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REVIEW ARTICLE

Extracellular vesicles and lipoproteins – Smart messengers of blood cells in the circulation

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Abstract

Blood cell-derived extracellular vesicles (BCEVs) and lipoproteins are the major circulating nanoparticles in blood that play an important role in intercellular communication. They have attracted significant interest for clinical applications, given their endogenous characteristics which make them stable, biocompatible, well tolerated, and capable of permeating biological barriers efficiently. In this review, we describe the basic characteristics of BCEVs and lipoproteins and summarize their implications in both physiological and pathological processes. We also outline well accepted workflows for the isolation and characterization of these circulating nanoparticles. Importantly, we highlight the latest progress and challenges associated with the use of circulating nanoparticles as diagnostic biomarkers and therapeutic interventions in multiple diseases. We spotlight novel engineering approaches and designs to facilitate the development of these nanoparticles by enhancing their stability, targeting capability, and delivery efficiency. Therefore, the present work provides a comprehensive overview of composition, biogenesis, functions, and clinical translation of circulating nanoparticles from the bench to the bedside.

KEYWORDS
biomarkers, drug delivery, extracellular vesicles, lipoproteins, therapeutic applications

1 | INTRODUCTION

The circulatory system not only distributes life-sustaining nutrients and removes waste products throughout the body, but also functions as an elaborate network for both short- and long-ranged intercellular communication complexes (Pos et al., 2018; Shah et al., 2018). The biochemical messages carried along the blood and lymphatic vessels may travel as lone molecules or as part of a complex involving extracellular vesicles (EVs), which are predominantly blood cell-derived EVs (BCEVs), and circulating lipoproteonucleotide complexes (Aday et al., 2018; Pos et al., 2018; Shah et al., 2018). The association with these circulating nanoparticles is significant as it helps to protect the bioactive cargo (e.g., a protein or nucleic acid), allowing it to persist and reach its destination. At the target site, proteins enriched at the surface of the nanoparticles may furthermore facilitate cargo uptake by the cells, inducing biological responses. In some cases, the nanoparticles themselves also have a modulatory effect on the recipient cell (Kuai et al., 2016; Pos et al., 2018). With multifaceted roles in various biological processes, BCEVs and lipoproteins hold great promise as a source of diagnostic biomarkers and therapeutic modalities.

BCEVs are mainly derived from red blood cells, platelets, and leukocytes (Arraud et al., 2014; Li et al., 2020). Red blood cells (RBCs), the most abundant cells in blood, are devoid of a nucleus, mitochondria, and DNA (Shi et al., 2014). RBCs participate in

the transportation of oxygen and nutrients, regulation of systemic nitric oxide metabolism, and redox balance (Kuhn et al., 2017). EV biogenesis and release during RBC maturation and lifespan is an important part of RBC physiology (Géminard et al., 2004; Zhang et al., 2009) and will be discussed in detail in the later sections. The second most abundant cells in blood are platelets, which are produced by megakaryocytes mainly in the bone marrow (Stegner et al., 2017). Platelets are highly responsive and secretory cells, and they participate in hemostasis, thrombosis, and inflammatory responses by interacting with other cell types and releasing regulatory factors and EVs (van der Meijden & Heemskerk, 2019). Another significant source BCEVs is leukocytes, including lymphocytes, monocytes, and granulocytes (Veerman et al., 2019; Zhang et al., 2014). These immune cells and their derived EVs modulate immune responses and facilitate intercellular communication to maintain cellular homeostasis and protect the host from pathogenic or harmful substances (Wen et al., 2017; Zhang et al., 2014).

Apart from BCEVs, the other major type of lipid-based nanoparticles in the circulation is lipoproteins. Although lipoproteins are likewise protein-containing complexes enclosed by a lipid layer, they are deeply distinct from EVs in both structure and functions. The generalized lipoprotein consists of a hydrophobic core (as opposed to EVs' aqueous core) of mainly triglycerides and/or cholesterol esters, surrounded by a phospholipid monolayer with integral free cholesterol and apolipoproteins (Feingold, 2000; Ramasamy, 2014). Apolipoproteins are specialized proteins that aid in the assembly of lipoproteins, increase their solubility in circulation, and mediate their interactions with various enzymes and receptors involved in lipid metabolism and transport (Feingold, 2000; Ramasamy, 2014).

This review aims to provide a general overview of circulating nanoparticles found in the blood, with a special focus on BCEVs and their known functions for the maintenance and progression of healthy and diseased states. We will also discuss the latest findings that support their potential in diagnosis and therapy as well as highlight recent advances in engineering methods that have revolutionized BCEV-based therapeutics. To create a more holistic view of circulating nanoparticles, we will also discuss the nature and roles of non-EV lipoproteins in the blood.

1.1 | Basic characteristics of extracellular vesicles

EVs are nano-sized, lipid membrane-bound particles that are released into the extracellular space by most cell types (Arraud et al., 2014; Galley & Besner, 2020; Iwai et al., 2016; Liu et al., 2018). EVs have been isolated from various biofluids in humans, including serum/plasma, urine, saliva, cerebrospinal fluid, and breast milk (Anderson et al., 2018; Galley & Besner, 2020; Iwai et al., 2016; Liu et al., 2018; Welch et al., 2018).

EV populations are highly heterogenous in size and molecular composition (Abels & Breakefield, 2016; Akers et al., 2013). EVs can be effectively classified into exosomes, microvesicles and apoptotic bodies based on their mode of biogenesis in the secreting cell. The biogenesis of exosomes is closely related to the endolysosomal system. Briefly, intraluminal vesicles (ILVs) progressively form via inward membrane budding of late endosomes, giving rise to multivesicular bodies (MVBs). This process is driven by the recruitment of the conserved endosomal sorting complexes required for transport (ESCRTs) machinery. ESCRT-0, -I, -II, and -III have been identified as key mediators involved in the recognition and sorting of protein cargos in endosomes. The tetraspanins CD9, CD63, and CD81 are involved in this ILV formation step in different cell types and are consequently enriched on the ILV membrane. ILVs are then released into the extracellular space as exosomes when the membrane of MVB fuses with the plasma membrane, under the regulation of various Rab GTPases (Abels & Breakefield, 2016; Akers et al., 2013). Other than the main biogenesis pathway involving ESCRT just described, an alternative, ESCRT-independent, ceramide-dependent mechanism has also been demonstrated in the formation of exosomes (Trajkovic et al., 2008).

Microvesicles arise from the outward budding and fission of the cell surface membrane (Akers et al., 2013; van Niel et al., 2018). This process involves changes in the composition of the plasma membrane and the restructuring of the actin cytoskeleton at the sites of vesiculation. Under normal physiological conditions, the membrane enzymes, flippase and floppase, maintain the asymmetric distribution of phospholipids between the two leaflets of the cell surface bilipid layer, including the confinement of phosphatidylserine (PS) to the inner leaflet. Disruptions to this asymmetry, induced by calcium influx or other cell activation signals, contribute to membrane bending and subsequent budding. Small GTPases are furthermore activated to trigger the localized contraction of the cytoskeleton, leading to the release of microvesicles carrying cellular contents (Akers et al., 2013; van Niel et al., 2018) (Figure 1). Not all cell types can produce both of these types of EVs; generally, platelets and leukocytes can produce both exosomes and microvesicles, but mature RBCs, which are devoid of membrane organelles, can only give rise to microvesicles (Aatonen et al., 2014; Arraud et al., 2014). Microvesicle biogenesis from RBCs involves the modification or degradation of band 3 molecules in RBC plasma membrane which are associated to oxidative damage, aging, and elevated level of intracellular calcium influx (Bosman et al., 2012; Nguyen et al., 2016; Sudnitsyna et al., 2020). Other than exosomes and microvesicles, a type of relatively large vesicles termed "apoptotic bodies" are also released by cells undergoing apoptosis (Battistelli & Falcieri, 2020). Current knowledge on the significance of apoptotic bodies beyond being a consequence of programmed cell death is scarce and will not be discussed in this review.

There is no single definitive marker for EVs. According to the International Society for Extracellular Vesicles (ISEV), EV samples should be characterized by both the positive detection of transmembrane/glycosylphosphatidylinositol (GPI)-anchored proteins (e.g., the tetraspanins CD9, CD63, and CD81) and cytosolic proteins (e.g., enrichment of proteins associated with the

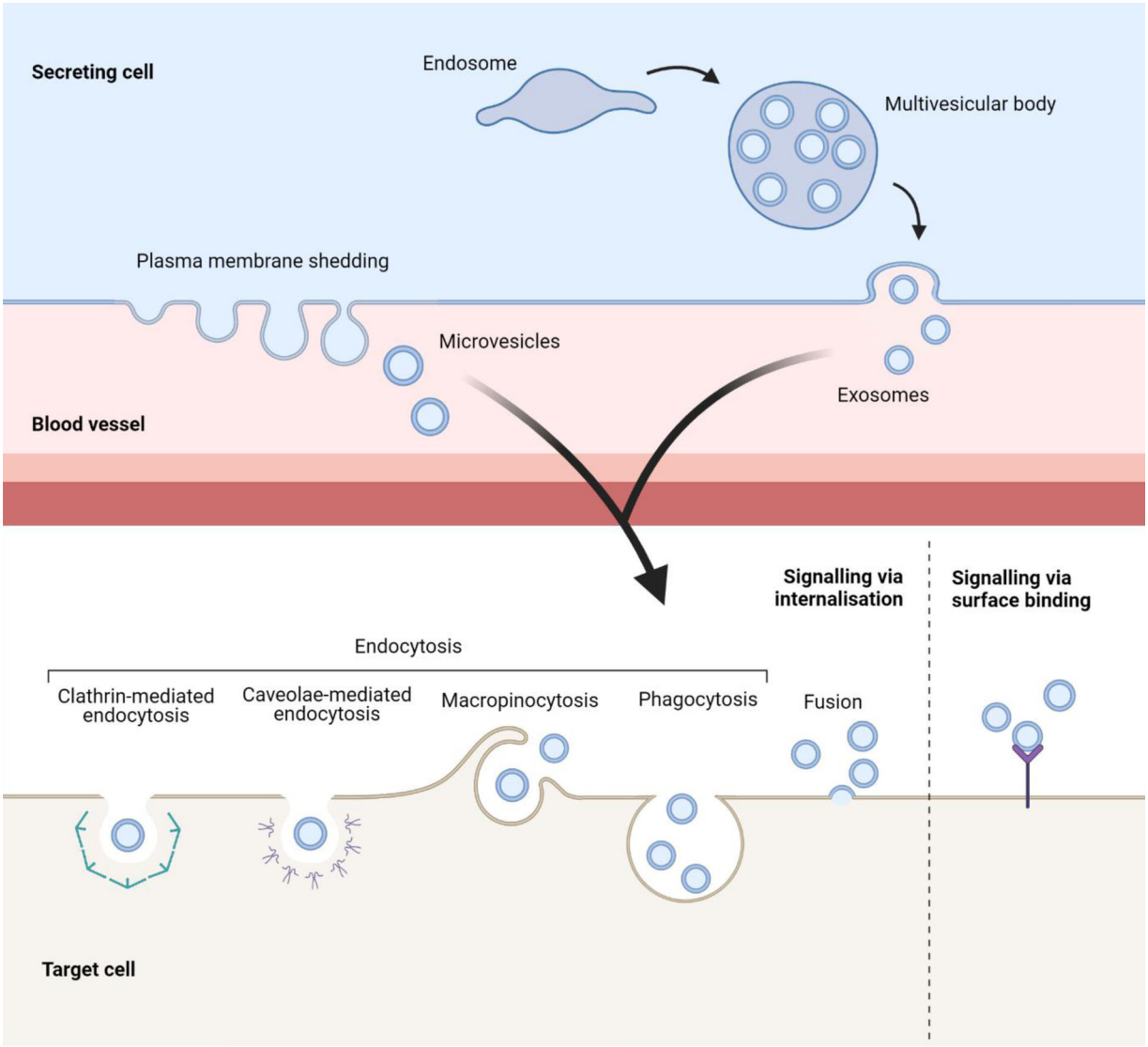

FIGURE 1 Overview of the biogenesis of blood cell-derived extracellular vesicles (BCEVs) and their modes of action on the target cells. Microvesicles are released via the outward budding and fission of the plasma membrane. Exosomes arise as intraluminal vesicles in the endolysomal system and are released by the fusion of the multi-vesicular bodies with the plasma membrane. The EVs are carried in the bloodstream to the target cells, where they might trigger a signalling cascade via binding to the cell's surface receptors. Alternatively, BCEVs might induce a biological change by transferring their bioactive cargos into the cell following internalization by membrane fusion or endocytosis (including clathrin-mediated or caveolae-mediated endocytosis, macropinocytosis, and phagocytosis)

ESCRT pathway, namely, ALIX and TSG101), as well as the lack of contamination from common co-isolates (e.g., lipoproteins and cellular membrane fractions like the endoplasmic reticulum and Golgi apparatus) (Thery et al., 2018). Further subtyping of EVs into exosomes or microvesicles might be possible by considering additional markers (e.g., high CD81 levels for exosomes and Annexin A1 for microvesicles) (Jeppesen et al., 2019). More details on the challenges and guidelines regarding the isolation and characterization of EVs will be discussed in a later section of this review.

Initially considered as merely a waste disposal mechanism, EVs have been demonstrated to deliver various types of functional proteins, nucleic acids, and lipids between cells (van Niel et al., 2018). Some cargos are carried inside the intraluminal space, while others are embedded in the membrane of the EVs. The transportation of these molecules by EVs has been shown to elicit responses in recipient cells in a variety of biological contexts, from regulating immune responses to cancer progression and even embryonic development (Gradilla et al., 2014; Zhang et al., 2021).

1.2 | BCEV sources and composition

The EVs found in the circulation are derived from a great diversity of cellular sources (Harding & Stahl, 1983; van Niel et al., 2018). Determining the relative contributions of the different cell types to the circulating EV pool has been a challenge, and the

numbers are under ongoing revision as both our understanding of EV biology and our toolbox of analytic techniques continue to develop (Arraud et al., 2014; Li et al., 2020; Shah et al., 2008). Despite variations in their estimates, most studies nonetheless agree that platelets account for the largest fraction of the total EV number in the plasma of healthy individuals (Arraud et al., 2014; Li et al., 2020; Shah et al., 2008; Weiss et al., 2018). Red blood cells, monocytes, and lymphocytes also contribute significant amounts of EVs in plasma, with smaller quantities attributed to other types of leukocytes (Arraud et al., 2014; Li et al., 2020). It should be noted that the literature on leukocyte-derived EVs (LEVs) reviewed here includes both studies which obtained the EV-producing cells directly from the blood and studies which obtained the cells from other sources, such as the bone marrow and the tumour microenvironment. Lastly, although EVs of non-blood cell origins are beyond the scope of this review, certain cell types such as endothelial cells and adipocytes also extrude EVs into the circulation in detectable amounts (Brahmer et al., 2019; Eguchi et al., 2016). According to a recent study which analysed the tissue-specific genes in long RNAs of EVs from 101 healthy plasma samples, only 0.2% of the circulating EVs could be traced back to non-blood cell types, while the other 99.8% were traced to the haemopoietic compartment (Li et al., 2020).

With regards to their molecular profile, BCEVs can be described in terms of three main components: the protein content, the nucleic acid content, and the lipid composition of the membrane. Carbohydrate is also an important component of EV and often presents as glycoconjugates such as glycoproteins and glycolipids. Detailed data on the identification of these biomolecules from a wide range of EV sources can be accessed through the Vesiclepedia (for EVs in general) and ExoCarta (for exosomes) depositories (Kalra et al., 2012; Keerthikumar et al., 2016).

As all EVs derive their luminal and membrane cargos from their parental cells, the protein markers for the parental blood cells are usually applicable for determining the origin of the BCEVs (Antwi-Baffour et al., 2015; Segura et al., 2005; Thangaraju et al., 2020). Platelet-derived EVs (PEVs) can be identified by the presence of platelet markers including CD41 and CD42, while red blood cell EVs (RBCEVs) contain high levels of haemoglobin A and express glycophorin A (GPA) found in abundance in RBCs (Grisendi et al., 2015). Data from electron microscopy reveals that EVs derived from reticulocytes, which are immature RBCs, express transferrin receptors (TfRs) or CD71 on their membrane (Harding & Stahl, 1983; Pan & Johnstone, 1983). EVs from DCs (DC-EVs) display the major histocompatibility complex I (MHC-I) and II (MHC-II), along with costimulatory molecules such as CD86 (Besse et al., 2016), characteristic of antigen-presenting cells on their surface. Lymphocyte-derived EVs could display various molecules depending on the immune subsets of their parental cells. For example, B cell-derived EVs express CD19, a marker of B cells, as well as the ectoenzymes CD39 and CD73 used by B cells to suppress immune response via adenosinergic pathway (Figueir, 2016; Zhang et al., 2019). EVs derived from human T cells inherit the T-cell receptor β, CD3 ε and ζ from their parental cells (Blanchard et al., 2002). Additionally, EV derived from regulatory T cells (Tregs) express functional molecules found on Tregs including CD25, CD39 and CCR4 (Tung et al., 2020). Studies on phenotypic switch of macrophages report that M1 macrophage-derived EVs contain the inducible nitric oxide synthase (iNOS) while M2 macrophage-derived EVs contain arginase (Gunassekaran et al., 2021; Kim et al., 2019). iNOS and arginase are typical markers of M1 and M2 macrophages, respectively. Both hematopoietic stem cells, which give rise to other blood cells, and their derived EVs express the lineage marker CD34 (Sahoo et al., 2011).

Furthermore, BCEVs harbour various glycosylated proteins and carbohydrate-binding proteins (i.e., lectins) on their surface, which mediate interactions with their surroundings and thus influence their properties, intercellular interaction, and biodistribution. For example, monocytic/macrophage-derived EVs display the P-selectin glycoprotein ligand-1 (PSGL-1), which is the binding partner of P-selectin expressed on platelets as well as PEVs. The presence of these adhesion molecules allows the EVs to bind to and possibly fuse with platelets to facilitate coagulation (Del Conde et al., 2005). For EVs isolated from the B-lymphoblastoid cell lines (B-CLC), the presence of transmembrane glycoproteins, which are integrins β1 and α4, is shown to be important for their binding to collagen type I and fibronectin - two major components of the extracellular matrix. Meanwhile, integrins β1 and β2 mediate the B-CLC EVs' adhesion to TNF-α-activated human fibroblasts *in vitro* via their binding with the upregulated intercellular adhesion molecule 1 (ICAM-1). This leads to an increase in strong adhesion of the B cell-derived EVs to the fibroblasts and triggers spikes in the level of cytosolic calcium ions in the fibroblasts, indicating the potential involvement of B cell-derived EVs in regulating inflammatory responses (Clayton et al., 2004). EVs from mouse B cells are also enriched in surface α2,3-linked sialic acid, a motif recognized by CD169 on the macrophages concentrated in the marginal zone of the spleen and in the subcapsular sinus of the lymph nodes. The EVs hence rapidly accumulate in at these sites following intravenous injection in C57BL/6 mice. In contrast, in CD169(-/-) knockout mice, the EVs are preferentially captured by different macrophage populations, namely, the SIGN-R1$^+$ and F4/80$^+$ macrophages in the outer marginal zone and red pulp of the spleen and the paracortex of the lymph nodes (Saunderson et al., 2014).

In general, the lipid profile of EV membranes is reflective of their parental cell's membranes, with some deviations that may confer new properties upon the EVs (Skotland et al., 2017). PS, which are usually confined to the inner leaflet of non-apoptotic cells, are found to be enriched on the outer leaflet of the membrane of many types of EVs. This exposure of PS is attributed to the action of the cell membrane-remodelling enzymes flippase and floppase during EV biogenesis and is thought to render the EVs vulnerable to recognition and uptake by phagocytes (Feng et al., 2010; van Niel et al., 2018). In human B cells, a mass spectroscopy analysis confirms that the four most abundant lipid classes in the B cell plasma membrane (cholesterol, sphingomyelin, phosphatidylcholine/similar phospholipids, and phosphatidylethanolamine/ether lipids) are also featured prominently on the

surface of B cell-derived EVs. However, the EVs display considerable enrichment in cholesterol and sphingomyelin and relative depletion in the other two lipid classes. These differences render the EVs more resistant to detergents (Wubbolts et al., 2003). EVs from several sources have also been found to be enriched in a certain class of glycan-conjugated lipids: glycosphingolipids (GSLs) (Phuyal et al., 2014; Skotland et al., 2017). GSLs have been linked to the sorting of cargos into EVs in the PC-3 prostate cancer cell line, where limiting the synthesis of GSLs by inhibiting the enzyme glucosylceramide synthase leads to increased levels of caveolin-1 and annexin A2 and reduced CD9 in the secreted EVs (Phuyal et al., 2014). It remains to be elucidated which mechanisms are responsible and whether glycolipids play a similar role in the context of BCEVs. Compared with protein studies on EVs, lipid-focused studies are currently limited in number and coverage, consisting of mostly lipidomic profiling efforts, and our understanding of the structural and functional significance of particular EV lipid species is only beginning to emerge (Sun et al., 2019; Williams et al., 2018).

Other than protein and lipid components, BCEVs are carriers of nucleic acids, including mRNAs, microRNAs (miRNAs) and a vast range of other non-coding RNAs, as well as mitochondrial and chromosomal DNA (Abels & Breakefield, 2016; Jeppesen et al., 2019). It is important and fascinating to note that the nucleic acid profile of EVs, including BCEVs, often show some divergence from that of the parental cells (Abels & Breakefield, 2016). Compared to the source cell, EVs tend to be enriched in small RNAs (≤200 nucleotides), while larger nucleic acids are present in lower amounts or as incomplete fragments (Abels & Breakefield, 2016). This observation might be partly attributed to the limits placed by the small physical size of EVs, but beyond sizes, the conformation and sequence of the nucleic acid also play a role. For instance, the circular isoform of ADP ribosylation factor-GTPase-activating protein containing SH3, Ankyrin Repeat, and PH domain (*ASAP1*) is preferentially retained in the platelets compared to its linear isoform, while the circular isoform of several other RNAs is preferentially released in EVs instead (Preusser et al., 2018). Sequence alignment analysis of miRNAs in primary human T cells and their exosomes identified short motifs overrepresented in either the cells (Clmotifs) or the exosomes (EXOmotifs) which regulate sorting of miRNAs containing these motifs (Villarroya-Beltri et al., 2013). These motifs are also found in hepatocyte, adipocyte, and skeletal muscle cell lines, as well as a colon cancer cell line (Garcia-Martin et al., 2022; McKenzie et al., 2016; Santangelo et al., 2016). The introduction or removal of EXOmotifs and Clmotifs changes the distribution of miRNAs into EVs derived from murine cell lines (Garcia-Martin et al., 2022). In this study, Alyref and Fus are identified as specific miRNA-binding proteins required for EXOmotif recognition and export of miRNAs into EVs. A better understanding of mechanisms that differentiate between miRNAs destined for release in EVs and those meant to stay inside the cells is not only of great scientific interest but also promises to inspire new strategies for loading miRNA drugs into EVs.

Even though EVs derived from the same type of blood cell tend to share similar characteristics, their molecular profile as well as rate of release are profoundly influenced by external factors. For instance, the number of EVs released by isolated platelets increases by 14.5 times following treatment of the cells with Ca^{2+} ionophore and 1.5 times after thrombotic activation relative to baseline levels (Aatonen et al., 2014). Compared to immature DCs, DCs stimulated by lipopolysaccharide (LPS) also release EVs with increased levels of MHC-II, CD86 and ICAM-1, which are involved in T-cell activation (Segura et al., 2005). As we will see in later parts of this review, the responsiveness to local and systemic changes not only facilitates the regulatory, and sometimes pathological functions of BCEVs but also makes them useful biomarkers.

Beyond the luminal and membrane cargos described so far, certain molecules present in the surrounding biofluid may also be adsorbed to the surface of BCEVs and thus be transported alongside these EVs. Lipoproteins in particular are frequently co-isolated with EVs, the details and implications of which will be discussed in later sections of this review. Other free plasma proteins may also associate with EVs. For instance, when THP-1 monocyte EVs are incubated with EV-depleted plasma and thereafter isolated for analysis, deposits of several proteins can be observed on the EV surface through immunofluorescence and immunogold labelling. These include fibrinogen alpha chain, haptoglobin, and the complement protein C3, where haptoglobin and C3 both co-localized with CD63 on the EV membrane (Tóth et al., 2021). On the other hand, DNAs are typically considered internal cargos of EVs, but they have been detected on the outer surface of EVs under special circumstances, such as when the EVs are released from Jurkat cells (immortalized cell line of T lymphocyte) following antibiotic treatment (Németh et al., 2017). As reviewed elsewhere, there is evidence suggesting the localization of functional enzymes to the EV surface, contributing to their ability to remodel the surrounding extracellular matrix and liberate growth factors, although it is not always clear how these enzymes end up on the outside of the EVs (Busatto et al., 2019). Given these complex associations, caution must be taken when interpreting data in BCEV studies, especially where *in vivo* experiments are concerned. Where possible, the presence of potentially confounding surface-adsorbed molecules should be characterized and controlled for.

1.3 | Modes of action of BCEVs on target cells

In some cases, BCEVs may trigger a signalling cascade in the target cells directly via binding to receptors on the surface of the cells (Mulcahy et al., 2014). This is well exemplified by the demonstration of Toll-like receptor (TLR) and T-cell receptor activation by macrophage- and DC-EVs respectively (Bhatnagar et al., 2007; Segura et al., 2005). In other cases, BCEVs are internalized and thereby transfer their cargos into the recipient cell in order to produce a biological effect. EVs can be taken up by cells

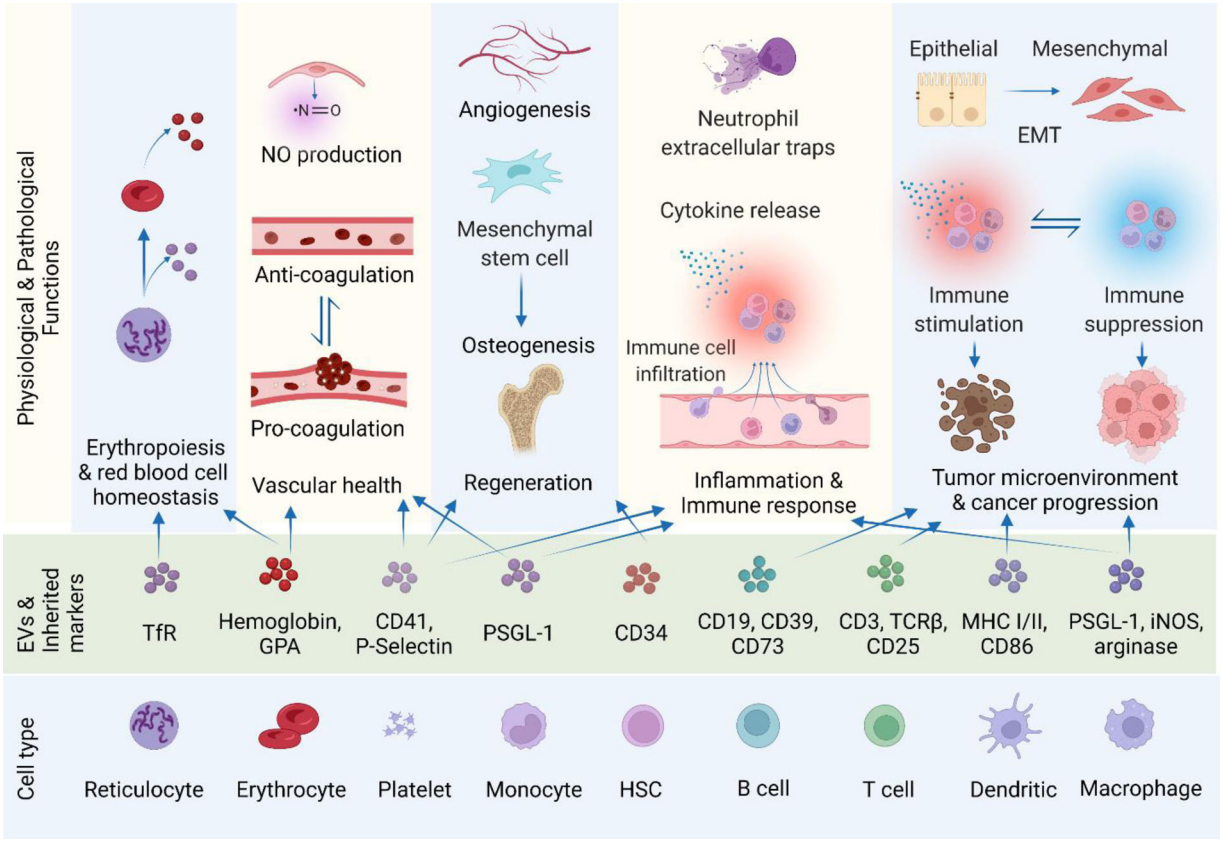

FIGURE 2 **Physiological and pathological roles of blood cell-derived extracellular vesicles (BCEVs).** BCEVs secreted by different cell types can inherit markers from their parental cells and exert variety of functions on the target cells. This includes (i) erythropoiesis and homeostasis of red blood cells, (ii) maintenance of healthy blood vessels by regulating angiogenesis, coagulation and nitric oxide level, and (iii) tissue regeneration which involves angiogenesis and cell differentiation. BCEVs also modulate immune responses under pathological conditions such as infection and cancer. BCEVs could enhance protective immune responses against pathogens and tumour cells. However, excessive immune activation could result in inflammatory diseases. On the other hand, BCEVs could support an immunosuppressive tumour microenvironment and promote epithelial-to-mesenchymal transition, which enable tumour growth and invasion. EMT, epithelial-to-mesenchymal transition; GPA, glycophorin A; HSC, hematopoietic stem cells; iNOS, nitric oxide synthase; NO, nitric oxide; PSGL-1, P-selectin glycoprotein ligand-1; TfR, transferrin receptor

via endocytosis (including clathrin-mediated endocytosis, caveolae-mediated endocytosis, micropinocytosis, and phagocytosis) and/or direct fusion between the EV membrane and the cell membrane (Mulcahy et al., 2014) (Figure 1). Using pharmacological inhibitors of micropinocytosis, lipid raft-mediated endocytosis, and phagocytosis, Pham et al. (2021) provide supporting evidence that the non-small cell lung cancer cell line H358 utilizes all three of these pathways to some extent to internalize RBCEVs, although the molecular details of the uptake process remain to be elucidated. The role of phagocytosis in the uptake of the RBCEVs is further demonstrated in the same study via the conjugation of the RBCEVs with peptides derived from CD47. This modification increases the number of "don't eat me" signals on the RBCEV surface and results in the decreased RBCEV uptake by monocytic cell lines, as well as the increased number of RBCEVs persisting in the circulation following injection into mice (Pham et al., 2021). For macrophage-derived EVs (MEVs), as mentioned in an earlier section, the expression of PSGL-1 allows the EVs to bind to P-selectin on the platelets, and the authors propose that a fusion event follows this binding based on a lipid-mixing assay (Del Conde et al., 2005). Beyond a few studies thus far that have probed the routes of uptake of BCEVs, the literature in this area is currently limited. Since the preferred mode of uptake may depend on the type and abundance of receptors and ligands on both the target cells and the EVs, the physical characteristics of the EVs, and even the prevailing environmental conditions such as pH, more dedicated studies need to be conducted to characterize the internalization of different BCEVs in different scenarios (Mathieu et al., 2019; Parolini et al., 2009; Rejman et al., 2004).

2 | PHYSIOLOGICAL ROLES OF BLOOD CELL-DERIVED EXTRACELLULAR VESICLES

The functional roles of BCEVs are greatly dependent on the vesicular contents inherited from their parental cells. Figure 2 is a summary of the various BCEVs and their roles in physiological and pathological conditions, the latter of which will be discussed in Section 3.

2.1 | Erythropoiesis; production of mature red blood cells

Erythrocytes are circular and biconcave-shaped with a cellular diameter of 5–7 μm (Diez-Silva et al., 2010). Their main functions include transporting oxygen and carbon dioxide as well as participating in nitric oxide (NO) homeostasis and coagulation (Thangaraju et al., 2020). In circulating blood, erythrocytes are one of the major cell types that releases vesicles, which occurs predominantly during the last stage of erythropoiesis, that is, reticulocyte differentiation (Vidal, 2010).

Vesiculation provides a means for reticulocytes, which are immature RBCs, to get rid of subcellular compartments and membrane proteins (e.g., transferrin receptor and acetylcholinerase), thereby mediating maturation to erythrocytes (Géminard et al., 2004; Zhang et al., 2009). Besides, there is a crucial need for reticulocytes to lose their adhesion capacities so that they can enter the microcirculation without any adverse consequences. Integrin $\alpha 4\beta 1$ is among the several adhesion molecules secreted in reticulocytes' EVs during reticulocyte maturation (Rabesandratana et al., 1998; Rieu et al., 2000). Additionally, a reduction in the transport of water, amino acids, nucleosides, sugars, and ions (calcium, potassium, and sodium) occur as reticulocytes progress to mature red blood cells which is mainly driven by the secretion of membrane transporters (Blanc et al., 2009; Jarvis & Young, 1982; Tucker & Young, 1980; Wiley & Shaller, 1977; Zeidler et al., 1976). Furthermore, EVs protect premature reticulocytes from removal by the reticuloendothelial system through sequestering and secreting complement membrane attack complexes, band 3 neoantigen, non-functional methaemoglobin, and other harmful membrane-associated components (Willekens et al., 2008). In the later stage, EVs also serves as a cellular defence mechanism in physiological aging of RBCs by continuously removing denatured haemoglobin and hazardous substances from RBCs and hence, prolonging their lifespan (Bosman et al., 2005). RBCs lose 20% of their haemoglobin content and cell membrane throughout their lifespan in the form of vesicles (Werre et al., 2004). Consequently, the haemoglobin composition in RBCEVs is similar to that of the oldest RBCs (Willekens et al., 1997). However, this vesicle formation system would eventually wear down and the whole RBC would be removed.

2.2 | Vascular health

The pro-coagulant properties of RBCEVs enable them to repair injured blood vessels by participating in the blood clotting process (Chung et al., 2007). Exposed PS on the surface of RBCEVs mediates the assembly of tissue factor (TF)/VIIa, tenase, and prothrombinase coagulation complexes. The coagulation cascade is thereby triggered to catalyse the conversion of inactive prothrombin to the active clotting enzyme thrombin (Chung et al., 2007).

Besides RBCEVs, PEVs play an important role in maintaining haemostasis during vascular injury and preventing excessive blood clotting in intact blood vessels (Kerris et al., 2020). Tans et al. (1991) tested the effects of various agonists, including thrombin, collagen, adrenalin, and calcium ionophore A23187, on platelet activation and vesicle shedding. Both platelets and their EVs possess pro-coagulation and anti-coagulation activities under non-stimulated and stimulated conditions, with PEVs accounting for 20%–30% of both activities. This study also shows that platelets and PEVs display the greatest anti-coagulant and pro-coagulant activities upon stimulation with calcium ionophore (Tans et al., 1991). This pro-coagulant property of PEVs is attributed to the presence of prothrombotic proteins, such as annexin-V, factor X, and prothrombin (Jy et al., 2010; Kerris et al., 2020; Morel et al., 2008; Owens & Mackman, 2011; Wang et al., 2018). Negatively charged PS is normally maintained on the inner leaflet of the platelet membrane by flippase under resting conditions (Morel et al., 2008). Upon platelet activation during an injury, flippase and floppase are inhibited and activated respectively, thereby increasing the number of PS on the outer leaflet of the platelet membrane prior to PEV formation (Morel et al., 2008). PS facilitates the interaction between annexin-V and factor X, thereby providing a site for prothrombinase complex assembly and amplifying thrombin production for coagulation (Morel et al., 2008). In the absence of an injury stimulus, small amounts of thrombin are still being produced which is mediated by the presence of CD41 highly expressed on the surface of PEVs (Berckmans et al., 2001; Kerris et al., 2020). These healthy levels of thrombin activate a natural anti-coagulant protein C, thereby preventing thrombophilia in healthy blood vessels (Berckmans et al., 2001; Kerris et al., 2020).

2.3 | Cellular differentiation, angiogenesis, and tissue regeneration

EVs secreted from various cell types have been shown to play a role in cellular differentiation, angiogenesis, and tissue regenerations. To further investigate the molecular mechanisms, several RNA profiling studies were conducted to explore vesicular RNA content. Sahoo et al. (2011) demonstrated that EVs released by $CD34^+$ hematopoietic stem cells carried pro-angiogenic miRNAs that induced neovascularization both *in vitro* and *in vivo*, in a paracrine signalling manner. Interestingly, EVs secreted by classically activated monocytes were reported to deliver osteogenic signalling cues, including bone morphogenetic protein-2 (BMP-2) and runt-related transcription factor 2 (RUNX2), to mesenchymal stem cells (MSCs) and induce osteogenic differentiation, thereby aiding tissue regeneration and integration at the bone-implant interface (Ekström et al., 2013). These studies

reveal that delivery of miRNAs and other regulatory RNAs can modulate gene expression in target cells, thereby promoting cell differentiation processes such as angiogenesis and tissue regeneration.

3 | PATHOLOGICAL ROLES OF BLOOD CELL-DERIVED EXTRACELLULAR VESICLES

3.1 | Thrombosis, coagulopathy, inflammation, and cardiovascular diseases

PEVs contribute to several proinflammatory characteristics traditionally ascribed to platelets (Kerris et al., 2020). They do so by releasing cytokines to recruit leukocytes, including B cells, T cells, and monocytes, to pathogen invasion sites, as well as interacting with different types of leukocytes to induce the release of leukocytic cytokines such as interleukin-6 (IL-6) (Aatonen et al., 2014; Dinkla et al., 2016; Šibíková et al., 2018). It was previously shown that pathogen-associated molecular patterns (PAMPs), including LPS and staphylococcal superantigen-like protein (SSL), could stimulate platelets via their surface TLR and induce PEV formation when added to platelets isolated from healthy blood (Bei et al., 2016; Brown & McIntyre, 2011). The released PEVs were loaded with interleukin-1 beta (IL-1β) which could upregulate the surface expression of vascular cell adhesion molecule 1 (VCAM-1) in endothelial cells, thereby promoting leukocyte binding to the endothelium and immune cell infiltration to infection site (Brown & McIntyre, 2011). Furthermore, P-selectin-PSGL1- and CD40L/CD40-mediated interactions between PEVs and monocytes lead to the activation of nuclear factor kappa B (NF-kB) signalling pathway and release of IL-1β and tumour necrosis factor α (TNF-α) (Bei et al., 2016). It has been shown that that the addition of PEVs and EV-isolated arachidonic acid increased monocyte adhesion to human umbilical vein endothelial cells (HUVEC) likely through the upregulation of surface antigens, including HUVEC-associated ICAM-1 and monocyte-associated CD11a (Barry et al., 1998). This indicates that PEVs modulate monocyte adherence to the endothelium via increasing the expression of surface adhesion molecules.

Furthermore, it was previously reported that PEV interaction with Tregs downregulated interleukin-17 (IL-17) production by Tregs, which in turn perpetuated inflammatory processes (Dinkla et al., 2016). This PEV-Treg interaction is mediated by P-selectin expressed on PEV surface and PSGL1 expressed on Tregs (Dinkla et al., 2016). Additionally, PEVs have the capacity to induce vasodilation and promote immune cell infiltration to infection site through upregulating cyclooxygenase-2, an enzyme that converts arachidonic acid to prostaglandin I2/prostacyclin (PGI2) (Barry et al., 1999).

Sepsis is a potentially life-threatening condition whereby an inflammatory immune response to a localized infection site goes into overdrive and attacks the body's own tissues and organs, often leading to multiple organ failure and, without medical attention, death (Levi et al., 2010; Semeraro et al., 2010). During an infection by *Neisseria meningitidis*, platelets are activated indirectly by the complement system protein C5a and release PEVs expressing excessive amounts of TF on their surfaces (Øvstebø et al., 2014). As a result, the level of coagulation activity is enhanced to an unhealthy level as seen in septic events (Øvstebø et al., 2014). Furthermore, dengue viral antigens activate platelets in a tyrosine kinase receptor CLEC-2-dependent manner and stimulate the release of PEVs (Sung et al., 2019). These PEVs, in turn, activate neutrophils and macrophages in a CLEC5A-dependent manner, thereby inducing neutrophil extracellular trap (NET) formation and cytokine release (Sung et al., 2019). Although NET is mostly thought of to be protective, it has been shown to contribute to airway obstruction in respiratory syncytial viral infection and acute lung injury in influenza pneumonia (Cortjens et al., 2016; Narasaraju et al., 2011).

Besides the exaggerated immune activation, PEVs can also boost viral replication and propagation through mediating viral entry (Rozmyslowicz et al., 2003). Human immunodeficiency virus (HIV) requires a specific surface antigen CD4 and a C-X-C chemokine receptor type 4 or type 5 (CXCR4 or CXCR5) for entry into target cells. While all these molecules are present on the surface of lymphoblasts, HIV has been demonstrated to infect CXCR4-negative cells such as cardiomyocytes, endothelial cells, and astrocytes (Rozmyslowicz et al., 2003). This vulnerability of CXCR4-negative cells to HIV is owed to PEV's capacity to transfer their CXCR4, a common receptor found on platelet surface, to CXCR4-negative cells (Rozmyslowicz et al., 2003).

EVs released by the THP-1 monocyte cell line upon LPS induction increase endothelial cell surface thrombogenicity and induce apoptosis in endothelial cells (Aharon et al., 2008). Three mechanisms can explain these observed effects (Aharon et al., 2008). Firstly, annexin V binding assay revealed that monocyte-derived EVs stimulate PS exposure on endothelial cell surface which, in turn, provide an assembly site for coagulation complexes. Secondly, adherence of TF-bearing monocyte-derived EVs as well as upregulation of endothelial TF mRNA expression contribute to the pro-apoptotic effects on endothelial cells. Lastly, anti-coagulant pathways are blocked by a decrease in the anticoagulant tissue factor pathway inhibitor (TFPI) and thrombomodulin antigen levels, which are stimulated by monocyte-derived EVs. Altogether, these data clarified the roles of monocyte-derived EVs in hypercoagulatory states and inflammatory diseases (Aharon et al., 2008).

In the last decade, several characterization studies of vesicular contents have uncovered proinflammatory properties of EVs originated from immune cells (Bhatnagar et al., 2007; Gunassekaran et al., 2021; Kim et al., 2019). Glycopeptidolipids (GPL) and TLR displayed on the surface of EVs secreted by *Mycobacterium avium*-infected macrophages (infected MEVs) are the main drivers of the proinflammatory effects in resting uninfected macrophages (Bhatnagar et al., 2007). Hence, infected MEVs play a crucial role in the immune surveillance during bacterial infections. Recent studies show that EVs derived from M1 macrophages express proinflammatory cytokines while those derived from M2 macrophages express immunosuppressive

cytokines (Gunassekaran et al., 2021; Kim et al., 2019). Therefore, harnessing the immunomodulatory properties of EVs presents a promising therapeutic strategy to regulate the balance between pro- and anti-inflammatory immune responses in diseases.

3.2 | Tumour microenvironment remodelling and cancer progression

MEVs promote tumour cell motility, breaking down and remodelling of the extracellular matrix, and angiogenesis mainly through releasing growth factors, matrix metalloproteases (MMPs), cytokines, and angiogenic factors (Condeelis & Pollard, 2006). Their cargo contents vary depending on the type of stimulus. For instance, aging-related formation of amyloid-β1-42 aggregates induces monocyte differentiation into macrophages, which simultaneously releases MEVs loaded with cytokine mRNA (Mitsuhashi et al., 2013). By delivering proteins, lipids, and nucleic acids, MEVs regulate cancer cells and cells of the tumour microenvironment (TME) at the post-transcriptional and post-translational levels (Liu et al., 2020). Interleukin-4 (IL-4)-activated macrophages secrete EVs containing microRNA-223 (miR-223) which activate Mef2c-β-catenin pathway and potentiate invasiveness in breast cancer cells. Additionally, MEVs were reported to transfer apolipoprotein E to gastric cancer cells, increasing their motility (Zheng et al., 2018). Interestingly, MEVs could also promote cisplatin resistance in gastric cancer cells (Zheng et al., 2017). MEV delivery of miR-21 suppresses apoptosis in cisplatin-treated gastric cancer cells by downregulating the phosphatase and tensin homolog (PTEN) and activating PI3K/Akt signalling (Zheng et al., 2017).

Angiogenesis is necessary for tumour progression to supply the actively growing TME with nutrients and oxygen and remove their waste products (Hanahan & Weinberg, 2011). Janowska-Wieczorek et al. (2005) demonstrated that PEVs upregulated the mRNA expression of *IL-8*, *MMP-9*, vascular endothelial growth factor (*VEGF*), and hepatocyte growth factor in A549 lung cancer cells. After the establishment of new blood vessels, cancer cells need to undergo epithelial-to-mesenchymal transition (EMT) so that they can migrate, intravasate, and form distant secondary metastases. The important role of PEVs in EMT is further validated in SKO3 ovarian epithelial cancer cells through miR-939 transfer (Tang et al., 2017) and breast cancer cells through TPM3 mRNA transfer to promote tumour invasion (Yao et al., 2019).

Besides, there are other studies highlighting the roles of certain LEVs in promoting an immunosuppressive TME to support tumour growth. Zhang et al. (2019) reported that CD39 and CD73 expressed on B cell-derived EVs hydrolysed adenosine triphosphate (ATP) released by tumour cells after chemotherapy treatment into adenosine. Adenosine bound to A2A adenosine receptors on T cells and suppressed T cell activation and effector functions. Importantly, the level of B cell-derived EVs in the serum of gastric and colon cancer patients is higher than healthy donors and the higher level of serum B cell-derived EVs is associated with shorter progression- free survival period after chemotherapy. The suppressive mechanism via anti-inflammatory adenosine is also found in EVs isolated from a murine Treg-cell line, which inhibited proliferation and cytokine production of non-Treg $CD4^+$ T cells (Smyth et al., 2013).

In contrast, certain BCEVs play a role in supporting an anti-tumorigenic immune landscape. DCs are the only antigen-presenting cells (APCs) that can elicit both primary and secondary immune responses as attributed to their ability to exist under two different states; immature DCs are efficient in antigen-mediated phagocytosis, while mature DCs are efficient in T-lymphocyte activation as they express many MHC class I/II and T cell costimulatory molecules on their surface (Dudek et al., 2013). Zitvogel et al. (1998) initially discovered that EVs isolated from DCs elicited a strong T-lymphocyte activation upon exposure to tumour antigens, which in turn resulted in the regression of established P815 mastocytoma tumours in mice. Subsequently, Zitvogel and colleagues further investigated the molecular contents of DC-EVs and found selective accumulation of heat shock protein hsc73 (Théry et al., 1999), which was reported to exert anti-tumour effect through immune activation (Srivastava et al., 1998).

3.3 | Dysregulation of nitric oxide homeostasis

Dysregulation of NO results in vascular damage. RBCEVs can regulate the NO production in endothelial cells by inhibiting NO synthase activity or upregulating NO synthase expression, thereby modulating NO bioavailability (Gladwin & Kim-Shapiro, 2009). However, this NO homeostasis can be disrupted by free haemoglobin (Hb) released from haemolysis due to the high binding affinity of NO to deoxygenated Hb, thereby reducing NO bioavailability and leading to endothelial dysfunction, haemostatic activation, and reactive oxygen species (ROS) generation (Doherty et al., 1998; Gladwin & Kim-Shapiro, 2009). Interestingly, the rate of NO scavenging is comparable between RBCEV-encapsulated Hb and cell-free Hb, which is a thousand times faster than RBC-encapsulated Hb (Donadee et al., 2011; Liu et al., 2013). Human blood stored under standard blood-banking conditions has elevated levels of RBCEV-encapsulated Hb and these RBCEVs can scavenge and deplete vascular NO, thereby leading to endothelial cell dysfunction in patients that have blood transfusion (Donadee et al., 2011).

A recent study showed that PEVs decreased the level of endothelial NO by 50% and suggested a possible mechanism of PEV-mediated endothelial injury via activating the mammalian target of rapamycin complex 1 (mTORC1) pathway in a diabetic rat model (Wang et al., 2019). Additionally, circulating T cell-derived EVs, isolated from patients with diabetes or infected with

human immunodeficiency virus, cause endothelial cell damage through downregulating endothelial NO synthase expression and overexpressing caveolin-1 expression (Martin et al., 2004).

4 | SEPARATION AND CHARACTERIZATION OF BLOOD CELL-DERIVED EXTRACELLULAR VESICLES

4.1 | Separation and concentration methods

Absolute isolation of pure EVs from non-EV components in the conditioned media, biofluids and tissues, and from other types of EVs is almost impossible. A more realistic goal is to separate and concentrate the EVs of interest. According to the MISEV2018, the techniques adopted greatly depend on the EV end use, and the yield and purity required for downstream experiments (Thery et al., 2018). The degree of separation and concentration can be assessed by characterization techniques, which are necessary to achieve conclusive data. Highly purified EVs are required for functional studies to attribute a function or biomarker to a specific EV subtype. This is because co-isolated contaminants may contribute to the EV function observed. Less pure EVs may be acceptable in other cases; for instance, in situations when a biomarker is effective without EV pre-enrichment. Hence, there is no one-size-fits-all approach, and a combination of separation and concentration techniques should be employed to achieve target EV yield and purity. The primary EV separation and concentration technique most used was reported, by a worldwide ISEV survey, to be ultracentrifugation (Gardiner et al., 2016). Additional techniques, including sucrose density gradients, filtration, size exclusion chromatography (SEC) and immunoisolation (Echevarria et al., 2014) were used to achieve various levels of recovery and purity. Some techniques are currently being developed to improve recovery and specificity while exerting minimal damage to EVs, and they include variations of tangential flow filtration (Watson et al., 2018), SEC (de Menezes-Neto et al., 2015), ion exchange chromatography, microfiltration (Merchant et al., 2010), fluorescence-activated sorting (Higginbotham et al., 2016), deterministic lateral displacement (DLD) (Wunsch et al., 2016), affinity isolation techniques, hydrostatic filtration dialysis, high pressure methods, and microfluidics devices (Contreras-Naranjo et al., 2017). Any newly developed techniques are required to indicate their degree of recovery and specificity.

There are differences in purification methods and detection techniques required for separation of each type of BCEVs. To isolate PEVs, blood samples are centrifuged to remove red blood cells and platelet-rich plasma (PRP) is obtained. Typically, platelets are isolated from PRP by centrifugation and then activated with thrombin or other stimuli to induce the release of PEVs. The culture is centrifuged to remove the residual platelets. The collected supernatants are subjected to ultracentrifugation to concentrate PEVs (Ma et al., 2020; Tang et al., 2021). To improve the purity of PEVs, Aatonen et al. (2014) layered platelets collected from PRP on a discontinuous iodixanol gradient and the gradient was centrifuged without a break. Compared to washing or gel-filtration, density gradient isolation yields a population of platelets with lesser contamination from lymphocytes, monocytes, macrophages, and granulocytes. Another important consideration is the different processes of platelet activation which could result in heterogenous populations of PEVs. Studies that investigated the strength of different inducers for PEV release report that Ca^{2+} ionophore- or calcium gluconate-mediated activation can generate more EVs than thrombin and LPS (Aatonen et al., 2014; Rui et al., 2021). However, thrombin activation yields EVs having higher concentrations of platelet-derived growth factor-BB (PDGF-BB) than the calcium gluconate-activated group (Rui et al., 2021). Interestingly, a mixture of an equal volume of calcium gluconate and thrombin is a more potent inducer than calcium gluconate or thrombin alone. The levels of cytokines including transforming growth factor-β (TGF-β), PDGF-BB, basic fibroblast growth factor (FGF), and VEGF in EVs generated from the mixture-activated PRP are higher than that of the other inducers (Rui et al., 2021).

To promote the development of BCEV-based therapeutics, there is an urgent need for a standardized protocol to produce EVs at high yield while preserving biological properties of EVs. Usman et al. (2018) devised a new approach to purify a large amount of RBCEVs at a low cost. They separated RBCs in the whole blood from leukocytes and plasma by centrifugation and the use of leukodepletion filters. RBCs are treated with calcium ionophore overnight to induce the release of RBCEVs. Removal of RBCs and cell debris is carried out by low-speed centrifugations. RBCEVs are concentrated by ultracentrifugation, then resuspended and layered above a frozen 60% sucrose cushion. After centrifugation at 100,000 × g for 16 h, a red layer above the sucrose containing RBCEVs is collected and washed again by ultracentrifugation. The sequential centrifugation including the use of sucrose cushion can remove protein contamination and generates a homogenous population of RBCEVs. These RBCEVs do not aggregate and still maintain their morphology, concentration, and size distribution after one to three freeze-thaw cycles, indicating the stability of RBCEVs (Usman et al., 2018).

There are various protocols for the production of LEVs. DC-EVs produced by classical differential centrifugation techniques often contain large contamination from cell debris, proteins, and media components, hence posing significant challenges for the use of DC-EVs in clinical trials (Lamparski et al., 2002). Lamparski et al. (2002) described an ultrafiltration-cushion ultracentrifugation method for rapid production of clinical-grade DC-EVs which are used in several clinical trials (Besse et al., 2016; Escudier et al., 2005; Lamparski et al., 2002; Morse et al., 2005). They enriched $CD14^+$ monocytes from washed PBMCs by allowing these cells to adhere onto the plastic surface of the flasks and then washed away non-adherent cells. Monocytes are induced to

differentiate into DCs by the addition of granulocyte-macrophage colony-stimulating factor and IL-4. Then the culture supernatant was harvested after seven days for EV purification. In this protocol, they incorporated a 500-kDa nominal molecular weight cutoff hollow filter cartridge during ultracentrifugation of the culture supernatant to discard large protein aggregates, followed by ultracentrifugation onto a 30% sucrose/D_2O density cushion. The recovery rate of EVs estimated by the relative concentration of MHC-II was reported at 40–50%, compared to 5–25% of the differential centrifugation method. To isolate T cell-derived EVs, T cells from peripheral blood are enriched by magnetic bead separation, followed by activation with plate-bound anti-CD3/CD28 antibodies in the presence of interleukin-2 (IL-2) (Seo et al., 2018). For Treg derived EVs, the culture medium is supplemented with both IL-2 and rapamycin for promoting the expansion of Tregs (Tung et al., 2020). The culture of NK cells to generate NK-EVs also requires the use of stimulatory cytokines including IL-2 and IL-15 (Di Pace et al., 2020). The culture supernatants are harvested, filtered, and subjected to sequential ultracentrifugation to concentrate EVs, followed by washing (Di Pace et al., 2020; Seo et al., 2018; Tung et al., 2020).

4.2 | Characterization methods

Several, complementary techniques for EV characterization are essential to ensure that the observed biomarkers and functions are associated with the EVs alone and not with other co-isolated non-EV components. ISEV recommendations for EV characterization include three parameters; the first two parameters involve quantitative assessments of EV source, such as number of secreting cells and volume of biofluids, and EV abundance, such as number of EV particles and protein or lipid contents. Lastly, the presence of components associated with EVs and the presence of non-EV co-isolated components should be tested.

Quantifying EV source may sometimes be challenging due to dynamic culture conditions, such as periodic collection of conditioned media in a continuous bioreactor-based culture (Mitchell et al., 2008). The MISEV2018 guidelines recommended that in such situations, the number of cells prior to initiation, expected doubling time, and the collection frequency should be reported.

There are a few ways to estimate the total amount of EV produced, namely, measuring the number of particles and total amount of proteins, lipids, and nucleic acids. Light scattering technologies such as nanoparticle tracking analysis (Dragovic et al., 2011; Gardiner et al., 2013), flow cytometry-based methods (Cointe et al., 2017; Krishnan et al., 2016) and dynamic light scattering (Van Der Pol et al., 2010) have been used to measure EV count. However, these techniques often result in an overestimation of particle number due to the inability to exclude contaminating particles such as lipoproteins and protein aggregates (Dragovic et al., 2011; Thery et al., 2018). Hence, particle number measurement is often coupled with other quantitative measures. Colorimetric assay (micro-bicinchonic acid), fluorimetric assay, and total global protein staining in denaturing polyacrylamide gel electrophoresis (SDS-PAGE) are common techniques used to measure total protein amount in EVs (Maiolo et al., 2015; Ramirez et al., 2018; Seo et al., 2018). Measurement of the total lipid content can be done using a lipid dye that fluoresces upon incorporation into the lipid bilayer such as DiO or DiR (Benmoussa et al., 2017); however, labelled EVs should be separated carefully from the dye micelles. Some studies propose the use of protein:particle ratio (Maiolo et al., 2015; Webber & Clayton, 2013) and protein:lipid ratios (Mihály et al., 2017) as a plausible purity indicator.

MISEV2018 proposed three major types of protein markers as positive and negative markers of EV. GPI-anchored transmembrane proteins localized to the external EV surface as well as cytosolic or peripheral proteins can be used as positive EV markers while lipoproteins, soluble albumin, and acetylcholinesterase are used as negative EV markers. Immunodetection by western blot represents a simple and efficient way to detect these positive and negative markers.

5 | DIAGNOSTIC APPLICATIONS OF BLOOD CELL-DERIVED EXTRACELLULAR VESICLES

As discussed in previous sections, physiological amounts of EVs are present in the bloodstream for the normal functioning of biological processes ranging from coagulation to cellular communication. Therefore, an elevation or reduction in the number of EVs or their contents could be indicative of a disease state and can be used for diagnostic purposes. Several clinical studies have been conducted to elucidate the relationship between the quantity of EVs or EV contents and disease progression.

Prolonged exposure to high shear stress or abrupt increases in shear stress, caused by abnormal blood flow in atherosclerotic vessels, trigger platelet activation and formation of PEVs (Holme et al., 1997). Increased numbers of PEVs found in various pathological conditions including cardio-pulmonary bypass, acute respiratory distress syndrome, and idiopathic thrombocytopenic purpura can be used as an indicator of disease (Abrams et al., 1990; Holme et al., 1997; Jy et al., 1992). Tan et al. (2016) investigated the role of thrombin-stimulated PEVs in vascular smooth muscle cells (SMCs) and proposed the use of miRNAs from PEVs as novel biomarkers for atherothrombotic events. Particularly, the levels of miR-223, miR-339, and miR-21 are higher in mouse PEVs before thrombosis. They are also elevated in thrombin-treated human platelets and PEVs compared to the control groups. These miRNAs can be transferred to SMCs via PEVs and inhibit the platelet-derived growth factor receptor-beta (PDGFRβ) in SMCs, which results in the increased apoptosis of SMCs and negatively affect their repair capacity during atherosclerosis.

In a recent study on hypertension, it was found that the level of T cell-derived EVs was positively correlated with high systolic blood pressure in angiotensin II-induced hypertension mice (La Salvia et al., 2020). However, there were no differences observed in the levels of EVs derived from B cells, neutrophils, monocytes, and macrophages between treatment and control groups. This work demonstrates the potential of T cell-derived EVs as a sensitive biomarker for hypertension. Besides, activated T cell-derived EVs may involve in the early initiation steps of Sjögren's syndrome (SS), a systemic autoimmune disease (Cortes-Troncoso et al., 2020). T cell-derived EVs could transfer miR-142-3p to salivary epithelial cells and impair their exocrine secretion by targeting intracellular Ca^{2+} signaling components. miR-142-3p is overexpressed in the salivary gland lesions of SS patients but not healthy donors, suggesting that miR-142-3p could serve as a critical biomarker and therapeutic target for SS.

6 | THERAPEUTIC APPLICATIONS OF BLOOD-CELL DERIVED EXTRACELLULAR VESICLES

Investigations into natural roles of BCEVs and their unique characteristics have flourished and established the groundwork for their therapeutic applications in a variety of diseases. EVs have been proposed as an ideal delivery system which could be a suitable alternative to cellular therapies and synthetic nanoparticles. BCEVs originate from cells and are found naturally in the blood circulation, and thus they are more biologically compatible compared to viral vectors and synthetic carriers. They also tend to have low toxicity and immunogenicity due to the lower abundance of membrane-bound proteins compared to parent cells (Murphy et al., 2019). BCEVs can be obtained with minimally invasive procedures and manufactured in a large-scale and thus more cost-effective manner (Usman et al., 2018), compared to cell-based delivery systems that are generally cost- and labor-intensive with a short shelf life (Weber et al., 2020). Notably, BCEVs possess an endogenous capability to permeate into tissues and cross cellular barriers, including the blood-brain barrier (BBB) in some instances (Banks et al., 2020; Saint-Pol et al., 2020), which makes them a potentially safe and efficient means to target brain diseases. Furthermore, some BCEVs display the ability to protect their cargo from lysosomal degradation, cytoplasmic proteases and nucleases, thus facilitating functional delivery of therapeutic cargos into the cytoplasm which in turn mediate phenotypic changes in recipient cells (Shelke et al., 2019; Yao et al., 2018). Possessing numerous advantageous features, BCEVs hold a great potential to outperform other synthetic delivery systems and have been successfully utilized in preclinical and clinical settings. To provide an overview of the therapeutic potential of BCEVs, we will discuss a selection of studies investigating their use for the treatment of a diverse range of disease conditions (Table 1). We also highlight engineering strategies for improving the therapeutic potential of BCEVs, that are summarized in Figure 3.

6.1 | Suppressing immune response in inflammatory diseases and transplantation

Possessing intrinsic affinity to sites of inflammation, PEVs have revolutionized the treatment of inflammatory diseases (Song et al., 2019). PEVs offer an alternative treatment to potent immunosuppressants which are often associated with high dosage and side effects including increased susceptibility to infection and malignant disorders due to nonspecific blockade of immune response (Dantal et al., 1999). Ma et al. (2020) developed a method to load an anti-inflammation agents called [5-(p-fluorophenyl)-2-ureido] thiophene-3-carboxamide (TPCA-1) into platelets by passive diffusion and hydrophobic interaction, followed by *in vitro* activation of platelets by thrombin to generate TPCA-1-PEVs. In mice challenged with LPS to induce acute lung injury, PEVs accumulate at the site of acute lung inflammation. Additionally, TPCA-1-PEVs significantly reduce the level of reactive oxygen species and lung oedema compared to the untreated and free drug treatment groups. Treatment with TPCA-1-PEVs also reduces immune cell infiltration into infected lungs and prevents potential cytokine storm as suggested by lower expression levels of TNF-α, IL-6, and IL-1β.

Immunosuppressants are normally prescribed post-operation to prevent tissue rejection in the patients. Due to the side effects of immunosuppressants, there is a need to develop new treatment strategies to prevent acute and chronic allograft rejection without causing nonspecific immune suppression (Dantal et al., 1999). EVs released from activated human Tregs exhibit immunosuppressive functions, highlighting their relevance in treatments of transplant rejection (Tung et al., 2020). Treg-EVs inhibit the proliferation of effector T cells and the production of inflammatory cytokines, leading to the suppression of immune response and prevention of graft rejection. The authors propose that CD25 displayed on the surface of Tregs deplete IL-2 in the microenvironment and hence decrease the survival of effector T cells. However, they did not validate this hypothesis. Furthermore, EVs secreted from B cells can potentially stimulate or suppress immune responses, depending on the functional status and mechanism of activation of the parental cell (Admyre et al., 2007; Klinker et al., 2014). For instance, EVs produced from lymphoblastoids, which are immortalized human peripheral blood B cells, induce *in vitro* apoptosis of antigen-specific $CD4^+$ T cells in a partially FasL-mediated fashion (Klinker et al., 2014). As such, HLA-DR^+ $FasL^+$ EVs are proposed for use in allograft transplantation, T cell-induced allergies, and autoimmune diseases. Despite being a biocompatible alternative for immunosuppressants, the

TABLE 1 Representative selection of studies investigating the use of blood cell-derived EVs in therapeutics

Condition	EV source	EV purification method	Models	Therapeutic agents	Administration	Main outcomes	Reference
Pneumonia	Platelets	Concentrated platelets from whole blood are activated by thrombin and centrifuged at 800 x *g* for 10 min. PEVs are collected from the supernatant by ultracentrifugation at 100,000 rpm for 2 h.	Acute lung injury mouse model	TPCA-1 is loaded into platelets by passive diffusion and hydrophobic interaction	intravenous	Treatment with TPCA-1-PEVs reduces the level of reactive oxygen species, lung oedema, and cytokine storm shown by the decrease in levels of TNF-α, IL-6, and IL-1β and infiltration of immune cells.	Ma et al. (2020)
Ischemia	Platelets	Concentrated platelets from whole blood are activated by thrombin and centrifuged at 5000 x *g* for 15 min. PEVs are collected from the supernatant by centrifugation at 20,000 x *g* for 90 min at 4°C.	Ischemic mouse model	Isolated platelet-derived EVs (PEVs) are incubated with adipose-derived stem cells (ADSCs).	Intramuscular	Precondition of ADSCs with PEVs promotes the survival of ADSCs, as well as their angiogenic and vasculogenic potentials which is essential in treatment of ischemic disease.	Tang et al. (2021)
Breast cancer and acute myeloid leukaemia (AML)	Red blood cells (RBCs)	RBCEVs from the supernatant of calcium ionophore treated RBCs are concentrated with sequential centrifugation including the removal of protein contamination using a 60% sucrose cushion at 100,000 × *g* for 16 h at 4°C.	Breast cancer and AML mouse models	miR-125b ASOs are electroporated into RBCEVs	intratumoral (breast cancer) intravenous (AML)	miR-125b ASO loaded RBCEVs could target tumour cells efficiently and suppress tumour growth *in vivo* with low toxicity.	Usman et al. (2018)
Lung cancer	RBCs	RBCEVs from the supernatant of calcium ionophore-treated RBCs are concentrated with sequential centrifugation including the removal of protein contamination using a 60% sucrose cushion at 50,000 × *g* for 16 h at 4°C.	Lung cancer mouse model	Chemotherapy drug (paclitaxel) is incubated with RBCEVs, followed by sonication and recovery at 37°C for 1 h	intravenous	Surface functionalization of RBCEVs with EGFR-targeting peptide and anti-EGFR nanobody enhances safe and specific drug delivery to cancer cells in a xenografted mouse model of EGFR positive lung cancer and results in improved tumour inhibition.	Pham et al. (2021)

(Continues)

TABLE 1 (Continued)

Condition	EV source	EV purification method	Models	Therapeutic agents	Administration	Main outcomes	Reference
Liver cancer	RBCs	RBCEVs from the supernatant of calcium ionophore-treated RBCs are concentrated with sequential centrifugation with a discontinuous density cushion composed of 25% sucrose/45% sucrose (pH 7.2) for 18 h at 100,000 × *g*	Nude mouse xenograft model of human liver cancer	RBCEVs are loaded with doxorubicin and sorafenib using electroporation	intravenous	Drug-loaded RBC-EVs inhibit angiogenesis and tumour growth without significant systemic adverse effects	Zhang et al. (2020)
Lung cancer, breast cancer, leukaemia	RBCs	RBCEVs from the supernatant of calcium ionophore-treated RBCs are concentrated with sequential centrifugation including the removal of protein contamination using a 60% sucrose cushion at 50,000 × *g* for 16 h at 4°C.	AML xenograft mouse model, lung metastatic breast cancer allografted mouse models	ASOs are loaded into RBCEVs using REG1 or Exo-Fect. Proapoptotic peptide is conjugated to RBCEVs using ligase.	intravenous, intratracheal	Conjugation of peptides, nanobodies and monoclonal antibodies increases RBCEVs uptake by targets cells. Surface functionalized RBCEVs specifically deliver miR-125b ASOs to tumour cells or induce apoptosis of tumour cells, and hence suppress tumour progression.	Jayasinghe et al. (2022)
Breast cancer	RBCs	RBCEVs from the supernatant of calcium ionophore-treated RBCs are concentrated with sequential centrifugation including the removal of protein contamination using a 60% sucrose cushion at 50,000 × *g* for 16 h at 4°C.	Breast cancer mouse model	RIG-I agonist RNAs are loaded into RBCEVs using REG1 or Exo-Fect	intratumoral, intrapulmonary	RIG-I agonist-loaded RBCEVs induce immunogenic tumour cell death, activate RIG-I pathway, and enhance tumour-specific immune response.	Peng et al. (2022)
Alzheimer's disease	Immature dendritic cells (DCs)	EVs from the culture supernatant of engineered DCs are centrifuged at 12,000 x *g* for 30 min to remove cell debris, followed by centrifugation at 120,000 x *g* for 1 h	Wild-type mouse (C57BL/6)	*BACE1* siRNA is loaded into EVs by electroporation	intravenous	EVs from engineered DCs expressing neuron targeting peptide could deliver siRNA specifically to neurons, microglia, oligodendrocytes in the brain, resulting in a knockdown of disease-associated gene.	Alvarez-Erviti et al. (2011)

(Continues)

TABLE 1 (Continued)

Condition	EV source	EV purification method	Models	Therapeutic agents	Administration	Main outcomes	Reference
Melanoma	Immature DCs	EVs from the culture media of monocyte derived-DCs are concentrated by ultrafiltration and diafiltered, followed by ultracentrifugation at 100,000 × *g* for 70 min at 4°C on a D_2O/sucrose cushion.	Metastatic melanoma patients with tumours expressing MAGE3 in a phase I clinical trial	MAGE3 peptides are loaded directly on EVs or indirectly on autologous DC cultures	intradermal and subcutaneous	Safe and well tolerated. There is no evidence supporting the induction of tumour specific immune response. There is an increase in NK cell activation.	Escudier et al. (2005)
Non-small cell lung cancer (NSCLC)	Immature DCs	EVs from the culture media of DCs are concentrated by ultrafiltration and diafiltered, followed by ultracentrifugation at 100,000 × *g* for 70 min at 4°C on a D_2O/sucrose cushion.	Advanced NSCLC patients with tumours expressing MAGE-A3 or A4 in a phase I clinical trial	MAGE peptides are loaded directly on EVs or indirectly on autologous DC cultures	intradermal and subcutaneous	Safe and well tolerated. There is no evidence supporting the induction of tumour specific immune response. There is an increase in cytolytic activity of NK cells.	Morse et al. (2005)
Multiple sclerosis	IFN-γ stimulated DCs	EVs from DCs are isolated by adding ExoQuick (SBI, Mountain View, CA) to the culture media overnight, followed by centrifugation at 2000 × *g* for 30 min.	Slice culture with lysolecithin-induced demyelination. Wistar rat (normal brain).	miR-219 enriched in stimulated DC-EVs	nasal	Treatment with IFN-γ DC-EVs promotes remyelination *in vitro* and *in vivo*. There is no depletion of neuron progenitor population.	Pusic et al. (2014)
NSCLC	Mature DCs	EVs from DC culture supernatants are purified using a process including ultrafiltration, diafiltration and ultracentrifugation through a 1.21 g/ml sucrose cushion.	Advanced NSCLC patients with inoperable tumours in a phase II clinical trial	Tumour associated antigen peptides are added in the culture of mature DC, followed by isolation of EVs from the culture supernatant.	intradermal	1/22 patient has a grade three hepatoxicity. The vaccine does not induce tumour specific T cell responses while boosting NK cell function likely through the expression of NK cell's ligand on EV.	Besse et al. (2016)
Melanoma	Mature DCs	The obtained supernatant from DC medium is filtered and centrifuged at 100,000 × *g* at 4°C for 120 min.	B16-OVA melanoma mouse model	DC-EVs are isolated from the culture of DCs conditioned with chicken ovalbumin and poly (I:C), followed by surface modification with anti-CTLA4 antibody via lipid anchoring method	subcutaneous	No critical toxicity is observed in mice. Modified DC-EVs could target T cells and increase T cell activation *in vitro* and *in vivo*. Effective priming of T cells results in better tumour control.	Dai Phung et al. (2020)

(Continues)

TABLE 1 (Continued)

Condition	EV source	EV purification method	Models	Therapeutic agents	Administration	Main outcomes	Reference
Transplantation	Tregs	The obtained supernatant from cultured media of activated T cells is centrifuged and filtered and EVs are isolated via ultracentrifugation or ExoQuick-TCTM (Systems Biosciences, California, United States).	Humanized mouse skin transplant model	Not studied	intravenous	Treg-EVs inhibit proliferation of effector T cells and modify cytokine profiles, promoting an immune suppressive microenvironment to protect the graft post-transplantation.	Tung et al. (2020)
Fibrosarcoma	$CD8^+$ T cells	The obtained culture supernatants are centrifuged, filtered, and concentrated via ultrafiltration. EVs are isolated from the concentrated supernatants via filtration and ultracentrifugation for 2 h at 100,000 × *g*.	CMS5a (fibrosarcoma) mouse model	miR-298–5p in $CD8^+$ T cell-EVs	intravenous	$CD8^+$ T cells derived-EVs infiltrate into tumor site. miR-298–5p in $CD8^+$ T cell-EVs potentially involves in the depletion of tumoral mesenchymal cell populations and hence, inhibiting tumour invasion and metastasis.	Seo et al. (2018)
Triple-negative breast cancer	$CD8^+$ T cells	EVs are isolated from cell culture supernatants and purified by sequential ultracentrifugation.	PY8119 (triple-negative breast cancer) mouse model	Not studied	intraperitoneal	PD-1 on EVs not only blocks the interaction between PD-1 on T cells and PD-L1 on tumour cells but also induces the internalization of PD-L1, thereby attenuating the suppressive tumour microenvironment and enhancing cytotoxic capacity of T cells.	Qiu et al. (2021)
Transplantation	B cell-derived lymphoblastoid cell lines (LCL)	EVs are isolated from concentrated culture medium by centrifugation at 100,000 x *g* for 1–4 h.	*In vitro* induced antigen-specific $CD4^+$ T cells by immunodominant peptide of tetanus toxoid	Not studied	$CD4^+$ T cells are incubated with autologous LCL-derived EVs	$HLA\text{-}DR^+$ $FasL^+$ EVs induce apoptosis of antigen specific $CD4^+$ T cells	Klinker et al. (2014)

(Continues)

TABLE 1 (Continued)

Condition	EV source	EV purification method	Models	Therapeutic agents	Administration	Main outcomes	Reference
Wound healing	Activated M2 macrophages	EVs are isolated from culture supernatant by sequential centrifugation including ultracentrifugation at 150,000 x *g* for 90 min.	Balb/c mice are used for skin wound model	Not studied	subcutaneous	M2-like macrophages-derived EVs induce phenotypic switch of M1-like to M2-like macrophage, displaying pronounced wound healing efficacy.	Kim et al. (2019)
Breast cancer	Activated M1 macrophages	The culture medium was collected and centrifuged, followed by centrifugation of the resulting supernatant at 12,000 × *g* at 4°C for 30 min and then at 120,000 × *g* 4°C for 70 min.	Breast cancer mouse model	NF- κB p50 siRNA and miR-511–3p are transfected into M1 EVs using 10 μl of Exo-Fect exosome transfection reagent (SBI system)	intravenous	Engineered M1 EVs having IL4R-binding peptide on the surface could target M2 macrophages and foster M1 polarization by downregulating genes promoting M2 phenotype while increasing the gene levels M1 markers. This promotes an anti-tumoral microenvironment and inhibited tumor growth.	Gunassekaran et al. (2021)
Acute lymphoblastic leukaemia	NK cells	The supernatant from NK cell culture is subjected to differential centrifugation, followed by filtration and high-speed centrifugation at 100,000 × *g* for 70 min	NALM-18 cancer cell line (Childhood B acute lymphoblastic leukaemia)	Not studied	NALM-18 cells are incubated with NK-EVs	NK-EVs are internalized into the tumour cells and mediate cytotoxicity against tumour cells. DNAM1 is expressed on NK-EVs and blocking of DNAM1-ligand on tumour cells by mAb results in a delay in apoptosis of tumour cells.	Di Pace et al. (2020)

TPCA-1, [5- (p-fluorophenyl)-2-ureido] thiophene-3-carboxamide.

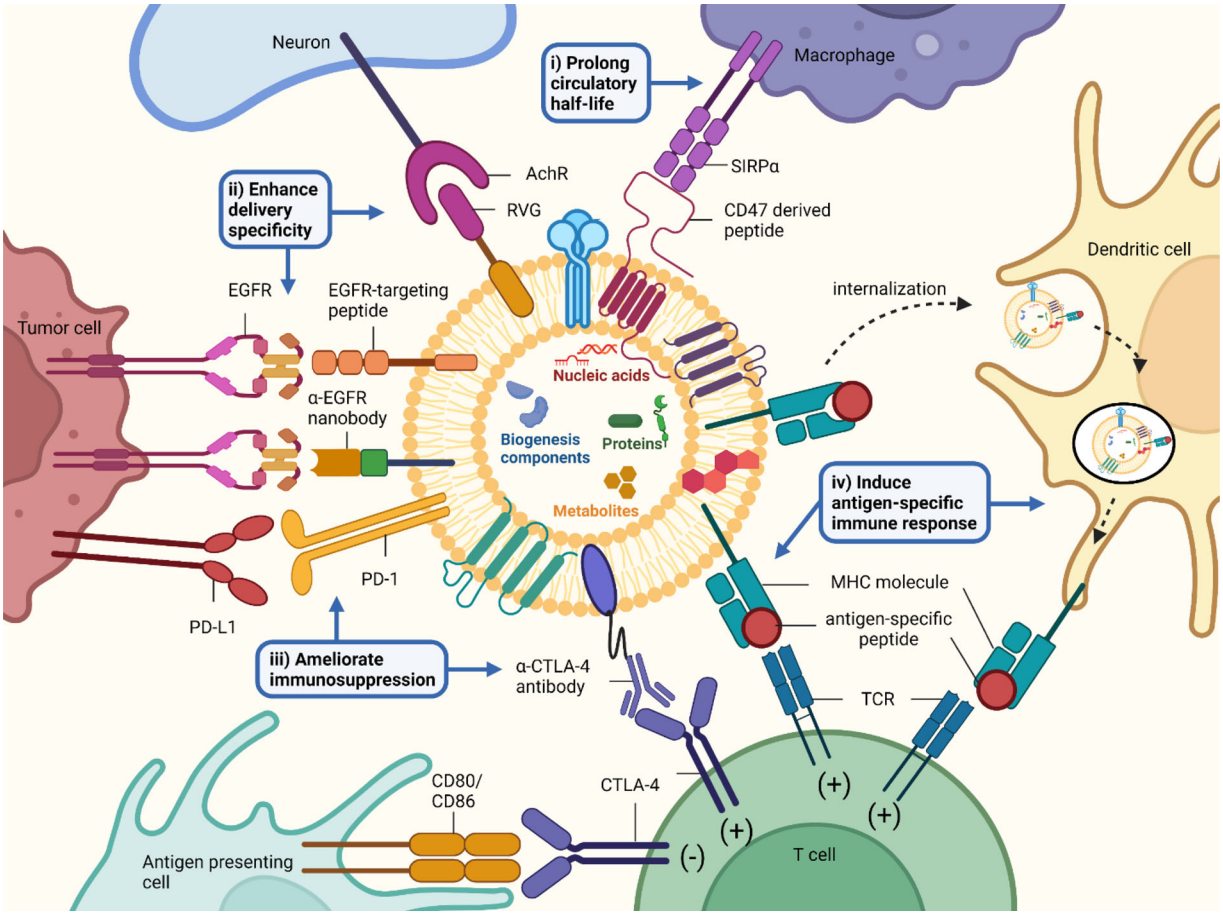

FIGURE 3 **Engineering strategies for improving the therapeutic potential of blood cell-derived extracellular vesicles**: (i) prolonging circulatory half-life by conjugating CD47 or 'self peptide', (ii) enhancing delivery specificity by conjugating targeting peptides/nanobodies (e.g. EGFR-targeting peptide/nanobody, RVG peptide) for targeting cells expressing corresponding surface antigens or molecules (iii) ameliorating immunosuppression by conjugating antibodies targeting immune inhibitory molecules (e.g. CTLA-4/PD-L1) and (iv) inducing antigen-specific immune responses by loading specific antigens (OVA, MAGE) to generate peptide-MHC complexes for antigen presentation. AchR, acetylcholine receptors; CTLA-4, cytotoxic T-lymphocyte antigen 4; EGFR, epidermal growth factor receptor; MAGE; MHC, major histocompatibility complex; OVA, ovalbumin; PD-1, programmed cell death protein 1; PD-L1, programmed death-ligand 1; RVG, rabies viral glycoprotein; SIRPα, signal regulatory protein; TCR, T-cell receptor

biodistribution and safety of these EVs are not well-addressed in these studies. More in vivo experiments are needed to evaluate their safety profiles.

6.2 | Wound healing

PEVs inherit various proangiogenic growth factors from their parent cells, which could be beneficial to the wound healing process as shown in a recent preclinical study (Tang et al., 2021). In this study, PEVs were used to pre-condition adipose-derived stem cells (P-ADSCs) in an ischemic mouse model. The data showed that PEVs could be internalized by ADSCs and P-ADSCs, followed by decreased apoptosis and increased expression of angiogenic-related markers such as VEGF and angiopoietin 1 (ANG1) under $CoCl_2$-induced hypoxic conditions. Mice receiving P-ADSCs have more extensive vessel structures and improved blood reperfusion recovery. Although the mechanisms of how PEVs act on ADSCs are not reported, these findings represent a proof-of-concept that PEVs exert therapeutic effects on engrafted ADSCs against ischemic injury. The proangiogenic property of serum-derived EVs containing mostly PEVs is being investigated in an ongoing clinical trial (NCT04652531). For this study, the authors propose the use of autologous serum-derived EVs to treat venous ulcers in patients unresponsive to conventional treatments.

Besides PEVs, the therapeutic applications of MEVs have received considerable attention due to their multifaceted roles in immune modulation. MEVs having immunosuppressive effects could be employed in treatments involving tissue repair and wound healing, while those having immunostimulatory effects could serve as a potential tool in treatments of infectious diseases and cancer (Wang et al., 2020). Taking advantage of the remarkable degree of phenotypic plasticity of the macrophage lineage, a hallmark which enables macrophages to adapt to changing environment, Kim et al. (2019) isolated EVs derived from

anti-inflammatory M2 macrophage to reprogram the proinflammatory M1 into the M2-like phenotype. Reprogrammed M2 macrophages enhance migration of fibroblasts and formation of endothelial tubes *in vitro*, which are required for wound repair. Subcutaneous injection of M2 EVs around the wound in mice displays pronounced primary wound closure.

6.3 | Neurodegenerative diseases

Among LEVs, DC-EVs have been extensively studied in various disease models both *in vitro* and *in vivo* for their ability to initiate and regulate immune response. In a landmark study on gene therapy in neurodegenerative disease, immature DCs from murine bone marrow were cultured and transfected with a plasmid encoding a neuron-specific rabies viral glycoprotein (RVG) derived peptide fused with a membrane protein (Alvarez-Erviti et al., 2011). Engineered DCs endow their EVs with targeting capabilities towards cells expressing acetylcholine receptors in the central nervous system. EVs are electroporated with siRNA against *BACE1*, a therapeutic target in Alzheimer's disease, and then intravenously injected into wild-type mice, resulting in a decrease of BACE1 at both the mRNA and protein level without causing significant changes in the level of proinflammatory cytokines. However, the therapeutic effect of BACE1-siRNA loaded EVs is investigated in normal C57BL/6 mice, but not in an *in vivo* Alzheimer's disease model. Moreover, the effects of the binding of RVG-derived peptide to acetylcholine receptors and the downstream signaling cascades are not studied. To validate if this EV-mediated siRNA delivery system is a promising alternative treatment in Alzheimer's disease, more evidence should be obtained to demonstrate if the EVs could cross the BBB and are immunologically well tolerated. A later study on multiple sclerosis, an inflammatory disorder of the nervous system, demonstrated that EVs from IFN-γ stimulated DCs were able to enhance myelination *in vitro* and *in vivo* (Pusic et al., 2014). The authors suggest the transfer of miR-219 from DC-EVs to oligodendrocytes as a possible mechanism. However, the effects of DC-EVs on remyelination *in vivo* were investigated using rats with normal brains and the uptake route of DC-EVs by oligodendrocytes was not elucidated.

6.4 | Cancers

Evidence is emerging that RBCEVs offer a robust and novel therapeutic delivery platform with additional advantageous features. Not only are RBCEVs relatively easy to obtain, they lack both nuclear and mitochondrial DNA (Shi et al., 2014). Unlike EVs originating from immortalized cells, RBCEVs are free from oncogenic DNAs and hence, do not pose a significant risk of gene transfer and tumorigenesis (Balaj et al., 2011).

Numerous efforts have been devoted to make RBCEVs a more efficient, scalable, and versatile platform for the delivery of various types of drugs and gene therapies. Our group developed a novel RBCEV delivery platform for RNA drugs (Usman et al., 2018). We demonstrate that RBCEVs loaded with anti-miR-125b antisense oligonucleotides (ASOs) are taken up by leukemic and breast cancer cells at high efficiency *in vitro* and *in vivo*, resulting in the knockdown of oncogenic miR-125b and the suppression of tumour growth without causing any observable side effects. We also validated the ability of this platform to deliver a functional CRISPR–Cas9 genome editing system into leukaemia cells. This work presents a new approach for cancer treatment that is suitable for clinical translation due to the versatility of the system, as well as the low cost of production of RBCEVs on a large-scale basis. Zhang et al. (2020) also demonstrate the potent therapeutic effects of drug-loaded RBC-EVs in a murine model of liver tumour. RBCEVs loaded with doxorubicin and sorafenib are taken up by the liver macrophages and accumulate in the liver. These drugs likely induce apoptosis of macrophages and then diffuse into other cells, including tumour cells, leading to the killing of tumour cells. The mouse model used in this study is deficient in T cells, hence the anti-tumour adaptive immune response could not be evaluated.

To further promote the utilization of RBCEVs in therapeutic contexts, we have developed a new approach to enhance the targeting specificity of RBCEVs via surface functionalization (Pham et al., 2021). Since RBCEVs cannot be genetically modified, we use a gentle enzymatic method to stably conjugate either an epidermal growth factor receptor (EGFR)-targeting peptide (ET) or anti-EGFR nanobody onto RBCEVs. As mentioned in the previous section, we also conjugate a CD47-mimicking 'self-peptide' on RBCEVs to decrease phagocytic clearance of RBCEVs and hence, improving their bioavailability in circulation. This ligation approach enhances specific uptake of RBCEVs by EGFR-positive cancer cells, both *in vitro* and *in vivo*, thereby increasing drug efficacy and decreasing side effects. Particularly, treatment with ET-RBCEVs loaded with paclitaxel (PTX) shows better anti-tumour response in a mouse model of EGFR-positive lung cancer compared to free PTX or uncoated PTX-loaded RBCEVs. This method could be applied to conjugate peptides or nanobodies targeting other surface receptors that are expressed by specific cell types, thus expanding the utility of this technology. We continue to extend the scope of RBCEV targeting by utilizing the enzymatic conjugation method in combination with the streptavidin-biotin system for surface functionalization of RBCEVs with peptides, nanobodies and even large and complex monoclonal antibodies (mAb) (Jayasinghe et al., 2022). Conjugation of the epithelial cell adhesion molecule (EpCAM) mAb onto RBCEVs specifically increases their uptake by human lung cancer cell line expressing EpCAM. Furthermore, bifunctional RBCEVs ligated with the CXCR4-targeting peptide and a pro-apoptotic peptide generated by this method can target CXCR4-positive leukemic cells and attenuate the disease progression *in vivo*.

In addition to the delivery of therapeutic agents to tumour cells, boosting anti-tumour immunity is one of the strategies to suppress tumour progression, for example, by fostering anti-tumoral M1 phenotypes in macrophages. EVs isolated from M1 macrophages were transfected with NF-kB p50 siRNA and miR-511-3p, followed by surface modification with an interleukin-4 receptor (IL-4R)-binding peptide (Gunassekaran et al., 2021). The engineered EVs targeted IL4R on pro-tumoral M2 macrophages and downregulated gene expression level of *NK-kB p50* and other M2 markers while increasing the levels of *iNOS* and *IL12p40* found in M1 macrophages. Reprogramming M2 TAMs into M1-like phenotype inhibited immunosuppressive immune subsets and, hence, suppressed tumour growth in a breast cancer mouse model more efficiently than other treatment formulations. In a recent study, we develop a new strategy for efficient and safe RIG-I agonist delivery by RBCEVs to suppress tumour growth and enhance anti-tumour immunity in breast cancer (Peng et al., 2022). Targeted delivery of immunomodulatory RNA or anti-miR-125b ASO with a 5′ triphosphate modification (3p-125b-ASO) by RBCEVs triggers RIG-I pathway and type I IFN production which lead to immunogenic cell death of tumour cells. The treatment also promotes immune cell infiltration and tumour-specific $CD8^+$ response.

Cancer vaccines are another application of BCEVs which has fuelled an immense interest in the field of cell-free therapeutics. Based on the hypothesis that EVs could activate antigen-specific immune responses via the MHC-antigen complex, Escudier et al. (2005) conducted a pioneering phase I clinical trial on autologous DC-EVs immunotherapy in stage IIIB and IV metastatic melanoma patients. Tumours from patients included in this study express the HLA-restricted Melanoma-associated antigen 3 (MAGE3) assessed by RT-PCR. DC-EVs are purified from immature DCs and loaded with MAGE3 epitopes before being intradermally and subcutaneously administered into patients. Another phase I clinical trial employed a similar approach to treat advanced non-small cell lung cancer (NSCLC) patients with tumour expression of MAGE-A3 and A4 antigens (Morse et al., 2005). Data from both studies indicate that DC-EV vaccine is safe and well-tolerated. An increase in NK cell functions was reported, but the treatments failed to induce antigen-specific T cell responses. To enhance the immunostimulatory effect of DC-EVs on T cells, unresectable NSCLC patients in a phase II clinical trial were vaccinated with tumour-associated antigen-loaded DC-EVs produced from mature DCs induced by IFN-γ (Besse et al., 2016). While the capacity of DC-EVs to promote NK cell cytolytic functions was reported, this treatment was still not able to generate T cell responses against specific tumour antigens. This could be due to the insufficient costimulatory signals on DC-EVs required for *in vivo* T cell priming and the presence of immunosuppressive immune subsets or molecules. Dai Phung et al. (2020) takes a further step to increase T cell activation by functionalizing the DC-EV membrane. EVs are isolated from ovalbumin (OVA)-pulsed mature DCs and decorated with anti-cytotoxic T-lymphocyte antigen 4 (CTLA-4) antibody to block CTLA-4, a checkpoint molecule involved in downregulation of immune responses. The modified DC-EVs can target T cells and induce their activation in mice bearing B16-OVA tumours, resulting in tumour growth suppression. Treatment with modified DC-EVs generates an increased proportion of effector memory T cells and proinflammatory cytokines, as well as a higher ratio of cytotoxic T cells/Tregs, suggesting effective T cell priming. Identification of new strategies to boost adaptive immune responses will continue to present opportunities to advance DC-EV cancer vaccines.

Compared to T cells, their derived EVs are still at their infancy in terms of clinical applications. More studies are required to investigate their diverse functions in immune regulations and explore possible modification strategies for therapeutic purposes. $CD8^+$ T cell-derived EVs have been mainly reported to elicit immunostimulatory or anti-tumour responses. In addition to direct cytotoxicity against tumour cells, $CD8^+$ T cells could remodel tumour microenvironment in an EV-mediated fashion (Seo et al., 2018). Activated $CD8^+$ T cells in healthy mice were reported to secrete EVs containing miR-298-5p to kill mesenchymal stem cells and destroy tumour stroma, preventing tumour invasion and metastasis in the CMS5a fibrosarcoma mouse model. However, this study did not elucidate the mechanism of actions of $CD8^+$ T cells-EVs or the target genes of miR-298-5p. In a triple-negative breast cancer model, the programmed cell death protein 1 (PD-1) on the membrane of EVs secreted by stimulated $CD8^+$ T cells can bind to the programmed death-ligand 1 (PD-L1) and, thereby, alleviate the immune suppression caused by the interaction between the PD-1 on $CD8^+$ T cells and PD-L1 expressed on tumour cells (Qiu et al., 2021). Interestingly, the binding of EV-PD-1 to PD-L1 induces PD-L1 internalization via clathrin-mediated endocytosis, which reduces PD-L1 burden in the tumour microenvironment while enhancing anti-tumour cytotoxicity of tumour-infiltrated lymphocytes.

In view of the critical role of NK cells in innate immunity, phenotypic and functional properties of NK cell-derived EVs have been explored for therapeutic applications. Although less rigorously tested in disease models *in vivo* than NK cells, NK-EVs have been shown to elicit cytotoxicity against several hematologic and solid cancer cell lines with limited toxicity towards normal resting cells (Jong et al., 2017; Lugini et al., 2012). These early studies reveal that NK-EVs contain typical tumour-targeting receptors such as NKG2D; cytotoxic proteins including perforin, granzyme A and granzyme B; and FasL on the surface, making them capable of inducing cancer cell death. A recent study on NK-EVs derived from IL-2 or IL-15- activated human NK cells revealed an additional mechanism of NK-EV-mediated cytotoxicity via the interaction of DNAX Accessory Molecule-1 (DNAM1) on the EV surface and its ligand, CD155, expressed by tumour cells (Di Pace et al., 2020). Taken together, these data highlight the potential use of NK-EVs in cancer immunotherapy.

TABLE 2 Classification of lipoproteins. The parameters for EVs are included for comparison

Lipoprotein	Size (nm)	Density (g/ml)	Major core lipids	Major protein (s)
Chylomicron	75–1200	< 0.930	Triglycerides	Apo B-48, Apo C, Apo E, Apo A-I, A-II, A-IV
VLDL	30–80	0.930–1.006	Triglycerides	Apo B-100, Apo E, Apo C
IDL	25–35	1.006–1.019	Triglycerides, Cholesterol	Apo B-100, Apo E, Apo C
LDL	18–25	1.019–1.063	Cholesterol	Apo B-100
HDL	5–12	1.063–1 .210	Cholesterol, Phospholipids	Apo A-I, Apo A-II, Apo C, Apo E
EVs	30–1000	1.13–1.19	No major core lipid; core is aqueous	Highly varied

7 | NON-EV CIRCULATING LIPID-BASED PARTICLES: LIPOPROTEINS

7.1 | Basic characteristics, diagnostic and therapeutic applications of lipoproteins

Lipoproteins can be classified based on their size and density into chylomicrons, very low-density lipoprotein (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL) (Table 2) (Feingold, 2000; Ramasamy, 2014; Simonsen, 2017). Unlike the classification of EV subtypes based on modes of biogenesis, the lipoprotein classes can be thought of as different nodes in the same network of metabolic pathways, where the members of some classes can be converted to another. In brief, chylomicrons transport dietary triglycerides from the intestines and are in the process catabolized to form chylomicron remnants, which are taken up and utilized by the liver. The liver synthesizes VLDL, which carries triglycerides to peripheral tissues. VLDL can be sequentially metabolized to IDL and then LDL, which are responsible for distributing cholesterol to both the liver and peripheral tissues. Meanwhile, HDL facilitates reverse cholesterol transport, absorbing cholesterol from peripheral tissues and delivering it back to the liver. Each step in these metabolic pathways is regulated by specialized enzymes and receptors and requires specific apolipoproteins (Table 2) (Feingold, 2000; Kwan et al., 2007; Ramasamy, 2014).

The balanced and steady systemic flow of lipids enabled by the lipoprotein network is essential for health, and disruptions to it may cause a variety of diseases. For instance, mutations in Apo C-II are known to compromise the hydrolysis of the triglyceride-rich VLDL and chylomicrons, increasing the risks of hypertriglyceridemia (Wolska et al., 2017). In atherosclerosis, one of the early stages of disease progression involves the oxidation of LDL due to oxidative stress. The oxidized LDL is prevented from being exported from cells, contributing to the formation of atherosclerotic plagues in blood vessels (Poznyak et al., 2020; Zeibig et al., 2011).

Beyond their natural importance in the body, lipoproteins have also received attention for their diagnostic and therapeutic potential. Due to the relative ease of obtaining blood samples for testing, circulating lipoproteins make for good biomarker candidates, particularly for cardiovascular diseases. Lowered level of LDL-cholesterol, for instance, may serve as a reliable indicator of acute coronary syndrome following myocardial infarction, and extent of the decrease in LDL-cholesterol corresponds to the severity of the myocardial injury (Sandhu et al., 2016). In patients with systemic lupus erythematosus, measurements of proinflammatory HDL show significant positive correlation with atherosclerosis occurrence (McMahon et al., 2006). The Apo B/Apo A-I ratio, as a proxy of the relative concentrations of their associated lipoproteins, has also been demonstrated to accurately predict the occurrence of cardiovascular diseases (Sniderman et al., 2006).

As described before in the context of BCEVs, one problem with the isolation of lipoproteins for use as biomarkers as well as research purposes is the considerable overlap in densities between lipoproteins, particularly HDL, and BCEVs (Table 2). As a result, methods of isolation that rely solely on density differences between components of the blood/plasma are likely to obtain a mixture of the particles instead of the desired pure EV or lipoprotein sample, leading to confounding conclusions (Simonsen, 2017; Thery et al., 2018). Sódar et al. (2016) used either density gradient ultracentrifugation or SEC to isolate PEVs. They found lipoproteins, detected by apoB-positive signals, in all fractions, including the fraction containing the purest PEV population. Therefore, it is suggested to combine different approaches to purify EVs and lipoproteins (Karimi et al., 2018; Mørk et al., 2017; Simonsen, 2017; Vickers et al., 2011). For instance, after centrifugation steps, lipoproteins can be collected from EV-containing mixtures using fast protein liquid chromatography or SEC fractionation and further separated using immunoprecipitation using antibodies against apolipoproteins. Once a sample is obtained, nanoparticle tracking analysis as well as conventional and cryo-electron microscope images can be highly informative due to the distinctly smaller sizes of some lipoprotein classes compared with EVs and the appearance of lipid monolayers instead of bilayers (Mørk et al., 2017; Takov et al., 2019; Vickers et al., 2011).

Finally, data showing the absence of EV markers and the detection of apolipoproteins would also serve as important evidence to demonstrate satisfactory purification of lipoprotein samples (Simonsen, 2017; Vickers et al., 2011). On the other hand, Karimi et al. (2018) demonstrated a two-step isolation procedure using density-based separation followed by SEC to isolate human plasma EVs from lipoproteins. Specifically, they designed a density cushion to remove lipoproteins and other components having lower densities than EVs. Then, lipoproteins having similar density to EV but differ in size can be removed by SEC. To further identify and differentiate EVs from lipoproteins, antibody-based strategies such as tetraspanin labelling for EVs and apoB labelling for lipoproteins has been used in flow cytometry to account for the extent of the confounding factors or to remove them in subsequent analysis (Botha et al., 2022). Additional validation and investigation are warranted to ascertain the effects of these isolation methods on the yield, purity, and functional activity of isolated EVs and lipoproteins.

In terms of therapeutic applications, HDL is the class of lipoproteins that have received the most interest by far. Due to their role in relieving cholesterol burden in peripheral tissues via reverse cholesterol transport, HDL is sometimes called the carrier of "good cholesterol" (Navab et al., 2009). HDL has been shown to have antioxidant and anti-inflammatory properties, allowing it to exert protective effects in several pathological conditions, including ischemia, atherosclerosis, and systemic inflammation (Navab et al., 2009; Thiemermann et al., 2003). Findings like these have motivated the development of reconstituted HDL (rHDL), where particles are artificially assembled from the major HDL apolipoproteins and phospholipids, usually via detergent dialysis or direct conversion (Ryan, 2010). The incorporation of HDL structures is meant to mimic HDL's native function in stimulating cholesterol efflux from cells and ferrying this excess cholesterol to the liver for processing. Indeed, infusion of different rHDL formulations in animal models as well as humans has been shown to improve cholesterol flux and endothelial functions, thus protecting against hypercholesterolemia, atherosclerosis, and diabetes type 2 (Drew et al., 2009; Ryan, 2010).

Beyond mimicking the natural HDL, studies have also explored the use of rHDL to package and deliver drugs. In a study by Song et al. (2016), Apo E and alpha-mangostin are incorporated into rHDL to target Alzeimer's disease. Apo E is a facilitator of cholesterol efflux in the central nervous system and Apo E-rHDL has been shown to bind to amyloid-beta (Aβ) with high affinity, while alpha-mangostin is a compound which blocks the formation of Aβ and facilitates Aβ degradation (Song et al., 2014, 2016). This rHDL formulation allows faster uptake and degradation of Aβ by the mouse microglia cell line BV-2 *in vitro*. Intravenous administration of the alpha-mangostin loaded Apo E-rHDL in an Alzheimer's disease mouse model demonstrates that Apo E improves the distribution of the nanoparticles to the brain. Both Apo E and alpha-mangostin contribute to a significant increase in degradation of Aβ, partially rescuing memory defects in the mice (Song et al., 2016). These results show promise for the development of a rHDL-based platform for treating Alzheimer's disease, pending a more comprehensive characterization of the systemic changes and safety profile of the treatment, as well as engineering efforts to further enhance brain targeting.

7.2 | Impact of BCEVs interactions with lipoproteins in uptake and functional studies

It is worth noting that many *in vitro* studies of EVs or lipoproteins are carried out in isolation using serum-free media or EV/lipoprotein depleted serum (Delenclos et al., 2017; Kaur et al., 2014; Schneider et al., 2017). However, BCEVs and lipoproteins may interact *in vivo*, hence leading to conflicting results in terms of cellular uptake and functional effects on recipient cells. Transmission electron microscopy (TEM) images obtained after *in vitro* mixing of LDLs and microvesicles reveal the extensive binding of LDLs on the surface of microvesicles (Sódar et al., 2016). Cryogenic TEM images of crude human plasma also show the association of EVs and lipoprotein-like structures, suggesting their interaction in an *in vivo* environment (Busatto et al., 2022).

The EV biomolecular corona in blood comprising lipoproteins could impact the EV-mediated effects on recipient cells. The presence of LDL preferentially enhances uptake of cancer cell-derived EVs by monocytes (Busatto et al., 2020). A recent study shows that the exposure of THP-1 monocytes to various cancer cell-derived EVs increases the level of TNF-α compared to untreated cells (Busatto et al., 2022). However, this effect is abrogated in the presence of physiological levels of LDL. Moreover, studies using media containing bovine serum components should also consider the difference between bovine and human lipoprotein profiles. Taken together, the inevitable interaction between lipoproteins and EVs *in vivo* should not be overlooked when designing, characterizing, and testing BCEVs for diagnostic and therapeutic applications. Additional studies are required to fully understand the functional significance of the association of lipoproteins around EVs, as well as exploring how to use this association to our advantage.

8 | CONCLUSION AND PERSPECTIVES

The clinical use of synthetic nanoparticles is constrained by their toxicity, limited ability to cross biological barriers, and poor biodistribution and bioavailability. This has led to more interest in naturally occurring structures to overcome these major hurdles. In this respect, circulating nano-scale particles in blood hold excellent prospects as biomarkers and cell-free therapeutic agents thanks to their biocompatibility and amenability to modifications, along with other advantageous innate properties. In this review, we described the basic biology, functions, and roles of circulating nanoparticles including BCEVs and lipoproteins in

physiological and pathological conditions. We highlighted strategies to characterize different physiochemical properties of both native and engineered circulating particles from different sources for diagnosis and treatment of diseases.

Investigations into the biology of circulating particles have flourished and paved the way for their clinical applications. Despite their enormous potential, there are several issues to be addressed before clinical translation. The lack of clinical-grade, scalable and standardized protocols remain the biggest challenge. There are only a few studies demonstrating effective and cost-efficient purification protocols for large-scale manufacturing. For successful translation of EV-based therapeutics, it is critical to select the donor cells for EV production with a complete risk-based analysis of their potency, immunogenicity, engineering capacity, and targeting specificity. In terms of productivity, industrial equipment can be employed for large-scale culture, such as enclosed bioreactors or platform-rocker wave bags (Whitford & Guterstam, 2019). Large-scale manufacturing of clinical-grade EVs requires a suite of quality control measures including pH, hydrodynamic force of the equipment, batch variance of media and supplements, cell growth and key parameters of EV's quality such as size, purity, and molecular composition. Moreover, storage temperature and numbers of freeze-thaw cycles need to be optimized to ensure the stability and potency of EVs. Last but not least, there is a need for dosing harmonization of EVs and the units of their payloads to establish a consistency in therapeutic assessments (Powell et al., 2021).

Moreover, there are many surface molecules that have been described to mediate binding and endocytosis of circulating vesicles, but the mechanisms underlying cellular uptake, endosomal escape, and intracellular cargo delivery are currently poorly understood. It is critical to characterize and validate these processes in different disease states using suitable *in vivo* models. This could lead to the discovery of new biomarkers and help to realize the full therapeutic potential of circulating nanoparticles. Advanced technologies and multidisciplinary research are required to overcome the limitations in this area. Integration of multi-omics data including proteomics, transcriptomics, and metabolomics, in combination with powerful imaging approaches, will certainly shed light on the intracellular fate and cellular processes governing physiological and pathological functions of these vesicles, as well as identifying components that determine targeting and interaction. The broad and increasing interest in BCEVs will continue to drive the development of novel engineering approaches to maximize targeting capability and delivery efficiency of circulating nanoparticles for their use in high-precision medicine.

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CONFLICTS OF INTEREST

Minh T.N. Le is a scientific co-founder, advisor and shareholder of Carmine Therapeutics, a company that develops extracellular-vesicle-based therapies. Other authors declare no conflict of interest.

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