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Emerging Prospects of Exosomes for Cancer Treatment: From Conventional Therapy to Immunotherapy

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Exosomes are a class of extracellular vesicles of around 100 nm in diameter that are secreted by most cells and contain various bioactive molecules reflecting their cellular origin and mediate intercellular communication. Studies of these exosomal features in tumor pathogenesis have led to the development of therapeutic and diagnostic approaches using exosomes for cancer therapy. Exosomes have many advantages for conveying therapeutic agents such as small interfering RNAs, microRNAs, membrane-associated proteins, and chemotherapeutic compounds; thus, they are considered a prime candidate as a delivery tool for cancer treatment. Since exosomes also provide an optimal microenvironment for the effective function of immunomodulatory factors, exosomes harboring bioactive molecules have been bioengineered as cancer immunotherapies that can effectively activate each stage of the cancer immunity cycle to successfully elicit cancer-specific immunity. This review discusses the advantages of exosomes for treating cancer and the challenges that must be overcome for their successful clinical development.

1. Introduction

For several decades, astronomical amounts of research funding and efforts have been invested into cancer research with the main objective of ultimately comprehending and eradicating cancer. Despite significant progress over the last 40 years in the discovery of new diagnostics, therapeutics, and preventive measures, cancer remains the second leading cause of death worldwide.[1] Studies have observed that genetic mutations mainly cause carcinogenesis and cancer progress; however, the other factors involved are not yet fully understood and it remains unclear how and why cancer occurs and progresses.

Using an evolutionary approach, the concept of a complex adaptive system (CAS) was developed to describe the behaviors of cancer tumorigenesis,[2] which may suggest a frameshift away from the limitations of current approaches that mainly address the importance of alteration in specific target molecules in cancer cells. This change in perspectives toward tumors, not simply as a disease to be cured but also as CAS, is expected to be the cornerstone of the paradigm shift toward innovative cancer therapies.[3]

The human immune system can also be viewed as a CAS and therefore expected to initiate self-defense mechanisms against cancer in a complex adaptive manner as long as it recognizes the cancer cells as the non-self-signals. Consequently, the key to controlling cancer could lie in understanding how to manipulate the immune system and strengthen its defenses against cancer. The concept of facilitating the immune system to fight against cancer was first suggested in the late 1800s by Dr. Wiliam Coley, who was the first to observe anti-tumor effects after intratumoral injection of microbe-derived toxins.[4] Since then, the field of cancer immunotherapy research has flourished, resulting in clinical achievements such as immune checkpoint blockades and chimeric antigen receptor T cell (CAR-T) therapy.[5] However, immune suppression resistance mechanisms have simultaneously been identified that have impeded favorable response to cancer immunotherapy.[6,7]

Exosome-based cancer therapies have emerged as a potential option for overcoming these limitations to the effects of current cancer therapies due to their pathophysiological efficacy against tumors.[8] Exosomes are secreted externally by cells and are found ubiquitously in blood, urine, saliva, cerebrospinal fluid, pleural fluid, and breast milk.[9,10] The distinction between different types of extracellular vesicles (EVs) is unclear; however, they are conventionally classified as either ectosomes (microvesicles or microparticles) or exosomes.[11] While ectosomes are formed by the outward budding of the plasma membrane, exosomes are formed from multivesicular bodies (MVB) containing intraluminal vesicles via inward budding of the late endosome, which later fuses to the membrane. The formed vesicles are then secreted via a process known as exocytosis (**Figure 1**). The two types of vesicle also differ in diameter,

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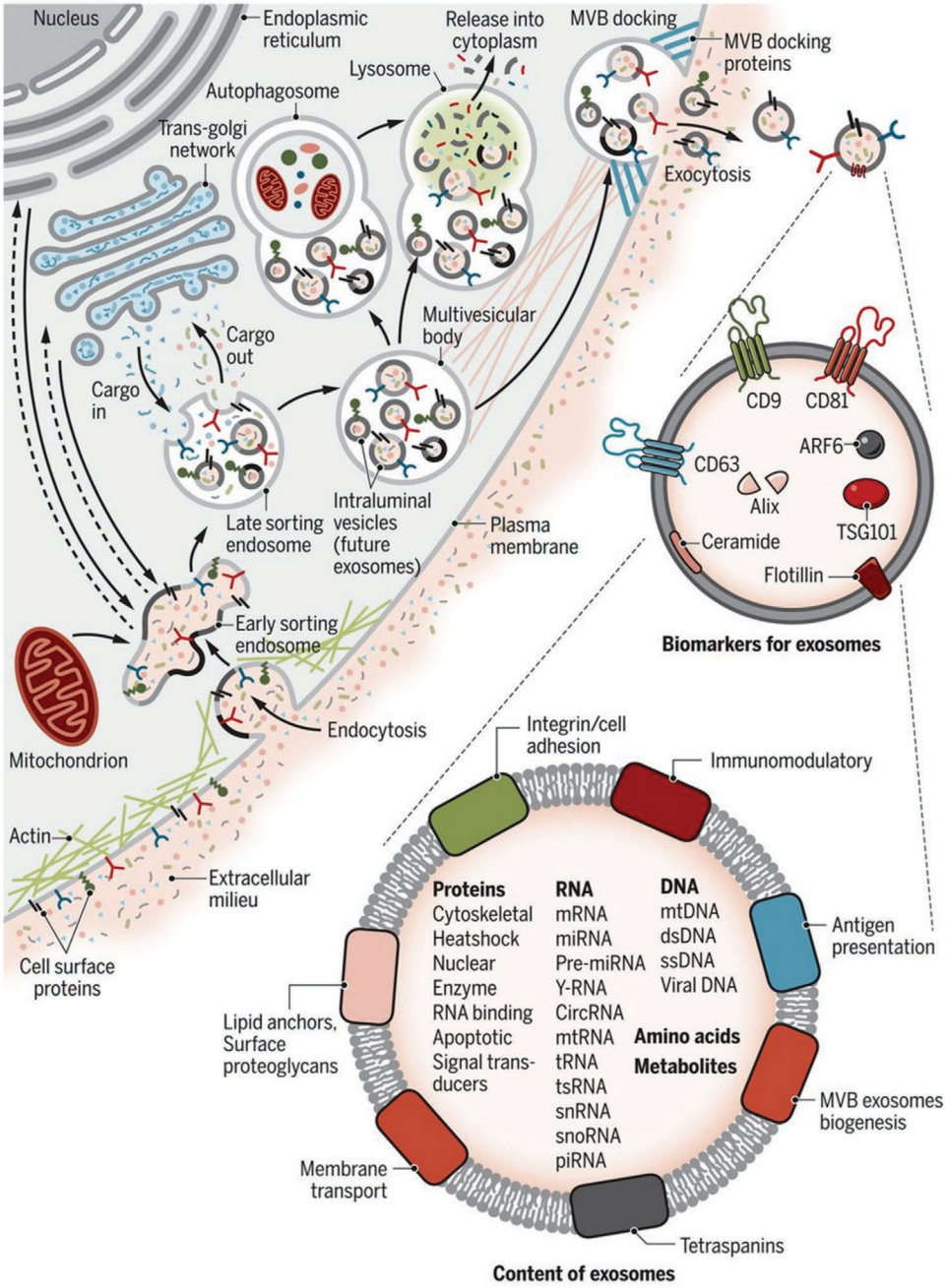

Figure 1. Exosome biogenesis. Exosomes are formed by the inward budding of the late endosome, known as a multivesicular bodies, which fuses to the membrane, and is followed by the exocytosis of exosomes. Thus, exosomes can represent the original characteristics of their parent cells, such as proteins, RNAs, DNAs, lipids, amino acids, and metabolites. Reproduced with permission.[11] Copyright 2020, The American Association for the Advancement of Science.

with ectosomes generally being larger in size with a diameter of ≈50 nm to 1 µm and exosomes displaying a diameter of 30–150 nm.

Exosomes have attracted a significant amount of attention as they can retain the original characteristics of their parental cells. Soon after their discovery, exosomes were shown to be involved in bone mineralization and platelet function,[12] and were later suggested as a mechanism for discarding cellular waste.[13] Numerous subsequent studies have demonstrated that exosomes contain or express various bioactive molecules such as proteins, RNAs (mRNA, microRNA, and other non-coding RNAs), DNAs (mitochondrial DNA [mtDNA], double-stranded DNA [dsDNA], single-stranded DNA and viral DNA), lipids, amino acids, and metabolites. These diverse constituents play crucial roles in intercellular signaling and modulate adjacent or distant cellular microenvironments.[14]

Exosomes can represent the complexity of their parent cells and have the intrinsic ability to control complex biological

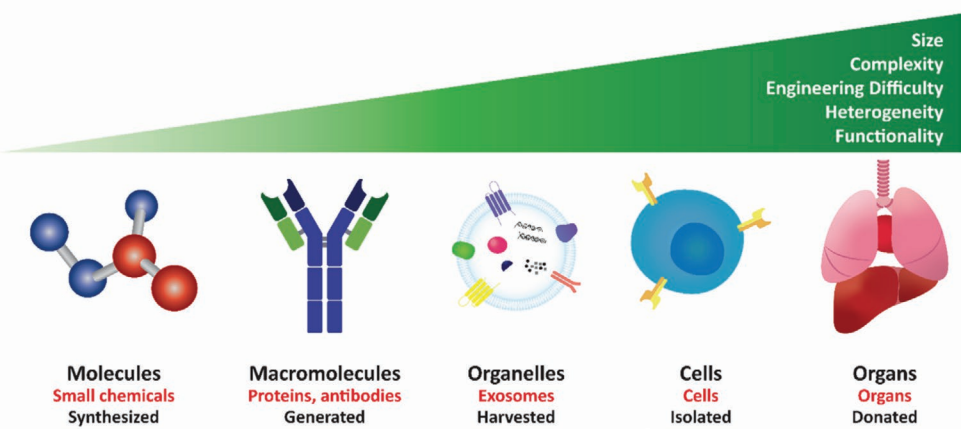

Figure 2. Levels of organization. Biological levels of living organisms, ranging from the simplest to the most complex, including molecules, macromolecules, organelles, cells, organs, and organisms. The simplest level, molecules can include small chemicals that can be synthesized as therapeutic tools. Macromolecules include proteins and antibodies that can be generated. In the middle, exosomes are located at the level of organelles that can be harvested as therapeutics. Cells and organs can be isolated and donated as therapeutic tools, respectively. Along with levels of the organization become complex, their size, engineering difficulty, heterogeneity, and functionality elevates. Exosomes can have distinct advantages as they are involved in an intermediate level of organization.

functions.[15] These advantages have increased the feasibility of the use of exosomes in diagnostic and therapeutic cancer management.[11] In addition, exosomes have many advantages over other small molecules and can efficiently deliver cancer therapeutic agents, thereby inducing successful anti-tumor responses.[16] Consequently, exosomes are considered to be candidate therapeutic delivery tools that have the potential to overcome the complex adaptive nature of tumors.

Endosomal markers can be used to distinguish exosomes of endosomal origin from other EVs; however, current exosome isolation methods, such as tangential flow filtration (TFF) and ultracentrifugation, are limited as they cannot completely exclude other EVs. Therefore, the results of any subsequent experiments may reflect a mixture of EVs, including exosomes. Several biological exosomal markers are currently under development, and exosome isolation strategies are continually being developed; thus, previous exosome studies require re-evaluations based on modern criteria.

This review introduces the strengths of exosomes in the field of cancer therapy and describes the momentous progress that has been made in cancer therapies that harness exosomes, particularly the recent achievements of an emerging class of exosome-based cancer immunotherapies. Finally, we discuss issues that must be solved for the clinical application of these methods and the future direction of exosomes in cancer therapy.

2. Benefits of Using Exosomes to Treat Cancer

Based on their size, complexity, engineering difficulty, heterogeneity, and functionality, cancer treatments can be classified as molecules (small chemical), macromolecules (protein and antibody), cells, and organs. For decades, researches have been conducted within these categorizations, from small molecules blocking specific signaling pathways to organ transplantation. Although current research focuses on protein/antibody drugs, more sophisticated and complex therapeutic modalities must be developed.

Exosomes can be categorized as organelles in between macromolecules and cells, and have attracted increasing attention as diagnostic markers and therapeutic agents. Unlike a sole protein or small molecule, exosomes contain molecules of heterogeneous function but lack the complexity of cells and organs; therefore, exosomes are considered appropriate tools for treating various diseases, including cancer (**Figure 2**). In addition, exosomes display many benefits with respect to biocompatibility, immunogenicity, stability, pharmacokinetics, biodistribution, and cellular uptake mechanism, making them potential options for anticancer treatment. These strengths can improve the therapeutic index of exosome-based cancer therapies by preferentially targeting tumor cells while minimizing unexpected side effects. Here, we illustrate these advantages for cancer therapeutics with relevant examples.

2.1. Messengers Reflecting Tumor Heterogeneity

Exosomes released from cancer cells can act as messengers to regulate both cancer cells and their microenvironment, from initial tumor generation to tumor progression.[17] Cancer cells are known to secrete more exosomes than normal cells, even in the initial stages of cancer development; therefore, tumor-derived exosomes (TEXs) are thought to reflect the unique molecular signatures of various cancers. Considerable research efforts have attempted to elucidate the roles of TEXs and tumor-associated cell-derived exosomes and have rapidly expanded the field of cancer diagnosis.[18] Although TEXs are multi-functional, they have been found to play predominantly pro-cancer roles.[19] Consequently, strategies to prevent excessive exosome production by cancer cells have been developed as cancer therapies, many of which have demonstrated anti-cancer efficacy.[20] In addition, TEXs harbor diverse antigens from their parental

cells and induce various T cell clones that can effectively control metastasis and/or resistance due to tumor complexity.[21] Further studies would improve our understanding of the complex functions of TEXs and discover methods of selective regulation that may help deduce novel strategies to overcome cancer complexity.

2.2. Immunogenicity

Non-self-substances are extremely antigenic and may elicit immune responses;[22] therefore, exosomes derived from allogenic or heterologous cell sources have the potential to provoke unwanted immune responses. However, blood transfusion, which is widely practiced in clinical settings, involves injecting patients with more than one trillion exosomes from other individuals without matching inter-patient human leukocyte antigen (HLA) and show no immune-related toxicity in the recipient.[11] Thus, allogenic exosome injection may not cause noticeable complications in terms of immunogenicity.

In mice, no severe immunotoxicity was reported following the repeated injections of exosomes from mouse or human (HEK293T) cell lines over a given period[23,24] (**Figure 3**). In particular, the systemic administration of exosomes ten times (8.5 µg per dose) induced no substantial differences in pro-inflammatory cytokine levels, blood chemistry panels, complete blood count, or the frequencies of immune cells in lymphoid organs, and no significant changes in body weight between the exosome- and non-exosome-treated groups.[24]

Microbe-derived membrane vesicles are commonly found in the blood,[25] and it is known that milk- or plant-derived exosomes do not cause noticeable immunotoxicity.[26] However, the contexture of the exosomes may be altered when DNA constructs are introduced into the parental cell to produce engineered exosomes. Accordingly, the immunogenicity of exosomes developed for specific purposes must be thoroughly and individually evaluated.

2.3. Stability

Exosomes are known to display good stability as they retain the nature of their parental cells with the maintenance of their inherent integrity for a long time. Sokolova et al. used nanoparticle tracking analysis to confirm that exosomes from HEK293T cells, endothelial colony-forming cells, or mesenchymal stem cells (MSCs) stored at −20 °C in phosphate-buffered saline (PBS) showed no size conversion or degradation.[27] Notably, multiple freeze–thaw cycles did not affect their size, confirming that freezing does not affect the quality of stored exosomes.[27] To confirm the stability of exosomes in plasma, Kalra et al. stored LIM1863 colon cancer cell-derived exosomes at 37, 4, −20, and −80 °C (**Figure 4**), finding that most samples retained their integrity for 3 months, even in the absence of protease inhibitors, and displayed the greatest stability at −80 °C.[28] Furthermore, a recent study reported that therapeutic exosomes sustained anti-tumor efficacy even after a minimum of 5 months of frozen storage.[29] Since exosomes enclose vulnerable bioactive molecules within a lipid bilayer membrane, they can protect therapeutic nucleic acids and proteins from degradation by RNases or proteinase.[30] Thus, the stability of exosomes is a considerable advantage not only for cancer treatment but also for diagnosis.

2.4. Biodistribution

To develop exosomes as cancer therapeutic agents, the biodistribution of administered exosomes from various sources must be evaluated. Peinado et al. evaluated the biodistribution of intravenously (IV)-injected B16F10 melanoma-derived TEXs,[31] which were detected in the spleen, liver, lung, and bone marrow. Similarly, Wiklander et al. investigated the biodistribution of IV-injected exosomes derived from murine B16F10 cancer cells, human HEK293T embryonic kidney cells, bone marrow-derived dendritic cells, and C2C12 myoblasts. The administered exosomes mainly accumulated in the gastrointestinal tract, lung, liver, and spleen, while B16F10-derived TEXs were distributed more in the lung than the other exosome types.[32] In addition, another study found that IV-injected MSC-derived exosomes were mainly distributed in the liver and spleen.[33]

The surfaces of exosomes are commonly modified to regulate their biodistribution when developing exosome-based cancer therapies. To achieve active accumulation at the tumor site, exosomes can be engineered to express specific surface molecules that can selectively bind to a molecule overexpressed on cancer cells. For instance, immature dendritic cell (DC)-derived exosomes genetically modified to express Lamp2b fused to the iRGD peptide have been shown to target αv integrin$^+$ tumor tissues.[34] To selectively target breast cancer cells with high epidermal growth factor receptor expression, Ohno et al. developed HEK293 cell-derived exosomes expressing the GE11 peptide fused to the transmembrane domain of the platelet-derived growth factor receptor.[35]

The treatment of brain-related diseases, such as Alzheimer's, Parkinson's, and brain cancer, is generally complicated because most molecules cannot pass through the blood–brain barrier (BBB) unless they are very small.[36] However, recent studies have demonstrated that TEXs can cross the BBB by suppressing Rab7 expression in brain endothelial cells via a mechanism known as transcytosis.[37] Yang et al. demonstrated the in vivo BBB penetration of drug-loaded exosomes in a zebrafish model with a similar BBB to humans, alongside the induction of anti-tumor responses (**Figure 5**).[38]

Based on the advantages of overcoming the BBB, various attempts have been made to treat brain cancer using exosomes. Hamideh et al. packed exosomes with microRNA (miRNA)-21-sponge constructs and injected them into a rat brain tumor model, observing potent tumor-suppressive effects.[39] In addition, exosomes extracted from MSCs transfected with miRNA-124a were found to reach GBMs and inhibit cancer growth in a mouse model, thereby improving the survival rate.[40] Exosomes containing chemotherapeutic agents, such as doxorubicin and paclitaxel, have also demonstrated anti-tumor efficacy against brain cancer.[41] To promote active accumulation at the tumor site, Alvarez-Erviti et al. engineered exosomes expressing the lysosome-associated

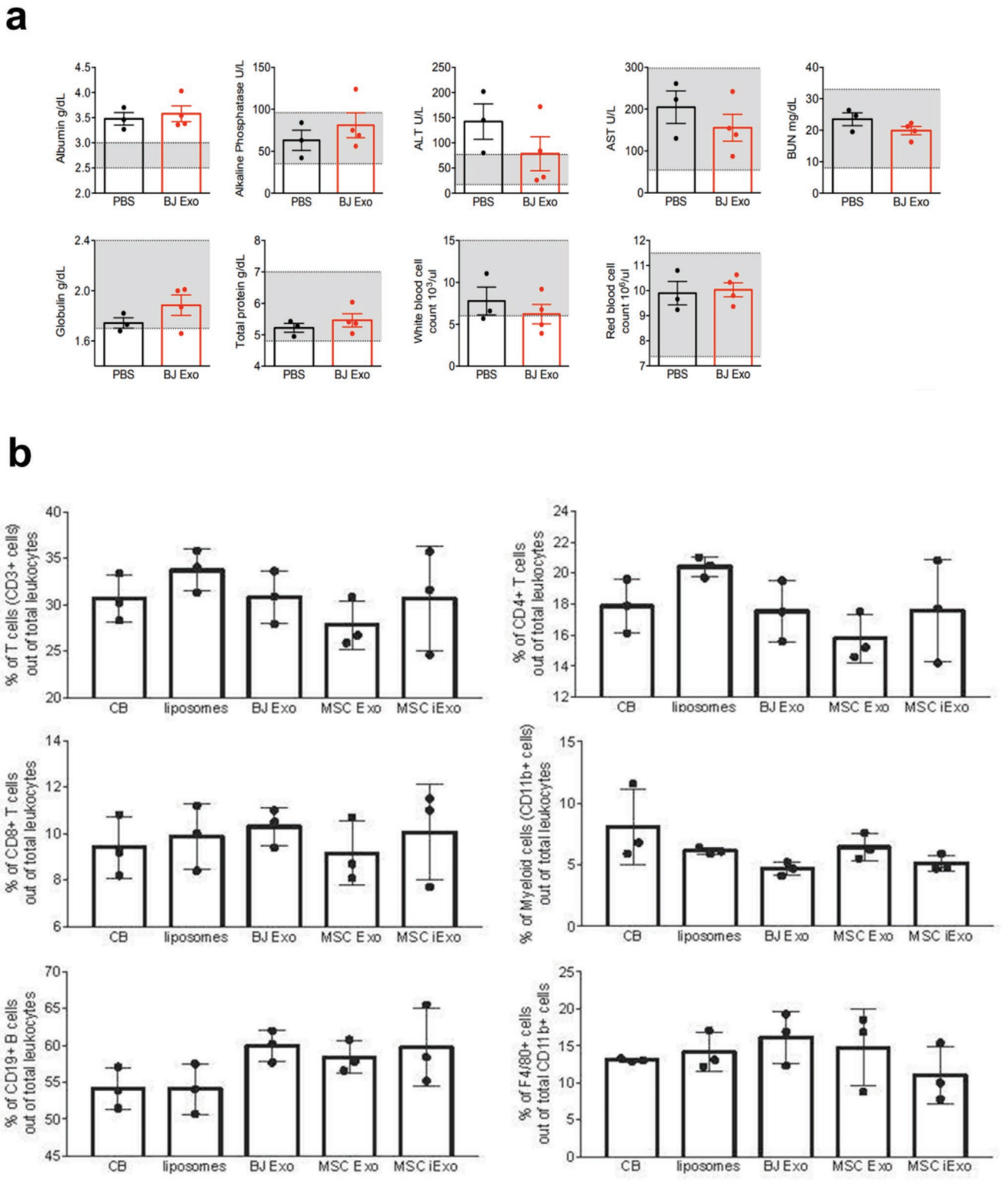

Figure 3. Exosome immunogenicity. a) Cytotoxicity of human foreskin fibroblast-derived exosomes in C57BL/6J mice as measured in the blood. The normal range is highlighted in grey. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen. Reproduced with permission.[29] Copyright 2018, American Society for Clinical Investigation. b) Immune cell profile demonstrating no significant differences in the spleen of five mouse groups after exosome or liposome treatment. PBS, phosphate-buffered saline; CB, Clinical buffer; BJ Exo, BJ fibroblast-derived exosomes; MSC Exo, MSC-derived exosomes; MSC iExo, MSC-derived exosomes loaded with KRAS siRNA. Bars and error bars demonstrate the mean and standard deviation, respectively. $*p < 0.05$. Reproduced with permission.[29] Copyright 2018, American Society for Clinical Investigation.

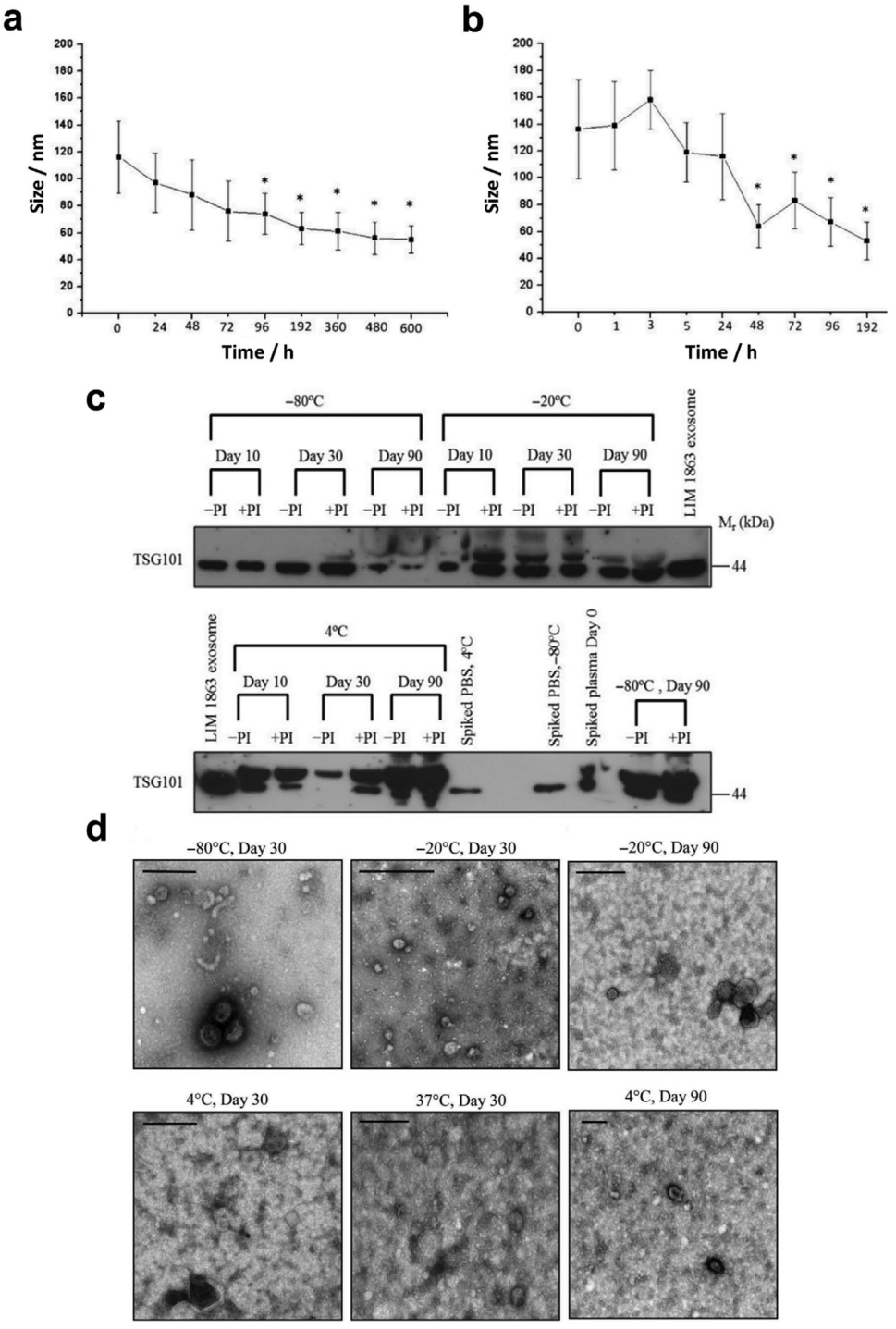

Figure 4. Exosome stability. Nanoparticle tracking assay (NTA) of size change HEK cell-derived exosomes stored at a) +4 °C and b) +37 °C Reproduced with permission.[27] Copyright 2011, Elsevier. b) Exosomes from LIM1863 cells spiked with plasma and PBS were stored at −80, −20, and 4 °C for 10, 30, and 90 days. c) Exosome stability demonstrated by TSG101 expression. Reproduced with permission.[28] Copyright 2013, Wiley-VCH. d) TEM images of stored plasma exosomes spiked with LIM1863 exosomes and stored at different temperatures for 30 and 90 days. Scale bar: 200 nm. Reproduced with permission.[28] Copyright 2013, Wiley-VCH.

membrane protein 2 (LAMP 2) and rabies virus glycoprotein (RVG) fusion proteins capable of targeting neural cells.[42] These exosomes effectively passed through the BBB to reach neurons, microglia, and oligodendrocytes.

2.5. Pharmacokinetics

Numerous studies have attempted to accurately measure exosome pharmacokinetics. Fluorescence-labeling-based approaches

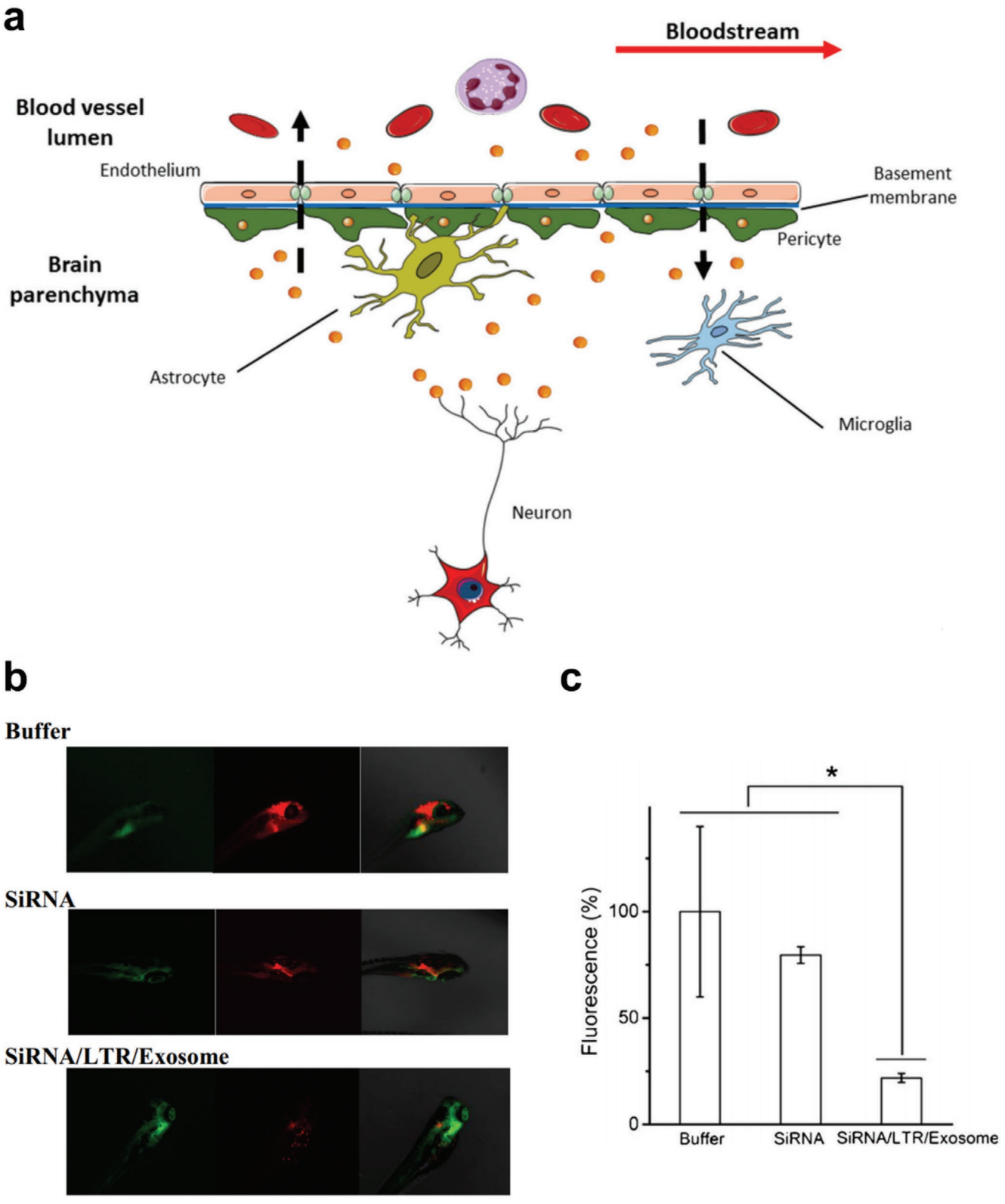

Figure 5. Exosomes can cross the BBB in both directions. a) Schematic diagram of this phenomenon. Orange circle indicates exosome. Reproduced under the terms of the CC-BY license.[260] Copyright 2019, D'Anca, Fenoglio, Serpente, Arosio, Cesari, Scarpini, and Galimberti. Published by Frontiers. b) Images of exosome-delivered VEGF siRNA in a Tg(fli1:GFP) zebrafish tumor model. Tumor cells are indicated in red. c) Statistical analysis of quantified DiD labeled (red) tumor cells in a zebrafish brain. Reproduced with permission.[38] Copyright 2017, Springer Nature.

using fluorescent dyes, such as PKH, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiD), or 1,1′-dioctadecyltetramethyl indotricarbocyanine iodide (DiR), have revealed the accumulation of systemically administered exosomes in vivo.[31,32] However, a lack of sensitivity and the release of free dyes means that these methods are unable to verify pharmacokinetic characteristics, that is, accumulation profiles at the organs or blood elimination profile.

To overcome these limitations, exosomes can be conjugated with luciferase as bioluminescence detection ensures high sensitivity.[43] For instance, one study used a Gaussia luciferase (gLuc) and lactadherin (LA) fusion protein (gLucLA) to demonstrate that B16-BL6 exosomes were promptly degraded in the blood with a half-life of 2 min when IV injected in a mouse model.[44] In addition, this method was used to verify the pharmacokinetics of exosomes derived from several mouse-cell lines with a 2–4 min half-life.[45] Thus, these approaches facilitate the interpretation of exosomes behavior in vivo.

Another suitable approach for analyzing exosome pharmacokinetics uses radiotracers such as a streptavidin (SAV)–biotin system with iodine-125 (^{125}I) labeling, which has enabled exosome accumulation profiles to be evaluated. The ^{125}I labeled B16-BL6 exosomes at the organs were analyzed quantitatively; the radioactivity of the ^{125}I labeled exosome was measured in the liver, spleen, and lung, which showed concentrations of 28%, 1.6%, and 7% of the injected ^{125}I labeled exosomes, respectively.[46] In addition, Smyth et al. quantified the pharmacokinetics of human prostate adenocarcinoma PC3-derived exosomes by labeling them with ^{111}In, revealing that the labeled exosomes were rapidly eliminated from blood circulation and that around 12% of the IV injection dose was primarily distributed in the liver after 24 h.[47]

Recent reports have demonstrated that the half-life of exosomes can be improved significantly by using their unique features to prolong their blood retention (**Figure 6**).[23] MSC-derived exosomes generally overexpress CD47, a "don't eat me" signal that can neutralize detection by phagocytic cells. A study found that iExosomes, small interfering RNA (siRNA)-loaded exosomes targeting K-RAS mutants, were present in the circulating blood of C57BL/6 or nude mice 24 h after intraperitoneal (IP) injection, unlike siRNA-loaded liposomes.[23] The iExosomes with high CD47 expression were highly detected for a long time; however, CD47-knockout diminished exosome retention, indicating that their retention was dependent on CD47. Thus, MSC-derived exosomes with these features could be used as anticancer therapies, while the modification of the exosomal surface to evade phagocytes and the reticuloendothelial system may extend exosomal half-life and improve therapeutic efficacy.

2.6. Cellular Uptake Mechanism

Exosomes are internalized via various mechanisms, including phagocytosis, micropinocytosis, fusion, and receptor- or lipid raft-mediated endocytosis.[48] Phagocytosis is the crucial mechanism for removing pathogens and cell debris.[49] Phagocytosis of exosomes by professional phagocytes, such as macrophages and DCs, and non-professional phagocytes, including $\gamma\delta$ T cells, are dependent on phosphatidylinositol 3-kinase (PI3K), dynamin 2, and actin cytoskeleton.[50] Further research is required to determine whether the purpose of the exosomal phagocytosis is merely for garbage disposal or intercellular communications. During micropinocytosis, the protrusions of cell membranes, which are dependent on Na^+ and PI3K activity non-specifically swallow extracellular fluid and particles, including exosomes.[51] Besides, it has been found that phosphatidylserine on exosomal surface activated micropinocytosis of macrophages.[52] Several studies have found that exosomes could enter the cells by using a fusion mechanism akin to certain viruses.[53] Parolini et al. demonstrated that exosomes could deliver their cargos into human melanoma cells via their fusion with cell membranes.[54] Receptor-mediated endocytosis, called clathrin-mediated endocytosis, requires specific ligands on the exosomal surface to interact with receptors on the plasma membrane.[48] Likewise, lipid raft-mediated endocytosis, including caveolae-mediated endocytosis and ARF6-, CDC42-, and RhoA-regulated endocytosis needs to express ligands on exosomes to engage with cholesterol- and sphingolipid-rich microdomains, on the cell membrane.[55]

These distinct cellular uptake mechanisms offer therapeutic benefits for exosome-based cancer therapies. For example, since oncogenic KRAS signaling promotes exosome micropinocytosis,[56] exosomes harboring KRAS siRNA can induce selective effects against KRAS mutant tumors.[23] In addition, the fusion of exosomes with cell membranes facilitates the transfer of therapeutic exosomal cargos into the cytoplasm of target cells while escaping lysosomal degradation.[57] Although the fusion ability of exosomes with the plasma membrane is required for the delivery of cargos into the cytoplasm, exosome is also able to release contents in acceptor cells via endocytosis.[57] The previous study demonstrated that exosomes facilitated membrane fusion between exosome and endosomal membrane to escape from endosomal maturation, leading to the release of exosomal contents.[57] However, only 24.5% of exosomes that were uptaken by cells during 12 h of incubation could expose their contents into the cytoplasm, showing that the efficacy of delivering exosomal cargos is limited.[57] To improve this efficacy, fusogenic exosomes expressing viral fusogen on their surface have been developed to increase the fusion ability of exosomes.[53] These fusogenic exosomes are expected to modify target cell membranes by inserting therapeutic membrane proteins as well as to efficiently deliver their cargos to recipient cells.[53] To date, detailed cellular uptake mechanisms for exosomes in specific cell types remain unclear, and further research is required to understand exosome internalization and allow the development of novel exosome-based cancer therapies.

3. Exosomes for Conventional Cancer Treatment

For decades, several drug delivery tools have been developed to treat cancer; however, the chances of effective drug delivery to the cancer tissues following administration in vivo are less than 0.7%, and a few drugs were clinically approved.[58] As discussed in the previous sections, exosomes exhibit excellent properties as drug carriers. Owing to their superiority over other carriers, exosomes are actively being used in many anti-cancer strategies to load or express various bioactive molecules and have

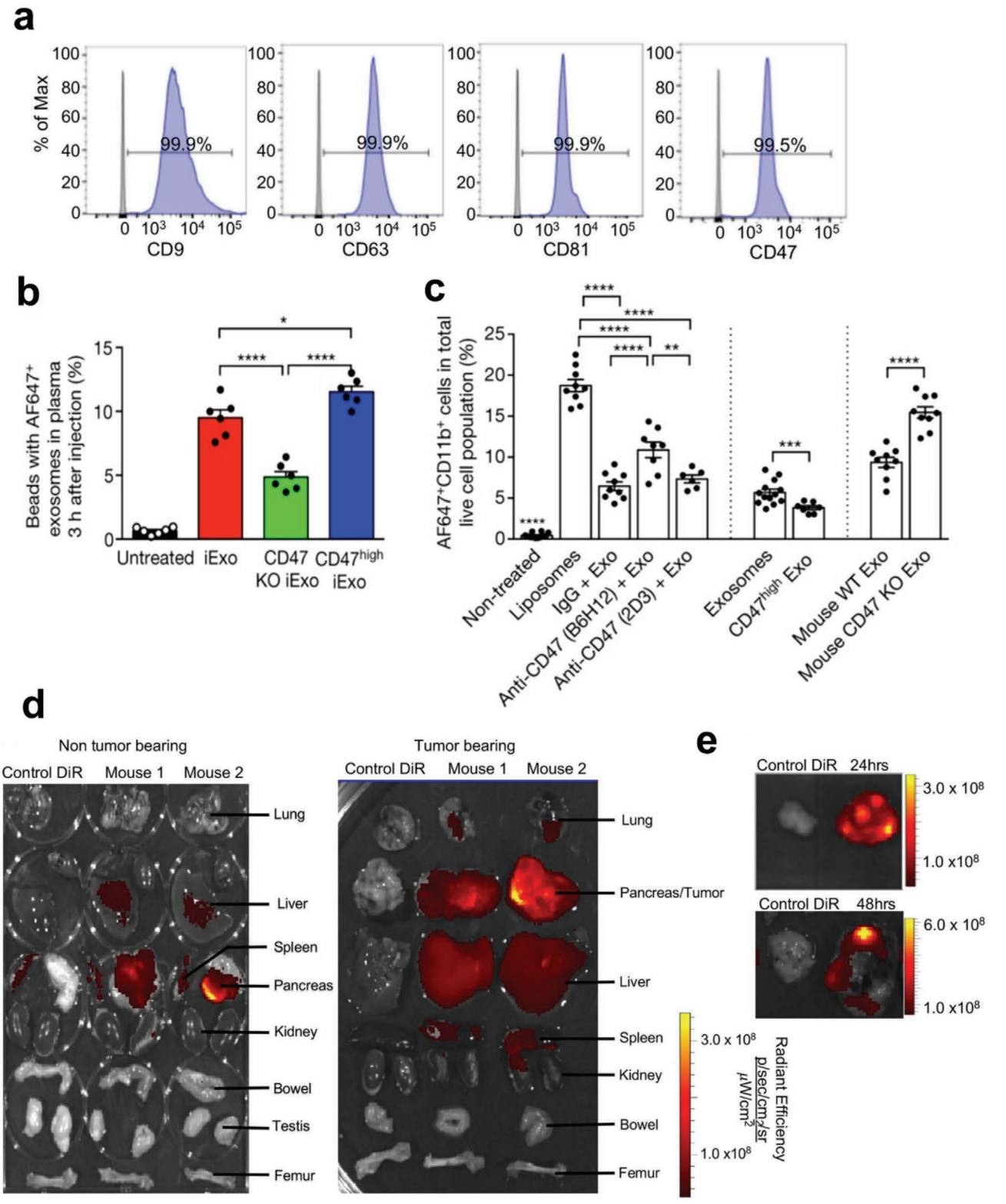

Figure 6. Exosome half-life. a) Flow cytometry analysis of exosomal markers (CD9, CD63, and CD81) and CD47 on MSC-derived exosomes. Reproduced with permission.[29] Copyright 2018, American Society for Clinical Investigation. Retention of exosomes by limiting phagocytic clearance: b) flow cytometry analysis of AF647-tagged iExosomes in plasma 3 h after intraperitoneal injection; c) estimation of circulating $AF647^{+}CD11b^{+}$ monocytes. Reproduced with permission.[23] Copyright 2017, Springer Nature. d) Detection of MSC-derived exosomes labeled with DiR in non-tumor-bearing and KPC689 tumor-bearing mice. e) Images of DiR-labeled MSC-derived exosomes 24 and 48 h after i.p. injection. Reproduced with permission.[29] Copyright 2018, American Society for Clinical Investigation.

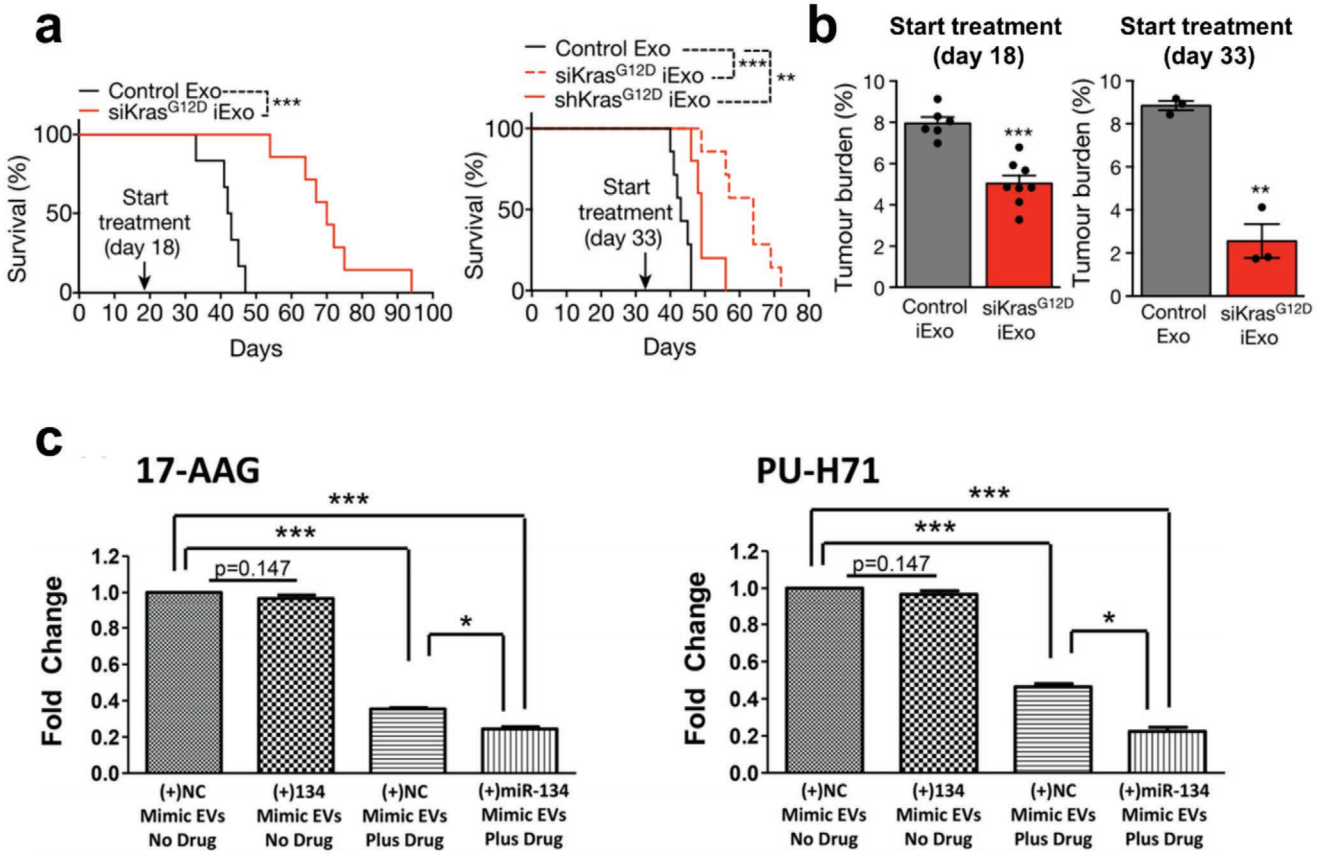

Figure 7. Exosome-mediated siRNA and miRNA delivery enhances anti-tumor efficacy. siKRAS-G12D-iExosome a) increase the survival rate and b) reduce the tumor burden in KPC murine tumor models. Reproduced with permission.[23] Copyright 2017, Springer Nature. c) Exosomes (a class of EVs) enriched with miR-134 increased anti-proliferative effects via the anti-HSP90 compounds 17-AAG (left) and PU-H71 (right). Reproduced under the terms of the CC-BY license.[64] Copyright 2015, K. O'Brien, M. C. Lowry, C. Corcoran, V. G. Martinez, M. Daly, S. Rani, W. M. Gallagher, M. W. Radomski, R. A. MacLeod, L. O'Driscoll. Published by Impact Journals, LLC.

recently been reported in clinical trials.[59] Here, we summarize the findings of studies using exosomes to achieve noteworthy effects against a wide range of cancer types, including exosomes manipulated to contain bioactive molecules, such as nucleic acids, proteins, and chemotherapeutics. These relevant examples indicate the potentials of exosomes to overcome the limitations of conventional anti-cancer drug carriers.

3.1. Exosomal Delivery of siRNAs and miRNAs

SiRNAs and miRNAs are small, double-stranded, and non-coding RNAs that regulate gene expression by suppressing mRNA expression and modulate cellular processes such as proliferation, division, and death.[60] Thus, miRNAs and siRNAs have the potential to be exploited for cancer treatment (**Figure 7**). ONPATTRO (Patisiran) is an siRNA therapy for the rare polyneuropathy caused by transthyretin-mediated amyloidosis that first received FDA approval in August 2018.[61] Over 20 siRNA-based treatments are currently in clinical trials and although no miRNA drugs have yet been approved, biotechnology companies, including Miragen, Synlogic, and Regulus Therapeutics are currently reaching miRNA-related drugs.

Unfortunately, siRNAs and miRNAs have several limitations that should be addressed before being used as therapeutics, such as low stability, unexpected immune responses via toll-like receptor (TLR) signaling, poor cell membrane penetration ability due to physical properties, and off-target gene-silencing-mediated toxicity. These limitations that hamper the full efficacy of nucleic acids in vivo have aroused interest in developing appropriate delivery tools for precise conveyance.[62] Therefore, studies are investigating the development of carriers, such as lipid nanoparticles.

Cell-derived exosomes show a high degree of biocompatibility, safety, and low immunogenicity, and are taken up by host cells via several methods, such as phagocytosis, micropinocytosis, endocytosis, and fusion.[48] Exosomal fusion with host cells can evade the endo-lysosomal pathway, enabling bioactive molecules within exosomes to be conveyed to the cytoplasm without endosomal trapping.[63] Consequently, exosomes have attracted a considerable attention as carriers for therapeutic small RNA molecules. For instance, miRNA-134 delivered to breast cancer cells via exosomes was shown to inhibit the tumor cell proliferation and increase their sensitivity to anti-heat shock protein 90 (Hsp90) drugs.[64] Studies have also shown that loading exosomes with cell-cycle-related let-7a miRNA affects their therapeutic efficacy against breast cancer.[35] Engineered

exosomes loaded with let-7a and expressing GE11 or AS1411 can target epithelial growth factor receptor (EGFR) or nucleolin, respectively, which are both highly expressed on breast cancer cells, thereby achieving sufficient anti-tumorigenic effects when systemically administrated.[35,65] Taking the advantage that exosomes can penetrate the BBB,[66,67] studies have shown that exosomal miRNA-21 or miR146b had potent tumor-suppressive effects when delivered to glioma.[67] Moreover, MSC-derived exosomes containing miRNA-124a have been found to pass through the BBB and retard brain cancer cell propagation, thus improving overall survival.[39,40]

Similar strategies are also being implemented to treat cancer by delivering siRNA using exosomes. K-RAS mutations are the most common mutations in pancreatic, non-small cell lung, and colorectal cancers; however, they remain untargetable despite numerous efforts.[68] Kalluri et al. developed iExosome, an MSC-derived exosome loaded with K-RAS G12D-specific siRNA, which showed therapeutic efficacy against pancreatic cancer. Due to CD47 expression on their surface, the iExosomes could avoid host immune clearance when circulating in the blood, while RAS-mediated macropinocytosis enhancement accelerated exosomal uptake by pancreatic cells to improve siRNA delivery.[23] Similarly, exosomes loaded with vascular endothelial growth factor siRNA have been shown to prevent brain tumor angiogenesis and exhibit anti-tumor effects.[38] Likewise, GRP78 is highly expressed on sorafenib-resistant cancer cells; therefore, it has been found that siGRP78-loaded exosomes are capable of overcoming resistance to sorafenib treatment.[69]

The advent of the novel gene-editing method CRISPR-Cas9 has considerably altered in the field of gene therapy[70] and has produced unprecedented results; however, current CRISPR-Cas9 approaches remain challenging in vivo, particularly in clinical settings.[71] Kim et al. reported that exosomes derived from ovarian cancer cells could target ovarian tumors more effectively than those derived from normal epithelial cells. Exosomes derived from SKOV3 ovarian cancer cells were engineered by electroporation to carry CRISPR/Cas9 targeting poly (ADP-ribose) polymerase-1 (PARP-1). Systemic treatment with the engineered exosomes successfully targeted PARP-1 and reduced its expression in tumor tissue, thereby significantly reducing tumor growth.[72] Another approach involved the exosome-liposome hybridization for functional modification and effective delivery to improve their ability to encapsulate large nucleic acids. Compared to liposomes or exosomes alone, these particles were able to effectively convey CRISPR-Cas9 genes to MSCs.[73] Collectively, these findings suggest that exosomes could be attractive candidates as gene therapeutic agent carriers for treating cancer.

3.2. Exosomal Delivery of Membrane-Associated Proteins

Membranes protect the internal components of living cells from their exterior and are an integral part of the cellular environment. To maintain cellular homeostasis, vital molecules, nutrients, and metabolic waste products pass through cell membrane via passive (diffusion and osmosis) or active (endocytosis, exocytosis, and ion channels) transport. In addition, membrane proteins play key roles in intermembrane survival signaling by regulating various biological signaling processes.[74] Therefore, abnormal biological activities due to membrane defects cause various diseases, including cancer, infectious diseases, and genetic disorders.

Numerous studies have attempted to utilize membrane proteins as therapeutic agents due to their beneficial attributes; however, structure complexity and difficulty producing their hydrophobic regions can cause technical difficulties. The utilization of membrane proteins as therapeutic agents involves a solubilization step during the purification process which requires detergents that may destabilize, inactivate, or denature the membrane proteins.[75,76] Similarly, the highly hydrophobic amino acid content of membrane proteins causes difficulties when reproducing their structures.[77] It has been suggested that liposomes (lipid-based nanoparticles), or nanodiscs (high-density apolipoprotein particles) can be used to reconstitute membrane proteins and resolve these limitations.[76,78] However, it is difficult to accurately control their size, safety, and structure by optimizing the artificial nanoparticles, which requires a lot of time and effort.[76,78,79]

Exosomes can provide a proper environment for membrane proteins[80] and have the potential to carry them [81]; indeed membrane proteins can be naturally expressed on the exosome surface during exosome biogenesis, enabling them to retain their inherent stability and dynamics.[77] Exosomal membranes also facilitate the oligomerization or clustering of membrane proteins, which helps to maintain their original structures and functionalities.[82] Thus, exosomes could be used to maximize the biological activities of membrane proteins in addition to simply acting as a carrier.

Recent developments in genomic engineering methods have enabled functional membrane proteins to be expressed on exosomal surfaces; thus, exosome surface display modification has emerged as a state-of-the-art technique for utilizing membrane proteins as therapeutic agents.[83] For instance, exosomes with signal-regulatory protein alpha (SIRPα) or hyaluronidase PH20 on their membrane can potentiate anti-tumor responses by enhancing immunity against cancer[84–87] (**Figure 8**). Besides, exosomes highly expressed with the co-stimulatory molecules and the major histocompatibility complex (MHC) peptide, like the antigen-presenting cell (APCs), are demonstrated to activate immune cells, including T cells.[88] Codiak Biosciences (Cambridge, MA, USA) has induced the overexpression of the glycoprotein PTGFRN in producer cells to increase exosomal PTGFRN expression by a 150-fold. Thus, they were able to develop a variety of membrane-based drugs by genetically fusing proteins to the N terminal of PTGFRN and demonstrated the therapeutic efficacy of each candidate drug. Consequently, the PTGFRN-mediated display of bioactive molecules at a high density on the exosome surface can enable the production of potent exosomes to treat various diseases.[89] The results mentioned above suggest that exosomes that enable membrane proteins to function properly can be promising options for delivering therapeutic membrane proteins.

3.3. Exosomal Delivery of Chemotherapeutics

Chemotherapeutics, such as doxorubicin and paclitaxel, have traditionally been used to target the characteristics of rapidly

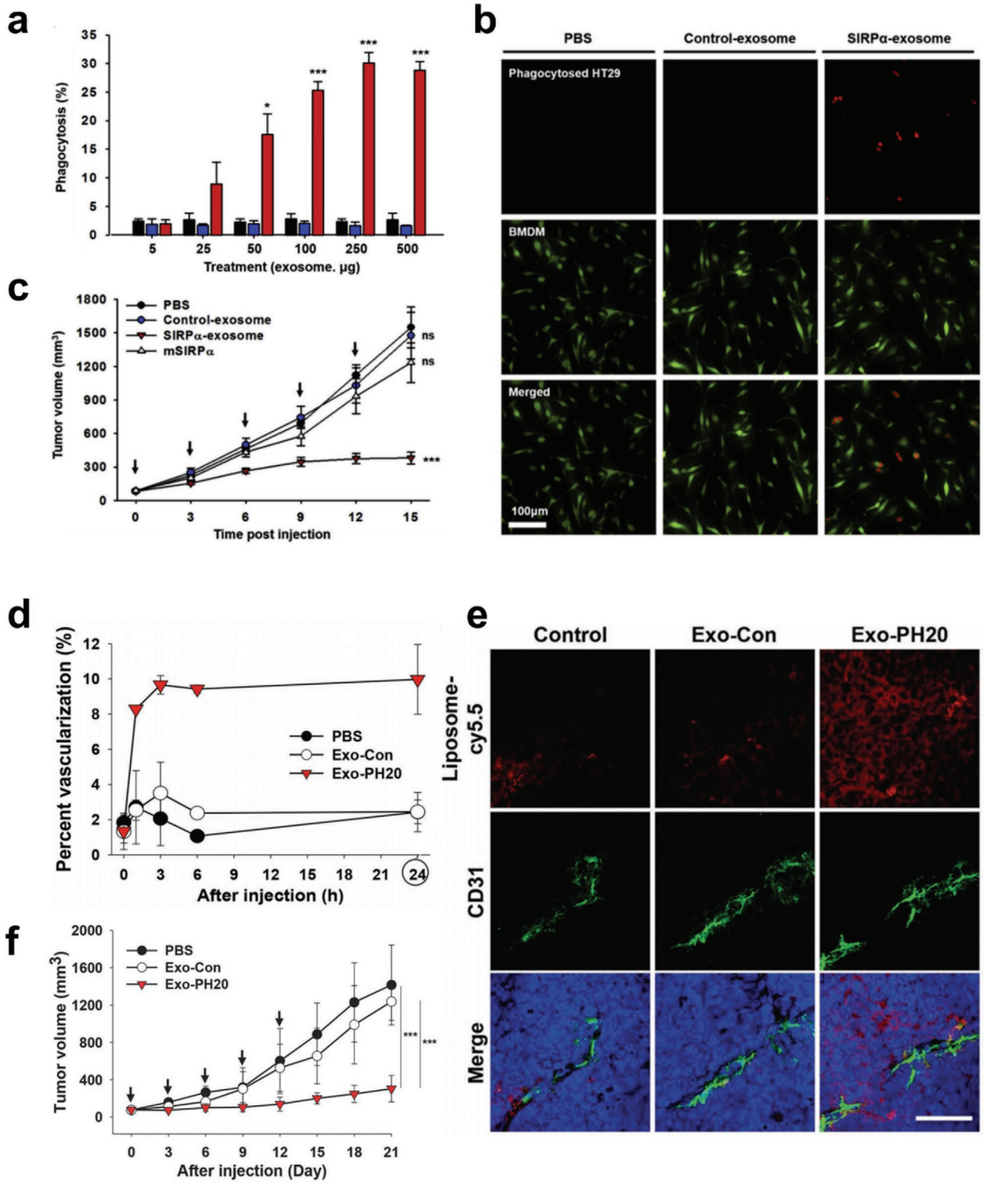

Figure 8. Exosomes expressing membrane-associated proteins with anti-tumor activity. a) SIRPα exosomes enhanced the phagocytosis of HT29 cancer cells by bone marrow-derived macrophages (BMDMs). b) Fluorescence microscopy images of HT29 cancer cell phagocytosis by BMDMs. c) Tumor growth suppressive effects of SIRPα exosome treatment in a CT26.CL25 tumor model. Reproduced with permission.[84] Copyright 2017, Elsevier. d) Percentage vascularization in PC3 tumor-bearing mice imaged by high-resolution ultrasound after treatment with PH20 exosomes (Exo-PH20), control exosomes (Exo-Con), and PBS. e) Intratumoral distribution of liposomes-Cy5.5 (red) indicating the activity of PH20 exosomes in the tumor tissue of PC3 tumor models. Blood vessels were stained with CD31 antibodies (green). f) Anti-tumor effects of PH20 exosomes in PC3 tumor models. Reproduced with permission.[86] Copyright 2017, Wiley-VCH.

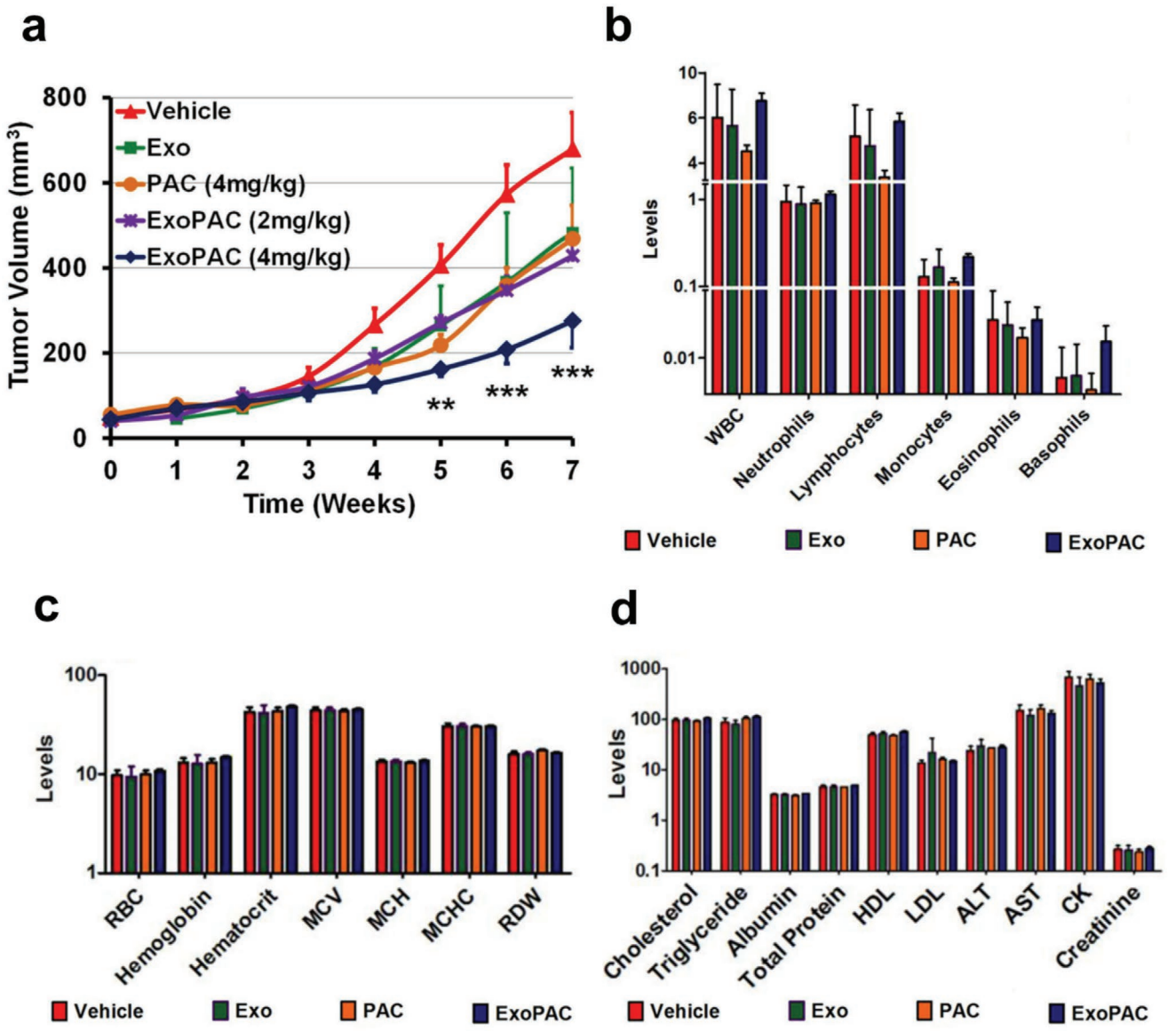

Figure 9. Efficacy of exosomes harboring chemotherapeutic agents for cancer therapy. a) Enhanced therapeutic efficacy of paclitaxel-loaded milk-derived exosomes. PAC, paclitaxel. Systemic toxicity profiles of paclitaxel-loaded milk-derived exosomes in mice against b) leukocytes, c) erythrocytes, and d) liver and kidney function enzymes. Reproduced with permission.[229] Copyright 2017, Elsevier.

dividing cancer cells[90]; however, cell proliferation is not a unique feature of cancer cells. The unwanted death of normal cells is therefore inevitable and is often accompanied by systemic toxicity, limiting the maximum dosage of therapeutic agents that can be used to treat cancer.[91,92] Furthermore, low bioavailability often restricts outcomes and prolonged chemotherapy may cause continuous genetic mutations in cancer cells that promote resistance and yield disappointing results.[92,93]

Recent studies have shown that exosomes can be used as carriers for chemotherapeutic drugs (**Figure 9**), and several methods have been used to load these drugs into exosomes. Exogenous methods using co-incubation, electroporation, or sonication can efficiently load chemotherapeutic agents into exosomes for cancer therapy,[59] while endogenous approaches reliant on cellular machinery enables drugs to be spontaneously encapsulated via sequential procedures from cellular isolation to incubation with chemotherapeutic reagents. For instance, doxorubicin-loaded exosomes were reported to reduce cardiotoxicity, the main side effect of doxorubicin, by 40% and exert considerable anti-cancer effects in vivo and in vitro.[94] Similarly, Akhil et al. loaded exosomes with nanosomes containing doxorubicin-conjugated gold nanoparticles to promote the active accumulation of doxorubicin in tumor tissue, finding that the nanosomes displayed preferential cytotoxicity on cancer cells and minimal activity against non-cancerous cells.[95] Moreover, paclitaxel containing exosomes have been shown to effectively cause cell death in LNCaP and PC-3 prostate cancer, A549 lung cancer, SKOV3 ovarian cancer, and MDA-hyb1 breast cancer cells.[96,97]

Since the components displayed on exosome surfaces can affect their biodistribution, several strategies have been developed to increase the tumor-targeting efficiency of the chemotherapeutics using exosomes: 1) iRGD (peptide) targeting αv-integrin expressing breast cancer, 2) hyaluronic acid grafted with 3-(diethylamino) propylamine targeting $CD44^+$ tumor cells at low pH (pH 6.5), and 3) brain tumor-targeting methods based on the ability of exosomes to penetrate physical barriers.[98] Moreover, the cellular uptake of paclitaxel-loaded exosomes has been reported to induce 50-fold higher levels of apoptosis than free paclitaxel in MDCKMDR1 (Pgp^+) cancer cells that initially displayed paclitaxel resistance.[99] Thus, exosomes have the potential to act as a clinical platform for the delivery of chemotherapeutics to treat cancer.

4. Exosomes for Cancer Immunotherapy

Cancer immunotherapy is a novel anti-cancer strategy that activates immune cells, unlike conventional anti-cancer treatments which primarily target tumors themselves, and has gained considerable attention from researchers and clinicians as a third form of cancer therapy after chemotherapy and targeted therapy. Promising clinical responses have been observed in terminally ill cancer patients with no other therapeutic options, providing the basis for several ongoing clinical trials of various novel cancer immunotherapies.[5,100–102] This movement has been led by researchers such as James P. Allison and Tasuku Honjo, who discovered the roles of cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed cell death protein 1 (PD-1) in cancer immunotherapy, respectively. However, the clinical effects of this remarkable approach are only valid in 20–30% of all cancers.[6,7]

To achieve successful and continuous anti-tumorigenic immune responses, the sequential procedures via which immune cells fight cancer cells must be initiated and expanded iteratively. In 2013, Chen and Mellman first introduced the cancer immunity cycle detailing the stepwise processes required to appropriately eradicate cancer cells, which are primarily initiated by the release of cancer antigens (**Figure 10**).[103] The first step involves cancer cell death causing the release of tumor-specific immunogenic antigens (or neoantigens) which are later recognized as an adversary by APCs, such as DCs or macrophages, which present the antigens. To produce potent tumor-specific T cell clones, this must be accompanied by signals that trigger the immunogenicity of the tumor antigens in APCs. DCs migrate to the lymph nodes where they mature, allowing them to process and present the captured antigens on MHC-I and/or MHC-II, and enabling T cell priming and activation into effector T cells, which can actively respond to cancer-specific antigens. The degree of the immune response is mainly determined by the success of this step as it determines the ratio of effector and regulatory T cells. Effector T cells later act as cytotoxic T cells that migrate to and infiltrate tumor tissue and recognize tumor cells via interactions between T cell receptors (TCRs) and cognate antigens bound to MHC-I. The cytotoxic T cells then kill cancer cells which release more tumor-associated antigens, providing a cue for additional immune responses. Ultimately, the whole cancer immunity cycle reiterates from the very first step, resulting in amplified and continuous immune responses.[103]

Unfortunately, cancer cells develop diverse mechanisms to evade each step of this cycle and evade attack from immune cells;[104] therefore, anti-cancer immunotherapy strategies that target the single steps are bound to suffer from limited efficacy. Consequently, researchers have attempted to evoke adequate immune effects by targeting multiple steps simultaneously. Recently, numerous studies have demonstrated that exosomes can effectively initiate each step of the cancer immunity cycle. For example, exosomes have been shown to act as therapeutic cell-free vaccines to induce sufficient anti-tumor immune responses.[8] In addition, exosomes offer a favorable environment for bioactive molecules to function properly in tumors, thereby producing substantial tumor-suppressive effects.[77] Since these features support exosomes as a promising option for cancer immunotherapy, this review examines the use of exosomes as the anti-cancer therapeutics against different steps of the cancer immunity cycle.

4.1. Tumor Antigen Source

Cancer formation is the result of a complex multistage process influenced by a variety of factors, predominantly epigenetic or genetic mutations produced by external/intrinsic stimuli which can initiate cancer and promote its malignization.[105] Cancer genome sequencing has established that somatic mutations are present in all cancers.[106] Even during initiation and growth, cancer cells acquire genetic (driver mutation) or cellular (passenger mutation) transformations due to genomic probability to protein-altering mutations.[107] Ironically, these genetic variants excessively increase the production of specific proteins that are then perceived as non-self-antigens by immune monitoring systems.[108]

Innate immune cells, such as macrophages and DCs, can initiate anti-cancer immunity by taking up cancer cell-derived antigens, including cancer-specific neoantigens.[109] In the early 20th century, several research groups observed that, following the surgical removal of carcinogen-induced tumor tissue from mice, the cancer was not re-formed when identical tumor cells were challenged.[110,111] The development of molecular biology techniques later revealed that proteins formed by specific genomic mutations in cancer acted as neoantigens to activate cytotoxic T cells.[112] Knuth, Old, and Rosenberg classified the clones within the peripheral blood and tumor of a patient via the genomic analysis of T cells and identified T cell clones that were reactive to tumor cells but not to normal cells.[113] Their findings suggested that tumor-specific antigens or tumor-associated antigens exist not only in mice but also in humans. In 1991, a cancer vaccine strategy was developed using human tumor antigen (MAGEA1), which is overexpressed in melanoma, followed by numerous antigen targets discovered for each cancer.[114]

For decades, clinical trials have attempted to treat numerous cancers by utilizing the potential anti-cancer immune efficacy of tumor antigens. The most common strategy involves the external specification and generation of cancer-specific antigens that are injected back into the patient's lymphatic system

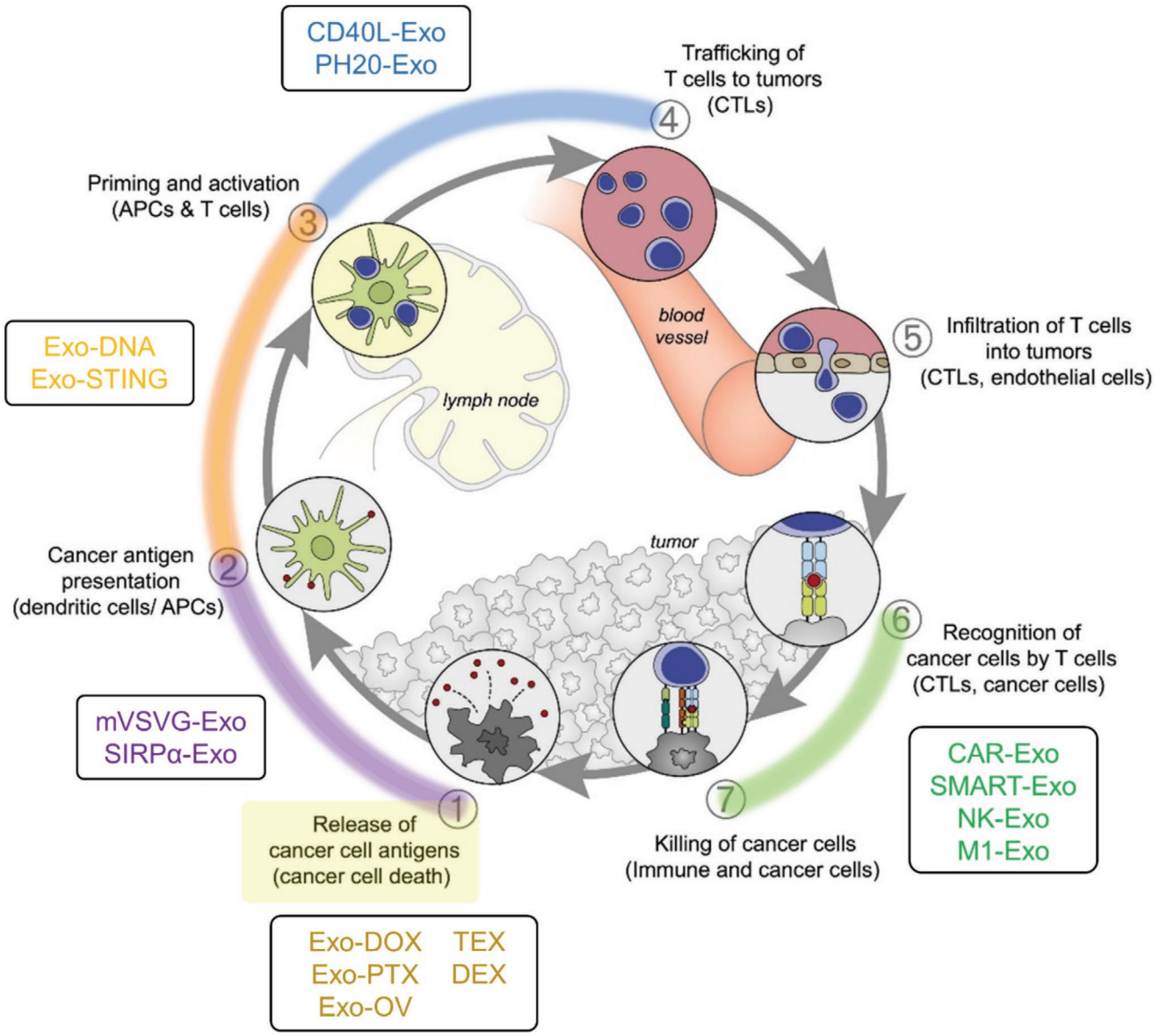

Figure 10. Cancer immunity cycle. The multistep process for generating anti-cancer immunity includes: (1) cancer antigen release from dying cancer cells, (2) cancer antigen presentation by APCs, (3) priming and activation of T cells by APCs in lymph node, (4) T cell trafficking to tumor microenvironment, (5) T cell infiltration into tumors, (6) cancer cell recognition by T cells, and (7) killing of target cancer cells by T cells. Various engineered exosomes can trigger each step of the cancer immunity cycle. Exo-DOX, exosome containing doxorubicin; Exo-PTX, paclitaxel-loaded exosome; Exo-OV, oncolytic virus-loaded exosome; TEX, tumor-derived exosome; DEX, dendritic cell-derived exosome; mVSVG-Exo, mVSVG protein expressing exosome; SIRPα-Exo, SIRPα expressing exosome; Exo-DNA, DNA-loaded exosome; Exo-STING, STING-loaded exosome; CD40L-Exo, CD40L expressing exosome; PH20-Exo, PH20 expressing exosome; CAR-Exo, CAR-T cell-derived exosome; SMART-Exo, synthetic multivalent antibodies expressing exosome; NK-Exo, NK cell-derived exosome; M1-Exo, M1-like macrophage-derived exosome. Adapted with permission.[257] Copyright 2019, Elsevier.

to boost their anti-cancer immune response.[115] However, the identification of a specific antigen that is present only in cancer cell is time-consuming and the likelihood of effectively inducing T cell-mediated immunity in vivo is relatively low.[116] Recent advances in mass spectroscopy, computation, and bioinformatics have made it possible to identify immunogenic neoantigens within weeks. These methods can selectively filter-specific antigens that exist only in tumor cells and display a high binding affinity with HLA class 1 or 2, thus increasing the likelihood of inducing T cell immunity when patients are vaccinated.[102] These technological advances have resulted in clinical trials for personal neoantigen vaccines in melanoma patients with a high mutational burden. Peripheral blood mononuclear cells and melanoma tissue were collected from each patient, evaluated by whole-exome sequencing to confirm immunogenic neoantigens, and then long synthetic peptides reflecting the specified tumor antigens were injected along with Poly-ICLC. Vaccination with neoantigens and adjuvants eventually induced successful $CD8^+$ and $CD4^+$ T cell-mediated immune responses against cancer and improved survival;[117]

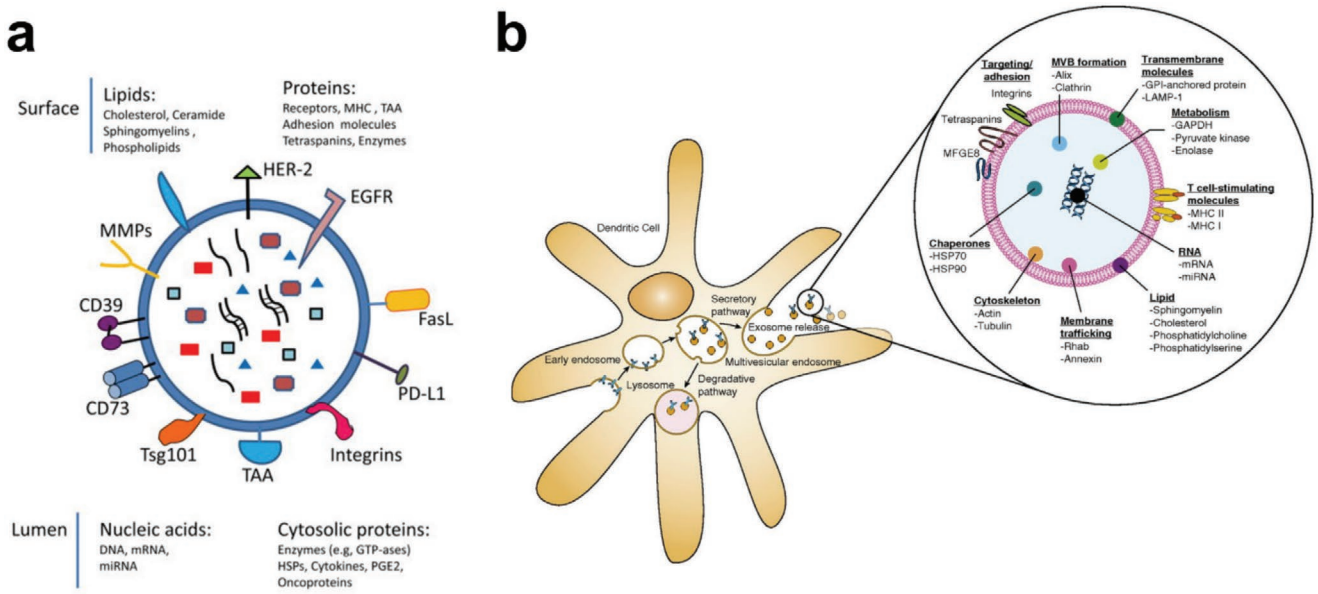

Figure 11. Exosomes derived from tumor cells and dendritic cells. a) Schematic diagram of exosomal contents derived from tumor cells Reproduced with permission.[261] Copyright 2016, Elsevier. b) Schematic diagram of exosomal contents derived from dendritic cells. Reproduced with permission.[262] Copyright 2014, American Association of Immunologists. Diagrams indicate the presence of various molecules on the exosomal membrane surface (lipids and proteins) and in the vesicle lumen (nucleic acids and proteins).

however, this approach has limited efficacy as it is exceptionally costly, labor intensive, and can only detect a small portion of the immunogenic antigens produced from cancer tissues.

In addition to utilizing proteins or peptides as cancer vaccination agents, nucleic acids, such as DNA and RNA are also being tested as these approaches can deliver multiple antigens with ease. Furthermore, dsDNA can activate the cGAS-STING pathways in the innate immune cells to augment the functions of APCs.[118] Consequently, DNA or RNA was directly injected into lymph node cells to generate immunogenic antigens and was found to not only effectively induce T cell immune responses in clinical patients but also significantly reduce the incidence of metastatic cancer.[119] Indeed, total cancer eradication was observed following co-treatment with PD-1 blockade.[120] However, RNA and DNA vaccines are poorly immunogenic, require specific transportation and storage conditions, have limited therapeutic efficacy, and are only effective against the tumor at a high mutational load. Therefore, the alternative breakthrough is required to overcome the problems of conventional vaccine strategies.

4.1.1. Tumor-Derived Exosome

Tumor cells are known to actively emit more exosomes into plasma and body fluids than normal cells.[121] TEXs affect surrounding cells and even organs at a considerable distance and contain various antigens possessed by the original cancer cells; thus, they have the potential to act as an antigen source for cancer vaccines (**Figure 11**). In 2001, Zitvogel et al. first reported that TEXs effectively induce cancer-specific T cell immunity.[122] Surprisingly, TEXs have demonstrated better cancer vaccination effects than tumor cell lysates, with several studies showing that TEXs are a source of tumor antigens that can be taken up and processed by DCs to induce active anti-cancer immune responses.[123]

TEXs can also directly convert innate immune cells, such as DCs or macrophages, into pro-inflammatory cells. Innate immune cells exposed to TEXs at tumor-draining lymph nodes have been found to increase the production of pro-inflammatory cytokines (i.e., IL-6, IL-12, and interferon (IFN)-γ), and reduce the production of anti-inflammatory cytokines (i.e., IL-10).[124] Cancer vaccines generally involve the application of both tumor neoantigens and adjuvants to enhance antigen processing; therefore, TEXs could be a potent cancer vaccine strategy as they act as both an antigen source and immune adjuvant. Consequently, clinical trials have attempted to use TEXs from malignant ascites to cure malignant pleural effusion [NCT02657460, NCT01854866].

Despite exerting remarkable vaccination effects, TEXs also display pro-tumorigenic characteristics as they resemble their original cancer cells; the role of TEXs in cancer progression is heterogeneous and highly dependent on cancer type, genomic characteristics, and stage. Exosomes derived from human pancreatic cancer cells have been reported to cause mutations in normal NIH/3T3 cells that initiate cancer cell transformation,[125] while TEXs from hypoxic GBM cells have been shown to accelerate endothelial cells' pro-angiogenesis in the brain tumor microenvironment.[126] Furthermore, TEXs from pancreatic cancer have been found to inhibit complement-mediated cancer cell lysis to support cancer growth.[127]

Various studies have demonstrated that the unique characteristics of TEXs that affect cancer metastasis are directly related to patient mortality. The expression patterns of integrins on TEXs have been found to differ depending on the metastatic potential of the tumor and play an essential role

in organotropic metastasis. Indeed, research has shown that ITG$\alpha v\beta 5$ expressed on TEXs from pancreatic cancer selectively binds to Kupffer cells and mediates liver metastasis, whereas ITG$\alpha 6\beta 4$ and ITG$\alpha 6\beta 1$ on TEXs from breast cancer formed a pre-metastatic niche by binding to lung fibroblasts and epithelial cells to cause lung metastasis.[128] Moreover, gastric cancer-derived TEXs expressing EGFR can integrate the membrane of Kupffer and hepatic stellate cells and foster a pre-metastatic liver microenvironment by activating hepatocyte growth factor signaling.[129] Webber et al. showed that TEXs expressing transforming growth factor-β (TGF-β) affect fibroblasts at the metastatic site by increasing α-smooth muscle actin and fibroblast growth factor 2 expression to induce microenvironmental remodeling.[130] Furthermore, exosomes from pancreatic cancer and melanoma can recruit bone marrow-derived macrophages related to metastasis,[131] while ovarian cancer cell-derived TEXs bear matrix metalloprotease 1 and increase the peritoneal dissemination of tumor cells.[132] Conversely, non- or pre-metastatic melanoma-derived TEXs expressing pigment epithelium-derived factor have been shown to promote the differentiation of bone marrow monocyte precursors to Ly6C low patrolling monocytes. These cells actively recruited NK cells and TRAIL-positive tumor-reactive macrophages to induce the immunogenic clearance of metastatic cancer cells at the pre-metastatic niche.[133]

Since TEXs contain bioactive molecules such as nucleic acids or signaling proteins, they may mediate neoplasia formation and modulate the surrounding tumor environment. TEXs derived from breast and prostate cancers contain a variety of miRNAs that are involved in neoplasia and tumor metastasis.[134,135] For instance, prostate cancer-derived TEXs have been found to contain miR-125b, miR-130, miR-155, H-RAS, and K-RAS mRNAs, which can affect the neoplasia reprogramming of tumor tropic adipose stem cells.[135] TEXs derived from metastatic breast cancer cells containing miR-200 can promote metastasis by stimulating the epithelial to mesenchymal transition of cancer cells,[136] while breast cancer-derived TEXs harboring miR-122 can hamper glucose uptake in pre-metastatic niche cells to facilitate breast cancer metastasis.[137] TEXs containing miR-105 have been shown to suppress endothelial tight junction ZO-1 (zonular occludens 1) expression and damage the integrity of normal blood vessels, thereby increased vascular permeability to promote active metastasis.[138]

The bioactive molecules within TEXs can also affect various immune cells in the tumor microenvironment. For instance, miR-212-3p in pancreatic cancer-derived TEXs downregulates regulatory factor X-associated protein and MHC-II transcription factor in DCs to increase cancer growth.[139] miR-222-3p in epithelial ovarian cancer-derived TEXs converts monocytes into M2 macrophages by downregulating SOC3 expression and activating the STAT3 signaling pathway.[140] In addition, exosomal miR-21 and miR-29a have been shown to activate nuclear factor-κB signaling in macrophages, resulting in the release of pro-metastatic cytokines.[141] Chalmin et al. showed that exosomal Hsp72 activates STAT3 signaling to expedite the immunosuppressive activity of myeloid-derived suppressor cells.[142] Furthermore, Gabrusiewicz et al. demonstrated that TEXs derived from GBM stem cells contain molecules that can simultaneously activate the STAT3 pathway, promote the differentiation of monocytes into M2 macrophages, and enhance PD-L1 expression.[143]

The immune regulatory molecules on the surface of TEXs have also been shown to modulate the immune response of the tumor environment. For example, TEXs derived from melanoma express PD-L1, which blocks DC maturation and migration to limit the anti-tumor activity of $CD8^+$ T cells.[144] $PD\text{-}L1^+$ TEXs secreted from cancer cells have also been shown to accelerate T cell exhaustion at the draining lymph node of tumor-bearing mice and reduce the efficacy of PD1:PD-L1 blockade.[145] In addition, melanoma or prostate cancer-derived TEXs expressing Fas ligand (CD95L or CD178), a well-known immune regulatory molecule, were shown to cause T cell apoptosis.[146] Moreover, TEXs expressing CD39 (ectonucleoside triphosphate diphosphohydrolase 1) and/or CD73 (5′ nucleotidase), which converts ATP to ADP (adenosine), were found to limit T and B cell immunity.[147]

Several recent studies have reported that TEXs can induce resistance to anti-cancer therapeutic strategy of antibodies specifically targeting overexpressed tumor antigens. Rituximab, which targets CD20 overexpressed in B cell lymphoma, and herceptin, which targets HER2 expressed on breast cancer cells, are typical therapeutic agents that can induce ADCC.[148] However, since TEXs partly represent the original characteristics of their parental cells, the secretion of $CD20^+$ TEXs from B cell lymphoma and $HER2^+$ TEXs from $HER2^+$ breast cancer may also neutralize antibodies against cancer cells.[149] Qu et al. observed that long non-coding RNA in TEXs from renal cell carcinoma competitively blocked miR-34 and miR-449 binding to their targets in the cancer cells, thereby conferring sunitinib resistance.[150]

Considerable efforts have been made to investigate the properties of TEXs as tumor antigen sources and immune adjuvants; however, several reports have suggested that TEXs also play pro-tumorigenic roles. The complex functions of TEXs must be considered thoroughly depending on their context, and in-depth research into TEX biogenesis and characterization will broaden their utility by allowing TEXs to be optimized to alleviate their disadvantageous features and reinforce their strengths.

4.1.2. Dendritic Cell-Derived Exosome

TEXs are thought of as a double-edged sword as they contain both cancer neoantigens to initiate anti-cancer immunity and factors that can expedite cancer progression. To solve this problem, several reports have used exosomes derived from tumor antigen-exposed DCs (dendritic cell-derived exosome [DEXs]; Figure 11) that can evade the pro-tumorigenic effects of TEX while acting as an efficient source of immunogenic antigens. Such an approach could successfully evoke anti-tumor immunity and DEXs have been found to be sufficient to arouse anti-tumor response.

DEXs express MHC-I and MHC-II bound antigen peptides along with ICAMs, adhesion molecules, integrins, docking molecules, and co-stimulatory signals such as CD40, CD80, and CD86,[151] indicating that DEXs could represent the functionality of DCs. Indeed, Zitvogel et al. demonstrated that a single intradermal injection of DEXs was able to hinder tumor

growth.[152] It has also been reported that DEXs can activate $CD4^+$ T cell immunity and result in Th1 and Th2 immune responses, irrespective of the maturity of the original human DCs.[153] Furthermore, DEXs have been shown to evoke antigen-specific T cell immunity more efficiently than microvesicles from DCs.[154]

Recently, two phase I clinical trials have investigated whether DEXs can generate anti-cancer effects by T cell priming in a clinical setting. First-generation DEXs isolated from DCs exposed to autologous tumor-associated antigens were enough to activate MHC-1-independent cancer cell cytotoxicity, whereas not to effectually induce T cell immunity.[155,156] Unlike APCs, DEXs displayed a limited ability to directly induce T cell priming in vivo[157]; however, additional experiments revealed that DEXs could provoke a NK cell immune response by stimulating IL-15/IL-15Rα and NKG2D ligand expression on NK cells.[158]

Second-generation DEXs were later acquired from DCs challenged by IFN-γ and melanoma antigen recognized by T cells 1 (MALT-1) peptides[122] and intradermally injected into IIIB/IV non-small cell lung cancer patients four times with an interval of 7 days. These DEXs were able to induce anti-tumor responses depending on DC maturation, with disease stabilization observed over 4 months in 7 of the 22 patients, other than one severe case of liver toxicity.[151] Taken together, these results suggest that DEXs can reduce tumor growth in end-stage cancer patients by generating anti-tumor immune responses.

4.2. Exposing Innate Immune Cells to Danger Signals

To activate the early stages of the cancer immunity cycle, cancer antigens must be released and signals that activate innate immune cells are required. Unlike viruses or external pathogens, cancer cannot easily be surveilled by immune cells as cancer cells secrete or express factors that establish local and systemic immunosuppressive surroundings and critically influence clinical success. The expression of PD-L1 on the surface of cancer cells has been shown to cause $CD8^+$ T and NK cell exhaustion.[159] The cancer cells also express ecto-5′-nucleotidase (NT5E, also known as CD73) that turns extracellular ATP, which generally exerts chemotactic effects for DC infiltration, into immunosuppressive ADP that hampers the induction of immunity against cancer.[160] Immunosuppressive environments are also known to form around tumors to undermine anti-cancer immunity. For instance, immunosuppressive immune cells or cancer-associated fibroblasts secrete immunosuppressive cytokines such as IL-10 and TGF-β into tumor microenvironment to silence immune responses.[161]

In general, apoptotic cells are known to be intrinsically tolerogenic and cannot induce dying cell-specific immunity.[162] Like normal cells, most cancer cells undergo tolerogenic cell death which suppresses the surrounding immune response rather than activating it[163]; therefore, the signals produced by cancer cells must be altered so that immune cells can sensitively detect them. Chemotherapeutics, such as doxorubicin, mitoxantrone, oxaliplatin, cyclophosphamide, and other anti-cancer treatments, including photodynamic therapy, oncolytic virus, and conventional radiotherapy, have been shown to induce the "immunogenic cell death (ICD)" rather than tolerogenic cell death.[164] These treatments can induce endoplasmic reticulum (ER) stress in cancer cells, leading to ICD and exposure to or the secretion of danger signals, known as danger-associated molecular patterns (DAMPs).[165] DAMPs consist of calreticulin (CRT), an "eat me signal" that enhances cancer cell phagocytosis[166,167]; ATP, "find me signal" that recruits phagocytes into the tumor microenvironment[168]; and HMGB-1 or Type 1 IFN, which are the "activating signals" that promote the processing of tumor antigens in phagocytes.[169,170] Furthermore, the ICD of cancer cells can effectively activate innate immune cells, particularly DCs, which in turn leads to acquired T cell immunity.

4.2.1. Exosomal Delivery of ICD Inducers

The efficacy of ICD inducers has been experimentally confirmed by directly injecting the inducers into tumors[166,168,169]; however, intratumoral injection is clinically challenging so most ICD inducers are administered systemically, which can cause toxicity against normal cells, including immune cells. Despite several clinical trials of ICD inducers, their ability to elicit anti-cancer immunity remains controversial.[49] To overcome these limitations, attempts are being made to load ICD inducers into nanoparticles for efficient delivery to the tumor site.

Exosomes are promising candidates for evoking anti-tumorigenic responses by conveying ICD inducers to the tumor site. For instance, Toffoli et al. showed that doxorubicin-loaded exosomes could alter the biodistribution of free doxorubicin to reduce its toxicity and produce sufficient anti-tumor responses.[34,94] The first-line chemotherapeutic paclitaxel is a well-known ICD inducer that can induce CRT exposure,[171] and paclitaxel-loaded MSC-derived exosomes have been shown to efficiently target both primary and metastatic tumors in a highly metastatic MDA-hyb1 breast tumor model.[97] In addition, oncolytic viruses loaded onto exosomes can be preferentially delivered to the tumor site due to the inherited cancer-homing nature of exosomes, causing cancer cell deaths.[172]

4.2.2. Reinforcement of Enemy Signals by Fusogenic Exosomes

Until recently, cell surface membrane proteins could not be edited and diseases caused by membrane-protein deficiency constituted a medical blind spot without proper treatments.[173] Yang et al. reported a de novo exosome-based membrane-editing technique focusing on the fact that exosomes mimic the membrane structures of their parent cells, allowing them to fuse to the recipient cell membrane. The membrane of the engineered exosomes displayed improved fusion efficiency under specific conditions, enabling desired membrane proteins to be inserted into the target cell membrane (**Figure 12**).[53]

Fusogenic exosomes can deliver viral proteins to the surfaces of cancer cell membranes and thus expose immune cells to danger signals. Kim et al. developed a mutant vesicular stomatitis virus G protein exosome (mVSVG-Exo) that can fuse to cancer cell membranes at pH 6.4–8.[174] Since VSVG is the G protein of the vesicular stomatitis virus and is a known TLR-4 agonist, their study demonstrated that exosomes expressing this

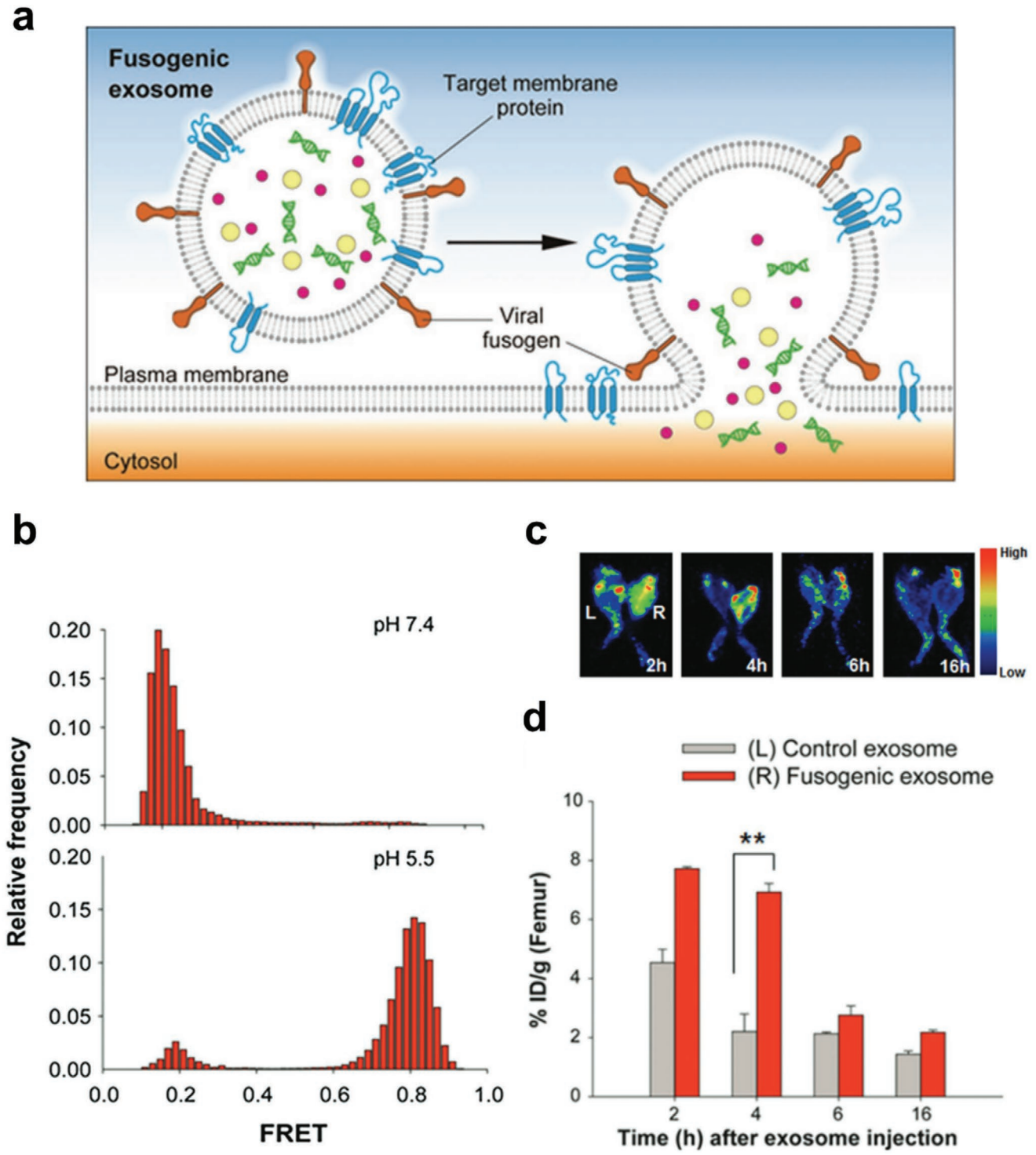

Figure 12. Membrane engineered fusogenic exosomes transferred membrane proteins to recipient cells by fusion. a) Representative schematic illustrations of fusogenic exosome platform. b) Normalized FRET efficiency distribution of the fusogenic exosomes measured at different pH conditions (upper, pH 7.4; lower, pH 5.5). c) Representative illustrations of PET scan indicated the ability of glucose uptake as time changes. Enhancement in glucose uptake was observed at the femur muscles of BALB/c nude mice after fusogenic exosome treatment (right) compared to control exosome treatment (left). d) Accumulation of [18F] 2-fluoro-2deoxy-d-glucose (18F-FDG) in skeletal femur regions. Reproduced with permission.[53] Copyright 2017, Wiley-VCH.

protein could act as a danger signal upon binding to the TLR-4 receptor of immune cells. Moreover, immune cell pathogen recognition receptors were able to recognize the viral mVSVG presented by cancer cells and identify them as non-self, thus enhancing their phagocytosis. This xenogenization strategy strengthened the danger signals on the surface of cancer cells,

suggesting that effective anti-cancer immunity can be induced in a wide range of tumors with synergistic tumor suppression by immune checkpoint blockades.

4.3. Enhanced Tumor Antigen Uptake

Innate immune cells, such as macrophages and DCs, have a high probability of encountering cancer cells and can rapidly engulf them via phagocytic receptors, which is a prerequisite for eliciting anti-tumor immunity.[175] However, cancer cell phagocytosis can be suppressed via diverse resistance mechanisms at different stages of tumor progression.

Immune cell phagocytosis is mainly determined by the overall balance between "eat me" and "don't eat me" signals. Cancer cells overexpress "don't eat me" signals, such as CD47,[176] B2M (beta 2 microglobulin),[177] PD-L1,[178] and CD24[179] and can suppress their engulfment by interacting with SIRPα, leukocyte immunoglobulin-like receptor subfamily B member 1, PD-1, and sialic acid-binding Ig-like lectin 10, respectively, on innate immune cells. Thus, impaired cancer cell phagocytosis dampens the induction of tumor-specific immunity.

4.3.1. Suppression of "don't eat me" Signals by Engineered Exosomes

CD47 is a "don't eat me" signal that is expressed on tumor cells around threefold more than on normal cells.[180] Since the interaction between CD47 and SIRPα can act as a myeloid immune checkpoint, researchers have begun to utilize these molecules as a new form of cancer immunotherapy that controls tumor cell phagocytosis.[181] Therapeutics targeting the CD47:SIRPα axis, particularly anti-CD47 antibodies, have demonstrated remarkable tumor-suppressive effects in preclinical models by enhancing cancer cell phagocytosis. Notably, CD47 blockade was reported to enhance the cross-priming ability of DCs linking to T cell immune responses; thus CD47 blockade can induce the activation of both innate and adaptive immunity to promote effective anti-cancer immunity.[182] Clinical trials are currently underway to investigate the regulation of the CD47:SIRPα signaling axis against diverse tumor types, including hematological malignancies and solid cancers.[181]

Native SIRPα proteins on the cell membrane dimerize when they interact with CD47. Since exosomes can retain their parental cell membrane, exosomal SIRPα proteins can form membrane-spanning SIRPα clusters that enhance their binding affinity with CD47. Koh et al. designed SIRPα expressing exosomes that efficiently block the interaction between CD47 on cancer cells and SIRPα on phagocytes to reinforce the phagocytosis of various cancer cells. These SIRPα exosomes displayed remarkable anti-tumor effects and successfully induced adaptive T cell immune responses, resulting in $CD8^+$ T cell infiltration in the tumor.[84] Notably, the SIRPα exosomes were able to induce more effective anti-tumor immune responses than ferritin, a nanocage protein displaying 24 SIRPα molecules, indicating that exosomes maximize the therapeutic efficacy of membrane-associated proteins.[85]

4.4. Augmentation of T Cell Priming

Despite the uptake of tumor neoantigens by APCs, only around 1% of mutated proteins can evoke anticancer immunity.[183] Meanwhile, the tumor microenvironment hampers DC activation in multiple ways and reduces their ability to prime T cell,[184] yet the induction of an efficient anti-cancer immune response requires APC activation.[185] CTLA-4 antibodies, such as ipilimumab, a well-known immune checkpoint blockade, can block the interaction between CTLA-4, the primary negative regulator on T cells, and its APC ligands, such as B7.1 and B7.2 (CD80 and CD86), thus improving T cell priming.[186] Consequently, clinical trials have been carried out on the combination of CTLA-4 antibodies and PD1:PD-L1 blockades to enhance the responsiveness of pre-existing PD-1 blockades in clinical patients.[187] Thus, sufficient APC activation is required to elicit adequate tumor-specific T cell immunity.

4.4.1. Exosomal Activation of the cGAS-STING Pathway

Stimulator of IFN genes (STING) is an intracellular receptor within the ER of DCs whose activation is known to induce the release of type 1 IFN, upregulates DC cross-presentation and induces the secretion of various pro-inflammatory cytokines and chemokines for potent anti-cancer immunity.[188] The STING pathway can be actively potentiated by cyclic dinucleotides (CDNs), such as cyclic dimeric guanosine monophosphate (c-di-GMP), cyclic dimeric adenosine monophosphate (c-di-AMP), and cyclic GMP-AMP (cGAMP),[189] and by the production of cGAMP (a natural CDN) induced by cytosolic DNA binding to cyclic GMP-AMP synthase (cGAS).[190] Activated STING progresses from the ER to the Golgi apparatus where it recruits TANK-binding kinase 1 (TBK1) and the transcription factor IFN regulatory factor 3 (IRF3) to induce the release of type 1 IFNs, such as IFN-α and IFN-β,[191] which can accelerate cytotoxic T cell responses and type 1 T helper cell (Th1) responses.[192] STING-deficient mice cannot generate tumor-specific T cells and therefore exhibit accelerated tumor growth and resistance to cancer immunotherapy.[193] Consequently, numerous natural and synthetic STING agonists are currently under development or clinical trial to treat infectious diseases and cancers.[194]

For instance, Kitai et al. isolated exosomes from breast cancer cells treated with topotecan, a topoisomerase 1 inhibitor used for cancer therapy, confirming that the exosomes contained DNAs from the parental cells which could activate cGAS-STING signaling in DCs and promote the infiltration of $CD8^+$ T cells and anti-tumorigenic effects in tumor tissue.[195] Another study found that more exosomes containing EGFR, P-EGFR, and genomic DNA (exo-gDNA) were produced by $EGFR^+$ tumor cells treated with second-generation EGFR kinase inhibitors, with the TEXs harboring genomic DNA expected to activate the STING pathway in APCs.[196] In addition, when T cells are primed by direct interaction with DCs, they secrete exosomal genomic and mtDNAs that can be taken up by DCs to activate T cell immunity via type I IFN production due to the cGAS-STING signaling pathway.[197]

Recently, Codiak Biosciences (Cambridge, MA, USA) developed exoSTING overexpressing PTGFRN and loaded with a

CDN small-molecule STING agonist. exoSTING was shown to reinforce IFNβ production in APCs and elicit anti-tumor immunity, inducing tumor-suppressive effects in a B16F10 tumor-bearing mouse model. Thus, exosomes harboring STING agonists can support the amplification of the initial stages of the cancer immunity cycle to evoke tumor-specific immunity.[89]

4.4.2. Activation of DC Functions by Engineered Exosomes

CD40 ligand (CD40L; or CD154) is a co-stimulatory molecule commonly expressed by T cells that reinforces the functions of APCs by interacting with CD40. Wang et al. genetically modified Lewis lung tumor cells to overexpress CD40L and produce TEXs expressing the molecule, reporting that these CD40L expressing TEXs induced DC maturation in vivo and anti-cancer T cell immunity, thus improving vaccination efficacy against lung cancer.[198]

Recently, engineered exosomes expressing an enzyme that can degrade the extracellular matrix (ECM) of tumor tissues were reported to activate DCs and induce immunogenic responses. Hyaluronic acid (HA) is a major ECM component around tumor tissue that supports cancer progression and malignization.[199] The engineered exosomes expressed hyaluronidase (PH20), an HA degradation enzyme, and were able to degrade HA and enhance the infiltration of therapeutic agents and $CD8^+$ T cells into the tumor to reduce its growth.[86] The degradation of HA by PH20 exosomes produced low molecular weight HA that directly interacted with TLR-4 on DCs to increase their maturation and cross-presentation to induce cancer-specific T cell immune responses.[87]

4.5. Improved Tumor Susceptibility to Effector Immune Cells

The recognition of cancer cells by tumor-infiltrating effector immune cells is critical for successful cancer immunotherapy.[200] TCR on $CD8^+$ T cells can only recognize host antigens presented on MHC-I. Normal cells generally present self-antigens on MHC-I on their surface, whereas cancer cells reduce MHC-I expression to evade non-self-recognition by T cells. Mutation in B2M, the major component of MHC-I, can autonomously suppress MHC-I expression.[201] Genetic and epigenetic alterations in IFN-γ receptor signaling pathways, including JAK1, JAK2, and APLNR, can also inhibit MHC-I expression on the surface of cancer cells.[202] Furthermore, the immunosuppressive properties of the tumor microenvironment can reduce the infiltration of effector immune cells such as $CD8^+$ T cells and natural killer (NK) cells into tumors.[6]

In recent years, CAR-T therapy alongside immune checkpoint blockade has been noted as a successful anti-cancer treatment.[101] Indeed, the efficacy of CAR-T therapy against B cell lymphocytic leukemia is widely recognized and clinical trials have expanded to various other cancer types.[203] CAR-T involves external engineering to add CAR to T cells isolated from the blood of patients; thus, the approach is customizable for each patient. CAR-T injection has been reported to not only directly induce potent anti-cancer effect but also induce memory to suppress the recurrence and metastasis of cancer.[204]

The advantage of CAR-T therapy over general T cells is its MHC-I independence when recognizing tumor antigens and recombinant CAR can not only recognize tumor antigens but also stimulate T cell activation signaling. The extracellular domain of CAR is a single-chain variable antibody domain that enables the receptor to recognize cancer-specific antigens, while the hinge domain commonly consists of immunoglobulin superfamily members such as IgG of CD8 or CD28. The intracellular signal transduction region of CAR is generally composed of both the CD3ζ (CD3 zeta) chain of TCR and a CD28, 4-1BB (CD137), or OX40 (CD134) co-stimulatory signaling moiety that is necessary to elongate the duration of T cell proliferation and survival.[204] However, this region may also enable CAR-T to recognize cancer cells more easily, leading to inappropriate T cell proliferation and hyperactive immune responses that cause critical and lethal side effects such as cytokine storm.[205]

NK cells are the innate counterpart of $CD8^+$ T cells that can recognize and eradicate tumors regardless of MHC-I.[206] NK cells express inhibitory receptors that sense the missing "self" MHC class-I molecules on tumor cells, such as inhibitory killer Ig-like receptors (KIRs) and CD94/NKG2A heterodimers.[206] This lack of self-recognition due to the loss of MHC- I leads to NK cell-mediated cytotoxicity against escaped tumor cells. Recent clinical studies have found that the adoptive transfer of allogeneic NK cells is safe for treating both hematological malignancies and solid tumors but evoked poor anti-tumor responses.[207] Therefore, novel NK cell-based strategies are required with improved therapeutic efficacy.

4.5.1. Bridging Between Cancer Cells and T Cells by Engineered Exosomes

CAR-T therapy can induce rapid and consistent clinical responses; however it can also cause acute immune-related toxicities. In addition, CAR-T therapy is based on engineered T cells which may eventually become exhausted or undergo apoptosis due to the immunosuppressive tumor microenvironment. A recent study showed that CAR-T-derived exosomes (CAR exosomes) could be utilized as a cancer immunotherapy as they not only express CAR but also cytokine molecules that evoke significant anti-tumor effects. CAR exosomes were also found to have fewer side effects like cytokine release syndrome and lacked functional suppression by PD-L1.[208] These results suggest that exosomes could be an option for cell-free therapy as they retain the functions of conventional cell therapy such as CAR-T while reducing the drawbacks of cell therapy.

To effectively induce anti-cancer T cell functions, they must be activated and directed toward cancer cells. Cheng et al. developed SMART Exo, double targeting exosomes expressing synthetic multivalent antibodies that co-expressed two ligands recognizing EGFR on cancer cells and CD3 on T cells, respectively. These exosomes were able to crosslink EGFR-positive cancer cells and T cells to induce the death of cancer cells by T cells.[209]

Table 1. Clinical trials based on exosomes for cancer immunotherapies.

Source	Conditions	Usage	Exosomal cargo	Dose	Administration	Outcome	Patients	Phase	Recruitment Status	References
Human (ascites)	Colorectal cancer	Vaccination	—	100–500 μg of protein	Four subcutaneous immunizations at weekly intervals	Safe, well tolerated; tumor-specific anti-tumor CTL response in exosome and GM-CSF combination group	40	1	Completed	[267]
Human (DCs)	Non-small cell lung cancer	Tumor antigen delivery	MAGE tumor antigens	1.3×10^{13} MHC Class II molecules	Four doses of DEX at weekly intervals	Safe, well tolerated; four stable disease (where two had initial progression)	13	1	Completed	[268]
Human (DCs)	Non-small cell lung cancer	Tumor antigen delivery	MAGE tumor antigens	8.5×10^{11}–1.0×10^{13} MHC Class II molecules	Intradermal injections of DEX once a week during 4 consecutive weeks	32% with stable disease, primary	41	2	Completed	NCT01159288
Human (DCs)	Melanoma	Vaccination	—	4×10^{13} or 1.3×10^{13} MHC Class II molecules	Four exosome vaccinations intradermally and subcutaneously at 1 week intervals	Safe, well tolerated; two stable disease. One minor response, one partial response, one mixed response	15	1	Completed	[155]
Human (MSCs)	Metastatic pancreatic adenocarcinoma	siRNA delivery	siRNA against KrasG12D	Not available	Treatment on days 1, 4, and 10. Repeats every 14 days for up to three courses	Not available	28	1	Not yet recruiting	NCT03608631
Human (tumor cells)	Malignant pleural effusion, malignant ascites	Drug delivery	Chemotherapeutics	Not available	Perfused to the pleural or peritoneal cavity of patients with four times per week	Not available	30	2	Unknown	NCT01854866
Human (tumor cells)	Malignant Pleural effusion	Drug delivery	Methotrexate	Not available	Injected once in 2 days until the malignant pleural effusion are disappeared or the treatment cycle has been six times	Not available	90	2	Recruiting	NCT02657460
Plant (turmeric)	Colon cancer	Drug delivery	Curcumin	3.6 g	Curcumin-conjugated exosome tablets taken daily for 7 days	Not available	7	1	Active, not recruiting	NCT01294072

4.5.2. Other Cell-Free Therapies: NK Cell- or M1 Macrophage-Derived Exosomes

Unlike TEXs, normal cell-derived exosomes have not been well described. A few studies have reported that exosomes generated by NK cells harbor the cellular machinery for killing tumor cells.[210] For example, NK cell lines-derived exosomes (NK exosomes) have been shown to carry functional molecules such as Fas ligand, perforin, and tumor necrosis factor-α, that can exert cytotoxic effects against tumor cells.[211] NK exosomes were also shown to exert specific cytotoxic effects on tumor cells but not normal cells. Activated NK cells were found to produce numerous exosomes that expressed several activating receptors and yielded greater anti-tumorigenic efficacy.[212]

Another cell-free therapy has been developed using M1 macrophage-derived exosomes (M1 exosomes). Wang et al. demonstrated that M1 exosomes activate the NF-κB signaling pathway in macrophages to promote pro-inflammatory cytokine expressions and caspase-3-mediated apoptosis in tumor cells.[213] In addition, Cheng et al. found that M1 exosomes can serve as an adjuvant for cancer vaccines,[214] with their subcutaneous administration leading to preferential uptake in macrophages and DCs at the lymph nodes and provoking the release of pro-inflammatory cytokines. Furthermore, the combination of M1 exosomes and cancer vaccines exerted potent anti-tumor immune responses. These findings indicate that exosome-based cell-free immunotherapies display potential immunological effects.

5. Remaining Challenges Facing the Use of Exosomes for Cancer Therapy

Exosome-based cancer treatment strategies are actively being tested in virtue of its various advantages, leading to several clinical trials for cancer therapy (**Table 1**). For instance, autologous malignant pleural effusion-derived TEXs loaded with anticancer drugs have been used to treat malignant pleural effusion [NCT02657460, NCT01854866], while DEXs isolated from autologous DCs loaded

with tumor antigens have undergone a phase II clinical trial for non-small cell lung cancer [NCT01159288]. Moreover, strategies have been developed using exosomes derived from various types of fruits and vegetables, such as curcumin-loaded exosomes from plants which have undergone clinical trials against colorectal cancers [NCT01294072]. MSC-derived exosomes (iExosome) containing K-RAS G12D siRNA have demonstrated anti-tumorigenic efficacy in pancreatic cancer models without toxicity and have been successfully scaled-up at a clinical grade;[29] thus, iExosomes have entered phase I clinical trials in pancreatic cancer patients [NCT03608631]. Despite these advances, several challenges must be overcome before exosomes can be used as therapeutic agents. Here, we address the major factors that affect the successful clinical approval of therapeutic exosomes and the problems that currently remain unsolved.

5.1. Cell Sources for Exosome Production

The primary consideration when developing therapeutic exosomes is the cell source that will be used to produce the exosomes (**Figure 13**), with MSCs being a major candidate. Friedenstein first discovered a specific population of bone marrow stromal cells that supported mesodermal differentiation and hematopoiesis,[215] which were named MSCs in the 1990s by Caplan.[216] MSCs can be harvested as a subset of stromal regenerative cells from various adult tissues[217] and numerous studies have shown that they can be used to treat many diseases that require the regeneration of damaged tissues, including respiratory, renal, hepatic, neurological, musculoskeletal, and cardiovascular organs.[218] In addition, MSC-derived exosomes have a long half-life due to high CD47 expression.[219] Therefore, MSC-derived exosomes are currently undergoing clinical trials for several diseases, including pancreatic cancer and severe therapy-refractory acute GvHD.[220] Autologous DCs, patient cancer cells, and established cell lines are also considered candidate cell sources.[221] Further studies to validate the safety and advantages of each cell source may enable the clinical use of exosomes derived from human cell and non-human cell sources for anti-cancer therapeutics.

Some studies have attempted to utilize exosomes from different fruits and vegetables, such as ginger, grapes, and lemons.[222] Raimondo et al. showed that lemon juice-derived exosomes can induce TRAIL-mediated apoptotic cell death to produce an anti-tumorigenic response against chronic myelogenous leukemia.[223] Meanwhile, it was recently reported that milk-derived exosomes can improve the intestinal absorption of orally delivered exosomes via FcRN-mediated transcytosis.[224] Another study compared the yield of milk-derived exosomes to that of other cell sources, finding that milk-derived exosomes are produced at 1000 times higher levels.[225] In addition, exosomes isolated from milk can be loaded with naturally available molecules, such as *Tripterygium wilfordii*, *Celastrus regelii*,[226] curcumin (isolated from turmeric),[227] and aglycones (anthocyanidins),[228] which can exert tumor-suppressive effects without toxic side effects. Furthermore, Agarwal et al. reported that orally delivered milk-derived exosomes loaded with paclitaxel displayed therapeutic efficacy and diminished toxicity in A549 lung tumor-bearing mice.[229]

5.2. Exosome Isolation

Serial centrifugation is the most commonly used exosome isolation method whereby sequential centrifugation from 2000 to 10 000 relative centrifugal force (RCF) allows the removal of cells, cellular debris, and MVs, while ultracentrifugation (>100 000 RCF) allows the exclusion of proteins (**Figure 14**).[230,231] However, this method cannot tackle risk factors such as macromolecules contamination, disruption of exosomal integrity, or exosome aggregation, and displays limited scalability due to time- and labor-consuming procedures.[232] Although additional procedures, such as density gradient separation via iodixanol and sucrose cushions, can be carried out after isolation by ultracentrifugation,[230,233] the extraction of exosomes from bodily fluids containing a mixture of diverse molecules, such as blood, remains challenging.[234]

Various size-based methods have been developed to improve the yield, purity, and functionality of exosomes, including TFF,[234] ultrafiltration devices,[235] and size exclusion chromatography (SEC). TFF is considered the most appropriate method for mass-producing exosomes for clinical use as it provides a higher yield with less non-exosome molecule contamination or aggregation than serial centrifugation. In addition, high batch-to-batch consistency has prompted many companies dealing with exosomes to establish TFF-based exosome production facilities.[236] SEC is considered appropriate for extracting exosomes from small-volume media as it can enhance purity using different pore sizes, thereby maintaining exosome functionality and integrity better that ultracentrifugation. SEC is also a convenient diagnostic method; however, its inability to deal with large volumes remains challenging for mass production.[237]

Polymer-based precipitation methods, using commercially available exoquick or polyethylene glycol 6000 are also an option for isolating exosomes and are mainly used for the clinical evaluation of biomarkers.[220] Precipitation commonly achieves a higher yield with better exosome functionality and integrity; however, it is unable to exclude unexpected precipitants.[238] Other methods currently being researched include immunoaffinity capture targeting exosome-specific molecules using anti-EpCAM and anti-CD63 antibodies, which are expected to achieve high purity.[239] However, the inherent heterogeneity of exosomes may hamper separation effort that utilize specific marker, while exosomes without the targeted molecule are excluded and may distort the authentic functionality of the exosomes.[240] Currently, many approaches are being developed to improve exosome isolation, such as magnetic methods[241] and microfluidic techniques.[242]

Since no gold standard method for exosome isolation or production has yet been established, current methods must be optimized. For exosome-based cancer treatments to enter the clinical phase, optimized production methods must be established by either developing groundbreaking new methods or combining existing methods. Fundamental studies should also be conducted on exosomes themselves to reveal the unique characteristics that distinguish them from other EVs. These efforts will eventually produce a standardized exosome isolation method that can assure productivity, functionality, integrity, and purity.

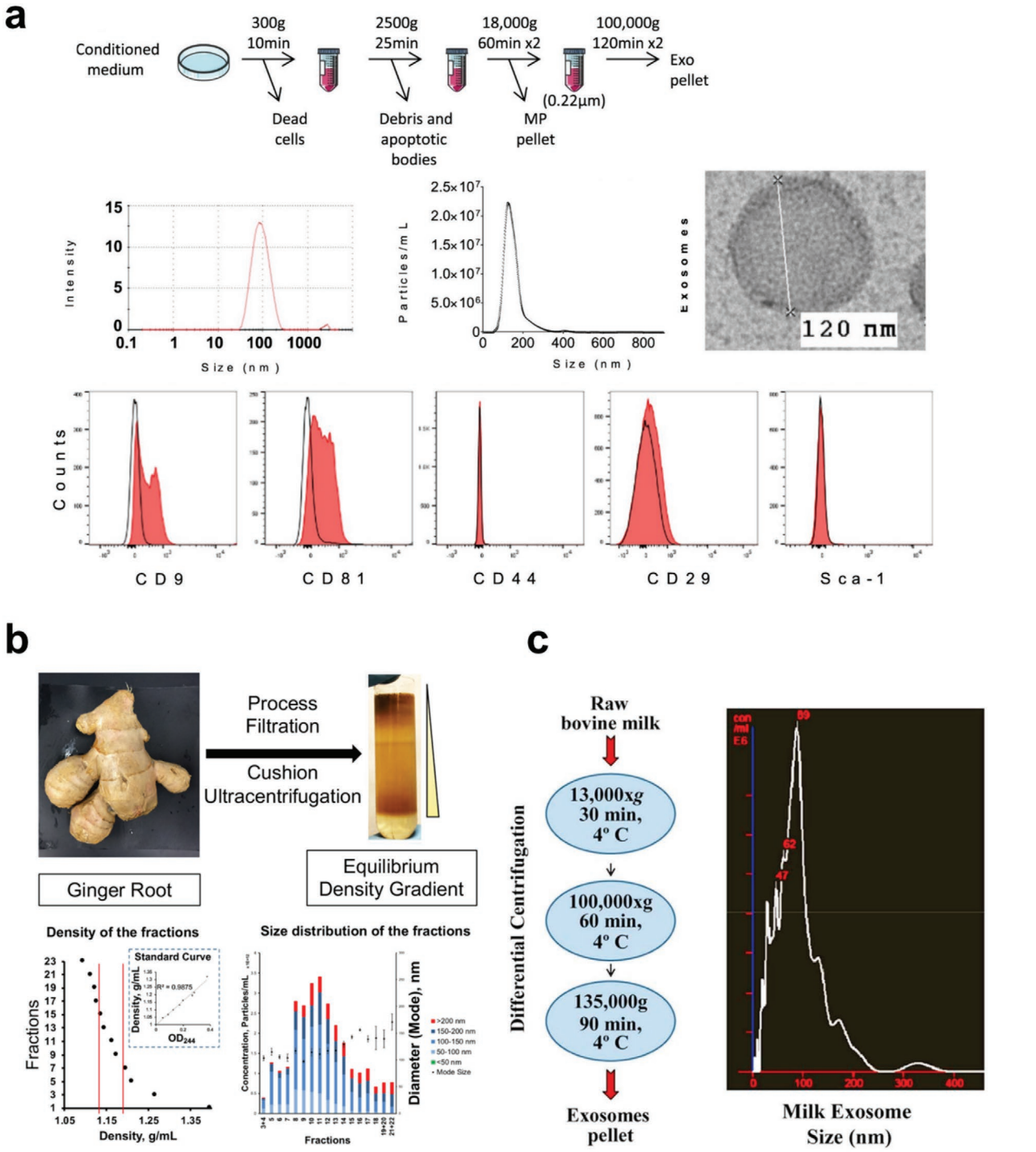

Figure 13. Isolation and characterization of exosomes from various cell sources. a) Characterization of murine bone marrow mesenchymal stem cell (BM-MSC)-derived exosomes. Experimental protocol for exosome isolation from MSCs by ultracentrifugation (top). Size and number of BM-MSC-derived exosomes detected by dynamic light scattering (DLS) analysis and nano tracking analysis (NTA), respectively (middle left). Representative transmission electron microscopy image of BM-MSC-derived exosomes (middle right). Expression of exosomal markers (CD9, CD81) and BM-MSC membrane markers (CD44, CD29, Sca-1) assessed with flow cytometry (bottom). Reproduced under the terms of the CC-BY license.[263] Copyright 2017, Stella Cosenza, Maxime Ruiz, Karine Toupet, Christian Jorgensen, Danièle Noël. Published by Springer Nature. b) Characterization of exosome-like nanovesicles extracted from ginger root. Workflow for exosome-like nanovesicle purification from ginger root by filtration, ultracentrifugation, and equilibrium density gradient ultracentrifugation (top). Density (bottom left) and size (bottom right) distribution of each equilibrium density gradient ultracentrifugation fraction. Reproduced under the terms of the CC-BY license.[264] Copyright 2018, Zhefeng Li, Hongzhi Wang, Hongran Yin, Chad Bennett, Huang-ge Zhang, Peixuan Guo. Published by Springer Nature. c) Exosome isolation from milk. Schematic workflow for exosome purification (left). Size of milk-derived exosomes analyzed by NanoSight (right). Reproduced with permission.[265] Copyright 2016, Elsevier.

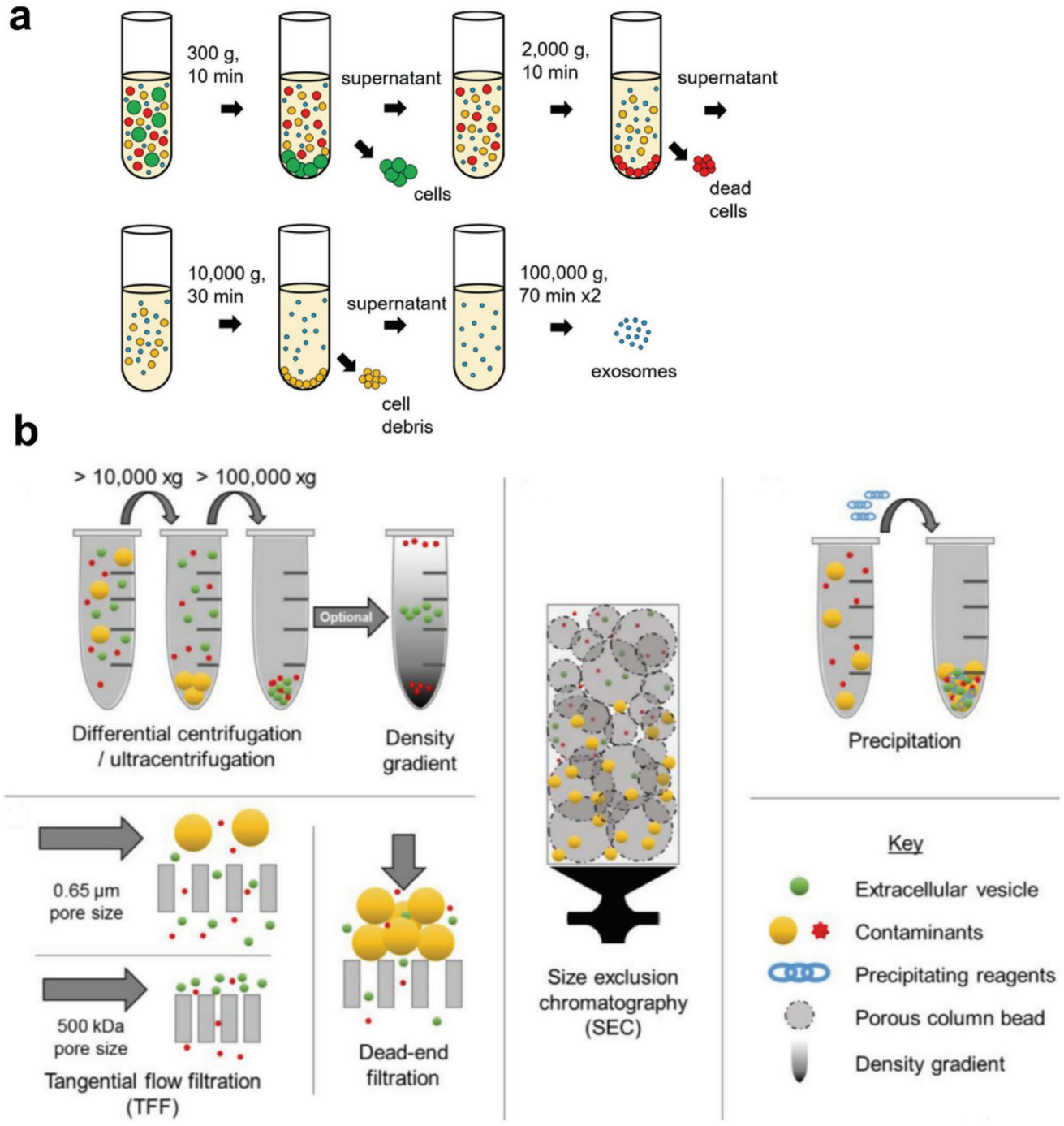

Figure 14. Exosome isolation. a) Exosome isolation via serial centrifugation and ultracentrifugation. Reproduced under the terms of the CC-BY license.[266] Copyright 2018, Yong Kyoung Yoo, Junwoo Lee, Hyungsuk Kim, Kyo Seon Hwang, Dae Sung Yoon, Jeong Hoon Lee. Published by MDPI. b) Types of exosome (a class of extracellular vesicles) isolation method. Reproduced under the terms of the CC-BY license.[59] Copyright 2019, Walker S, Busatto S, Pham A, Tian M, Suh A, Carson K, Quintero A, Lafrence M, Malik H, Santana MX, Wolfram.

5.3. Loading Bioactive Molecules on to Exosomes

Exosomes can be loaded with bioactive molecules to treat cancer at different intervention time points: direct loading is carried out after exosomes are isolated, whereas indirect loading involves the manipulation of producer cells before exosome isolation and takes advantage of the exosome biogenesis process to grant the desired trait (**Figure 15**).

Exogenous therapeutic agents can be loaded directly via the following process: 1) simple incubation, 2) electroporation, 3) sonication, 4) chemical conjugation, 5) permeabilization, 6) freeze–thaw cycles, and 7) extrusion.[243] However, excessive

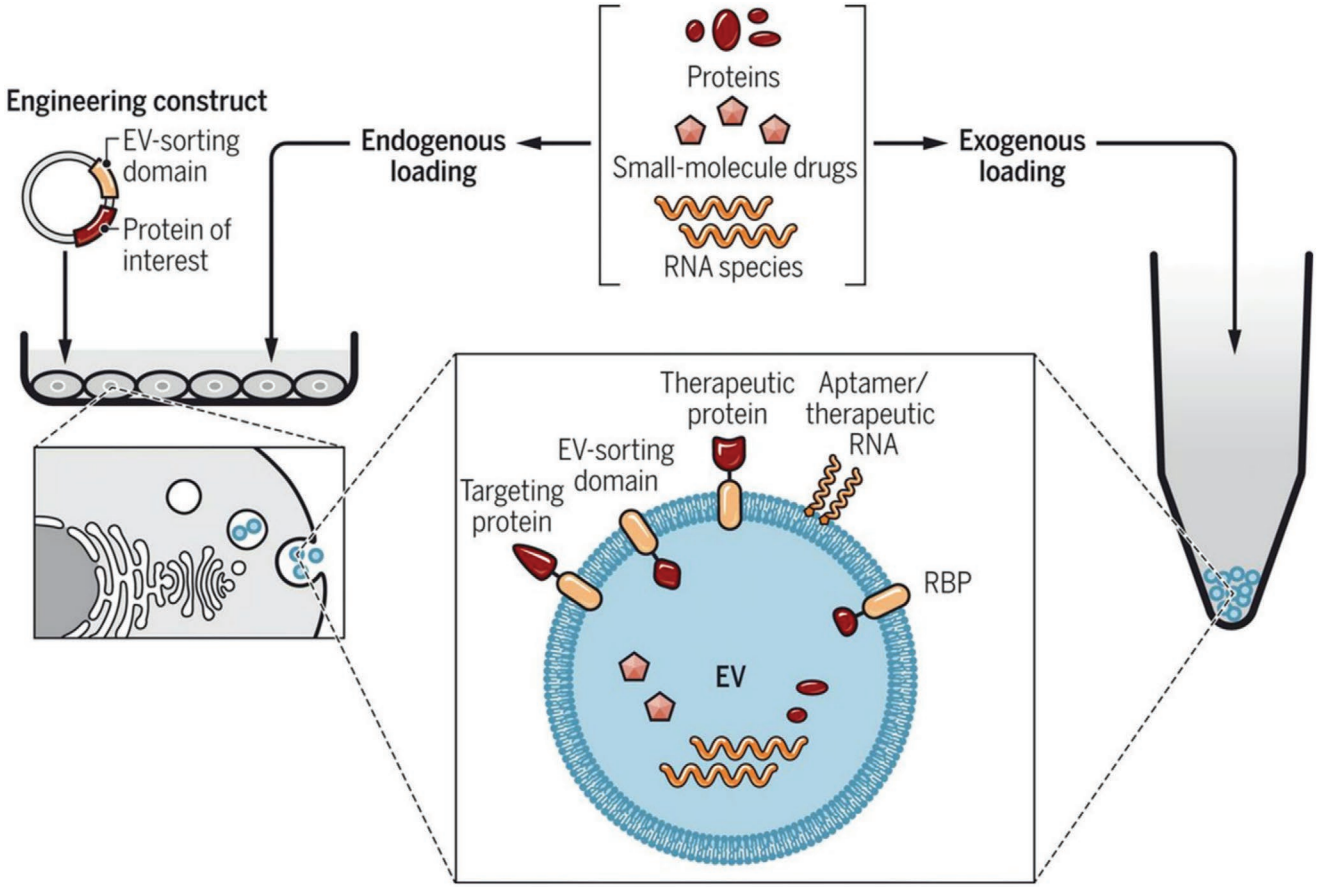

Figure 15. Loading of bioactive molecules into exosomes. Therapeutic substances such as proteins, small-molecular drugs, and RNA species can be loaded into exosomes (a class of EVs) using an endogenous (loading into producer cells) or exogenous (loading into isolated exosomes) approach. Reproduced with permission.[9] Copyright 2019, The American Association for the Advancement of Science.

loading may result in exosomal aggregation or reduce safety and integrity. Furthermore, there is no reliable way to determine whether exosomes contain the active molecule following direct loading and loading efficiency is reported to differ greatly between research groups, ranging from 85% to 0%.[244] The iExosome developed by Kalluri et al. recently entered phase I clinical trials for pancreatic cancer and the direct encapsulation of K-RAS G12D siRNA by electroporation was reported to load 1 μg of RNA into 10^8 exosomes, even after washing, and demonstrate potent anti-cancer effects in preclinical tumor models.[29] These results indicate that directly loading exosomes with cargoes can induce the desired responses.

The exogenous loading of bioactive molecules into producer cells involves similar methods to those mentioned above, such as simple incubation, electroporation, and sonication.[29] The expression of a specific protein can also be achieved by transfecting exosomes with commercially available cationic liposomes expressing designed plasmid constructs.[84] Optogenetic loading is another indirect approach, wherein transfection with an engineered construct can induce reversible protein–protein interactions in HEK293T cells stimulated with blue light via light-dependent loading feature.[245] Recently, the electrical cellular nanoporation method was reported to load 1000-fold more therapeutic mRNAs and targeting peptides with a 50 times higher yield of the desired exosomes. Yang et al. designed a device to electrically stimulate the producer cells focally and transiently thus increasing the loading of the desired PTEN mRNA. Moreover, following exosomal loading and systemic injection, the PTEN mRNA displayed potent anti-tumor efficiency in PTEN-deficient glioma tumors.[246]

5.4. Exosome Heterogeneity

Exosome populations are highly heterogeneous and can induce complex biological responses, making exosomal quality control difficult. Thus, considerable time and effort is required to optimize production conditions to produce exosome with high functionality and efficacy. Moreover, exosome heterogeneity also hinders a comprehensive understanding of their biogenesis, contents, biodistribution, and functions.

To explain exosome heterogeneity, complex factors, such as size, content, functional effect, and cell source, must be considered comprehensively (**Figure 16**). Uneven MVB formation during biogenesis has been shown to result in a wide range of sizes.[11] In addition, some isolation methods may degrade the structural integrity of exosomes and thus their quality.[247] Simply detecting specific bioactive molecules such as miRNAs

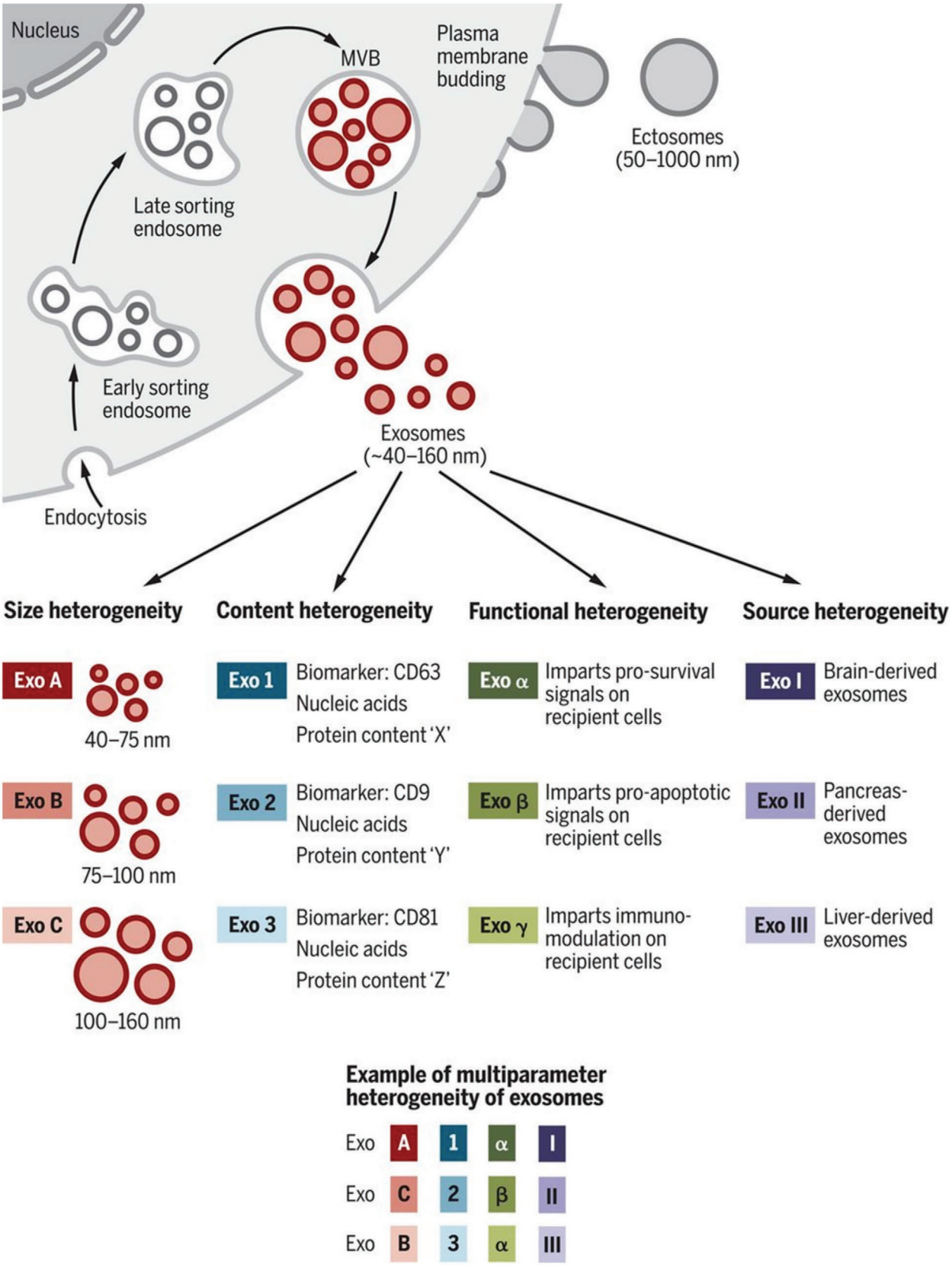

Figure 16. Exosome heterogeneity. Highly heterogeneous exosomes can be generated with varying size distributions, exosomal contents, functionalities, and producer cell sources. Reproduced with permission.[11] Copyright 2020, The American Association for the Advancement of Science.

in the bulk exosome samples cannot ensure that miRNA is contained in every single exosome[248] since current available analysis techniques lack the ability to evaluate the exosomes individually, while the cellular environment and biological characteristics may affect exosome contents.[248]

One of the main reasons for exosome heterogeneity is that there is still no established exosome isolation method. Exosomal contents may differ depending on the exosome isolation method; therefore detailed studies are required to investigate the specific isolation of genuine exosomes.[249] Haiying et al. demonstrated that two different isolation methods, asymmetric field-flow fractionation and differential ultracentrifugation, resulted in exosomes with two discrete sizes (large: 90–120 nm diameter, or small: 60–80 nm diameter) and non-membranous nanoparticles known as exomeres (≈35 nm). These three types of nanoparticles displayed disparate biophysical characteristics such as zeta potential (large exosomes: −12.3 to −16.0 mV; small exosomes: −9.0 to −12.3 mV; exomeres: −2.7 to −9.7 mV) and stiffness (large exosomes: 26–73 MPa; small exosomes: 70–420 MPa; exomeres: 145–816 MPa). Each type also contained different amounts and types of proteins, lipids, and nucleic acids, and all three were taken up by hematopoietic organs such as the liver, spleen, and bone marrow with larger exosomes accumulating more in the lymph node.[250]

In another example, Jeppesen et al. combined the direct immunoaffinity capture method targeting the classical exosome marker tetraspanin with high-resolution density gradient fractionation to separately assess exosome composition, small EV exosomes, and non-vesicular components. Exosomes and non-vesicular components were found to harbor different proteins and RNAs; for instance, exosomes did not contain miRNA processing proteins such as Argonautes (Agos), glycolytic enzymes, or cytoskeletal proteins. Nor did they contain the dsDNA or DNA-binding histones, which are traditionally known to exist in exosomes, suggesting that dsDNA or DNA-binding histones are released via an exosome-independent mechanism. These findings highlight the need for a detailed analysis method to understand exosomal heterogeneity and reassess exosome composition.

6. Conclusions and Outlook

This review focused on emphasizing the usefulness and potential of exosomes in cancer therapy. Until recently, cancer treatment has relied mainly on physical surgery, chemotherapy, target therapy, or radiotherapy; however, these therapeutic modalities are commonly accompanied by side effects, acquired resistance, frequent metastasis, and recurrences.[251] Cancer immunotherapy has emerged as a promising alternative to these conventional therapies to treat a variety of malignancies and has demonstrated remarkable clinical results, gaining considerable attention as a next-generation cancer treatment.[252]

Despite these positive clinical responses, many cancer patients remain unresponsive to current cancer immunotherapies[5] for several reasons: 1) immune cells may not detect tumor antigens due to intrinsic tumor resistance,[253] 2) DCs and T cells may recognize tumor antigens as their own, thereby promoting a tolerogenic immune response,[254] 3) educated T cells may not properly home to tumor tissues,[255] 4) inhibitory signals, such as PD-L1 and CTLA-4 may dampen immune surveillance against tumor cells,[110] and 5) unique properties such as hypoxia, stiffness, and dense ECM in the tumor microenvironment may suppress tumor-specific effector T cell immunity.[256]

These limitations emphasize the complexity and heterogeneity of cancer and support the concept of cancer as CAS.[3] Thus novel immunotherapeutic strategies should meet the following five requirements: 1) targetability for the selective recognition of neoantigens expressed on cancer cells; 2) adaptability to antigen diversity due to tumor mutations; 3) self-propagating immune system to improve anti-tumor immune responses; 4) durable immune effects; and 5) immune cell infiltration into tumor tissues.

Recently, we proposed the concept of intrinsic cancer vaccination (ICV), showing that the induction of immunogenic cell death and activation of APC function prompts intrinsic antitumor immunity that propagates and cycles efficiently when combined with current immunotherapies (**Figure 17**).[257] The human immune system belongs to the same category as jawed vertebrates and has evolved elaborately for over 500 million years to protect organisms from external or internal danger signals. ICV takes advantage of intrinsic aspects of this well-established immune system, thereby fulfilling the five immunotherapy prerequisites mentioned above. Thus ICV can promote the efficient processing of diverse tumor antigens in APCs to elicit sufficient tumor-specific immunity. Consequently, the amplification of T cell diversity and clonality may overcome tumor heterogeneity.[258] Unlike current cancer vaccines, this approach does not depend on selecting a single neoantigen and ex vivo cell manipulation, thus may be applicable to a wide range of cancers.

Exosomes can exert profound effects on the phenotype of their recipient cells as they can deliver contents from their parental cells.[15] Indeed, TEXs are known to affect surrounding immune cells and other cells in the tumor microenvironment, even in distant organs. Immunotherapeutic strategies targeting these properties of TEXs may awaken innate immune system against cancer, while exosomes engineered to regulate immune functions could fully amplify these anti-tumor immune responses at each stage of the cancer immunity cycle. Therefore, the development of an ICV strategy using exosomes could overcome the limitations of conventional immunotherapies.

Notably, exosomes have many advantages over other nanoparticles due to their excellent biocompatibility, low immunogenicity, high stability, prolonged half-life, ability to cross physical barriers such as the BBB, and targetability.[259] In addition, their ability to deliver functional biomolecules (therapeutic proteins, chemotherapeutics, and nucleic acids), and propensity for bioengineering has attracted significant attention recently.

Several exosome-based drugs are currently in the pipeline, a few of which have just started clinical trials; however, some critical problems remain unsolved. For instance, the natural heterogeneity of exosomes must be understood in detail to manufacture de novo exosome drugs with consistent quality and efficacy and a more refined method of exosome isolation is required. Further research is also necessary to determine which cells should be used for manufacturing and a new approach is needed to effectively increase the loading efficiency of bioactive

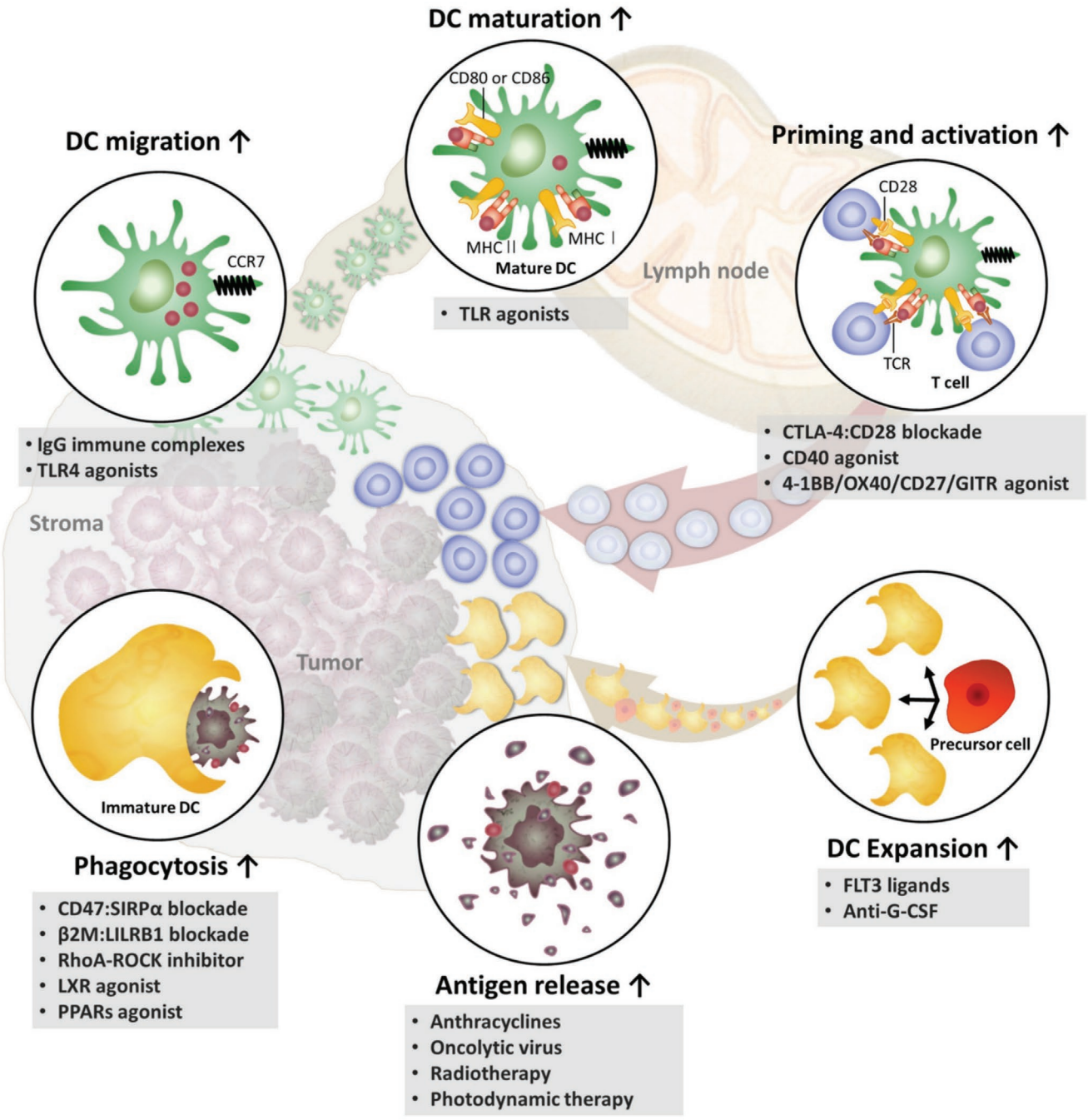

Figure 17. Sequential therapeutic strategies for intrinsic cancer vaccination. These strategies drive innate immune responses against cancer cells to produce cancer-specific T cell immune responses. Rational targets for APC activation are highlighted. Reproduced with permission.[257] Copyright 2019, Elsevier.

molecules without damaging exosome integrity. Nonetheless, the continued efforts will allow exosomes to emerge as the next generation of cancer treatments.

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Conflict of Interest

The authors declare no conflict of interest.

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