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REVIEW ARTICLE

The function and clinical application of extracellular vesicles in innate immune regulation

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The innate immune system plays a crucial role in the host defense against viral and microbial infection. Exosomes constitute a subset of extracellular vesicles (EVs) that can be released by almost all cell types. Owing to their capacity to shield the payload from degradation and to evade recognition and subsequent removal by the immune system, exosomes efficiently transport functional components to recipient cells. Accumulating evidence has recently shown that exosomes derived from tumor cells, host cells and even bacteria and parasites mediate the communication between the invader and innate immune cells and thus play an irreplaceable function in the dissemination of pathogens and donor cell-derived molecules, modulating the innate immune responses of the host. In this review, we describe the current understanding of EVs (mainly focusing on exosomes) and summarize and discuss their crucial roles in determining innate immune responses. Additionally, we discuss the potential of using exosomes as biomarkers and cancer vaccines in diagnostic and therapeutic applications.

Keywords: extracellular vesicles; exosome; innate immune; cancer diagnosis; immunotherapy

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INTRODUCTION

It is becoming increasingly evident that almost all living cells can secrete extracellular vesicles (EVs), including microvesicles (MVs), which are also known as microparticles and ectosomes, exosomes and apoptotic bodies.[1–3] The size of an exosome ranges between 30 and 150 nm in diameter, whereas that of a microvesicle (0.1–2 μm) and an apoptotic body (1–5 μm) is usually larger.[4–6] Recently, two novel subpopulations of exosomes (large exosome vesicles, Exo-L, 90–120 nm; small exosome vesicles, Exo-S, 60–80 nm) and an abundant population of nonmembranous nanoparticles termed 'exomeres' (~35 nm) were identified.[7] As a novel mediator of intercellular communication, EVs carry bioactive molecules such as proteins, lipids, multiple RNA species (microRNAs, mRNAs, and long non-coding RNAs), and even DNA fragments from donor to recipient cells.[8–15] The largely selective content packaged into EVs mostly reflects the aims and functions of the parent cells, and EVs are found in a number of human body fluids, such as blood plasma, urine, saliva, sputum, and breast milk.[16–20] In addition to eukaryotic cell types, EVs can also be secreted by plant cells and pathogens, including bacteria, archaea, and fungi, suggesting a highly evolutionarily conserved function as a mode of intercellular communication.

Exosomes were first identified in 1981, originally termed shedding vesicles, because of their 5′-nucleotidase activity, which is derived from various normal and neoplastic cell lines.[21] Exosomes not only originate from cells in the endosomal pathway via the formation of multivesicular bodies (MVBs) but also bud from the plasma membrane,[3,22,23] whereas microvesicles are secreted only by shedding or outward budding of the plasma membrane.[24,25] Exosomal budding of the plasma membrane has been observed and is supported by multiple experiments such as electron microscopy and atomic force microscopy, strongly supporting the argument against the widely accepted endosome-only model of exosome biogenesis.[22,23,26,27] This prevailing thought may be the result of observational bias caused by the use of electron microscopy, in which intact cells with MVBs are easily recognized, but exosomes budding from the plasma membrane may be undersampled. Based on their different cellular origins, exosomes play distinct roles in normal physiological processes, such as the immune response, cell proliferation, inflammation, metabolism and neuronal function, and in different stages of diseases, including cancer.[1,5,28–31] Moreover, other vesicles, such as oncosomes and melanosomes, also play roles in immune control and in cancer. Oncosomes are atypically large (1–10 μm diameter) cancer-derived EVs originating from shedding membrane blebs.[32] They contain abundant molecules and are associated with advanced disease.[33–35] For example, oncosomes containing Cav-1, a serum biomarker of metastatic prostate cancer, have been correlated with, and can serve to distinguish, patients with metastatic disease.[36,37] Melanosomal microvesicles, also called melanosomes, are specialized organelles in melanocytic cells and are devoted to melanin pigment synthesis and storage.[38,39] It was suggested that the release of FasL-bearing melanosomal microvesicles mediates the apoptosis of T lymphocytes at the tumor site, representing a possible mechanism by which tumor cells can eradicate antitumor T-cell reactivity.[40] In addition, exosomes are

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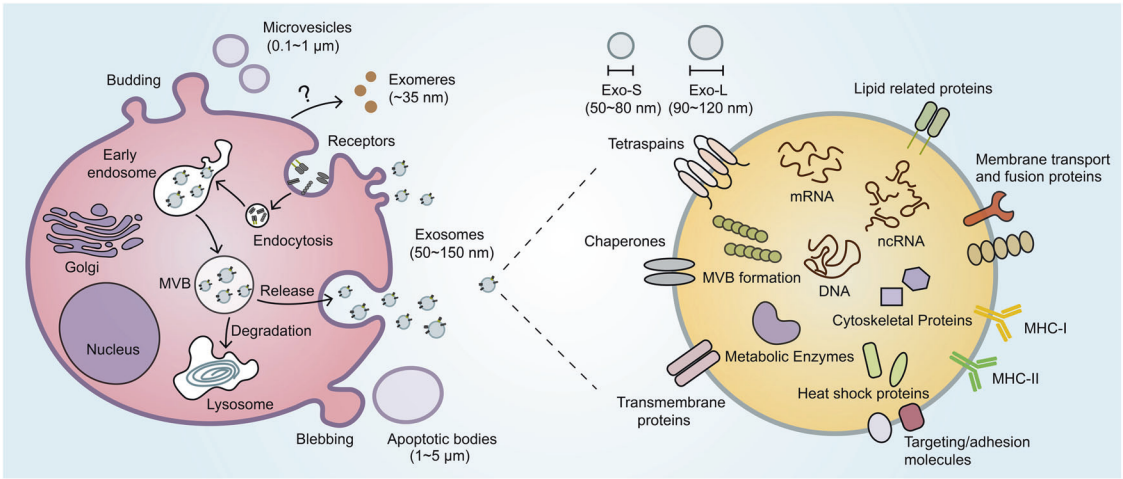

Fig. 1 Exosome biogenesis and composition. Exosomes originate not only from ILVs in MVBs but also from plasma membrane budding. Early endosomes maturate into late endosomes/MVBs, which follow either the secretory or the degradative pathway. Microvesicles are generated by budding from the cytomembrane. Apoptotic bodies are generated during programmed cell death (left). Exosomes have spherical structures consisting of a lipid bilayer and contain complex contents, including proteins, mRNA, miRNA, ncRNA, and DNA (right)

also being implicated as diagnostic biomarkers for diseases because they have high stability, reach sufficient concentrations in the circulation and contain a variety of content, such as proteins and RNAs, that reflect their parental cell.[20,41]

In recent years, studies about exosomes, immunity and their interplay in human diseases have received increasing attention. The innate immune system is composed of a network of cells, including monocytes/macrophages, dendritic cells (DCs), neutrophils and natural killers (NK) cells, that mediate the earliest interactions between host and pathogens.[42] Innate immunity is the first line of defense against any invading substances, including viral infections, and plays a key role in the elimination of viruses from a host. Pattern-recognition receptors (PRRs) that recognize viral nucleic acids include Toll-like receptors (TLRs), RIG-I-like receptors (RLRs) and certain DNA sensors, such as cGAS.[43–47] PRRs recognize various pathogen-associated molecular patterns (PAMPs), including DNA and RNA from bacteria and viruses and danger-associated molecular patterns (DAMPs).[48,49] Different PRRs respond to diverse PAMPs, activate specific signaling pathways, trigger the expression of antiviral type I interferons (IFNs) and pro-inflammatory cytokines, and lead to distinct antipathogen responses.[50–53] In this review, we impart basic knowledge of EVs and exosomes and describe the latest important findings on the mechanisms underlying EV and exosome involvement in innate immune modulation. We also summarize their therapeutic potential.

BIOLOGICAL CHARACTERISTICS OF EXOSOMES

Exosome biogenesis

In addition to direct outward budding from the plasma membrane, exosomes can also originate from intraluminal vesicles (ILVs) within MVBs in the endocytic pathway and be released from cells upon the fusion of MVBs with the plasma membrane.[24,54–56] First, plasma membrane- and cytosol-associated molecules such as nucleic acids, lipids, and proteins are endocytosed and transferred into early endosomes. During the endosome maturation process, early endosomes differentiate into late endosomes/MVBs. Then, late endosomes fuse with lysosomes, leading to their degradation, or fuse with the plasma membrane, releasing the vesicles into the extracellular space as exosomes[57–60] (Fig. 1). The Rab family of small GTPases controls different steps of vesicular trafficking. Rab27a and Rab27b were found to function in MVE docking at the plasma membrane.[61] Rab27a has a key role in the size determination of MVEs, whereas Rab27b mediates the transfer of MVEs from microtubules to the actin-rich cortex and their retention at the cell periphery.[62] Ultimately, exosomes interact with recipient cells by direct signaling through ligand/receptor molecules on their respective surfaces or are taken up by recipient cells in unique fashion, such as direct membrane fusion, endocytosis, macropinocytosis, or even phagocytosis.[8,63–69]

The exosome generation pathway can be regulated by either the endosomal sorting complex required for transport (ESCRT)-dependent pathway or an ESCRT-independent pathway.[70] In the typical case, ESCRT machinery is required for MVB formation because it sorts ubiquitinated intracellular cargos that are destined for lysosomal degradation into MVBs.[71] The ESCRT machinery consists of four ESCRT proteins (ESRT-0, ESRT-I, ESRT-II, and ESRT-III) and accessory proteins (VPS4, VTA1, and the ALG2-interacting protein X (ALIX) complex).[72] Whereas the ESCRT-0 subunit, hepatocyte growth factor-regulated tyrosine kinase substrate (HRS), binds ubiquitylated proteins, ESCRT-I (comprising tumor susceptibility gene 101 protein (TSG101) and Vps28) is recruited to the endosomal membrane and incorporates ESCRT-II (Vps22).[73–75] ESCRT-I and ESCRT-II then facilitate the formation of reverse budding in MVB membranes and uptake of cytosolic cargo.[76] Next, ESCRT-II recruits ESCRT-III to catalyze vesicle cleavage inside the neck of nascent ILVs.[75–77] Although these ESCRT subunits are released into the cytosol for recycling, some ESCRT components and accessory proteins, such as TSG101, HRS, and ALIX, remain in exosomes. In general, the ESCRT machinery is predominantly involved in the sorting of ubiquitinylated proteins into ILVs.[70,78] However, not all proteins require ubiquitylation to be sorted into exosomes. In addition to the ESCRT-dependent formation of MVBs and exosomes, ESCRT-independent mechanisms of exosome formation and release were found to depend on neutral sphingomyelinase (nSMase)-dependent ceramide formation.[79] Thus, using the inhibitor GW4869, which blocks the generation of ceramide, has been shown to reduce the release of exosomes.[79] In addition, another mechanism of ESCRT-independent endosomal sorting involves tetraspanins (such as CD9, CD63, CD81, and CD82), which mediate the organization of particular proteins.[80] Presumably, these two alternative mechanisms of MVB and exosome biogenesis do not operate independently and may coexist within a cell or subset population of MVBs.

Exosome composition

Exosomes are heterogeneous and carry plasma membrane- and cytosol-associated molecules such as nucleic acids, lipids, and

proteins both inside and outside the vesicles (Fig. 1). As mentioned above, exosomes are highly enriched in ESCRT machinery-associated proteins (such as TSG101, HRS, and ALIX), which are involved in MVB synthesis, and tetraspanins (such as CD9, CD63, and CD81), which are frequently recognized as exosome markers.[9,81] In addition, various metabolic enzymes, such as ATPase, glyceraldehyde-phosphate dehydrogenase (GAPDH), enolase 1, pyruvate kinase type M2 (PKM2) and phosphoglycerate kinase 1 (PGK1), have been detected in exosomes by proteomic analyses.[82,83] Exosomes also include molecules that are involved in signal transduction, such as 14-3-3 and G proteins. Exosomal 14-3-3 can activate the oncogenic Wnt pathway in target colorectal cancer (CRC) cells in vitro.[84] Hepatocellular carcinoma-derived exosomal 14-3-3 impairs the antitumor function of tumor-infiltrating T lymphocytes.[85] Furthermore, heat shock proteins (such as HSP70 and HSP90) and major histocompatibility complex (MHC) molecules are also found in exosomes derived from most cell types and are involved in antigen presentation.[86–88] Interestingly, compared with exosomes, exomeres have unique proteins and are highly enriched with metabolic enzymes and signature proteins involved in glycolysis and mTORC1 signaling.[7,89] In addition to the abovementioned evolutionarily conserved and commonly found proteins, exosomes also contain proteins that are involved in specific functions. For instance, tumor cell exosomes usually contain both tumor antigens and immunosuppressive proteins. Programmed death-ligand 1 (PD-L1), a membrane-bound ligand on many cancer cells, was found to be specifically enriched on exosomes from plasma samples of patients with a variety of cancers, suggesting circulating exosomal PD-L1 as a biomarker for the clinical outcomes of anti-PD-1 therapy.[90,91] In addition to proteins, the exosome membrane contains a total of 1116 lipids, according to the exosome database (www.exocarta.org), such as phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidyl inositols (PIs), phosphatidic acid (PA), cholesterol, ceramides, sphingomyelin, glycosphingolipids, and a number of others at lower abundance.[11,12,92] Importantly, lipidomic analyses revealed cell type-dependent differences in the total lipid level and composition among different subpopulations of EVs.[7] Future exploration of this issue will undoubtedly contribute to the understanding of exosome formation and secretion and greatly advance the understanding of the tissue/organ specificity of exosome function. Exosomes also contain mRNAs and noncoding RNAs (ncRNAs), including microRNAs (miRNAs), small nuclear RNAs (snRNAs), transfer RNAs (tRNAs) and Y RNAs.[93,94] Exosomal RNA transmission can alter the epigenetic characteristics of target cells, largely through gene regulation. An increasing number of studies have indicated that tumor-derived exosomes (TEXs) contain high levels of miRNAs, which are thought to be potential circulating diagnostic biomarkers in many types of cancer, such as glioblastoma, ovarian cancer and prostate cancer.[41,95,96] Although several studies have reported the presence of DNA in exosomes, including single-stranded DNA, double-stranded DNA (dsDNA), genomic DNA and even mitochondrial DNA,[13–15,97] there is an opposite view that suggests that dsDNA is not present in exosomes or any other type of small extracellular vesicle.[98] The debate on this issue is likely based on early studies that often did not discriminate between MVs and exosomes. The International Society for Extracellular Vesicles (ISEV) proposed Minimal Information for Studies of Extracellular Vesicles (MISEV) guidelines in 2014 and updated it in 2018 to guide and improve the EV field.[99,100] In future studies, there is an urgent need to improve the methodology for exosome isolation, which is expected to enable more precise determination of the molecular composition of classical exosomes.

FUNCTIONS OF EXOSOMES IN INNATE IMMUNE SIGNALING AND REGULATION

Innate immune responses

In response to infection, host cells sense invading viruses and initiate a series of signaling pathways that lead to the production of type I interferons and the expression of an array of interferon-stimulated genes (ISGs).[101,102] The recognition of foreign nucleic acids is a critical strategy by which the innate immune system recognizes many pathogens.[103] Several nucleic acid sensors have been identified, including cytosolic RNA sensors, such as TLR3, TLR7, TLR8, RIG-I and MDA5, and DNA sensors, such as TLR9, AIM2, and cGAS.[43,48,49,104–107] Following the recognition of viral nucleic acids, PRRs recruit downstream adaptors, including TRIF, MAVS, and STING, which subsequently activate downstream kinases such as inhibitor IκB kinase (IKK) complexes composed of either IKKα, IKKβ, and IKKγ or kinases TBK1 and IKKε.[108–110] The IKK complex and TBK1-IKKε activate transcription factors NF-κB and IRF3, which then are translocated to the nucleus, where they are involved in the production of pro-inflammatory cytokines regulated by NF-κB signaling and type I interferons (IFN-α and IFN-β) as mediated by IRF signaling.[50–53] IFN-β and IFN-α subsequently activate downstream signaling pathways that induce a diverse set of interferon-stimulated genes and protect host cells against the invading virus. We summarize the proposed roles of EVs and exosomes containing cargo, including dsDNA, virus RNA or a specific protein, in modulating innate immune responses (Fig. 2).

EV-mediated innate immune response activation

Consistent with previous reports, dsDNA or virus RNA-containing EVs or exosomes can trigger immune responses.[111–114] Upon activation by interactions with antigen-bearing dendritic cells, T cells transmit EVs that contain genomic and mitochondrial DNA back to the presenting DCs, further enhancing antiviral responses via the cGAS/STING cytosolic DNA-sensing pathway and the subsequent induction of IRF3-dependent interferon-regulated genes in vitro[111] (Fig. 2a). These results suggest a feedback mechanism by which T cells enhance the activity of an antigen-presenting cell (APC), priming it to respond more efficiently to subsequent infections by the same pathogen or a similar pathogen. As the changes in DCs induced by T cell-derived EVs occur in a specific antigen-dependent manner, the enhanced innate antiviral immunity triggered by DCs responds only to specific stimuli. Interestingly, oxidized mtDNA and genomic DNA, together with mtDNA-binding proteins, are present in T cell exosomes, suggesting that these EVs may maintain cellular homeostasis by releasing harmful or damaged components.[111,115] However, the evidence that signaling mediates the loading of DNA into late endosomes/MVBs is lacking, and thus further investigation is warranted. In addition to immune cells, cancer cells also release DNA-loaded exosomes/EVs under certain conditions, such as during radiation treatment or chemotherapy. For example, a recent study showed that treatment of breast cancer cells with the topoisomerase I inhibitor topotecan, an antitumor chemotherapy, significantly increased exosomal DNA production, which led to the activation of dendritic cells through cGAS-STING signaling in vitro and in vivo.[116] This finding suggests that exosomal DNA can also activate innate antiviral immune cell responses.[116,117] Interestingly, it was recently noticed that combinations of radiotherapy and immunotherapy can lead to more effective antitumor responses and the reasons for this synergy in cancer treatment has attracted attention.[118,119] Radiotherapy destroys cancer cells in the area where it is applied. Although normal cells in the area can also be damaged by radiotherapy, they are usually able to repair themselves, but cancer cells cannot undergo self-repair. Radiotherapy induces immunogenic cell death and the release of new antigens into the immune system, thereby affecting the immune response and

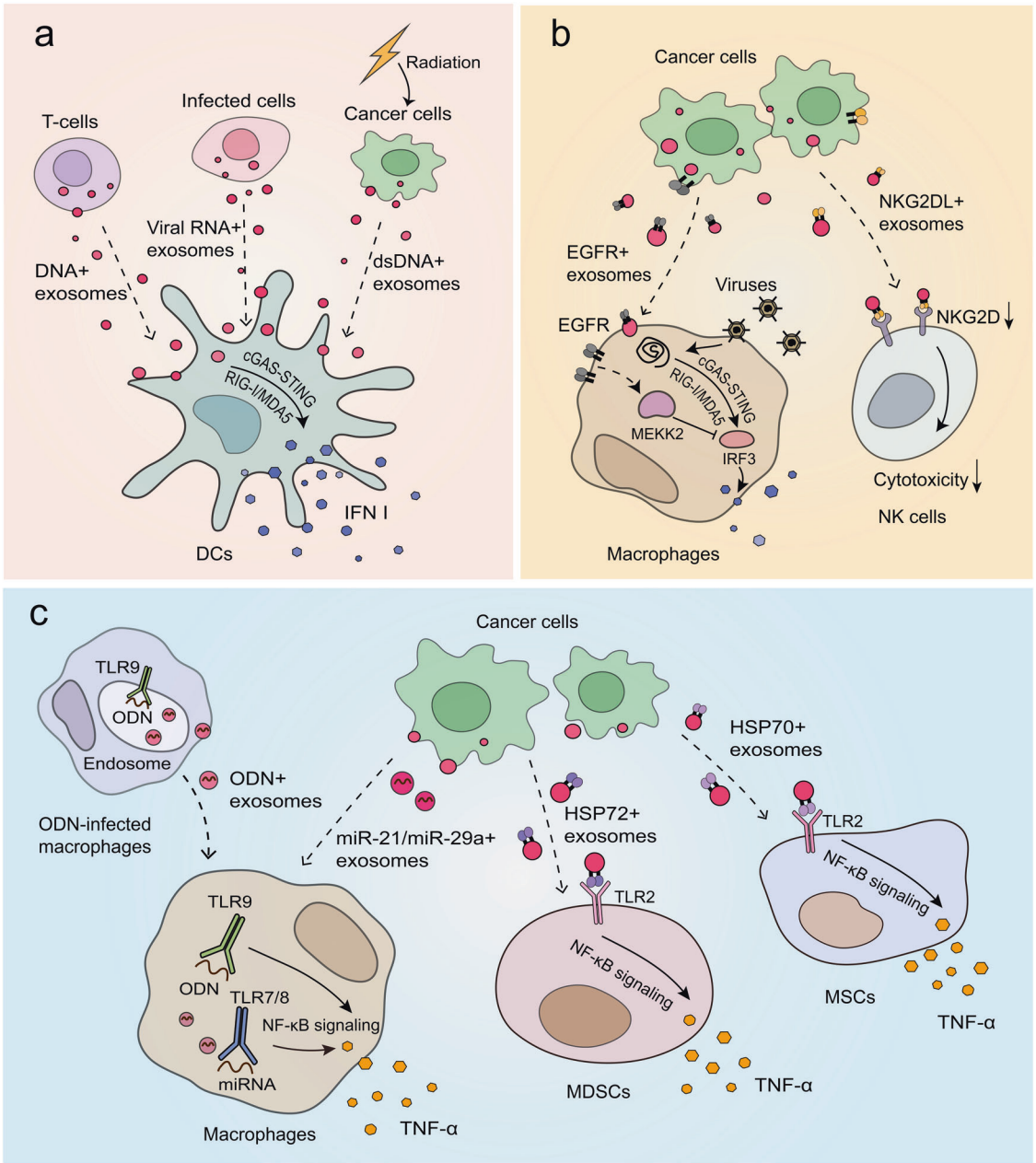

Fig. 2 Functions of exosomes in innate immunity. **a** Activated T cell-derived exosomes containing DNA are transferred to DCs, inducing an antiviral IFN response; RNA-bearing exosomes secreted by virus-infected cells activate the innate immune system of DCs; and irradiated cancer cells deliver tumor dsDNA to DCs via exosomes, leading to DC activation and IFN I release. **b** Tumor cells secrete and transfer $EGFR^{+}$ exosomes to macrophages, which interfere with innate antiviral immunity via MEKK2-mediated deregulation of IRF3, and tumor-derived microvesicles/exosomes containing a ligand for NKG2D downregulate NKG2D expression on DCs and inhibit the cytotoxic activity of DCs. **c** ODN-loaded extracellular vesicles derived from TLR9-activated macrophages are transported to naïve macrophages and induce the release of chemokine TNF-α; tumor-secreted miR-21 and miR-29a bind with TLR7 and TLR8 in macrophages, triggering a prometastatic inflammatory response; and TEXs containing HSP70/HSP72 activate NF-κB signaling through TLR2 on MDSCs or MSCs

improving the initiation and activation of effector T cells. At immunogenic doses, radiotherapy causes the accumulation of cytosolic dsDNA in cancer cells, which is sensed by cyclic GMP-AMP synthase (cGAS) in DCs, resulting in the production of IFN-β and the induction of several interferon-stimulated genes (ISGs) in vitro and in vivo[112] (Fig. 2a). EVs from cancer cell lines and patients contain DNA that reflects the mutational status of the parental tumor cells.[13,15] Irradiated cancer cells thus also release exosomes that carry the tumor dsDNA to the cytosol of DCs, leading to DC activation and antitumor T cell priming.[112] However, the following hypothesis needs further investigation: circulating TEXs in peripheral blood may be biomarkers that indicate RT-induced immunogenic changes in tumors. In fact, these findings provide a theoretical basis for suggesting a new vaccination strategy.

It is well known that viral nucleic acids often trigger an innate immune response in infected cells. In addition to DNA, various types of RNA have been identified in exosomes.[114] For example, latent Epstein–Barr virus (EBV)-infected cells can trigger antiviral immunity through dendritic cells (DCs) by the exosomal transfer of 5′ppp-RNA in vitro.[114] This finding suggests that 5′ppp-recognizing sensors such as RIG-I are more likely to have a role in the

recognition of exosomal small RNA. Consistent with this report, exosomes derived from breast cancer stromal fibroblasts carry 5′ppp-RNA RN7SL1, which activates RIG-I in tumor cells and results in STAT1 activation and ISG induction in vivo and in vitro[113] (Fig. 2a). Therefore, a conserved intercellular pathway transmits signals between cells in the form of small RNAs via exosomes. Upon recognizing invading viruses, host cells trigger signaling events that ultimately lead to type I interferon secretion. Despite the mechanism by which many viruses evade the pathogen-sensing pathway, there are alternative pathogen-sensing strategies that are not challenged by viral evasion mechanisms. For example, hepatitis C virus (HCV)-permissive cells can selectively package immunostimulatory viral RNA into exosomes and deliver it to neighboring plasmacytoid DCs (pDCs), which triggers an antiviral IFN response in vitro.[120] This finding describes a mechanism that was previously undiscovered in innate host responses, whereby infected cells with a pathogen-sensing mechanism that is inhibited by viral proteins can release viral RNA-containing exosomes to trigger an alternative host defense strategy.

EV-induced innate immunosuppression

Emerging evidence has shown that tumors can interfere with host immunity by secreting EVs or exosomes. By transducing different signals, TEXs can affect the proliferation, apoptosis, cytokine production and reprogramming of both innate and adaptive immune cells.[121–123] For example, TEXs enriched with miRNAs, such as miR-21-3p, miR-125b-5p, miR-181d-5p and miR-1246, potently reprogram neighboring macrophages into tumor-supportive agents.[124,125] Previous studies suggested that tumor-derived microvesicles/exosomes expressing TGF-β1 and a ligand for NKG2D can downregulate NKG2D expression and reduce the cytotoxicity induced by natural killer cells[126–128] (Fig. 2b). In addition, tumor-derived microvesicles/exosomes can also promote the differentiation of monocytes into myeloid-derived suppressor cells (MDSCs), which inhibit DC maturation.[129–131]

Epidermal growth factor receptor (EGFR), located on the cell membrane, belongs to the ErbB family of receptor tyrosine kinases (RTKs).[132–134] Highly or abnormally expressed EGFR is associated with the occurrence of various kinds of tumors.[135] In the tumor microenvironment, TEX-mediated delivery of EGFR and human epidermal growth factor receptor 2 (HER-2) to monocytes promotes tumor-derived monocyte survival prior to the formation of numerous tumor-associated macrophages (TAMs).[136] In line with this report, it was suggested that ALIX, a critical mediator of exosome biogenesis, modulates immunosuppression through the regulation of PD-L1 and EGFR in breast cancer cells.[137,138] ALIX depletion results in enhanced EGFR activity as well as reduced exosomal PD-L1 secretion and increased surface PD-L1 expression.[137] However, a recent study showed that exosomal PD-L1 contributes to immunosuppression in patients with metastatic melanoma.[90] Chronic lymphocytic leukemia (CCL)-derived exosomes and the Y RNA they contain can also induce PD-L1 expression and cytokine release in monocytes and thus contribute to a tumor-supportive microenvironment.[139] Similarly, EGFR is in the exosomes secreted by gastric cancer cells and is highly expressed in the exosomes of cancer patients; by stimulating paracrine HGF in liver stromal cells, EGFR-containing exosomes derived from gastric cancer cells may favor the development of a liver-like microenvironment promoting liver-specific metastasis in vitro and in vivo.[140] Recently, our group discovered previously unknown interplay between lung tumor cells and innate antiviral immunity in virus-infected mice by elucidating a TEX-mediated control mechanism of IRF3 signaling in recipient macrophages in vitro and in vivo.[141] By secreting and transferring $EGFR^{+}$ exosomes to the host macrophages, tumor-derived EGFR stimulated host MEKK2, which unexpectedly phosphorylated IRF3 at Ser173; this modification led to the repression of IRF3 and type I interferon activation, weakening the host's pathogen-defense ability (Fig. 2b). The results from this study explained a process by which malignant tumors can interfere with the innate antiviral system via exosomes and identified a mechanism by which cancer cells can dampen host innate immunity. In addition, these mechanistic studies helped to explain the diminished innate antiviral immunity frequently found in patients with cancer.[142]

Indeed, EV-innate immune system cross talk may evolve and thus may differ during tumor development. In our opinion, the functions of exosomes in cancer are determined by their specific cargos. Under certain physiological conditions or in a primary tumor, premetastatic niche and metastatic tumor sites, different TEXs containing specific contents may mediate either immunostimulatory or immunoinhibitory activity. As explained above, TEXs carrying tumor antigens can be transferred to DCs and elicit tumor-specific immune responses.[143] However, in most cases, TEXs containing immunosuppressive proteins such as PD-L1 and EGFR have been shown to cause innate immunosuppression and protumorigenic effects.[90,91,141,144]

EV-mediated regulation of TLR/NF-κB signaling

TLRs, a class of proteins that play key roles in the innate immune system, were the earliest discovered and most well-studied PRRs.[145] There are 11 TLRs (TLR1-11) that have been identified in humans, and these different TLRs specifically recognize distinct PAMPs and DAMPs.[146–149] TLR3, TLR7, TLR8 and TLR9 sense viral RNA and DNA in the endosome.[47,101,150] Among these TLRs, TLR9 recognizes CpG-containing oligodeoxynucleotides (CpG-ODNs), and the TLR9 agonist synthetic CpG-ODN is being investigated as a cancer vaccine adjuvant in clinical trials.[151,152] TLR3 in lung epithelial cells can be activated by exosomal small nuclear RNA secreted by primary tumors, resulting in the production of chemokines and the recruitment of neutrophils.[153] Once recruited in the premetastatic niche, neutrophils were shown to elicit a prometastatic inflammatory microenvironment by suppressing both innate and adaptive antitumor immunity.[154–157] Gastric cancer cell-derived exosomes containing high mobility group box-1 (HMGB1) also induce the tumor-promoting activation of neutrophils, via TLR4/NF-κB signaling.[158]

Recent studies demonstrated that EVs secreted by immune cells can also play immune regulatory roles.[159–161] For example, exosomes derived from mycobacteria-infected macrophages contain TLR ligands and thus can promote both innate and acquired immune responses.[159] Microvesicles released from infected red blood cells activate host monocytes and neutrophils.[161] In line with this finding, TLR9-activated macrophages transport ODN to naïve macrophages via EVs, thus inducing the release of chemokine TNF-α, resulting in a synergetic effect in the propagation of the intracellular immune response in vitro[162] (Fig. 2c). This study also elucidated the role of EVs in the internalization of different PAMPs and the subsequent activation of intracellular innate immune signaling. In addition to ODN, Cdc42 is transferred from EVs into naïve macrophages and further activated by TNF-α, leading to the enhancement of EV uptake.[162] Similar to TLR9, TLR7 and TLR8 localize to intracellular compartments such as endosomes, lysosomes and the ER.[150,152,163] Exosomal miR-21 and miR-29a secreted by lung cancer cells in BALB/c mice were transferred to macrophages where they could bind to TLR7/8, leading to TLR-mediated NF-κB activation and secretion of the prometastatic inflammatory cytokine TNF-α in vitro and in vivo[164] (Fig. 2c). These results suggest that the transfer of EVs with nucleic acids can activate TLR molecules to initiate innate immune responses. In addition to nucleic acids, proteins such as HSP72, which has been found on the surface of TEXs, can also activate NF-κB signaling and induce the production of interleukin-6 and TNF-α in myeloid-derived suppressor cells (MDSCs) in a TLR2/MyD88-dependent manner[165,166] (Fig. 2c). Similar results showed that TEXs that contained HSP70 activated

NF-κB signaling through TLR2 on mesenchymal stem cells (MSCs) in vitro and in vivo in nude mice.[167] During cancer progression, exosomes derived from breast cancer cells were reported to induce the secretion of TLR2- and TLR4-dependent pro-inflammatory factors by distant macrophages[168,169] (Fig. 2c). In summary, the latest evidence has revealed that TLR signaling can be the target of EVs and is required for EV-induced NF-κB activation, as well as the production of pro-inflammatory cytokines. However, we must recognize that the study of the EV-mediated regulation of TLR/NF-κB is still in its infancy; how this process is tightly controlled remains unknown. Future studies with more detailed elucidation of EV composition will help to reveal the intricate mechanisms of EV-induced immunomodulation.

EXOSOMES IN CANCER DIAGNOSTIC AND THERAPEUTIC APPLICATIONS

Liquid biopsy based on exosomes

Currently, liquid biopsy has emerged as a noninvasive and convenient approach for cancer diagnosis and prognostic monitoring. In contrast to surgical biopsy and puncture biopsy, liquid biopsy can be used to directly detect circulating tumor cells (CTCs), circulating tumor DNA (ctDNA) or cell-free tumor RNA from blood, saliva and other body fluids.

More recently, exosomes have become particularly valued for use in liquid biopsy because of their natural advantages over other samples.[170] For example, exosomes can protect nucleic acids from rapid degradation; the formation of exosomes is closely related to the state of parental cells; the detection of exosomes is more specific than that of traditional tumor markers; and exosomes are widely found in various body fluid samples. Compared with CTCs, exosomes circulate at higher concentrations in blood (e.g., $>10^9$ vesicles per mL of blood); therefore, only a small volume of blood is necessary for analysis.[170] Moreover, exosomes are highly stable in blood plasma, whereas ctDNA and cell-free tumor RNA are rapidly degraded. The standardization of sample collection, isolation and analysis methods for exosome isolation from small amounts of biofluids, such as blood plasma, has been reported in several previous ISEV position papers.[99,171,172] Ultracentrifugation is the most traditional and widely accepted technology used for exosome purification from blood plasma or cell culture supernatants.[6,173,174] Exosome-based diagnosis can also be used to monitor changes in molecular markers over time during the development of the disease. Owing to the ease and noninvasive nature of sample collection, exosome-based liquid biopsy provides clinical information, including that for diagnosis and prognosis, which contributes to clinical decisions (e.g., precision or personalized therapy, disease monitoring)[175] (Fig. 3a).

Exosomal nucleic acids, including microRNAs (miRNAs), mRNA, lncRNA, and DNA, are involved in cancer angiogenesis and metastasis and may be promising biomarkers for cancer diagnosis[13,15,176] (Fig. 3a). Over the past decades, various miRNAs in exosomes have been shown to be potential biomarkers for various types of cancer, including lung cancer, liver cancer, gastrointestinal cancer, pancreatic cancer, melanoma, breast cancer, ovarian cancer, and prostate cancer. For example, oncogenic mutated KRAS (KRAS G12D and KRAS G12V) mRNAs have been detected in serum exosomes of patients with pancreatic cancer. Interestingly, circular RNAs (circRNAs) were also abundant in exosomes derived from cancer cells and patient serum, and they may serve as a new class of exosome-based cancer biomarkers.[177,178] In addition to nucleic acids, exosomes also contain a variety of protein molecules that reflect the characteristics of their parental cells and thus can be used as molecular markers for tumor diagnosis. Although a large number of serum samples from pancreatic cancer patients showed that the proportion of glypican-1 (GPC1)-positive exosomes in the serum of pancreatic cancer patients was significantly higher than that in healthy patients,[179] the clinical application of GPC1-positive exosomes is still controversial. Some have voiced concern about diagnoses proclaimed to have 100% sensitivity, specificity, positive and negative predictive value.[180,181] Thus, it remains to be seen whether GPC1-carrying exosomes will be subsequently validated by other research groups. Recently, high GPC1 crExos were used to determine PDAC tumor size and disease burden, but they could not distinguish PDAC from benign pancreatic disease at the GPC1 levels carried by the crExos[182]. Furthermore, the combined detection of exosomal GPC1, exosomal CD82, and serum CA19-9 shows great promise as a standard method for PC detection.[183] Another study has shown that the determination of S100B and MIA in exosomes may be an alternative to their analysis in serum for the diagnosis and prognosis of melanoma patients.[184] In addition, exosomal cytoskeleton-associated protein 4 (CKAP4), a novel DKK1 receptor, may represent a biomarker for pancreatic cancer.[185] Therefore, these methods are claimed to accurately diagnose and distinguish early cancer, which may substantially change the fate of patients with cancer. However, little data concerning the expression of specific cancer-associated biomarkers for monitoring the outcome of their use in cancer patients are available, and data on prostate cancer and melanoma have been published without further clinical application.[184] The application of exosome-based cancer therapies is urgently needed.

Application of exosomes in cancer vaccines

Due to their high stability in circulation and low toxicity and immunogenicity, exosomes for use as vehicles in clinical practice are promising, and the idea of using them is inspiring.[186,187] Modified exosomes as drug delivery carriers loaded with tumor drugs or tumor-targeting RNAi have been designed for clinical applications.[188–190] In addition, as a typical immunotherapy for tumors, cancer vaccines are research hotspots that have increasingly attracted attention in recent years, and their clinical application has recently been greatly improved.[191–194] Cancer vaccines are most suitable for cancer patients receiving early or postradical treatment rather than patients with advanced disease. As mentioned above, exosomes derived from virus-infected cells can trigger an antiviral IFN response, which makes them attractive candidates for cancer vaccine development. NK cells are innate lymphoid cells that play a central role in the immune response against cancer.[195] Results from a recent study indicated that NK cell-derived exosomes mediate antitumor effects against aggressive melanoma in vitro and in vivo.[196] In addition, exosomes derived from M1-polarized macrophages may be used as a vaccine adjuvant.[197] Recently, engineered exosomes have emerged as novel approaches for cancer vaccine development in immunotherapy and have been applied in phase I clinical trials.[198] Large-scale production and purification of clinical grade exosomes from dendritic cells using good manufacturing practice have been reported, and these exosomes were found to enhance NK cell function in patients with non-small cell lung carcinoma or melanoma.[191,194] Dendritic cell-derived exosomes (DEXs) loaded with synthetic CTL-defined epitopes, such as melanoma antigen recognized by T cells 1 $(MART1)_{26–35}$ peptides, by nanotechnology were shown to elicit stronger immune responses toward cancer cells.[198] A phase II clinical trial tested the clinical benefit of IFN-γ-DEX loaded with MHC I/II-restricted cancer antigens as maintenance immunotherapy after induction chemotherapy.[199] However, a T cell response was not found in patients bearing inoperable non-small cell lung cancer (NSCLC) without tumor progression.[199] One reason for the limited efficacy for DEX immunotherapy is that the INF-γ used in the process of DEX production may upregulate PD-L1, an inhibitor of T-cell activation, in DCs and DEXs.[200–202] Previous studies have shown that PD-L1 is a marker of the immunosuppressive DC subset that accumulates as tumors progress.[203–206] Recent findings indicated that exosomal PD-L1 derived from cancer cells contributes to immunosuppression

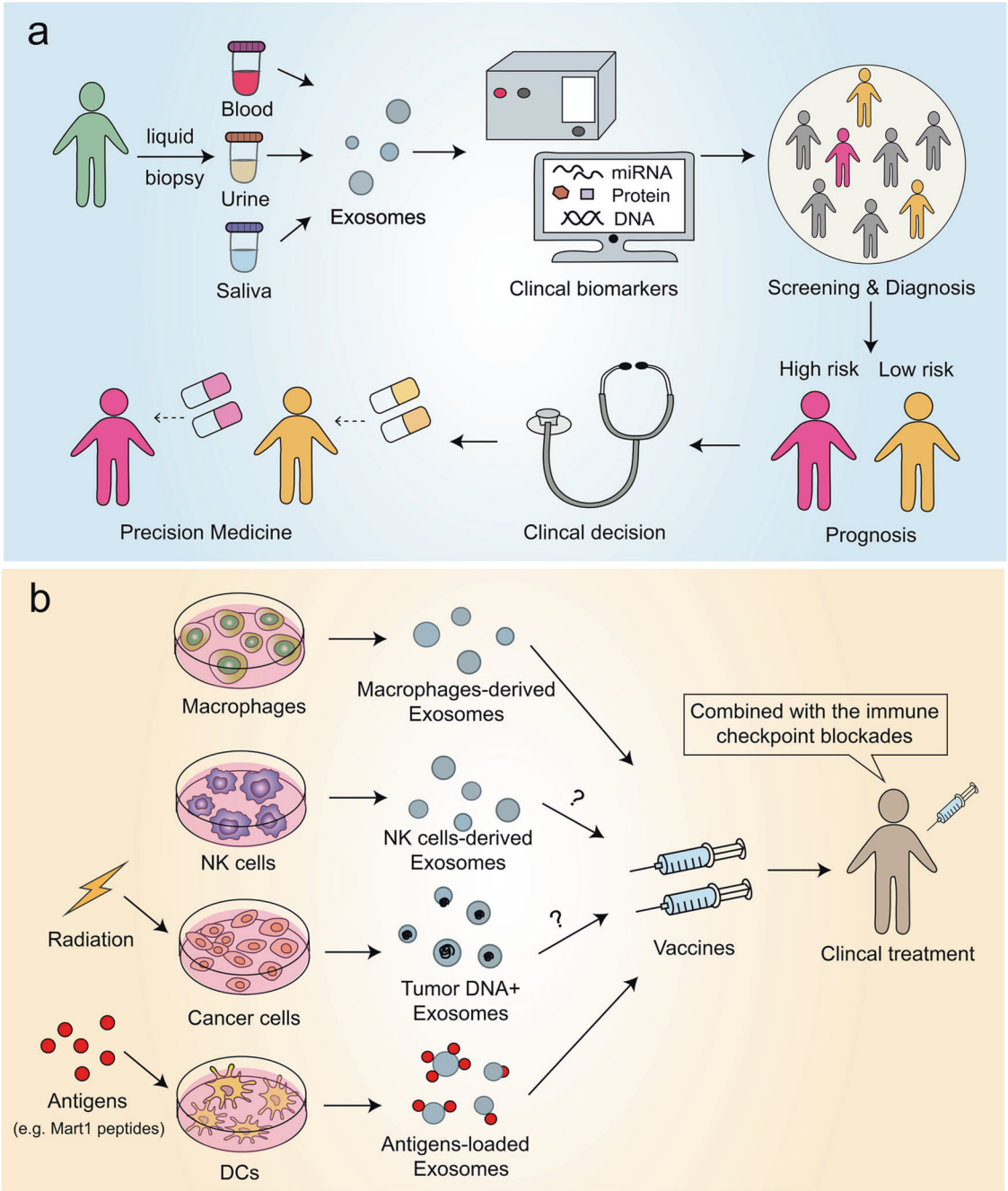

Fig. 3 Exosomes in cancer diagnostic and therapeutic applications. **a** Exosomes bearing certain proteins, miRNAs or DNA may be valuable as cancer biomarkers in liquid biopsy samples. The test results may provide meaningful guidance for disease screening, prognosis, diagnosis, risk assessment, clinical decisions, and personalized treatment. **b** Exosomes derived from M1-polarized macrophages or NK cells may be used as vaccine adjuvants, and radiation-induced tumor DNA-loaded exosomes or antigen-loaded DC exosomes are potential vaccines for cancer treatment

and mediates immune evasion.[90,91] It remains to be determined whether DEXs express functional PD-L1 or PD-L2 molecules that may restrict T cell responses. Therefore, using DCs with low PD-L1 expression to generate DC-based vaccines may be a strategy against cancer. Indeed, in line with this possibility, combining DC-based vaccines with the suppression of inhibitory signals, as with a PD-1/PD-L1 blockade or anti-CTLA4 therapy, has great potential for eliciting a better immune response against cancer.[207,208] Another study showed that targeting ovalbumin (OVA) and the G protein of vesicular stomatitis virus (VSV-G) to the same exosome-like vesicles improved the immunogenicity of exosomal vaccines in vivo.[209] Thus, incorporation of a viral fusion protein and targeting of antigens to DEXs are attractive strategies to enhance the immunogenicity of exosomal vaccines.[209] Therefore, as a new vaccine strategy for cancer immunotherapy, DEX remains promising with potential for improvement. It is important to combine DC-based vaccines with new approaches that overcome the immunosuppressive mechanisms in the tumor microenvironment and promote the activation of the immune system (Fig. 3b).

DISCUSSION

Over the past decade, the field of EVs, including that studying exosomes, has emerged as an exciting area of research. The evidence gathered from various sources has revealed that exosomes play unexpected functions in broad biological processes, including those of human diseases, such as antigen presentation, immune response, cancer metastasis, inflammation and drug resistance, through intercellular communication.[28,123,210–215] Although some breakthroughs have been made, many more unknown areas have emerged, and an increasing number of technical problems need to be resolved. Although the biogenesis of EVs or exosomes is relatively clear, the sorting and screening of exosome contents are usually linked to the ESCRT complex, and the delivery process is less clear. Questions abound on how specific proteins, RNAs and even DNA are selectively packaged into exosomes, and answers are needed for this apparent, urgent problem in the basic research field of EVs. Another inspiring question remains elusive: why do exosomes secreted by different cell types always play roles, albeit diverse, in mediating the innate immunity of recipient cells? EV populations are heterogeneous;[7,98,216–219] without doubt, the functions of exosomes must depend on the specific cargos loaded. It is conceivable that even exosome with identical content could be delivered to different recipient cells to produce different biochemical reactions and effects. In fact, it is an urgent task to comprehensively map the composition of exosomes from different origins, and great effort is required to achieve a consensus about the biochemical definition and classification of EV subpopulations and to determine the cellular signals or events that determine their size and cargo composition.

Currently, research in the field of EVs or exosomes is mainly focused on RNA. However, the mechanisms of loading and transduction, the fate after uptake and the working mode of exosomal RNA are still unclear. Considering that extracellular vesicles carry not only RNA but also a variety of proteins, lipids, and cell metabolites, most scholars believe that EV-associated components have corresponding functions as a whole structure, which definitely warrants further investigation. Interestingly, in a very recent study, adipocytes were found to release intact triglycerides packaged into small particles, called adipocyte exosomes (AdExos).[220] The uptake of these AdExos by macrophages in adipose tissue enabled the direct transfer of lipids, revealing a manner of EV-mediated exchange of signals and nutrients between adipocytes, immune cells, and metabolic organs.

By studying EVs in different disease states, we may find how their contents can modulate immune cell function to influence cancer progression. It would be very helpful to summarize the models and technologies currently used for EV-immune study and discuss their limitations. To date, most exosome studies in vitro have been established using a cell coculturing system to investigate the mechanism of EV delivery, uptake, transfer and regulation in immunity. The research of EV-immune system cross talk in vivo is mainly based on well-established transgenic or orthotopic mouse models under certain physiologically relevant experimental conditions. The use of other animal experiments, such as Drosophila or zebrafish models, to study the biogenesis, trafficking and cellular entry of EVs in vivo is currently being considered.[221] Another limitation of the current methods used to study EV-immune mechanisms likely involves the collection of pathological samples, which cannot reflect the dynamic changes in EVs. For example, the purification of EVs using ultracentrifugation methods takes a long time in the clinical setting. Therefore, novel models and analytical methods are being developed to improve the ability to characterize the behavioral dynamics and importance of these vesicles in different biological contexts. In addition, it is important to record the information obtained in samples from biofluids because donor age, diet, body mass index, medications, and other factors may affect the contexts of the EV samples. Because of the complexity of the composition and functional heterogeneity of EVs, single-vesicle identification, isolation and analyses are recommended, which will substantially accelerate our understanding of EV biology, EV-based therapies, and diagnostics. The application of exosomes as immunotherapeutics for cancer is promising, especially the use of exosomes derived from immune cells. Although studies have suggested that EVs are the main media of intercellular communication in the immune system, clinical trials have shown that using immune cell-derived EVs alone is often insufficient to induce an effective immune response in vivo.[222] To boost their immunogenicity, engineered exosomes with tumor antigens are being generated to make them more recognizable by the immune system.[223] Thus, the targeting specificity of EVs needs to be understood, and an effective strategy for loading nucleic acids, proteins and/or lipids into EVs needs to be developed. Although more work is definitely required to understand the complex functions of exosomes in innate immune regulation, their mysteries will eventually be unveiled.

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AUTHOR CONTRIBUTIONS

X.Z. and F.X. conceived and drafted the paper. X.Z., L.W., F.X., and F.Z. discussed the concepts presented in the paper. X.Z. generated the figures. F.Z. approved the version to be submitted.

ADDITIONAL INFORMATION

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