune response [222]. This “immunogenic stress” could induce cancer cells to prime their own microenvironment through release of EVs containing specific immunoregulatory mediators (including various RNAs, DNAs, proteins

(including cancer antigens), and lipids [223,224]), depending on the initiating stressor (reviewed [95]). sEV-containing HMGB1 (a primary regulator of inflammation, promoting $CD4^+$ T cell activation by stimulating cancer neoantigen presentation by DCs) has been shown to impact immune cell activation [225]. Further, tumour sEVs could transfer HMGB1 to B cells and stimulate TIM-1^+Breg cell expansion through MAPK and Toll-like receptor (TLR) 2/4 pathways [225]. Such infiltrating TIM-1^+Breg cells exhibit $CD5^{high}CD24\text{-}CD27^{-/+}$ $CD38^{+/high}$ phenotype and elevated immunosuppressive IL-10 and a strong suppressive effect on $CD8^+$ T cells [225]. There is a crucial balance between the stress state in cancer cells and the impact on stress-associated immunogenic EV cargo. These activities require the active participation of DCs to process and present EV-derived antigens to T cells. It is important to emphasize that Tu-sEVs composition, which is dynamically regulated by its microenvironment [63,226], is an important modulator for immune function and tolerance.

3.5. Pathogen and viral control

EV-mediated remodelling of the immune response is also driven by the host immune system and pathogen-associated molecular patterns (PAMPs) through activation of pattern recognition receptors (PRRs) and Toll-like receptors (TLRs). PAMPs could be released by various pathogen-infected cells through EVs, along with other immunomodulatory molecules to promote the infectious cycle. sEVs from bacterium-infected macrophages have been shown to be immunomodulatory and stimulate macrophages and neutrophils to secrete pro-inflammatory mediators, including TNF-α and RANTES (up-regulating iNOS expression) [227,228]. sEVs from infected macrophages can induce systematic inflammatory response (e.g., through TNF-α, IL-12) in infected mice, which was shown indispensable at the early phase of pathogen control [228,229]. Similarly, EVs from *mycobacterium*-infected macrophages transfer pathogenic RNA to naïve macrophages to activate its intracellular RIG-I/MAVS/TBK1/IRF3 RNA sensing pathway, promoting Th1 immune response through IFN release [230]. Transfer of *mycobacterial* RNA to EVs was highlighted as dependent on expression of *mycobacterial* SecA2 secretion system to facilitate bacterial pathogenesis and cellular responses to environmental stress. Moreover, these EVs from *mycobacterial*-infected macrophages also synergise with antibiotics to decrease bacterial burden within infected macrophages – providing an immunotherapeutic strategy for drug-resistant *mycobacterium* [230]. Indeed, sEV-carried PAMPs may also enhance immune surveillance [227,228]. EVs have been shown to synergize with antibiotics to promote bacterial clearance and limit pathology as a novel immunotherapeutic approach to promote host immunity [230]. Further, EVs from dengue virus-infected macrophages were shown to transfer NS3 protein to promote cytokine release in endothelial cells, activating the defence program against early-phase dengue virus infection [231]. EV-mediated inflammasome activation could also occur in the context of central nervous system (CNS) trauma. Keane and colleagues showed that CNS-derived inflammasome-containing sEVs (with siRNA to regulate caspase 1 activation) when treated on injured blood-spinal cord barrier, could deliver their cargo in vivo and activate innate immune response in peripheral tissues [232]. Such findings support a signalling cascade for sEV-mediated inflammasome activation and may give rise to new insights into biologically relevant targets to control inflammatory diseases, altered blood–brain barrier or infections.

In the context of cellular stress and infection, various types of RNA have been identified in and transferred by sEVs [233]. Epstein-Barr virus (EBV) encoded small RNA (EBER1) transcript produced in EBV latently infected B cells is packaged in sEVs (as 5'ppp-RNA) and recognized by DCs inducing an inflammatory response. Indeed, such transcript (EBER1) and EBV-miRNAs have also been identified in inflamed skin lesions infiltrated with DCs in autoimmune patients (systemic lupus erythematosus, SLE); while in contrast, limited EBV-DNA present in such tissues, suggesting that continuous intercellular EBER1 transmission via sEVs occurs in humans [233]. Such findings suggest that 5'ppp-recognizing sensors (such as RIG-I) are likely to recognise sEV-derived small RNAs. Such sEVs are distinct from those released by cells exposed to IFN-α as they carry a distinct antiviral proteome [234]. Nabet et al., identified stromal cell sEVs deliver 5'ppp-RNA to activate STAT1-dependent antiviral signalling in tumour cells and IFN-stimulated gene induction [222]. Moreover, antitumour chemotherapy resulted in altered sEV DNA composition, activation of DCs through cGAS-STING signalling, and an innate antiviral immune cell response [218]. Recently, to gain insights into composition of EVs as distinct from infected virions, a proteomic approach was employed using human T cells following HIV-1 viral infection [41]. EVs produced by HIV-infected T cells reflected specific markers to differentiate EVs from virions (e.g., CD45 and acetylcholinesterase (AChE) [235]); and 26 proteins differentially expressed upon HIV-1 infection included a key surface protein SERINC3 shown in host EVs while moloney leukemia virus 10 (MOV10) and sialophorin (SPN) in the virion [41]. Parasites have been shown to release EV subtypes (based on size) with signatures of different target sites and alter host response [236]. Indeed, recent identification of the surface landscape of EV subtypes has highlights not only differences in surface marker expression of mediators of immune regulation, but specific ligands for pro-inflammatory innate immune response between sEVs and lEVs [9].

4. Small extracellular vesicles: delivering immune suppressive cargo

A large body of evidence points towards the established role of Tu-sEVs in promoting a pro-tumorigenic phenotype and immunosuppression [63,76,77] as well as in parallel, hijacking mechanisms employed by cancer cells to perturb therapeutic response [237,238]. Tu-sEVs can compromise the anti-viral innate immunity of the host by transferring receptors (e.g., EGFR) from tumour cells to macrophages [239]. For instance, a kinase screen identified macrophage MEKK2 as an effector that negatively regulated antiviral immune response [239]. Indeed, MEKK2-deficient mice are more resistant to viral infection. As a key mechanism regulating innate immunity, transferred Tu-sEVs derived EGFR resulted in increased viral load and morbidity in an EGFR- and MEKK2-dependent manner triggering poly-ubiquitination and phosphorylation of IFN regulatory factor 3 (IRF3) [239]. Such findings provide new insights into how Tu-sEVs can dampen host innate antiviral immunity with potential for cancer patients to become immunocompromised. Dissection of signalling pathways dysregulated by Tu-sEVs leading to immune suppression [63,240–242] will provide valuable insights into identification and/or selection for cancer therapies.

Tu-sEVs have been shown to suppress T cell and NK cell activity and stimulate MDSCs [243,244] (Fig. 1**D**). Seminal findings by Poutsiaka and colleagues [245] showed membranous vesicles released by murine B16 melanoma cells repressed the IFN-γ-dependent class II expression on murine macrophages to impact antigen presentation to $CD4^+$ T cells. Tu-sEVs can also induce T cell apoptosis to evade immune surveillance. Andreola and colleagues demonstrated that in melanoma cells, Fas ligand (FasL) was restricted to MVBs that contain melanosomes [246]. These melanosome-positive MVBs were demonstrated to further release FasL-containing sEVs that induce apoptosis in Jurkat and lymphoid cells. Therefore, neoplastic modulation involving release of FasL-positive sEVs promote immune escape. sEVs expressing FasL and TNF were also shown to regulate T cell apoptosis in human cancer [247]. Patient hepatocellular carcinoma (HCC) infiltrating TIM-1^+Breg cells stimulated by Tu-sEVs (carrying DNA chaperone HMGB1) suppressed $CD8^+$ T cells [225]. Recently, cervical cancer sEVs (containing miR-1468–5p) were shown to remodel lymphatic endothelial cells (LECs) through JAK2/-STAT3 pathway activation, generating immunosuppressive cancer-associated LECs in the tumour microenvironment [242]. Further, miR-142–5p transferred by cancer sEVs into LECs was shown to exhaust $CD8^+$ T cells via the up-regulation of lymphatic enzyme activity

(indoleamine 2,3-dioxygenase) via ARID2-DNMT1-IFN-γ signalling, contributing to immune checkpoint development and tumour progression [242]. Such studies provide an emerging understanding of the immunosuppressive mechanisms of cancer-associated lymphatic cells to evade host immunity and enable the rational development of immunotherapeutic strategies based on EVs of tumour origin. An intriguing concept, recently reviewed [92], focuses that while Tu-sEVs suppress anticancer immunity and stimulate pro-tumorigenic processes, what is the function of physiological EVs and do they confer protection against tumour progression? sEVs could hijack the intrinsic ability of the host immune system to recognize and eliminate tumour cells, representing a promising therapeutic strategy. Indeed, as an effective immunomodulatory and potential anti-metastatic therapy, sEVs have been used in this capacity. Plebanek et al., showed sEVs from a low metastatic mouse melanoma stimulated $Ly6C^{low}$ patrolling monocytes, which inhibited metastatic dissemination to lung [78]. Importantly, surface expression of PEDF on sEVs from non-metastatic melanoma cells was critical for the activation of an innate immune response to inhibit melanoma metastasis.

Tu-sEVs expressing specific NK cell ligands can regulate their cytotoxic response and promote immune suppression and tumour escape (Fig. 1**D**). TGF-β1 was shown to downregulate NKG2D expression and reduce NK cell cytotoxicity [248,249]. Further, pre-treatment of mice with TS/A or 4 T.1 murine Tu-sEVs enhanced tumour growth in syngeneic and nude mice, while diminishing NK cytotoxic molecules and repressing IL-2 induced NK cell proliferation, overall contributing to tumorigenesis [250]. Ashiru et al., [251] observed truncated MHC class I-related chain (MIC) A (allele MICA*008) on sEVs induced down-regulation of its receptor NKG2D leading to decreased NK cytotoxicity. MICA and MICB are crucial for the induction of the NK activating receptor NKG2D. Detection of these molecules in sera is often associated with compromised immune response and tumour escape from immune surveillance. Functionally, MICA-carrying sEVs decreased the surface expression of NKG2D on NK cells and $CD8^+$ T cells in PBMC from healthy donors [252]. In addition, Tu-sEVs promoted monocyte differentiation into MDSCs, which in turn inhibited DC maturation [253].

sEVs produced by murine TS/A mammary tumour cells are internalised by $CD11b^+$ myeloid precursors in the bone marrow in vivo [253]. Myeloid cells uptake of Tu-sEVs can induce IL-6 production preferentially, which suppresses their differentiation into DCs [253]. Cooperation between MDSCs and Tu-sEVs from various murine cell lines has been suggested to promote MDSC-mediated repression of T cells [254]. The varied in vivo anti-tumour efficacy of the chemotherapeutic drug cyclophosphamide in various murine models was shown to be consistent with the involvement of sEV Hsp72 in potentiating MDSC activity [200]. Xiang et al. [255] reported that murine TS/A and 4 T-1 breast tumour sEVs induced bone marrow myeloid cells (BMMCs) to differentiate into MDSC, with a concomitant increase in tumour development and growth. In addition, for tumour sEVs to mediate neoplastic progression through MDSCs, TGF-β and prostaglandin E2 (PGE2) were found to play an important role [256]. MyD88 is a cytoplasmic adaptor molecule crucial for the propagation and integration of signals generated by the TLR family. Liu et al., used sEVs from metastatic lung cancer cells to promote IL-6, TNF-α, and chemokine CCL2 production and induce MDSCs in a myD88-dependent manner [256]. Together, these studies highlight the various strategies employed by tumour cells using sEVs to suppress tumour immune responses.

This area of research has gained significant attention recently with the fact that tumours can release sEVs containing tumour antigens and immunosuppressive proteins, including cancer-associated PD-L1 (Fig. 1**D**). In parallel, tumour cells can coordinate immune cell evasion [257,258] while modifying the production of immune suppressive mediators, tolerance and immune deviation as cancer progresses [63,250]. Supporting this, studies in tumour models (in vivo) have highlighted different phenotypes between EVs released at the tumour site and present in the tumour microenvironment and those detectable at distance in the periphery [63,226]. Recently it was suggested that ALIX, a critical mediator of exosome/sEV biogenesis, modulates immunosuppression through the regulation of PD-L1 and EGFR in cancer cells [259]. ALIX depletion results in enhanced EGFR activity as well as reduced sEV PD-L1 secretion and increased surface PD-L1 expression. However, further understanding of the composition of Tu-sEVs (luminal and surface composition, tumour staging/development) with respect to immune regulation is needed, given that a recent study has highlighted that sEV PD-L1 contributes to immunosuppression in patients with metastatic melanoma [260].

5. Immunorecognition and small extracellular vesicles: interactions with the complement system

The complement system, part of the phylogenetically ancient innate immune system, serves as a nonspecific enhancer for clearing invading pathogens and is comprised of a group of abundant proteins that react with each other to opsonize pathogens and induce inflammatory responses and lysis of pathogens [71]. Complement system is capable of driving systemic reactions through the release of anaphylatoxins, as well as of opsonizing target cells for phagocytosis, and plays a central role in immunosurveillance in cancer [261]. sEVs (containing MFGE8) from immature DCs act as an opsonin by binding to phosphatidylserine on the surface of dying cells, and thus tag them for phagocytosis [262] (Fig. 1**E**). Clayton and colleagues investigated the modulation of CD55 and CD59, two glycophosphatidylinositol (GPI)-anchored complement regulator proteins that prevent formation of the complement membrane attacking complex, on sEVs derived from APCs [263]. sEV-mediated complement lysis was enhanced by the CD55 and CD59 blocking antibodies, demonstrating that CD55 and CD59 carrying sEVs play a role in protecting exosomes from complement attract in the extracellular environment. Enrichment of cell surface CD9 and pMHC-II was also found in DC-sEVs [91]. Tetraspanin CD9 was suggested to direct membrane fusion, circumventing endosome-to-lysosome fusion [264]. In addition, Morelli and colleagues demonstrated that sEVs were internalised through ligand-mediated (for example, CD11a and its ligand CD54) mechanism by recipient splenic DCs and antigen presentation to $CD4^+$ T cells in vivo [265].

6. Small extracellular vesicles and immunoregulation impact tumour microenvironment

The sustained growth, invasion, and metastasis of cancer cells depend upon bidirectional cell-cell communication within complex tissue environments. Such communication predominantly involves the secretion of soluble factors and EVs from cancer cells and/or stromal cells within the tumour microenvironment [8,266–270]. Key studies in the field have shown tumour sEVs play critical roles in contributing to and regulating the tumour microenvironment through suppressing specific T cell immunity and mediating innate immune cells to support pro-tumour function [12,63,146,271,272]. Broadly, such functions include reducing immune surveillance for primary tumours locally while creating a remote, permissive metastatic environment (niche) that supports cell seeding, survival, and outgrowth [69,211,268]. Tu-sEVs have been shown to directly modify the tumour microenvironment by inducing N2 polarization and autophagic response of neutrophils (through HMGB1 transfer and interaction with TLR4) to promote gastric cancer cell migration [77]. Similarly, metastatic dissemination of primary tumours (lung, 4T1) was shown to require mobilization of neutrophils induced by Rab27a-dependent secretion of sEVs together with certain cytokines and/or metalloproteinases [76]. This regulatory effect was shown to be dependent on tumour type and involves sEV-dependent and -independent secretions, most likely due to immune system-dependent mechanisms [76]. Tu-sEVs have the capacity to not only mobilize pro-metastatic bone marrow cells (through transfer of active surface MET [63] or MIF [268]) but also regulate EV homing to

specific organs to establish pre-metastatic niches to enhance metastasis [44,78,268]. For example, human breast cancer-derived sEVs (expressing MET) were shown to promote vascular leakiness in the lung by upregulating specific S100 proteins and activating kinase signalling to induce molecular and cellular changes that regulate endothelial cell integrity [44]. Tu-sEVs can further contribute to tumour progression by sequestering tumour-reactive antibodies and decreasing their binding capacity/ability to tumour cells, thus inhibiting antibody-dependent anti-tumour cytotoxicity [273] and reducing the effectiveness of antibody-based anti-cancer drugs [274]. Recently, various types of EVs and extracellular particles (exomeres), from a large-scale, comprehensive cell line/tissue/biofluid analysis [275] showed that their proteome composition reflected the systemic effects of cancer in both the developing primary tumour and the tumour microenvironment, distant organs (e.g., liver), and the immune system. Here, specific proteins involved in eliciting immune responses, such as damage-associated molecular pattern proteins, were shown in tumour-derived EVPs, including the unique identification of S100A13, BSG, LGALS9, biglycan (BGN), and integrins (ITGs) α5 and αX compared to sEVs from non-cancer origin. Given that Tu-sEVs can transfer phenotypic features, and concomitantly result in a phenocopy of the tumour [276], this does provide clinical opportunities for their systemic monitoring [277] and understanding such reprogramming (for stromal [278] or immune cell functions). Clearly, based on the properties of cancer sEVs in modulating the immune system, this illustrates the clinical potential of sEVs or their engineered counterparts in immunotherapy, therapeutic targeting, and drug delivery.

7. Immuno EVs: Small extracellular vesicles as an immunogenic therapeutic strategy

Interests in sEVs for therapeutic use have been garnered by their ability to protect diverse bioactive cargo, inherent biocompatibility, nanosized structure and negative surface charge, ability to transverse and deliver cargo across biological membranes, and to target specific cell types (reviewed [7,279]). To overcome some limitations in therapeutic utility, EVs can be modified (bioengineered) to harbor specific pharmaceutical content, enhance their stability, and modify surface epitopes for improved tropism and targeting to cells and tissues in vivo (reviewed [7,279]). Natural sEVs hold significant therapeutic potential as immunomodulators, for eliciting or suppressing immune response [13], with structural similarities to that of viruses. Indeed, several reports demonstrate that viruses exploit mechanisms associated with EVs biogenesis/trafficking/release and intercellular transfer [280–283]. With their demonstrated capacity to tune and activate immune response in vivo against tumour antigens [110,119,284–288], various clinical applications of sEVs and EVs-like nanocarriers as antigen-carrying vehicles have gained significant attention (reviewed [7,289]). Here, we review current generation sEVs as cancer vaccines, their utility as next generation therapeutics (Table 2), and the technologies integrated in novel cell-free cancer vaccines.

7.1. sEVs: potential novel cancer vaccines

sEVs have garnered significant interest as potential novel cancer vaccines [98,317] (Fig. 2, Table 2). First generation cell-based (DCs) immunotherapies were limited in their clinical efficacy, high cost and quality control parameters in generation/processing/storage, despite their safety, tolerability and immunogenicity [98,318]. Subsequently, DC-sEVs were used as a cell-free strategy, providing promising results as a cancer vaccine [98]. Such sEVs were comprised of functional pMHC-I/II, co-stimulatory molecules (such as CD80, CD86) for T cells, and ligands (BAG6, NKG2D-L) for NK cells [98]. Zitvogal et al., demonstrated that DC-sEVs from tumour antigenic peptide-pulsed DCs mediated CTL priming and anti-tumour effect, providing proof of concept that such iDC-sEVs could serve as a cell-free cancer vaccine [110]. Seminal advances over the last two decades have led to several completed and ongoing clinical trials of DC-sEVs as cancer vaccines (from advanced NSCLS, phase I [285]; metastatic melanoma, phase I [284]; to advanced NSCLC, phase II [291]). Despite safety profiles of DC-sEVs in vivo and the ability to trigger cytotoxicity of NK cells, clinical efficacy of DC-sEVs remained limited due to their ability to efficiently activate T cells [284,285,291]. Interestingly, when IFN-γ- induced mature MoDCs were pulsed with various TAAs to produce DC-sEVs (IFN-γ-DC-sEVs), such DC-sEVs improved CTLs responses in vitro *and in vivo* [290] (Table 2). This second generation IFN-γ-DC-sEV vaccine, although improved clinical outcome (phase II), had limited efficacy due to limited stimulation of antigen-specific T cells in these patients [291], likely due to poor antigen presentation by DCs taken IFN-γ-DC-sEVs [318] and/or poor biodistribution/fate of IFN-γ-DC-sEVs in vivo [98]. Indeed, this limited efficacy was shown associated with a specific NK cell-dependent response due to IFN-γ stimulation of DCs, causing upregulation of BAG6, a specific NK cell ligand (reviewed [98]).

Recently, nanotechnology, and molecular engineering have been utilised to develop third generation DC-sEV-based cancer vaccines [319–321] (Table 2). For example, DC-sEVs-AFP derived from DCs transfected with a highly expressed hepatocellular carcinoma (HCC) antigen, α-fetoprotein (AFP) induced CTL against diethylnitrosamine (DENA)-induced orthotopic HCC in mice [294]. Further, an innovative engineered strategy forms a "designer" DC-sEV-P-A2-N vaccine using DC-sEVs decorated with a HCC-targeting peptide (P47-P), an α-fetoprotein epitope A2 (referred to AFP212-A2) and a functional domain of high mobility group nucleosome-binding protein 1 (N1ND-N) as a DC immunoadjuvant, via an sEVs anchor peptide (CP05) [295]. Intravenous administration of DC-sEV-P-A2-N vaccine specifically promoted recruitment, accumulation, and activation of DCs ($CD103^+CD11c^+$ and $CD8\alpha^+CD11c^+$ DCs) in orthotopic HCC mice, resulting in enhanced cross-presentation of tumour-specific neoantigens and *de novo* T cell response [295]. Importantly, when AFP A2 epitope was replaced with a full-length AFP to form DC-sEV-P-A-N vaccine and supplemented with Fms-related tyrosine kinase 3 ligand (Flt3L), a DCs lineage development factor, this combination maintained strong immune response, tumour eradication and long-lasting antitumour effect, highlighting the potential of targeted, engineered DC-sEV vaccines for cancer immunotherapy [295].

In addition to immuno targeting, DC-sEV-based vaccines have further been developed to incorporate immune checkpoint inhibitors (Fig. 2, Table 2). Interestingly, a DC-sEV cancer vaccine with immune checkpoint blocking ability was designed, in which sEVs were collected from OVA-pulsed DCs conjugated with anti-CTLA-4 antibody (anti-CTLA-4-DC-sEV). This vaccine was shown to induce increased tumour infiltrating T cells in a B16 melanoma mouse model and significantly reduced tumour growth after 12 days [296]. Inspired by CAR-T cells and bispecific T cell-engager (BiTE), Fan and colleagues [293] developed antibody-decorated sEVs from tumour antigen-pulsed DCs (anti--CD3/anti-EGFR-DC-sEVs) to promote T cell-cancer cell interaction, resulting in crosslinking of cancer cells with T cells and enhanced tumour cell killing, similar to CAR T-cell strategy. Of note, this anti-CD3/anti-EGFR-DC-sEV cancer vaccine also up-regulated PD-L1 expression in tumour tissue, while combination therapy with anti-PD-L1 antibodies further enhanced the efficacy of such tumour-targeting sEVs that mimicked CAR T-cell strategy [293]. To further enhance anti-tumour effect, DC-sEV vaccines could potentially incorporate additional targeting molecules, co-stimulatory molecules (CD80), various tumour-associated antigens or neoantigens; It is also possible to use DC-sEV vaccines in a combination therapy with other antitumour vaccines and/or therapies, such as immune checkpoint inhibitors (such as anti-PD-L1) (Fig. 2).

In addition to DC-sEVs for vaccine development, Tu-sEVs have been utilised as a source of tumour antigens for cross-priming T cell by DCs [107]. Further, sEVs from biofluids, including malignant effusion (ascites)-derived sEVs (A-sEVs) could serve as an abundant source of

Table 2
Development and application of EV-based vaccines.

	Source / EV type	Engineering strategy	Application / disease	Phase (pre-clinical / clinical)	Key findings	
EV cancer vaccine	Dendritic cell-sEV (DC-sEV)	N/A (1st gen)	Mastocytoma	Preclinical	sEVs secreted by antigenic peptide-loaded dendritic cells (DCs), (first generation DC-sEVs), triggered tumour specific CD8+ T cell activity and elicit tumour suppression in a mouse model	[110]
			Metastatic melanoma	Phase I	Phase I trial of 1st generation DC-sEVs, loaded with tumour antigen (no IFN-γ stimulation) showed safety with no serious side effects in patients with metastatic melanoma	[284]
			Non-small cell lung cancer (NSCLC)	Phase I	Phase I trial of 1st generation DC-sEVs, loaded with tumour antigen (MAGE-A3,-A4, -A10, and MAGE-3DPO4 peptides) in advanced NSCLC patents. Outcome: DC-sEV therapy well tolerated, some patients showed MAGE-specific T cell responses and increase NK activation	[285]
			Melanoma	Preclinical	Mouse DC-sEV (first generation) promote NK activation *in vivo* and human DC-sEVs (first generation) carry NKG2D ligands to promote NKG2D dependent NK cell activation *in vitro* and in melanoma patients	[129]
		IFN-γ simulation (IFNγ-DC-sEVs) 2nd gen		Preclinical	Because of failure to stimulate T cell response in two clinical trials, second-generation DC-sEVs (IFNγ-DC-sEVs) were developed to enhance co-immunostimulatory properties for the purpose of CD8+ T cells dependent activation in a phase II trial.	[290]
		IFN-γ simulation (IFNγ-DC-sEVs) 2nd gen	NSCLC	Phase II	The phase II clinical trial of second-generation DC-sEVs vaccine (IFNγ-DC-sEVs) to test progression-free survival in unresectable NSCLC patients (n=22) following vaccination. IFNγ-DC-sEVs carry NKp30 ligand BAG6, induced NKp30-dependent NK cytotoxicity and did not induce tumour-specific T cell immune response.	[291]
	3rd gen engineered DC-sEV	DC-sEVs-phagocytosed pathogen		*In vitro*	Monocyte-derived DC (MoDC) incubated with phagocytosed *E. coli* and secreted sEVs containing phagocytosed pathogen; potential effective application for vaccine design	[292]
		DC-sEVs-OVA-anti-CD3 and anti-EGFR	Melanoma (B16-OVA cell line)	Preclinical	Tumour peptide-stimulated DC-derived sEVs (DC-sEVs) engineered with lipid anchored anti-CD3 and -EGFR antibodies to form DC-sEV-OVA-αCD3-αEGFR in B16-OVA melanoma mouse model. Targeted DC-sEV-OVA-αCD3-αEGFR enhanced T cell proliferation, and cytokine production and strong tumour suppression/increased survival. Biodistribution displayed initial spleen and lymph node homing, before localising to tumours (post 8h). DC-sEVs-OVA-aCD3/aEGFR demonstrated strong tumour growth inhibition and increased survival. Elevated CD4+ and CD8+ T cells in tumours, similar to CAR T therapy, as well as stimulation of humoral immunity as detected by the presence of tumour-specific antibodies. Tumour recurrence also reduced, along with exhibition of anti-metastatic properties	[293]
		DC-sEVs-AFP (trigger)	Hepatocellular carcinoma (HCC)	Preclinical	α-fetoprotein, a fetal liver protein expressing DC secreted sEVs displayed enhanced antigen-specific immune responses, elevated IFNγ+ CD8+ cells/reduced CD25+Foxp3+ Treg, suppressed tumour growth in an *in vivo* HCC model	[294]
		DC-sEVs-P&A2&N (trigger)	Hepatocellular carcinoma (HCC)	Preclinical	Third generation DC-sEVs developed using HCC-targeting peptide (P47-P), an α-fetoprotein epitope A2 (AFP212-A2) and a functional domain of high mobility group nucleosome-binding protein 1 (N1ND-N), an immunoadjuvant for DC recruitment and activation, via sEV anchor peptide (CP05) to form a "trigger" DC-sEVs vaccine (DC-sEVs-P&A2&N). DC-sEVs-PP&A2&N showed greater liver tumour accumulation, and DC recruitment, activation, T cells response, reduced tumour growth. Interestingly, combination with Fms-related tyrosine kinase 3 ligand significantly enhanced anti-tumour immunity	[295]
		DC-sEVs-anti-CTLA-4	Melanoma (B16-OVA cell line)	Preclinical	Mature BMDC (activated by Poly(I:C)) OVA loaded sEVs, painted with a checkpoint blockade CTLA4 mAb to form DC-sEVs-anti-CLTA4. DC-sEVs-CLTA4 enriched in MHC I/II molecules, displayed increased tumour infiltrated T cells in a melanoma mouse model	[296]
		DC-sEVs-MUC1	Melanoma (B16-MUC1 tumour cells)	Preclinical	DC-sEV surface coated with MUC1 antigen (DC-sEVs-MUC1) [copper-free click reaction] triggered high MUC1-specific IgG antibody. DC-sEVs-MUC1 induced MUC1 positive tumour specific CD8+ T cell cytotoxicity, reduced tumour growth and prolonged survival in preventative and therapeutic mouse models	[297]

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Table 2 (*continued*)

	Source / EV type	Engineering strategy	Application / disease	Phase (pre-clinical / clinical)	Key findings	
		DC-sEVs-RAE-1γ	Chronic myeloid leukemia (CML)	Preclinical	Retinoic acid early inducible-1 γ (RAE-1γ), a major ligand of NKG2D loaded into CML specific DC pulsed with lysate of RAE-1 γ expressing CML cells or T315I-mutant CML cells to form CML-DC-sEVs-RAE-1γ. CML-DC-sEVs-RAE-1γ displayed NK cells and T cell activation, also supressed BCR-ABL or BCR-ABLT315I CML tumour growth in mouse model	[298]
	Tumour cell derived sEVs (Tu-sEVs)	Tu-sEVs 1st gen	Colon cancer, breast cancer, mastocytoma	Preclinical	Human tumour sEVs (Tu-sEVs) contained shared full-length tumour antigens, stimulated DCs to activate tumour specific CD8+ T cells *in vitro*. Further, Tu-sEVs induced CD8+ T cell dependent tumour rejection in mouse tumour models	[107]
		TPT treated Tu-sEVs 2nd gen	Breast cancer	Preclinical	Topotecan (TPT), antitumor agent and an inhibitor of topoisomerase I, induced DNA sorting into Tu-sEVs, triggered STING-dependent anti-tumour immunity	[218]
	3rd gen engineered Tu-sEVs	Tu-sEVs-C1C2-OVA	OVA bearing cancer cells	Preclinical	OVA antigen fused with the lipid-binding domain C1C2 of MFGE8 presenting on surface of Tu-sEVs as Tu-sEVs-C1C2-OVA induced OVA specific CD8+ and CD4+ T cells and efficiently suppressed tumour growth of OVA-expressing tumours	[299]
		Tu-eNV-FAP	Colorectal cancer, Melanoma, lung cancer, breast cancer	Preclinical	Cancer-associated fibroblasts overexpressed fibroblast activation protein-α (FAP). Tu-sEV-based vaccine engineered to overexpress FAP in tumour cells to form Tu-sEV-mimetic nanovesicles carrying FAP; resulted in suppression of tumour growth and strong specific CTLs in various *in vivo* cancer models	[300]
		Tu-sEVs-ODN-p(I:C)	Breast cancer	Preclinical	Breast cancer 4T1/HER2 cell sEVs loaded with ODN, a TL9 ligand, and p(I:C), a TL3 ligand using lyophilisation/rehydration approach, termed Tu-sEVs-ODN-p(I:C). These modified sEVs stimulated antigen specific T cell response, triggered Th1-based immunity, suppressing tumour growth in 4T1 tumour-bearing mice	[301]
		Tu-sEVs-N1ND pulsed DCs	HCC, Pancreatic cancer, breast cancer	Preclinical	All cancer groups showed consistent tumour growth suppression upon BMDC(Tu-sEVs-N1ND) treatment. Resultant increase in CD8+ cells and reduced CD4+/CD25+ cells observed. Orthotopic HCC groups treated with DC(Tu-sEVs-N1ND) showed prolonged survival (60% survival wk-9 vs 0% other groups), as well as supressing tumour growth. This prolonged survival could be associated to induction of humoral immunity initiated by DC(Tu-sEVs-N1ND). Indeed, a direct impact on stimulation of memory T cells was shown, due to localisation of sEVs to lymph nodes. Serum sEVs from healthy and cancer patient samples (human) coated with N1ND also induced cytolytic activity *in vitro*	[302]
		Tu-sEVs-IFN-γ	Prostate cancer	Preclinical	sEVs from mouse prostate cancer RM-1 cells were surface coated with biotin, incubated with streptavidin-IFN-γ to form Tu-sEVs-IFN-γ: engineered sEVs displayed anti-tumour vaccine suppressing tumour growth in prostate cancer mouse model	[303]
		Tu-sEVs-IRF-1	HCC	Preclinical	IRF-1 overexpressed or IFN-γ treated tumour secreted sEVs in combination with CpG adjuvant suppressed tumour growth, highlighting patient-specific tumour autologous EV vaccine	[304]
	Ascitic derived sEVs (A-sEVs)	A-sEVs 1st gen	Melanoma	*Ex vivo*	sEVs from melanoma ascites (A-sEVs) contained Mart1/Melan A tumour antigen could promote DCs to induce Mart1/Melan A-specific, HLA-A2 restricted CD8+ T cell responses *ex vivo*.	[108]
		A-sEVs + GMCSF (A-sEVs+GMCSF) 2nd gen	Advanced CRC	Phase I	In phase I clinical trial, patients received a total of four subcutaneous immunizations at weekly intervals. Both therapies were safe/well tolerated: A-sEVs+GMCSF but not A-sEVs alone shown to induce tumour-specific anti-tumor CTL response; A-sEVs can serve as an alternative in immunotherapy in advanced CRC	[305]
EV vaccine	sEVs-CD24 COVID19 vaccine	HEK-sEVs-CD24 2nd gen	Viral	Phase I	CD24, an immune checkpoint protein, enriched HEK-sEVs as a novel therapy for hyper-immune activation in COVID19 patients (a phased Ib/IIa study n =35, 10^8-^10 per dose, inhalation, 5 days) showed no adverse events observed (up to 443-575 days). HEK-sEVs-CD24 reduced inflammatory markers and cytokine/chemokine storm. HEK-sEVs-mCD24 (mouse homologue version) demonstrated to prevent cytokine storm in lungs	[306]
	ACE2-sEVs	Circulating-sEVs- ACE2 2nd gen		Preclinical	Circulating sEVs-ACE2 from plasma of COVID19 patients has broad protection for SARS-CoV-2 variants, and also showed promising protection against SARS-CoV-2 induced lung injury and mortality	[307]

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Table 2 (*continued*)

Source / EV type	Engineering strategy	Application / disease	Phase (pre-clinical / clinical)	Key findings	
Ba-sEVs (OMV) vaccine	Pertussis-sEVs (pOMV) 1st gen	Bacterial	Preclinical	Pertussis sEV vaccine (pOMV) activated tissue-resident memory CD4+ T cells in lung of mice post vaccination post 2 dose of pOMV	[308]
	OMV meningococcal B vaccine (MeNZB) 1st gen		Case-control clinical study	Vaccination with MenZB showed significantly reduced infection and estimated 31% vaccine effectiveness of MeNZB against gonorrhoea	[309]
	1790GAHB 1st gen		Phase IIa	Bacterial sEVs (OMV), *Shigella sonnei* vaccine (1790GAHB), two dose 1.5/25 μg of O antigen per protein, or 5.9/100 μg at day 1/day 29 in a phase 2 study (n =72 vaccinated). Robust anti-*S. sonnei* LPS serum IgG responses observed in two vaccinated groups, sero-response was 96% in high dose with 5.9/100 μg. Together, Ba-sEVs-based (1790GAHB, OMV) vaccine well tolerated and highly immunogenic in a population of African adults	[310]
	4CMenB 2nd gen		Phase I/II/III	4CMenB comprises three surface-exposed recombinant proteins (fHbp, NadA, and NHBA) and MenZB. 4CMenB offers 66-91 % coverage against *meningococcal* serogroup B strains	[311]
	Native-OMV-FHbp mut (R41S) 3rd gen		Preclinical (macaques)	A native *meningococcal* secreted Ba-sEVs overexpressed FHbp mutant (R41S) as a third generation Ba-sEVs based vaccine shown to be superior to detergent treated Ba-sEV MenB-4C vaccine (also known D-OMV), eliciting higher and broader serum bactericidal antibody response	[312]
Salmonella infected macrophages	M-sEVs-*Salmonella* 2nd gen		Preclinical	sEVs from *Salmonella*-infected macrophages (M-sEVs) contained *Salmonella* proteins, triggered anti-Salmonella IgG antibodies such as anti-OmpA, also stimulate CD4+ T cells secreting Th1 type cytokines	[313]
Circulating-sEVs	aEAU-sEVs 2nd gen	Autoimmune uveitis	Preclinical	Circulating sEVs isolated after IRBP R16 peptide induced experimental autoimmune encephalitis (aEAU) in Lewis rats (termed aEAU-sEVs) suppressed inflammation	[314]
DC-sEVs	DC-sEVs-immunoregulatory cargo 3rd gen	Chronic degenerative bone disease	Preclinical	DC-sEVs loaded with TGFB1 and IL10 after purification, along with immune stimulatory (LPS induced; stimDC-sEV) and immune "null" immature (iDC-sEV) unmodified after purification, delivered via I.V. route or locally into tissue overlying alveolar bone. Local administration: DC-sEVs=high affinity for inflamed sites, and taken up by DCs and T cells in situ; stimDC-sEV protected immunoregulatory cargo from proteolytic degradation; stimDC-sEVs promoted inflammation	[315]
T-sEVs	CD4+ T-sEVs (T cell microvilli particles/TMP) 1st gen		Preclinical	Particles/EVs shown to directly bud/release from CD4+ T cell membrane by trogocytosis. T cell microvilli particles (TMPs) enriched in TCRs, LF-2/CD2 and various cytokines, activate APCs *in vitro* and *in vivo*	[316]

tumour-rejection antigens, and EV-based cancer vaccine [108]. For instance, A-sEVs combined with GM-CSF were shown feasible, safe, and superior in inducing tumour-specific CTLs compared to A-sEVs alone in advanced colorectal cancer patients from a phase I clinical trial [305]. Recently developed second generation Tu-sEV-based vaccines, such as sEVs surface coated with IFN-γ (Tu-sEVs-IFN-γ) [303], could directly activate immune cells to inhibit tumour. Furthermore, IRF-1 protein when incorporated on Tu-sEVs (Tu-sEVs-IRF-1) enhanced antitumour immune response compared to sEVs expressing IFN-γ only. This functional response was mediated through elevated expression of IL-15Rα and MHC-I, resulting in increased tumour infiltrating $CD4^+$ and $CD8^+$ T cells [304].

Circulating sEVs can be a novel source for sEV vaccine for autoimmunity. Circulating sEVs isolated from autoimmune patients were shown to contain autoantigens that may differentially regulate (suppress) immune response. sEVs from synovial fluid of rheumatoid arthritis (RA) patients contain citrullinated proteins, which are known to be autoantigens [322]. sEV-based immunotherapy and allergenic peptide-encapsulated in liposomes have been shown to prevent allergic reactions in vivo [323,324]. Circulating sEVs from Lewis rats with autoimmune uveitis, when used as a vaccine, were demonstrated to prevent recurrent intraocular inflammation [314]. Importantly, vaccination with plasma-derived sEVs 3 days prior to antigen-specific (IRBP-specific) T cell transfer failed to prevent intraocular inflammation, as direct and/or indirect autoreactive T cell inhibition took up to 14 days. Instead, the transferred antigen-specific, uveitogenic T cells reduced IFN-γ but increased the production of IL-10 [314].

Bacterial sEVs, known as outer membrane vesicles (OMV) (Ba-sEVs), are an excellent vaccine against bacterium-induced infectious disease [325]. Ba-sEVs from *Neisseria meningitidis* serogroup B (MenB), also known as MeNZB™, were shown effective against gonorrhoea, as vaccinated healthy individuals were more protected than unvaccinated individuals in a population study [309]. Due to limited strain-specificity of Ba-sEVs (MeNZB™) vaccine, a second-generation Ba-sEV vaccine containing three highly immunogenic recombinant MenB antigens (GNA2091-fHbp, NadA, and NHBA-GNA1030) and MeNZB™, referred to 4CMenB (Bexsero®), provided broader protection against multiple *meningococcal B* strains [311,326]. Engineered OMV (Ba-sEV) vaccines have further led to new developments, for example, Ba-sEVs from MenB ΔABR strain, which deleted major outer membrane proteins (such as PorA, PorB and RmpM), showed a wider protection than those from wild-type stain in a murine GI disease model [327]. Advances in understanding genetically engineered variant strains associated with Ba-sEV-stimulated immune response will likely promote further development of next generation Ba-EV vaccines [325,326].

Recently, sEVs have further been expanded in their use as a vaccine platform for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Tsai and colleagues demonstrated that mRNA loaded HEK293F-sEV-based SARS-CoV-2 vaccine, compared to a lipid nanoparticle vaccine incorporated the same SARS-CoV-2 spike mRNAs, stimulated antigen-specific $CD4^+$ and $CD8^+$ T cell responses to nucleocapsid and spike proteins [328]. Circulating sEVs expressing angiotensin-converting enzyme 2 (ACE2), an entry receptor for SARS-CoV-2 (sEV-ACE2) showed protection against SARS-CoV-2 induced lung injury and mortality in hACE2 transgenic mice [307]. Of note, this sEV-ACE2 displayed activity against broad viral variants (α, β, and δ), supporting a broad-spectrum antiviral mechanism. Further studies employing modified sEVs (from HEK293 cells) expressing a novel fusion tetraspanin protein, CD63 (containing the bivalent VHH72 known to sterically block spike engagement with the ACE2 receptor) embedded within an anti-CoV-2 nanobody, showed broad variant blocking in vitro of infectious SARS-CoV-2 through the spike protein [329]. Further, an OMV-based vaccine expressing SARS-CoV-2 spike receptor-binding domain (RBD), when delivered intranasally (3 doses 3E+10 particles/ dose or 18 μg, 14 days apart) in a *Mesocricetus auratus* model, demonstrated a decreased body weight loss viral titres in the bronchoalveolar lavage fluid, and lung pathology (i.e., reduced inflammation, alveolar collapse, and haemorrhagic areas) compared to unvaccinated controls [330]. A phase Ib/IIa study employed sEVs expressing CD24 (sEV-CD24), an immune check point protein, as a novel therapy to control hyper-immune activation in COVID-19 patients [306]. Anticoli and colleagues used sEVs engineered to incorporate specific viral antigens, such as HIV Nef mutant (Nef^{mut}) to elicit strong and effective antigen-specific CTLs in syngeneic mouse models (C57 BL/6) [331]. Importantly, this novel and flexible vaccine platform was expanded to incorporate different viral antigens from HIV, Ebola, Influenza, hepatitis B (HBV) and C (HCV) viruses [331].

7.2. Engineered sEVs: next generation therapy platforms

Besides DCs, other immune cell-derived sEVs have also been investigated as potential cancer vaccines. For instance, B cell-derived sEVs (B-sEVs) have been shown to induce antigen specific T cell response [80]. Additionally, NK cell-derived sEVs (NK-sEVs) displayed cytotoxic and anti-tumour effect [165,166], while M1 macrophage-derived sEVs (M1-sEVs) showed a pro-inflammatory effect [332]. Activated T cell-derived sEVs (T-sEVs) exhibited immune-mediated tumoricidal effect (e.g., Vδ2-T-sEVs induce EVB-associated anti-tumour function) and showed effective anti-tumour activity [333]. Further, CAR-T cell-derived sEVs (CAR-T-sEVs) carried functional surface CAR and displayed potent anti-tumour activity [334,335]. Modified CAR-T-sEVs-RNA7SL1, using immuno-activating RNA (i.e., RN7SL1, an immunostimulatory RNA), were shown to activate RIG-I in immune cells and reject tumours with resistance caused by the loss of CAR recognized antigen in vivo [336] (Fig. 2, Table 2).

sEVs are less immunogenic, capable of crossing biological barriers such as the blood-brain barrier and flexible for modified cargo loading and tissue specificity [8,279]. Oligonucleotides are nucleic acid polymers designed for precision and/or personalized gene therapy, which are divided into several major categories: antisense oligonucleotides (ASOs), interfering RNA (RNAi), microRNAs (miRNAs), and anti-miRNAs. However, their properties present challenges to deliver across biological membranes [337,338] (Fig. 2, Table 3). sEVs are natural carriers of various RNA and DNA and the delivery of their contents can instil functional changes in either donor (depletion of specific cargo) or recipient cells following transfer [8,339]. Interestingly, accumulation of intracellular anti-KRAS ASOs (AZD4785) into $CD63^+$ late endosomes mediated the secretion of ASO-loaded sEVs [340]. In addition, sEVs loaded with ASO or siRNA targeting STAT3 to form sEVs-siRNA-STAT3, and sEVs-ASO-STAT3, respectively. Both engineered sEVs displayed STAT3 suppression compared to the control in an in vivo liver fibrosis model, while sEVs-ASO-STAT3 showed preferential restoration in liver function [341]. Electroporation has been successfully used to load various molecular cargo into sEVs, including siRNA [342]. For modifying delivery of sEVs, Kamerkar et al., showed CD47 modified sEV-s-sh/-siRNA-$KRAS^{G12D}$ (including shRNA or siRNA targeting $KRAS^{G12D}$) were more potent in inhibiting tumour progression compared to the lipid nanoparticle loaded with matching siRNA/shRNA [176]. Moreover, let-7a miRNA-loaded sEVs coated with GE11 peptides were shown to target $EGFR^+$ tumours and successfully suppress tumour growth in a xenograft breast cancer tissue in $RAG2^{-/-}$ mice [105]. More recently, viral-encoded artificial miRNA 4 (amiR-4) was employed, in combination with target moiety ARID1A (a key player in resistance to oncolytic virus replication) and methyltransferase EZH2 inhibitor, to sensitize cancer cells to oncolytic virus [343]. Here potent anti-tumour activity on uninfected tumour cells was shown. Such bystander killing was (likely) mediated by intercellular transfer of amiR-4 by sEVs [343]. These oncolytic viruses provide novel anti-cancer therapeutics when combining deliverable small molecule inhibitors and/or immune checkpoint inhibitors. Recently, Li et al., developed a macrophage-sEV-coated nanoplatform for targeted doxorubicin (DOX) chemotherapy of cancer cells, where sEV surface was modified with a

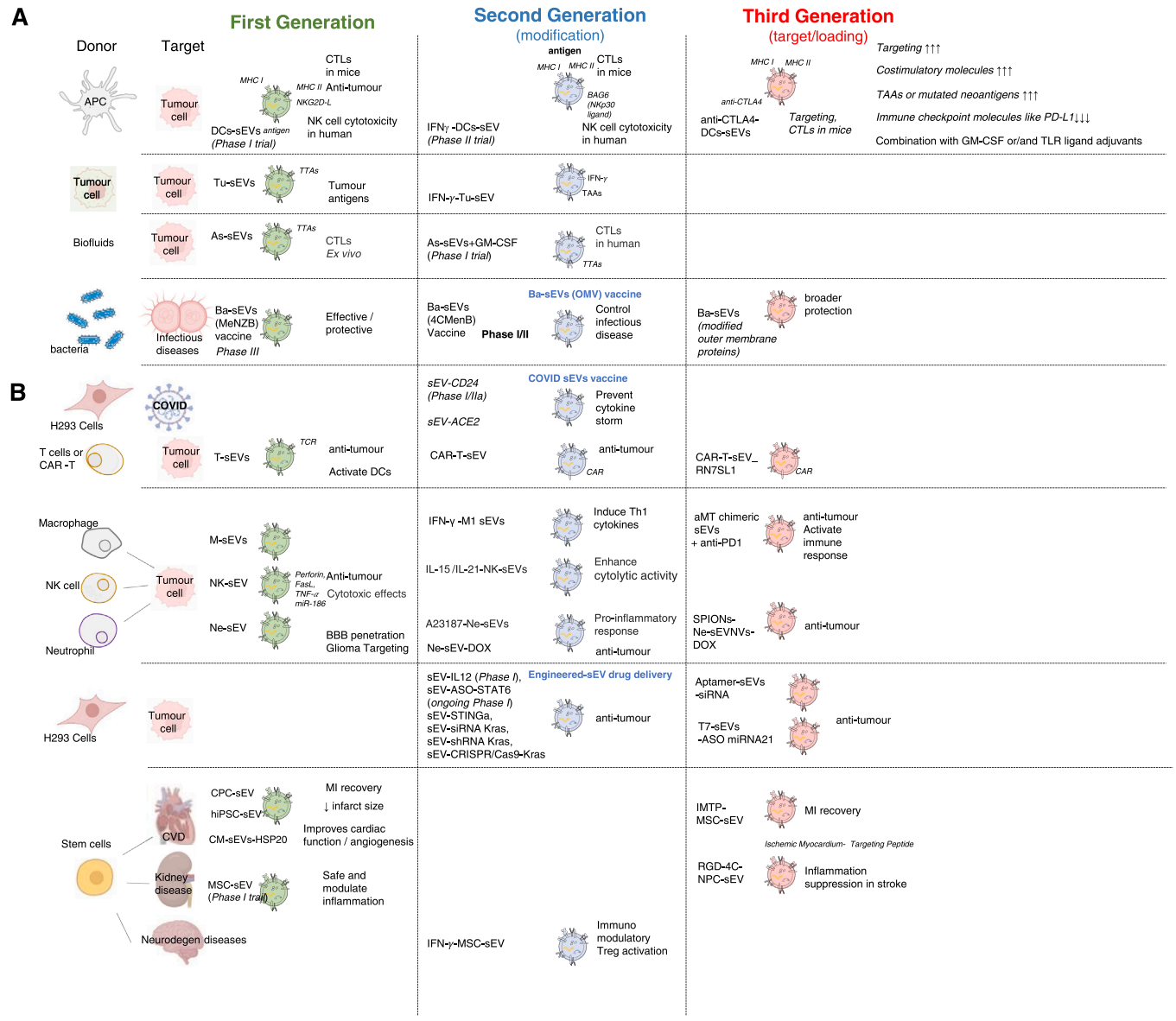

Fig. 2. Evolution of sEV vaccines and sEV-based drug delivery systems. (A) Current development of sEV vaccines experienced from first generation (native sEVs), second generation (activation or loading) towards third generation (loading and targeting, combination therapy). sEV vaccines comprise DC-sEVs-based vaccine, tumour cell derived sEVs (Tu-sEVs)-based vaccine, biofluids derived sEVs based vaccine, and bacteria derived sEVs (Ba-sEVs, also known as outer membrane vesicles/OMV) based vaccine, and more recently, sEVs-CD24 as COVID vaccine. (B) Recent advances in immune cell-sEVs for anticancer therapy (CAR-T-sEV-, M1-sEV-, NK-sEV-, Ne-sEV) and sEV-based cancer therapy for delivery of various molecular/synthetic factors (antisense oligonucleotides, ASOs, RNA interference, RNAi, miRNAs, anti-miRNAs), recombinant proteins (rIL-12), and STING agonists (cGAMP and cyclic dinucleotides, CDNs). Interestingly, various applications of stem cell derived sEVs for treatment of various diseases such as chronic kidney diseases (CKD), neurodegeneration disease, cardiovascular disease, and stroke.

peptide targeting c-Met [344]. While significantly improving cellular uptake efficiency and antitumour efficacy of DOX, modified sEVs exhibited high tumour-targeting efficacy (while un-coated sEVs resulted in no change in non-tumour organ distribution) and reduction in tumour growth. Further, 'coated' macrophage-derived sEVs could significantly prolong circulation time of DOX, likely due to the immune-evasive ability of source (macrophage) membrane proteins and membrane encapsulation of chemotherapeutics.

Other advances in developing sEVs as immunomodulatory drug delivery vehicles include reengineered 293 T-sEVs-IL-12 (IL-12 fused with sEV surface protein PTGFRN), which was shown to reduce substantial toxicity of recombinant IL-12 (rIL-12) in vivo [348]. Further, such therapeutic effect was shown to prolong tumour retention and improved anti-tumour activity compared to rIL-12 alone in a murine MC38 colon cancer model (complete responses 63% vs 0%, respectively). This study also highlighted that following the second sEVs-rIL-12 delivery, the responder mice showed no tumour regrowth, and the observed anti-tumour activity was $CD8^+$ T cell-dependent [348]. 293 T-sEV-rIL12 was retained locally when subcutaneously delivered in primates [348]. Currently, a phase I/IIa trial is assessing such 293 T-sEV-rIL-12 vaccine in patients with cutaneous T cells lymphoma (CTCL) (NCT05156229). To boost T cell response to cancer immunotherapy, various approaches incorporating the STING pathway have been used; for example, cytosolic DNA was used to stimulate DCs and further activate CTLs. Initial clinical trials involving STING agonists (STINGa) including cGAMP and other cyclic dinucleotides (CDNs) showed limitations in pharmacological profile and poor bioavailability [349,351]. STING agonist cyclic GMP-AMP externally loaded into sEVs (sEVs-cGMP-AMP) suppressed B16F10 tumour growth and were more efficiently internalised by DCs for activating CTLs [349]. Following intratumoral injection,

Table 3
Advances in EV-based drug delivery platform.

Source / EV type	Engineering strategy	Application	Phase (pre-clinical / clinical)	Key findings	
T cell sEVs (T-sEVs)	γδ2T-sEVs	EVB-associated tumour	Preclinical	γδ2T cells (T cells) have innate-like phenotype with lytic activity restricted by MHC. γδ2T-sEVs transport FasL and TRAIl, NKG2D, CD80 and CD86, MHC I and II to kill EBV-associated tumour cells and also reduced EBV-associated tumour in a Rag2-/- γc-/- and humanized mice. More interestingly, allogeneic γδ2T-sEVs induced greater antitumour immunity compared with autologous γδ2T-sEVs, NK-sEVs and DC-sEVs.	[333]
CAR-T cells sEVs (CAR-T-sEVs)	CAR-T-sEVs	Breast cancer, HCC,	Preclinical	Chimeric antigen receptor (CAR) T cells released sEVs, carried functional surface associated CAR suppressed tumour growth in breast and HCC tumour xenografts, and displayed safer efficacy compared with CAR-T therapy in a preclinical *in vivo* model of cytokine release syndrome	[334]
	CAR-T-sEVs	Breast cancer	Preclinical	Intravenous administration of target mesothelin CAR T cells secreted sEVs showed suppression of tumour growth of both endogenous and exogenous MSLN positive triple negative breast cancer	[335]
	CAR-T-sEVs-RN7SL1	Melanoma	Preclinical	RN7SL1, an endogenous RNA activating RIG-I/MDA5 signalling, loaded into CAR-T-sEVs, promoted DC mediated T cell killing and suppressed myeloid cells, rejecting solid tumour	[336]
B-sEVs	B-sEVs		*In vitro*	sEVs from human and murine B lymphocytes induced antigen-specific MHC class II-restricted T cell responses; suggest a role for sEVs in antigen presentation *in vivo*	[80]
NK-sEVs	NK-92-sEVs	Melanoma	Preclinical	NK-92MI cells release sEVs (NK-92-sEVs) comprising markers Alix/TSG101 and functional NK proteins (perforin and FasL) and TNF-α. NK-92-sEVs kill melanoma B16F10/effuc cells (enhanced firefly *luciferase) in vitro*. In a B16F10/efflux cell xenograft model, NK-92-sEVs inhibited tumour growth progression (monitored using bioluminescence/ultrasound imaging systems)	[165]
	Primary NK-sEVs	Neuroblastoma	Preclinical	Human peripheral blood mononuclear cell-derived NK cells release sEVs that result in anti-tumour effect of MYCN-amplified neuroblastoma *in vitro* and *in vivo*	[166]
Ne-sEVs	Ne-sEVs-DOX	Glioma	Preclinical	Neutrophil sEVs (Ne-sEVs) loaded with DOX to form Ne-sEVs-DOX, successfully inhibit tumour growth in a glioma mouse model. Interestingly, Ne-sEVs displayed neutrophil-like chemotactic function and penetration of blood-brain barrier	[345]
	SPIONs-Ne-NVs-DOX	Gastric cancer	Preclinical	Cell nanovesicles from neutrophils generated by cell extrusion (Ne-NVs) at high yield, and decorated sEV-NVs with superparamagnetic iron oxide nanoparticles (SPIONs). Ne-NVs loaded with DOX to form SPIONs-NE-NVs-DOX, which displaced a selective accumulation at tumour site under an external magnetic field, and an effective anti-tumour response in an *in vivo* gastric cancer model	[346]
M1-polarized macrophages derived sEVs (M1-sEVs)	M1-sEVs	Melanoma	Preclinical	IFN-γ simulation M1 macrophage secreted sEVs (M1-sEVs) showed lymph node retention after subcutaneous injection and triggered stronger release of Th1 cytokines by residential macrophages and DCs. Interestingly, M1-sEV were associated as a vaccine adjuvant, enhancing activity of lipid calcium phosphate nanoparticles encapsulated Trp2 vaccine *in vivo*, in contrast to M2-sEVs or CpG oligonucleotides	[332]
Chimeric-sEVs	Macrophage-tumour chimeric sEVs (MT-sEVs)	lymphoma, breast cancer, melanoma	Preclinical	sEVs from macrophage-tumour hydride cells prime T cell activation and homing ability to tumour sites, mediating immunosuppression. Interestingly, MT-sEVs inhibited tumour growth when combined with anti-PD1 treatment, and further limited metastases and postsurgical tumour recurrence in various mouse models	[347]
DCs+Tu-sEVs	Alarmin-painted sEVs+DCs	Liver cancer	Preclinical	High mobility group nucleosome-binding protein 1 (HMGN1) is a potent adjuvant, containing a functional domain, NIND. Tu-sEVs coated with NIND using an sEV/exosomal anchor peptide (termed Tu-sEVs-N1ND); then pulsed DCs used to generate DC-Tu-sEVs-N1ND. This engineered EV strategy resulted in suppression of tumour growth and displayed homing capacity to lymphoid tissues and established memory T cells	[302]

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Table 3 (*continued*)

Source / EV type	Engineering strategy	Application	Phase (pre-clinical / clinical)	Key findings	
Tu-sEVs	sEVs-IL12	CRC and melanoma	Preclinical	sEVs surface display of rIL12 using PTGFRN-rIL12 (sEVs-rIL-12) approach suppressed tumour growth having limited systemic toxicity compared with recombinant IL12. In MC38 model, complete responses were observed in 63% of mice treated with sEVs-rIL12 compared with 0% complete response at equivalent IL12 dosed. Dose-dependent increase in tumour antigen-specific CD8+ T cells observed	[348]
	sEVs-shRNA Kras	Pancreatic cancer	Preclinical	Engineered sEVs containing CD47 and electroporated loading *KRASG12D* siRNA or shRNA (CD47-sEVs-siRNA *KrasG12D* or CD47-sEV-shRNAKrasG12D) resulted in reduced tumour size compared with liposome loaded siRNAKrasG12D or shRNAKrasGD in mouse KRASG12D pancreatic cancer models (orthotopic PANC-1, KTC mice and orthotopic KPC)	[176]
	sEVs-CRISPR/Cas9-Kras G12	Pancreatic cancer	Preclinical	CRISPR/Cas9 plasmid DNA loaded sEVs by Exo-Fect transfection kit supressed *KrasG12D* gene in pancreatic cancer. BM-MSC-sEVs associated with CRISPR/Cas9-KrasG12G decreased tumour growth *in vivo*	[349]
	sEVs-let7a	Breast cancer	Preclinical	GE11 peptide (an EGFR binding peptide) coated sEVs loaded with let-7a miRNA (GE11-sEVs-let-7a) reduced tumour growth in EGFR-expressing xenograft in RAG2(-/-) *in vivo* model	[105]
	sEVs-STINGa	Melanoma	Preclinical	sEVs modified to deliver STING agonist cyclic GMP-AMP (sEVs-cGMP-AMP), resulting in reduced melanoma tumour growth and enhanced APC activation, with consequent increase in tumour specific activated CD8+ T cells	[350]
	sEVs-CDN/STINGa)	Melanoma		A STING analogue loaded in sEVs (sEVs-CDN/STINGa) promoted CD8+ T cell infiltration and reduced tumour growth while displayed no systemic cytokine storm after intratumonal injection of sEVs-CDN	[351]
	sEVs-ASO-STAT6	CRC, HCC	Preclinical	ASO-STAT6 loaded onto the surface of sEVs to form sEVs-ASO-STAT6, suppressed transcription factor STAT6 in immunosuppressive M2 tumour-associated macrophages and their reprogramming towards proinflammatory and anti-tumour M1-TAMs ($NOS2^{+}$). Interestingly, sEVs-ASO-STA6 reduced > 90% tumour growth and 50-58% complete remission	[75]
	sEVs-OV-amiR-4	Pancreatic cancer	Preclinical	Viral-encoded artificial miRNA 4 (amiR-4 targeting ARID1A, a key player in resistance to oncolytic virus replication, combined with EZH2 inhibitor for the potent killing of both infected and uninfected tumour cells. sEVs carried amiR-4 to mediate cell death of uninfected cells	[343]
	Aptamer-sEVs-siRNA	Prostate cancer; Breast cancer; CRC	Preclinical	siRNA loaded sEVs surface coated with aptamer shown to mediate tumour targeting and reduced tumour growth in different cancer models (aptamer-PSMA-sEVs-siRNA survivin for prostate cancer; aptamer-EGRF-sEVs-siRNAsurvivin for breast cancer; aptamer-folate-sEV-siRNAsurvivin for colorectal cancer)	[352]
	T7-sEVs-ASOmiR21	Glioblastoma	Preclinical	T7, a transferrin receptor-binding peptide, fused with Lamp2b for decoration on sEV surface (T7-sEVs). T7-sEVs loaded with ASOmiR21 to form T7-sEV-ASOmiR21, targeting glioblastoma after intravenous injection. T7-sEV-ASOmir21 suppressed tumour miR21 expression and consequently upregulated tumour PDCD4 and PTEN expression and inhibition of tumour growth	[353]
	αCD3- αEGFR-PD-1-OX40L GEMINI-sEVs	Breast cancer (TNBC)	Preclinical	Multifunctional immune modulating sEVs (GEMINI-sEVs) engineered in Expi293F cells by genetically introducing programmed death 1 (PD1) and OX40 ligand (OX40L) and monoclonal antibody against CD3 and EGFR. These engineered GEMINI-sEVs displayed candidate agents for immunotherapy; (i) redirect and activate T cells for killing EGFR-positive cancer cells (ii) potent anti-cancer immunity	[354]
syBV+Ti-sEVs	Synthetic bacterial vesicles (syBV) and melanoma tissue sEVs (Ti-sEVs)	Melanoma, CRC	Preclinical	Applied lysozyme and high pH treatment, density gradient, and sonication to generate synthetic bacterial vesicles (syBV). Subcutaneous injection of both syBV and (tumour) tissue sEVs (Ti-sEVs) triggered activation of BmDCs and suppressed tumour growth through Th1-type T cells immunity	[355]

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Table 3 (*continued*)

Source / EV type	Engineering strategy	Application	Phase (pre-clinical / clinical)	Key findings	
Stem cell derived sEVs (SC-sEVs)	Ca-sEVs-Hsp20	CVD/Diabetes	Preclinical	Cardiomyocytes from HSP20 transgenic mice secreted sEVs (Ca-sEVs-HSP20) containing HSP20, p-Akt, surviving and SOD1. Prevented *in vitro* hyperglycemia-triggered cell death, and protected STZ-induced cardiac adverse remodelling	[356]
	hiPSC-CC-sEVs	Myocardial infarction	Preclinical (swine)	Human induced pluripotent stem cells (hiPSCs) differentiated into cardiomyocytes, endothelial cells and smooth muscles cells. A mixture of those cells secreted sEVs (2:1:1 ratio) improved myocardial recovery in swine	[357]
	Cardiosphere-derived cells (CDCs) and c-kit+ cardiac progenitor cells (CPCs)	Ischemic myocardium	Preclinical	Circulating sEVs from transplanted progenitor cells aid the functional recovery of ischemic myocardium	[358]
	IMTP-Lamp2b-BMSC-sEVs	Myocardial infarction	Preclinical	An ischemic myocardium-targeting peptide (IMTP) fused with Lamp2b to engineer bone marrow derived stem cells to form IMTP-BMSC-sEVs. IMTP-sEVs enriched in the injured H9C2 cells, also accumulated in ischemia heart area reducing inflammation and apoptosis, while enhancing cardiac function	[359]
	RGD-4C-NPC-sEVs	Ischemic stroke	Preclinical	A new strategy using RGD-4 peptide fused to PS binding domain of lactadherin (C1C2) to transfect neural progenitor cells, forming RGD-4C-NPC-sEVs; these engineered sEVs suppressed inflammatory response in MCAO stroke mice model after IV injection	[360]
	UC-MSC-sEVs	Kidney diseases	Phase II/III Clinical trail	After UC-MSC-sEVs treatment in chronic kidney disease patients, while no significant adverse events observed, with mixed efficacy. Transient effect of MSC-sEVs treatment, with efficacy peaking at 3-6 mths. MSC-sEVs also had anti-inflammatory effect, observed (at 12 wks) by increase in TGF-B1 and IL-10 levels and decrease in TNF-α levels. CD133 and Ki67 mRNA expression increased in the treatment group indicating regeneration of tubular cells, in contrast to no changes in control group	[361]
	IFNλ-MSC-sEVs	Autoimmune and neurodegenerative diseases	Preclinical	sEVs from MSCs following IFN λ stimulation (IFNλ-MSC-sEVs) suppressed inflammatory response and promoted Treg activation in an autoimmune encephalomyelitis (EAE) murine model after IV injection	[362]

CDNs-loaded sEVs (sEVs-CDNs) were retained within the tumour and enhanced local Th1 and $CD8^+$ T cell responses (noting 100–200-fold improvement in potency by sEVs-CDNs). Importantly, such tumour localisation/retention generated systemic anti-tumour immunity without the often-associated systemic inflammatory cytokine storm in clinical trial patients [351].

As a source of sEVs, mesenchymal stem cells from various early phase clinical trials have been approved. Two dose treatments of MSC-sEVs in grade III-IV chronic kidney disease (CKD) patients (dose one: intravenous, dose two: intra-arterial) showed improved kidney function, reduced inflammatory cytokine (TNF-α) and increased immunoregulatory cytokines (TGF-β1 and IL-10) [361]. IFN-γ treated human MSC-sEVs (IFN-γ-a-sEVs) carrying anti-inflammatory RNAs and proteins improved neurological response in a murine experimental autoimmune encephalomyelitis (EAE) model [362]. It was suggested that because of low engraftment rate of transplanted cells (such as MSC), that the observable functional benefit is associated with paracrine signalling of transplanted cells [362]. Cell-free delivery strategies using sEVs from induced pluripotent stem cells (iPSCs) and their cardiac derivatives emerged as scalable therapeutic strategies [363] (single dose treatments; human cardiomyocytes, primary cardiac fibroblasts, and endothelial cells) and novel treatments for purine post-myocardial infarction (MI) heart failure (HF) [357]. Human iPSCs differentiated into cardiac cells and released sEVs (hiPSCs-CC-sEVs) which at 7.5 mg dose, reduced MI scar size compared with control [357]. Interestingly, transplanted human cardiac progenitor cells (CPC) released sEVs into circulation in a xenogeneic rodent MI model, and those CPC-sEVs contained miRNAs promoting myocardial recovery [358]. Furthermore, Hsp20-mediated activation of sEV biogenesis in cardiomyocytes improved cardiac function and angiogenesis in a diabetic mouse model [356]. A salient protein-replacement therapy study recently employed human dermal fibroblast-derived sEVs with encapsulated mRNA encoding extracellular-matrix α1 type-I collagen (COL1A1) formulated with hyaluronic acid (HA) for microneedle (COL1A1-sEV-MN) intradermal delivery and retention [364]. You and colleagues demonstrated dose-dependent synthesis and replacement of dermal collagen (from 2.7×10^9 copy number of *COL1A1*) and provided an improved wrinkle treatment in photoaged skin using a cellular nanoporation (CNP) mRNA loading strategy. Important findings included the dynamic mRNA and protein presence over time in vivo, with corresponding COL1A1 protein detected as early as 12 h, peaked at 4 d, and sustained for several weeks after delivery [364]. Moreover, COL1A1-sEV-MN treatment reduced wrinkle number and size compared with encapsulated lipid nanoparticles (COL1A1-LNPs), the latter further induced notable inflammatory infiltrate in the local tissue [364]. Taken together, emerging advances on how source sEVs could potentially be manipulated to gain therapeutic advantage (e.g., different MSC cellular microenvironments impact composition and function of derived sEVs; immunomodulation, anti-fibrotic, anti-inflammatory properties [365]) as well as improved EV-delivery systems may help us better design next generation targeted drug delivery approaches with considerations for their clinical manufacture.

7.3. Technologies for next generation immuno EV therapeutics

Technologies that have led advances in understanding and developing cancer vaccines include mRNA platforms [366,367], high-throughput screenings (interaction), immunopeptidomics [368], and various mass spectrometry (MS)-based proteomic strategies [369], including mass cytometry and single cell proteomics. Such technologies could also directly assess the repertoire of immune cells, their presented peptides and neo tumour antigens. The acquired knowledge on cancer cell heterogeneity and immune cell signature could help to improve diagnosis and predict recurrence and potential drug-resistance [368, 370–372].

Tumour antigens are potentially useful for cancer immunotherapy, including neo-antigens derived from mutations unique to the tumour cells as well as cancer/testis antigens (CTA), often absent from somatic cells. Neo tumour antigens enable T cell recognition of tumour cells [373,374]. However, identification of HLA-bound peptides from tumour antigens and candidates for immunotherapy is complicated by the difficulty of detection of their presentation on tumour cells [375]. Here, identifying tumour-specific antigens can be aided through selective enrichment strategies, including immunoaffinity MHC capture with peptide elution with MS [376]. The advances made in MS technologies, mass cytometry, neoantigen discovery and cancer immunotherapy have catalysed the launch of the Human Immunopeptidome Project (HIPP) with the goal to provide a complete profile and map of the human immunopeptidome [377]. Other effective strategies to identify immunotherapeutic targets on tumour cells have comprehensively surveyed the landscape of peptides accessible to T cells by performing MHC capture with MS [375]. This approach identified tumour-specific antigens from non-mutated oncoproteins including peptide QYNPIRTTF, shown to induce potent tumour killing across multiple HLA alleles and expand the diversity of immunotherapeutic targets.

sEVs are increasingly viewed as promising vectors to deliver peptide-based cancer antigens to the human immune system. Heck and colleagues showed that HLA-I peptide ligandomes of sEVs compared to matched donor cells were enriched in HLA-B complexes, peptide ligands (~3500), surface CD proteins, tetraspanins, and cysteinylated peptides that may modulate immune responses [378]. These findings suggest that vesicular HLA complexes may be utilized to carry peptide vaccines, as well as different peptide and post-translationally modified ligands to be presented on or transferred by sEV HLA-I complexes. Composition of sEVs has been shown regulated by ESCRT proteins to subsequently activate pathogen-specific cytotoxic T cell response [379] or regulate immunosuppression through regulating PD-L1 [259]. Hu et al., [380] showed presentation of antigen on sEVs using viral transmembrane domains enhanced antigen immunogenicity. Here, the efficiency of sEV incorporation was dependent on transmembrane domain to regulate both humoral and cell-mediated responses in vivo; such findings have implications in vaccine design for displaying antigens onto EV surface. A recent finding has also demonstrated the presence of complex EV surface proteins may provide a unique advantage in preferential uptake and activation of immune cells. Importantly, EV surface proteins including glycoprotein PTGFRN with their sialic acid glycoprotein receptors could facilitate internalization and preferential delivery of STING agonists for preservation of viability for APCs [351]. Although molecular mechanisms of agonist delivery and how surface recognition/delivery are yet to be established, such findings show that EVs could target and enhance immune cell activation and in vivo anti-tumour activity, which may be contributed by cellular tropism and immune cell signaling activity of specific surface factors on EVs.

sEVs are increasingly being used to enhance immunogenicity. Immunogenic sEVs containing black phosphorus nanoparticles were shown under laser irradiation an effective anticancer photo-nanovaccine targeting lung cancer in vivo [381]. This premise is based on Tu-sEVs could possibly present a variety of tumour antigens following ablation, activate host immunity, and increase the number of tumour-infiltrating T cells, eventually eliciting a tumoricidal immune response. In comparison with vehicle (nanocarrier, non-sEVs) alone, sEV treatment resulted in improved long-term outcomes, greater elevation of tumour temperature and tumour targeting efficacy in vivo. Specifically, the group immunized with sEVs (without laser irradiation) showed a significantly larger percentage of $CD8^+$ T cells in the spleen indicating that the sEV approach was effective at activating the immune system in vivo [381]. Therefore, vaccination with sEVs in combination with this photothermal therapy demonstrated therapeutic efficacy against established lung cancer and promoted infiltration of T lymphocytes within the tumour tissue [381]. Likewise, Zhang's group constructed synthetic multivalent antibodies retargeted sEVs (SMART) that express CD3 and epidermal growth factor receptor (EGFR) antibodies for the immune attack of $EGFR^+$ triple-negative breast cancer cells [272]. Results in vitro and in vivo indicate that such SMART sEVs can not only induce cross-linking of EGFR-expressing tumour cells and T cells but also elicit anti-tumour immune responses. Thus, the development of this technology is of great significance for enhancing and modulating cancer immunotherapy. Further modifying sEVs as direct therapeutic agents may lead to reprogramming of the tumour microenvironment. This is highlighted recently for macrophage-derived sEVs loaded with metformin and modified to express CD206 mannose receptor for targeting and reprogramming M2 tumour-promoting macrophages to M1 type [382]. Therefore, advances in cell-specific tumour/stroma microenvironment characterisation, with nanotechnology and combination therapy are required to further advance sEV-based targeting, immunoregulation, and anti-cancer function [27].

8. Perspective

The immune system is a dynamic and complex system. The realization that different immune cells can exchange proteins, lipids, and nucleic acids through EV transfer has provided emerging developments in immunoregulation. Tumour cells release EVs that can dynamically regulate immunogenicity through either inhibiting tumour growth by eliciting anti-tumour immune responses [383] or promoting tumour growth by inhibiting anti-tumour immunity or enhancing angiogenesis and/or metastases As a first step towards defining a context-dependent sEV response, an in-depth biophysical characterisation of sEVs and appropriate biological responses are crucial. Different EV types from different cellular origins, or even the activation state of the cell-derived vesicle population, will produce a specific response on target cells. Indeed, a complication of the molecular and functional analyses of immune cell-derived EVs is extremely difficult, if not impossible, to obtain EVs from populations exclusively comprising only one immune cell population (i.e., immature DCs or only activated DCs, conventional or non-conventional DCs, migratory or residential DCs [146]). Cultures of un-stimulated DCs always contain some activated DCs, as determined by the expression of activation markers, while ligand-based stimulation often is asynchronous and incomplete in cell activation within a defined population. This heterogeneity in source and response should be considered.

To develop novel approaches for cancer therapy that promote antigen presentation and T cell activation we need detailed characterization of such vesicles and their biogenesis during steady state or initial tumour development, as well as deciphering the precise mechanism and requirements of vesicle transfer between different DCs based on their location (i.e., residential and migratory DCs [146]). Indeed, efforts to expand tumour infiltrated APCs with Flt3L [384,385] and delivery of intratumoral immune modulatory stimuli to promote DC maturation and migration [386] are recent developments in enhancing anti-tumour immunity. Clinical trials assessing the therapeutic efficacy of immature DC-sEVs although scheduled, are yet to complete, undoubtably will provide further context in autoimmune diseases or transplant settings. Generation of allograft-rejecting immune response has already been linked to cDC1 subtypes, specifically capable of cross-presentation in

cancer immune regulation. Challenges remain in determining optimal antigen presentation required to prime endogenous T cells [387] and whether targeting (versus non-targeted/non-modified) sEV or hybrid-sEV vaccines are efficacious warrants future investigations. Tu-sEVs containing endogenous tumour antigens and immune stimulatory CpG DNA, could activate DCs for tumour-specific T cell activation [221,388], and thereby potentially be incorporated in novel vaccination strategies for effective anti-tumour immunity [389,390].

Presently, Tu-sEVs in tumour progression has focused on their innate ability to sequester tumour-reactive antibodies, inhibit anti-tumour cytotoxicity [273] and limit the effectiveness of antibody-based anti--cancer drug interventions [274]. Given their ability to transverse biological membranes and be found in biofluids such as blood, Tu-sEVs could further be systemic candidate biomarkers, containing an abundance of components involved in cell-cell communication and membrane exchange, tumour (enriched or specific) antigens, and cell-specific markers, or otherwise as evaluating responses to therapy [391–393]. The prospect of enlisting sEVs or hybrid nanocarriers as immuno-therapeutic agents remains attractive, with the advent of vesicle–mediated therapeutic platforms for efficient and targeted delivery [394,395]. In addition to exhibiting distinct advantages over cell-based approaches [396] (e.g., owing to their small size, low toxicity, high biocompatibility, and efficient penetration of solid tumours), vesicle therapeutics also feature the ability to cross tissue barriers as well as their non-tumorigenic potential [397]. Drawbacks of systemically administered sEVs, such as rapid clearance via phagocytes and systemic dilution remain therapeutic challenges [141]. Surface expression of CD47 on sEVs can facilitate their evasion of phagocytosis and consequently increase circulating half-life [398,399]. Belhadj et al. [399] revealed that incorporation of CD47 on the surface membrane of sEVs reduced endocytosis by macrophages, enhanced uptake to target tumour cells, and reduced off-target localisation to the liver and spleen, while increased (>120%) tumour localisation. Further, genetic engineered multifunctional sEVs comprising both surface antibodies for activating and directing T cells for anti-tumour cell activity and immune- checkpoint modulators have been described [354]. Such studies provide important strategy for camouflage immunosurveillance [400] and targeted, active anti-cancer therapy through development of EV engineering strategies. Both CAR-T-sEVs and modified CAR-T-sEVs-RNA7SL1 showed promising therapeutic outcomes [334–336], alternatively, CD5 targeted lipid nanoparticles (LNPs) containing CAR T mRNA, have further shown transient and effective in vivo in cardiac repair [401]. Impacting on the field of immunotherapies, loss of PD-L1 in Tu-sEVs, was shown to inhibit tumour growth and promote T cell activation in draining lymph nodes [260]. Stimulation with IFN-γ was shown to increase PD-L1 on sEVs from metastatic melanoma, suppressing the function of $CD8^+$ T cells and promoting tumour growth. Recent studies demonstrated that PD-L1 is highly expressed on the surface of Tu-sEVs, with this being used as an antibody-tethered immune-biochip to selectively capture $PD\text{-}L1^+$ sEVs from patient sera and diagnosis of lung cancer [402]. Understanding and altering the mechanism of selective PD-L1 packaging into and on sEVs could provide important strategies for resistance to antibody therapy. Recent findings of the functional importance of EV transmembrane proteins and their interaction with corresponding receptors on recipient cell membrane has been highlighted in targeting tumour-draining lymph nodes and activating T cell immunological reaction and inhibition of T_{reg} cell induction [403]. Additionally, engineered EVs (N-terminal functional Gasdermin D (GSDMD-N) mRNA encapsulated sEVs armed with chlorin e6 (Ce6, a hydrophilic sensitizer) (2 ×100 μg delivery dose) and HER2 antibody)) in combination with ultrasound treatment and anti-PD1 antibody have recently been highlighted to directly induce tumour cell pyroptosis, robust anti-tumour immune response in breast tumour mouse models (SKBR3- and HER2 transfected 4T1 models) resulting in reduced lung metastasis, and prolonged mouse survival [404]. Such findings demonstrate a promising strategy for EV engineering toward pyroptosis induction for immunotherapy.

This review has focused on the importance of sEVs in the context of tumorigenesis in immunoregulation, mechanisms including modulating antigen presentation, immune activation, immune suppression, immune surveillance, and intercellular communication throughout the extracellular environment. sEVs from tumour cells can impact the proliferation, apoptosis, cytokine production and reprogramming of both innate and adaptive immune cells. Our knowledge of immune cell derived EV cargo remains limited, hindering our understanding of their function. We have discussed DNA, RNA, protein and even mtDNA cargo and critical surface regulations on EVs, but very little is known about the role of sEV derived DNA in selecting an immune repertoire, establishing tolerance during normal immune development, and driving immunopathologies. A current challenge in the field is addressing signalling mechanisms involved in EV-mediated target cell reprogramming. Understanding mechanisms involved in how EVs regulate immune responses at a cellular level would enable the engineering of EVs that could efficiently tune effector immune cell populations, for example, stimulating/priming anti-tumour immune responses [272]. Further understanding of sEV biology related to above-mentioned areas, especially the molecular mechanisms involved in immune cell interactions, and biodistribution and immune cell targeting through advanced labelling/tracking/imaging [405], is likely to provide significant insights into tumour-related immune suppression and novel therapeutic intervention. With seminal studies providing significant progress in the sequential development of engineered EVs as therapeutic anti-tumour platforms, we now require direct assessment to tune and improve the efficacy of resulting immune responses [406] - essential in their translation into the clinic. We are in an exciting yet challenging times in the use and rational design of extracellular vesicles as next generation immunotherapeutics.

Conflict of interest

The authors declare no competing interests.

Data Availability

No data was used for the research described in the article.

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Author contributions

DG and XU designed, discussed, and evaluated all aspects of this article, with critical input from WC. All authors contributed to researching the data for the article. All authors reviewed/ edited the manuscript before submission.

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