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Extracellular vesicles in vaccine development and therapeutic approaches for viral diseases

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ABSTRACT

Extracellular vesicles (EVs) are lipid bilayer nanovesicles generated by almost all living cells which possess various size ranges depending on producer cells and biogenesis mechanisms. Several EV markers were determined including tetraspanins (*e.g.,* CD9, CD63 and CD81), heat shock proteins (HSP70 and HSP90), some 14–3–3 proteins (a family of conserved regulatory molecules), major histocompatibility complex molecules (MHC-I/-II), and enzymes (Glyceraldehyde 3-phosphate dehydrogenase and enolase-1). EVs are known as an abundant source of antigens and immune molecules that can be used for vaccine development in human and animals. EV-based immunization could significantly activate immune responses in different infections such as Porcine reproductive and respiratory syndrome virus (PRRSV), Lymphocytic choriomeningitis virus (LCMV), Marek's disease virus (MDV), and SARS-CoV-2 infections. The engineered and modified EVs showed a promising potential in development of anti-tumor vaccines and therapeutics, protection against parasitic diseases (*e.g.,* Eimeria, and Plasmodium yoelii) and viral diseases (*e.g.,* COVID-19), and improvement of biomarkers. Also, EVs possess a crucial role in antigen presentation *in vivo*. In this review, we describe the roles of EVs in vaccine development and therapeutic approaches for viral diseases.

1. Introduction

Extracellular vesicles (EVs) are a heterogeneous group of natural membrane vesicles released from different types of cells. They are found in body fluids and adipose tissue. Extracellular vesicles containing biomolecules (*e.g.,* proteins, metabolites and nucleic acids) play a major role in intercellular communication, regulation of tissue repair mechanisms (*e.g.,* acute lung injury caused by the SARS-CoV-2 virus), and remodeling activities [1–4]. Generally, EVs attach to the surface of target cells by adhesion molecules, and enter their contents into cytosol by endocytosis, phagocytosis and macropinocytosis, and/or direct fusion with the membrane. The nano-sized vesicles are structurally similar to enveloped viruses. Both EVs and viruses release nucleic acids into recipient cells by endocytosis [5,6].

For the first time, EVs were found to determine the fate of the transferrin receptor during the process of red blood cells (RBCs) maturation [7,8]. The next study indicated that B-cells-released EVs containing MHC-class II molecules on their surface could elicit antigen-specific MHC-II-restricted T-cell responses [9]. Generally, EVs (30–5000 nm in size) are divided into ectosomes, microvesicles, exosomes, and apoptotic bodies (generated by cell death) based on the physical and biochemical properties, and the biogenesis process [10,11]. Exosomes (30–100 nm vesicles in diameter) are secreted by fusion of multivesicular late endosome (MVB, endocytic compartment) with the cell membrane, whereas ectosomes and microvesicles (100–1000 nm vesicles in diameter) are released by the budding of the cell membrane. The largest EVs are apoptotic bodies (1000–5000 nm vesicles in diameter) [11–14].

EVs have different functions in therapeutic approaches. In general, EV-based therapies regulate/diminish inflammation (by EVs-carried biological cargos including proteins, growth factors, miRNAs, and cytokines) [15], increase/modulate immunity (by secretion of chemokines and cytokines) [16,17], and enhance tissue regeneration (by secretion of interleukins or by release of growth factors) [18–21]. For instance, mesenchymal stem cells (MSCs)-derived EVs could produce similar or better therapeutic effects than MSCs in preclinical studies. These EVs

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were safely stored for a long time without losing activity [22,23]. MSCs and MSC-derived EVs were used in clinical trials for the treatment of COVID-19 patients [22]. Moreover, the normal and pathological conditions can be determined by EV contents [24]. For example, Epstein-Barr virus (EBV)-infected cells released EVs containing the latent membrane protein 1 (LMP-1) for immune escaping and virus survival [25]. Also, $CD81^+$ EVs released by hepatitis C virus (HCV) into the extracellular space could hide the viral RNA for detection and neutralization by immune cells [26]. The findings showed that EVs transfer viral entry receptors (*e.g.,* CCR5 receptor in HIV) to non-susceptible cells leading to later human immunodeficiency virus-1 (HIV-1) infection [27]. In addition, Ebola virus-derived $VP40^+$ exosomes induced cell death/apoptosis [28]. On the other hand, EVs deliver host-derived antiviral compounds, and immune enhancers [29,30]. For instance, cytidine deaminase protein (APOBEC3G)-loaded exosomes showed antiviral effects against the HIV-1-infected cells [31]. Furthermore, exosomes secreted from lymphoblastoid B cell line (LCL1) expressing EBV structural protein gp350 selectively targeted B-cells to elicit strong antiviral immunity [32].

EVs play an important role in vaccine development. The reports showed that immunogenicity of adenoviral vaccines was improved by directed targeting of an antigen to the exosome surface in preclinical studies [11,33]. Indeed, EVs are an abundant source of antigens and immune responses-related molecules for development of human and animal vaccines [34–36]. For instance, gram-negative bacteria-derived immunogenic spherical nanoparticles known as outer membrane vesicles (OMVs) were used as useful tools for therapeutic approaches. Several promising vaccine strategies were designed using decoration of OMVs with heterologous proteins and glycan antigens for stimulating the immune system, and reducing the toxicity [37]. In addition, OMV-based vaccines stimulated protective immunity against influenza viruses [38–40]. On the other hand, various studies indicated that monocytes-derived EVs loaded with viral peptides from Influenza virus, Epstein-Barr virus, and Cytomegalovirus could induce the IFN-γ secretion from antigen-specific $CD8^+$ T cells [41]. Main components of EVs and their functions are indicated in Table 1.

Recent studies have indicated that the extracellular vesicles are used for viral drug/vaccine delivery and also the diagnosis of inflammatory diseases. Indeed, EVs have a major role in cellular communication and pathogenesis [21]. The contents of EVs (or EV cargos) can enter the target cells, and promote or suppress the viral infection [6,42]. For example, the delivery of specific microRNA (targeting sialoadhesin or CD163 receptors involved in the attachment and internalization of viral particles) to Sus scrofa cells using EVs could suppress the infection of porcine reproductive and respiratory syndrome virus (PRRSV) [43]. Also, tumor cells/host cells/bacteria/parasites-derived exosomes could mediate the communication between the invader and innate immune cells, and modulate host innate immune responses [44]. The roles of extracellular vesicles are shown in Table 2. As shown in Table 2, exosomes harboring nucleic acids (*e.g.,* microRNAs, mRNA, lncRNA and DNA) or containing various protein molecules are involved in cancer angiogenesis and metastasis. These vesicles were used as promising biomarkers for tumor diagnosis [45–47]. Moreover, EV-carried antigens after DNA vaccination generated higher immunogenicity and stronger activities of CTLs (cytotoxic T lymphocytes) than soluble antigens in tumor mouse model [48]. Therefore, the characterization of EVs' composition can provide novel diagnostic information in a variety of diseases [49]. Characterization of exosomal cargo is of interest because it affects biogenesis, targeting, and cellular effects of exosomes, and may be a source of biomarkers for disease diagnosis, prognosis and response to treatment. The contents of exosomes change when transitioning from health to disease [50]. In this review, we describe the roles of EVs in vaccine development and therapeutic approaches for viral diseases. Fig. 1 describes the biogenesis of EVs.

2. General description of EVs in therapeutic approaches

EVs have a major role in cellular communication, protection of their cargo from degradation in targeted delivery of drugs and active molecules (*e.g.,* proteins, RNA, and lipids), signal transduction, cell survival and diagnosis (*e.g.,* EVs as a carrier for transferring infectious material). EVs are used for modulation of inflammatory pathway, induction of immune response, and treatment of cancer, cardiovascular, lung and neurological diseases [51]. EVs are considered as key mediators of immunopathogenesis in bacteria, fungi, and protozoa [52]. Extracellular vesicles (EVs) released by pathogens (*e.g.,* bacteria, fungi, and parasites) indicate the importance of EV molecules in various infections. Pathogen-derived EVs (harboring proteins, lipids, nucleic acids, and glycans) contribute to modulation of the immune responses in their host [53]. For instance, bacterial EVs-carried toxins modulate antigen presentation, and pathogen clearance. In contrast, EVs-related polysaccharides induce protective immune responses as potential vaccine targets [53]. The studies indicated that polysaccharides induce differentiation of memory B cells that are different from immune responses

Table 1
Major components and markers of extracellular vesicles derived from normal and tumor cells, parasites, fungi, and bacteria.

Cell types	Tumor cells	Mammalian/ Health cells	Bacteria	Protozoa	Fungi
Markers/ Molecules for Delivery	MHCI & II; miRNA & mRNA; CXCR4 & MMP-9; TrkB, EGFR & TES complex; Rab22A, Pabp1& PSA; CD40, CD80, CD86 & CD54; GTP_{ase} & Rab27a; FADD, P-glycoprotein, MPPs, PS & TF	MHCI & II; FasL; mRNA, miRNA, Caspase3, Signaling components, Complement proteins & Cytokines	OmpQ & pertactin; Gene transfer; Gentamycin; RNAs	tGPI-mucin; Tc85; Gp63 & LPG; TS	α-gal; GXM & GlcCer
Functions	Antigen presentation; Oncogenic activity, drug resistance, metastasis and angiogenesis; Invasion and migration; Angiogenesis; Metastasis; Immunity; Up-regulation of immune system and inhibition of tumor growth; Immune suppression; Matrix degradation; Coagulation	Antigen presentation; Immune suppression; Communication; Inflammation; Cellular homeostasis; coagulation	Immunogenicity; Communication; Cell death; Virulence factor; Modulation of the immune system; Antibiotic resistance	Activation; Invasion; Adhesion; Virulence factor	Immunogenicity; Virulence factor; Serological markers; Immunomodulatory activity
References	[170–182]	[183,184]	[185–189]	[190–196]	[197–200]

Abbreviations: MHC, major histocompatibility complex; miRNA, microRNA; CXCR4, CXC motif chemokine receptor 4; MMP-9, matrix metalloproteinase; TrkB, tropomyosin receptor kinase B; EGFR, epidermal growth factor receptor; TES, testin; Rab22A, Ras-related protein; Pabp1, polyadenylate-binding protein 1; PSA, prostate-specific antigen; CD40, cluster of differentiation 40; GTPase, guanosine triphosphatase; Rab27a, ras-related protein; FADD, fas-associated protein with death domain; MPP, mitochondrial processing protease; PS, phosphatidylserine; TF, transcription factor; FasL, fas ligand; OmpQ, outer membrane porin protein; tGPI, toxoplasma gondii protease inhibitor-1; Tc85, trypanosome cruzi surface glycoprotein; Gp63, glycoprotein 63; LPG, lipophosphoglycan; TS, thymidylate synthase; α-gal, α-galactose; GXM, glucuronoxylomannan; GlcCer, glucosylceramide.

Table 2
The roles of extracellular vesicles and exosomes in clinical trials.

EVs in clinical trials		Ref.
Biomarker		
Source	**Application**	
Bronchoalveolar lavage fluid	Effect of cigarette smoking on EV miRNA profiles	[201]
Serum	Tumor-associated hypoxia (prognostic value)	[202]
Cerebrospinal fluid	Specific PD-associated mutations in LRRK2	[203]
Blood & Urine	Expression of the HSP70 protein in cancer patients	[204]
Treatment		
Blood	Acute myocardial infarction	[205]
Autologous platelet- and EV-rich plasma	Chronic inflammation of temporal bone cavities	[206]
Dendritic cells	Non-small cell lung cancer	[207]
Glioma	Malignant glioma	[208]
Fruit	Colon cancer	[209]
Fruit	Mucositis	[210]
Dendritic cells	Metastatic melanoma	[211]
Dendritic cells	Non-small cell lung cancer	[212]
FetA modified strain 44/76	Meningitis	[213]
B:4: P1.7–2; 4 strains	Meningitis	[214]
Ascites fluid	Colorectal cancer	[215]
Umbilical cord-blood derived MSC	Type 1 diabetes mellitus	[216]
MSC	GVHD	[217]
B:4: P1.7–2; 4 strains	Meningitis	[218]
Exosomes in clinical trials		
Biomarker		
Source	**Application**	
Urine	Prostate cancer	[219]
Bronchoalveolar lavage fluid	Non small Cell Lung Cancer	[220]
Blood and Urine	PD-susceptibility, progression and therapy effectiveness	[221]
Blood	*In vitro* effects on blood coagulation and platelet function	[222]
Blood and Urine	Sepsis, septic shock or multiple organ failure	[223, 224]
Urine	Kidney transplanted patients with calcineurin inhibitors	[225]
Blood	Pre-adolescents with high risk for development of type 2 diabetes	[226]
Treatment		
Mesenchymal stromal cells	SARS-CoV-2 infection	[227]
Mesenchymal stromal cells	SARS-CoV-2 infection	[228]
Wharton's Jelly-derived Mesenchymal stromal cells	Chronic skin ulcer healing	[229]
Dendritic cells	Immunotherapy in Non Small cell lung cancer	[230]

Abbreviations: PD, programmed cell death protein 1; LRRK2, leucine rich repeat kinase 2; HSP70, heat shock protein 70; MSC, mesenchymal stem cell; GVHD, graft *versus* host disease; SARS-CoV-2, severe acute respiratory syndrome coronavirus-2.

related to protein antigens. A polysaccharide-specific IgG response results from memory B cells that act as T-independent type II immune responses in naive B cells sensitive only to polysaccharides [54]. Indeed, conjugation of polysaccharides to a carrier protein induced a T-cell--dependent immune response to the glycan moiety [55]. It was reported that polyvalent polysaccharide vaccines may act through caveolae-mediated memory extracellular vesicles resulting in prolonged signaling to B cells, and the lack of cell-mediated stimulation [54]. Moreover, the lipid A moiety of lipopolysaccharide (LPS) in bacteria-derived EVs elicits potent pro-inflammatory responses [53].

On the other hand, parasite EVs-associated microRNA may increase parasite survival through suppression of host gene. Thus, study of molecules associated with pathogen-derived EVs and their effects on the host immune system show the importance of EV molecules in infection biology, and open new ways to prevent and control infectious diseases by immune intervention [53]. The findings demonstrated that virus-infected cells secrete viral proteins, RNA, cellular proteins and miRNA in exosomes. For example, miRNA and viral proteins of Epstein-Barr virus (*e.g.,* LMP1, Galectin-9 and dUTPase) in exosomes derived from infected cells generally led to cell growth, migration by the PI3K/Akt pathway activation, apoptosis, and inflammation. Furthermore, human herpes virus 6-infected cells release exosome-enveloped virions leading to distribution of the virus between cells. On the other hand, Kaposi's sarcoma-associated herpes virus-derived exosomes change the metabolism of the recipient cells, promote latency, and help in generation of tumor [56].

EVs are applied as a drug delivery system in preclinical studies [57]. For instance, bacterial membrane vesicles (BMVs) play an important role in the regulation of the host immune system (immunomodulation) due to delivery of small RNA, and other types of noncoding RNA [58]. These vesicles transport microbial derived substances, and thus vary widely in their composition and function [59]. For example, natural outer membrane vesicles (OMVs) generated by all gram-negative bacteria can deliver several antigens in their native environment for vaccine development. Moreover, they contain host defense peptides (*e.g.,* cathelicidins), CATH-2, PMAP-36 and K9CATH that can modulate immune responses. For example, cathelicidins interact with lipopolysaccharide (LPS), and neutralize LPS-induced TLR4 activation resulting in reduction of undesired immune responses related to LPS. It was shown that TLRs 2, 4, 5 and 9 (especially TLR-4) were involved in stimulation of macrophages by OMVs, and cathelicidins could modulate these immune responses [60]. On the other hand, bacterial membrane vesicles are responsible for biofilm formation, bacterial colonization in the host tissue, and survival (/antibiotic resistance) leading to pathogenesis and virulence [58]. Fig. 3A shows bacteria-infected cells release vesicles that modify T-cell and macrophage function. In addition, therapeutic potential of EVs derived from MSCs were reported in a variety of tissues in preclinical trials. Other findings showed that regenerative and immunomodulatory cells-derived EVs including amniotic epithelial cells, endothelial progenitor cells, induced pluripotent stem cells, embryonic stem cells, cardiosphere-derived cells, and dendritic cells (DCs) have therapeutic effects in wound healing, vascular repair, myocardial infarction, pulmonary fibrosis, and finally vaccination in preclinical studies [61].

3. General description of EVs in vaccine development

EV-based vaccination strategies against pathogens include pathogen antigen-pulsed EVs, EVs derived from infected cells, and pathogen-released EVs [61]. For instance, both gram-negative and gram-positive bacteria release EVs that are similar in size to mammalian-derived EVs, and mediate bacteria-host communications by transporting various bioactive molecules [62]. Successful use of gram-negative bacteria-derived EVs to induce antigen-specific humoral and $CD8^+$ T-cell responses was primarily described [63,64], which are essential in therapeutic vaccination against tumors and intracellular viruses. Recently, EVs released by gram-positive bacteria were identified as promising vaccine components. Immunization with EVs from gram-positive bacteria was reported to be successful in stimulation of antigen-specific antibodies and T-cell responses such as *S. aureus*-derived EVs [62,65]. Rivera et al. showed that *Bacillus anthracis*-derived EVs or *Mycobacterium tuberculosis*-derived EVs stimulated strong immune responses leading to higher survival rates in mouse model [66, 67]. On the other hand, the first vaccine based on pathogen-derived EVs was OMVs released from *Neisseria meningitidis* serogroup B (a gram-negative bacterium) [68–70]. Four licensed OMV vaccines are available [69–71] such as Meningococcal group B OMV vaccine (Bexsero) containing multiple antigens for broad protection against bacteria [71].

4. Extracellular vesicles and immunity

The immunomodulatory properties of EVs are presently under

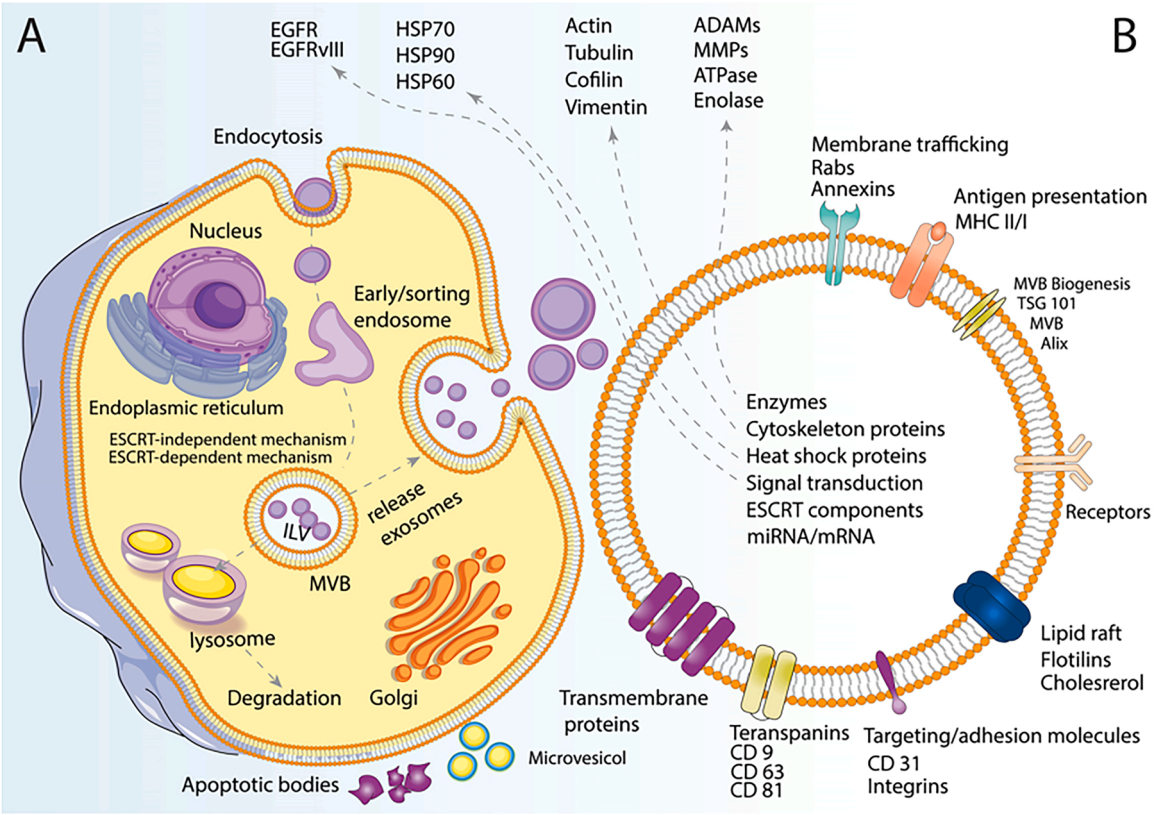

Fig. 1. A) The biogenesis of extracellular vesicles (EVs): EVs can be divided into three subtypes: exosomes, microvesicles and apoptotic bodies. Exosomes are formed as intraluminal vesicles (ILVs) in the multivesicular bodies (MVBs). This process requires the involvement of the ESCRT (endosomal sorting complexes required for transport) components and it also occurs in ESCRT-independent pathways. After ILVs formation, MVBs are transported to the plasma membrane or the lysosome. Finally, MVBs fuse with the plasma membrane using SNARE (SNAP REceptor) complex and exosomes are released. Microvesicles are released directly after the outward budding from the plasma membrane. Apoptotic bodies only generate from apoptotic cells and shed from the cell surface; B) Structure of exosomes with different markers and contents: Exosomes are made up of lipid bilayers, and enriched in proteins, nucleic acids and lipids (B).

investigation. Fig. 2 shows internalization mechanisms and signalling pathways for exosomes. Functional effects of EVs and also dendritic cells-derived exosomes (dexosomes/DEX as a major member of EVs) in modulation of immune system are described individually as follows.

4.1. *Functional effects of EVs in modulation of immune system*

The markers (molecules for delivery) and functional effects of EVs are special in different strains and cell types. Generally, EVs affect the presentation of antigens, suppression of immune system, inflammation, intercellular interaction/communication, coagulation, and cellular homeostasis in normal and tumor cells [49]. For instance, the mammalian cells-derived EVs carry biological molecules including mRNA, miRNA, major histocompatibility complex (MHC) classes I/II, cytokines, caspase 3, signaling factors, and structural proteins. The tumor cells-derived EVs include FasL, MHCI/II, mRNA, miRNA, Fas associated *via* death domain (FADD), P-glycoprotein, Matrix metalloproteinases (MMPs), prostaglandin (PS), and transferrin (Tf) [49]. On the other hand, EVs transport virulence factors in different infections. For example, bacteria-derived OMVs carry adaptation factors, and resistant markers to antibiotics. Also, fungi-derived EVs contain serological markers and immunomodulatory molecules [49].

EVs (especially exosomes) derived from host cells, tumor cells, bacteria or viruses modulate host innate immune responses. They transfer antigens and activate $CD4^+$/$CD8^+$ T cells directly *via* cross-dressing (*i.e.,* without intervention of peptide-MHC complex) or indirectly *via* antigen presenting cells (APCs) [72–75]. Exosomes play a dual role (activation or inhibition of the immune system) depending on the conditions [76,77]. For instance, the intestinal epithelial cells-derived exosomes showed immunosuppressive potency [78]. In contrast, the retinal pigment epithelium (RPE)-derived EVs demonstrated immunomodulatory effects [79–81]. In general, immune cells-released exosomes are strongly immunogenic with low side effects [82–84].

Therapeutic strategies based on the immunomodulatory properties of EVs are presently in progress such as EV-mediated delivery of anti-tumor drugs/vaccines for cancer treatment in clinical trials [82–85]. NK- and dendritic cells-derived exosomes were tested in aggressive melanoma [86] and in non-small cell lung carcinoma, respectively [87]. EVs can even neutralize SARS-CoV-2-related cytokine storm [88]. Currently, the treatment of inflammation is a critical step in a variety of diseases. As known, the danger-associated molecular patterns (DAMPs) generate inflammation through activation of the inflammasomes in immune cells [89]. Thus, the inflammasome-induced EVs reduce cytokine activity, and elicit the process of tissue repair [89,90]. The role of EVs in regulating inflammation and immune responses is crucial for development of therapeutic drugs [91]. Recent researches have indicated that viral components are found on the surface of infected cells-released EVs (*e.g.,* functional importance of EVs in influenza virus distribution) [92]. Moreover, EVs are used in cell-free vaccine platforms; because they are stable in circulation, and immunogenic with low toxicity [93]. EVs are able to deliver antigens and elicit both humoral and cellular immune responses. For example, *Mycobacterium bovis*-derived exosomes harboring bacterial antigens generated memory $CD4^+$ and $CD8^+$ T cells [94–96]. On the other hand, the host-derived exosomes generated during macrophage infection could promote activation of Th1 cells. For instance, the exosomes released during *Salmonella* infection induced protective immune responses against bacteria in mice [97].

Regarding the roles of EVs in immunity, EVs-mediated effects in viral

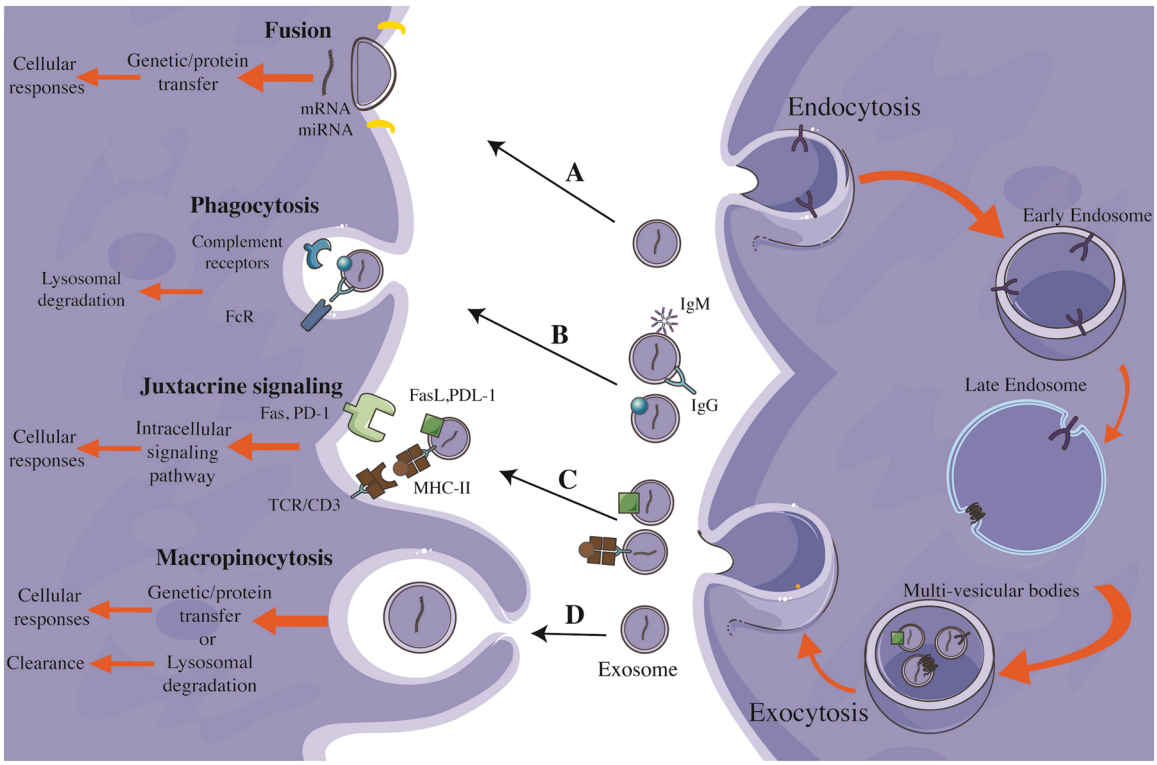

Fig. 2. Internalization mechanisms and signalling pathways for exosomes: Despite of considerable data about exosome biogenesis and secretion, but there is few data about the uptake of exosomes by immune and non-immune cells and the internal signalling pathways. It is not clear whether exosomes must be internalized by immune and non-immune cells for inducing cellular responses or not. Based on different opinions, the internalization of exosomes can occur by fusion (A), macropinocytosis (B) or phagocytosis (D). The macropinocytosis or phagocytosis methods further show mechanisms for the clearance of exosomes, rather than the induction of cellular immune responses. On the other hand, RNA species (*e.g.,* mRNA, miRNA) internalized through fusion could elicit cellular immune responses. In contrast, membrane-bound apoptosis-inducing ligand (FasL), and tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) molecules on the surface of exosomes are not internalized, and induction of cellular immune responses depend on location and temporary adhesion for juxtacrine (juxtacrine signalling; C). In general, some molecules on the surface of extracellular vesicles such as PDL1 (immune-checkpoint molecules programmed death ligand 1), CTLA4 (cytotoxic T lymphocyte antigen 4), FasL, and TRAIL interact with receptors expressed by T cells and natural killer (NK) cells leading to inhibition of their activity and/or induction of apoptosis.

pathogenesis are summarized such as: a) Enhancement of *picornavirus* replication and packaging using phosphatidylserine lipid-enriched EVs [98]; b) Enhancement of herpes simplex virus 1 (HSV-1) transmission to new host cells using delivery of microRNA and mRNA molecules by exosomes [99,100]; c) Activation of $CD4^+$ lymphocytes and decrease of EBV pathogenesis using EVs derived from EBV-infected B-lymphocytes harboring MHC II-antigen complex [101]; d) Induction of $CD4^+$T cell apoptosis, and evasion of EBV from host immune response using EBV-infected nasopharyngeal carcinoma cell-derived exosomes transporting immune-regulatory protein Galectin-9 [102]; e) Activation of innate immunity (natural killer cell: NK cells), activation of T cells and IFN-γ secretion, and decrease of hepatitis B virus (HBV) pathogenesis by induction of the NKG2D (natural killer group 2 member D) ligand expression in macrophages using exosomes generated from HBV-infected cells [103,104]; f) Enhancement of hepatitis C virus (HCV) spread, viral packaging, and stability of infection using exosomes containing viral particles [105]; g) Enhancement of HCV spread using transportation of viral regulatory elements (human protein argonaute-2 (Ago2) and microRNA-122 (microR-122)) by exosomes [106]; h) Enhancement of autophagy and Zika virus (ZIKV) resistance, reduction of virus replication, and antiviral effects using EVs carrying C19MC microRNA (chromosome 19 microRNA) [107,108]; i) Induction of placental pro-inflammatory cytokines and increase of ZIKV pathogenesis using exposure of placental cells to macrophage-derived exosomes [109]; j) Enhancement of human immunodeficiency virus (HIV)− 1 infectivity in lymphoid tissues using the release of HIV-1 Gp120-loaded EVs [110]; k) C-X-C motif chemokine receptor 4 (CXCR4)-mediated apoptosis, enhancement of $CD4^+$ T-cell tolerance to HIV-1, and increase of viral pathogenesis using delivery of Nef antigen through exosomes to target cells [111]; l) Upregulation of inflammation-related genes in human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CM) and increase of severe acute respiratory syndrome β-coronavirus 2 (SARS-CoV-2) pathogenesis using uptake of exosomes derived from SARS-CoV-2-infected cells by hiPSC-CMs [112]; m) Indirect infection of target and increase of SARS-CoV-2 pathogenesis using viral RNA packaged within exosomes derived from SARS-CoV-2-infected cells [113,114]; n) Generation of neutralizing antibodies in SARS-CoV-2 vaccine development, and decrease of pathogenesis using incorporation of spike (S) protein into exosomes [113,114].

4.2. *Functional effects of dexosomes in modulation of immune system*

Dexosomes are dendritic cells-derived symmetric heat-stable nanovesicles (/exosomes) harboring tetraspanins and all proteins for antigen presentation (*e.g.,* MHC-I/-II, and co-stimulatory molecules). Dexosomes induce antigen-specific cellular immunity through delivery of the antigen-MHC complexes to naïve DCs [115]. Indeed, dexosomes cannot interact with T cells until they are captured by other DCs for antigen processing and antigen-specific T cell priming [116]. For instance, antigen-loaded dexosomes could stimulate potent antitumor immunity in different *ex vivo* and *in vivo* studies [115]. These studies showed that immature DCs internalize dexosomes whereas mature DCs typically maintain dexosomes on their external surface [117]. Other studies indicated that dexosomes induce T cells against tumor cells [118,119].

For example, human breast adenocarcinoma cells treated by dexosomes (compared to untreated cells) re-stimulated the proliferation of formerly activated T cells leading to high IFN-γ secretion [118]. Moreover, dexosomes could induce the proliferation of splenic cells resulting in cytotoxic effects against L1210 tumor cells [119]. Fig. 3 shows functional effects of EVs in immunity.

5. Viral vaccine development based on EVs derived from different sources

EV-based vaccines have indicated hopeful results against various types of infectious diseases [120–122], Table 3]. The roles of extracellular vesicles and exosomes in vaccine development are shown individually in the next sections.

5.1. EVs in viral vaccine development

The roles of EVs are described in viral vaccine development based on the source of EVs. Several experimental studies have been performed for using EVs in vaccination against viral diseases such as: a) Stem cells-derived EVs (after transfection with plasmid DNA expressing Nef mutant/Influenza virus A-nucleoprotein (NP)) could significantly induce CTL activity in mouse model [123]; b) The S-glycoprotein-incorporated EVs (SGTM) followed by adenoviral vector vaccine (Ad-SGTM) could successfully elicit neutralizing antibodies against SARS-CoV infection in mice [124]; c) Mammalian cells infected with human cytomegalovirus (CMV), and HIV could generate antigenic EVs and stimulate immune responses. In fact, EVs harboring HIV Gag protein, and CMV gB protein could stimulate memory $CD4^+$ T cells in the presence of APCs [125,126]; d) The Spike (S) envelope protein presented on EVs could trigger humoral response against SARS-CoV infection [127]; and e) Peptides-loaded EVs from Epstein-Barr virus, Cytomegalovirus, and Influenza virus could directly induce IFN-γ secretion *in vitro* [128].

In EV-based vaccines, one strategy is the use of a specific EV subpopulation with pro-inflammatory properties. For instance, bone marrow-derived macrophages primed with lipopolysaccharide and adenosine triphosphate could generate EVs harboring caspase-1, IL-1β, and inflammasome components, and induce an effective immune response in naive animal models [22]. Other strategy is the use of viral vectors along with EVs. For example, this strategy was used for SARS-CoV treatment in mouse model. Indeed, two injections of EVs expressing the chimeric S protein without adjuvants effectively induced neutralizing antibodies [129]. Moreover, the highest neutralizing activity was observed when EVs expressing the chimeric S protein were used for priming followed by the adenovirus vector expressing the chimeric S protein. Generally, the use of EV-based vaccines was more potent than protein-based vaccines likely due to facilitating the crosslink between EVs harboring viral protein on their surface and B-cell receptors [129]. Moreover, EVs are utilized in vaccine as natural carriers for viral antigens. They present the antigens in their native form for stimulation of a potent immunity. For example, an intramuscular injection of DNA vector expressing HPV E7 oncoprotein fused to the C-terminus of protein Nef^{mut} exosome indicated high expression of target protein, and induced effective antigen-specific CTL responses [130]. Such approach was used to incorporate immunogenic antigens from different pathogens such as

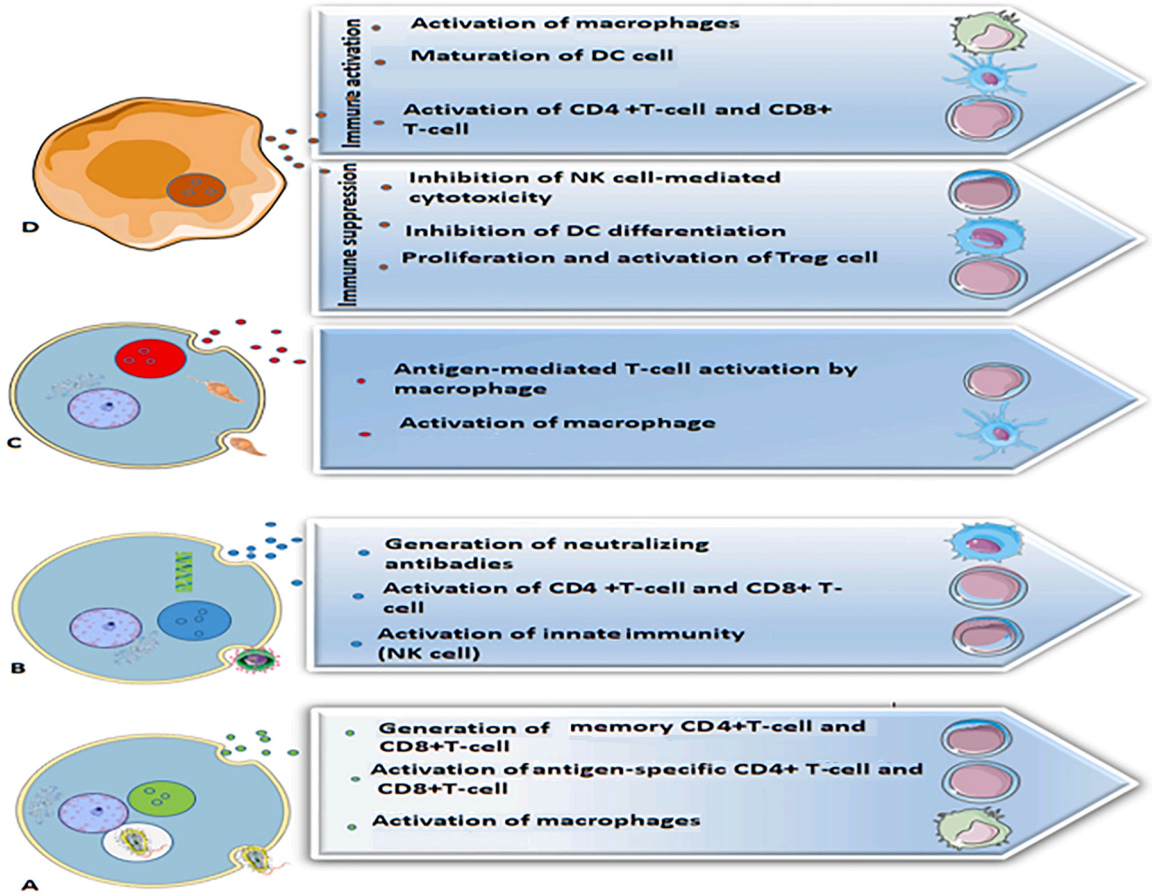

Fig. 3. Functional effects of EVs in immunity: EVs play a dual role (activation or inhibition of the immune system) depending on the conditions. EVs derived from tumor or infected cells modulate host innate and acquired immune responses through antigen presentation and cytokine production. Bacteria-infected cells release EVs that modify T-cell and macrophage function (A); Viral or parasitic-infected cells release EVs that modulate host immunity (B & C); EVs released from tumor cells can inhibit or promote immune system (D).

Table 3
Extracellular vesicles in vaccine development.

Vaccine strategy	Cell type	Model	Injection type	Results	Ref.
EVs isolated from DCs pulsed with antigens of *Eimeria tenella* sporozoites (First EV-based animal vaccine)	$CD45^+$ dendritic cells isolated from the intestine	Chickens	Intramuscular	The surface of EVs contained MHC-I/-II, CD80, flotillin and HSP70 proteins; High induction of IgG, IgA, IL-2, IL-16 and IFN-γ; Lower intestinal lesions and mortality	[231]
Serum EVs released from MDV infection	Serum EVs	Chickens	Subcutaneous	Serum EVs from CVI988 (Rispens)-vaccinated chickens included a high quantity of anti-tumor microRNAs	[232]
Dendritic cell-derived exosomes during murine LCMV infection (first vaccination trial using animal virus and EVs)	BMDCs	Mice	Subcutaneous and Intravenous	The surface of EVs showed CD11c, CD80, CD86 and MHC class I and II molecules; DC-derived EVs did not activate antiviral CTLs, and did not protect the mice in a challenge trial	[233]
EVs + Alum	*Heligmosomoides polygyrus* (nematode)	C57BL/6 mice	Intraperitoneal	Vaccination decreased worm burden by 82%; Induction of IgM, IgG1, IgA & IgE isotypes; Generation of EV-responsive IgM	[234]
EVs without adjuvant	*Trichuris muris* (nematode)	C57BL/6 mice	Subcutaneous	Vaccination decreased worm burden by 60%; Increase in IgG1 & IgG2a/c serum antibody	[235]
EVs without adjuvant	*Echinostoma caproni* (trematode)	Balb/c mice	Subcutaneous	No difference in worm burden; Delay in parasite development; Increase in survival rate of mice	[236]
EVs + Alum	*Opisthorchis viverrini* (trematode)	Hamsters	Intraperitoneal	Vaccination decreased worm burden by 27%; Average length of worms became shorter; Increase in IgG level; Antibodies from vaccinated hamsters blocked the uptake of EVs by cholangiocytes	[237]
EVs (ExoFlo™)	Bone marrow MSCs	Human/Phase II	Intravenous	Outcome:-; Status: Not recruiting; NCT04493242 (EXIT COVID-19)	[122,238]
EVs (ExoFlo™)	Bone marrow MSCs	Human/ Access	Intravenous	Outcome:-; Status: Active; NCT04657458 (COVID-19)	[122]
EVs (CAP-1002)	Cardiosphere	Human/-	Intravenous	Outcome:-; Status: Inactive; NCT04338347(COVID-19)	[122]
Exosome	T-cell	Human/Phase I	Aerosol	Outcome:-; Status: Not recruiting; NCT04389385 (CSTC-Exo; COVID-19)	[122]
EVs	Amniotic fluid (Zofin™)	Human/Phase I & II	Intravenous	Outcome:-; Status: recruiting; NCT04384445; COVID-19	[122]
Exosome	Adipose MCS	Human/Phase I	Aerosol	Outcome:-; Status: recruiting; NCT04276987 (treatment of COVID-19); NCT04313647 (healthy volunteers)	[122]
Exosome	MSC (EXO1 & EXO2)	Human/Phase I & II	Aerosol	Outcome:-; Status: complete; NCT04491240 (COVID-19)	[122]
EVs derived from MSCs	MSCs	Human/Phase I	Directly into the lung/ intravenous	Outcome: Awaiting completion (ChiCTR2000030261, ChiCTR2000030484); Treatment of pneumonia and recovering lung damage caused by SARS-CoV-2	[122]
Dexosome derived from Autologous MCDC cultures loaded with HLA-MAGE-A3, -A4, -A10, and MAGE-3DPO4 peptides (melanoma-associated antigens)	PBMCs	Human/Phase I	Combination of subcutaneous (90%) and intradermal (10%) injections	Production of the DEX vaccine was practical; DEX therapy was well tolerated in patients with advanced NSCLC; MAGE-specific immune reactivity was confirmed by DTH response; Some patients experienced long term stability of disease and activation of immune effectors	[239]
MCDC dexosome	Monocyte derived-DC culture supernatants	Human/Phase I	Intradermal and subcutaneous	MAGE-specific $CD4^+$ and $CD8^+$ T cell activation and DTH response were not detected in the peripheral blood of patients (with MAGE-3-overexpressed malignant melanoma); Production of exosomes was practical in large scale; Exosome administration was safe	[240]
Immature MCDC dexosomes expressing NKG2D ligands on their membrane which bind to NKG2D on NK cells	Monocyte derived-DC	Mice, Human/Phase I	Intradermal and subcutaneous	The improved control of tumor metastasis in B16F10 melanoma cell-inoculated mice by $NK1.1^+$ cells; Enhancement of the number of circulatory NKs; Induction of NK proliferation *in vivo* in an IL15Rα-dependent fashion	[241]
Dexosomes originated from LPS- or IFNγ-matured DCs	Matured DCs	Human/Phase II	Intradermal	The use of IFN-γ dexosomes was insufficient to manifest TAA-specific T cell reactivity; Progression-free survival at four months post-chemotherapy in advanced NSCLC patients	[242–244]
DEXs derived from DCs transfected with an adenoviral vector expressing HIV gp120 (Gp120-Texo)	DCs	Mice	Intravenous	Induction of strong and long-term HIV-specific $CD8^+$ T-cell responses independent of $CD4^+$ T cells and DCs	[143]
Exosomes purified from DC_{Gag} (EXO_{Gag})	DC expressing Gag (DC_{Gag})	C57BL/6 mice	Intravenous	Induction of specific immune response against Gag	[144]
EVs (HIV Nef protein as an EV-anchoring	DNA vector expressing fusion injected in mice	Mice	Intramuscular	Induction of high and specific $CD8^+$ T cell immunity for all tested viral proteins	[134]

(*continued on next page*)

Table 3 (*continued*)

Vaccine strategy	Cell type	Model	Injection type	Results	Ref.
protein in place of CD63, and its fusion with proteins from different viruses including HPV E7, Ebola VP24/VP40/NP, Influenza NP, Crimean-Congo Hemorrhagic Fever NP, West Nile NS3, and HCV NS3)					
Unmodified exosomes derived from THP-1 cell line	THP-1: Lipopolysaccharide-stimulated human monocytic cell line	Mice	Subcutaneous	Induction of Th1 immune response, Enhancement of the IFN-γ level, Adjuvant activity of EVs in HBV vaccines	[245]
Exosomes from mice infected with Influenza virus	Lung and serum	Mice	Inhalation	High levels of miR-483–3p associated with the release of pro-inflammatory cytokine	[246,247]
Natural EV engineering	HEK-293 T	Mice	Subcutaneous	Expression of the S protein of SARS-CoV-2 embedded in the EVs; Induction of potent neutralizing and cellular responses without any adjuvant	[248]
EV vaccines containing S-glycoprotein boosted with adenoviral vector vaccine	HEK-293 T	Mice	Subcutaneous	Production of relatively efficient antibody response in the SARS patients	[124,249]
EVs loaded with viral peptides from Influenza virus, Epstein-Barr virus, and Cytomegalovirus	Monocyte-derived DCs	Peripheral blood from human/*in vitro*	-	The release of IFN-γ from $CD8^+$T cells in an antigen-specific manner	[41]
EVs engineered to present M/NS/L antigens of Respiratory syncytial virus	Dendritic cells	*In vitro*/Mice	Subcutaneous	*In vitro*: Induction of IFN-γ production and antigen-specific T cell proliferation; Mice: Stimulation of antigen-specific $CD8^+$ T cell activation, without side effects	[132]

Abbreviation: EVs, extracellular vesicles; SARS-CoV-2, severe acute respiratory syndrome β-coronavirus 2; DEX, dendritic cell-derived exosomes; MHC, major histocompatibility complex; IL-2, interleukin-2; MDV, Marek's disease virus; LCMV, lymphocytic choriomeningitis virus; BMDCs, bone marrow-derived dendritic cells; MSC, mesenchymal stem cell; MCDC, merocytic dendritic cell; MD-DC, monocyte derived-DC; TAA, tumor-associated antigen; LPS, lipopolysaccharide; NKG2D, natural killer group 2 member D; MAGE, melanoma antigen gene protein; HEK, human embryonic kidney, PBMCs: peripheral blood mononuclear cells; HBV, hepatitis B virus.

Ebola virus, Influenza virus, West Nile virus, Crimean-Congo hemorrhagic fever virus, and Hepatitis C virus [29].

Two major strategies were applied in the EV engineering for vaccine development [131] including: a) Direct modification of cells-isolated EVs using electroporation or bio-conjugation; b) The most used method is the engineering of the EV donor cells for continuous production of the engineered EVs. In this way, the proteins of interest are loaded into the EV lumen or displayed on the EV surface as fusion with the EV-specific proteins (*e.g.,* lysosome-associated membrane protein 2 (Lamp2b), tetraspanins, platelet-derived growth factor (PDGFR), and C1C2 domain of lactadherin for surface display; Nedd4 family interacting protein 1 (Ndfip1), and ubiquitin tags for lumen loading). Moreover, RNAs can be loaded into EVs using fusion of the EV-specific proteins with RNA-binding proteins (*e.g.,* TAT, HuR and L7Ae) [131]. For instance, dendritic cells-derived EVs were engineered to present M, NS, and L antigens of respiratory syncytial virus (RSV). These EVs induced antigen-specific CTL activation without side effects in mice [131,132].

5.2. Exosomes in viral vaccine development

Among EVs, exosomes play an important role in the pathogenesis of infection, and also induce immune responses for protection against pathogens. Exosome-based vaccines were used as a novel strategy in development of Influenza vaccines [133]. For instance, the exosomes isolated from murine muscle cells transfected with DNA vectors expressing the Nef $_{mutant}$/Influenza virus ANP (Nef $_{mutant}$/Flu-NP) induced antigen-specific $CD8^+$ T cell response in mice [134]. On the other hand, a chimeric SARS-CoV S protein was produced by replacing the transmembrane and cytoplasmic domains of the S protein with *Vesicular stomatitis virus* G protein. HEK-293 T cell line was used to generate exosomes harboring chimeric SARS-CoV S protein [135]. This exosomal S protein-based vaccine free of adjuvant induced high neutralizing antibodies as compared to the adeno-associated virus (AAV) vaccine expressing chimeric S protein. The exosomal S protein-based vaccine generated higher immunological responses after boosting with AAV vaccine expressing chimeric S protein [135]. Furthermore, reports showed that exosomes released from SARS-CoV-2-infected cells induced effective immune responses [136,137]. Also, EBV-infected Raji cells-released exosomes containing deoxyuridine triphosphatase (dUTPase used as an adjuvant for exosomal vaccines) could induce NF-κB activation, and cytokine secretion [138].

The immunomodulatory functions of immune cells-derived exosomes have been known very well [139]. For instance, dexosomes-based vaccines possess simpler management and lower cost than DCs-based vaccines. Furthermore, cancer cells-derived exosomes-based vaccines induce strong anti-tumor immune responses, as well [120]. Induction of effective innate and adaptive immunity using dexosomes was studied in preclinical trials [115]. Dexosome-pulsed DCs were more potent in stimulation of $CD8^+$ T cells than peptide-loaded DCs [140]. Mature DCs-derived exosomes were more efficient in activation of $CD8^+$ T cells than immature DCs-derived exosomes, indicating the importance of co-stimulatory molecules presented on the mature DCs-derived exosomes. Some strategies could improve antigen-specific $CD8^+$ T cell responses induced by dexosome such as the use of dexosomes loaded with IFN-γ cytokine [141], and/or dexosomes containing a danger signal (*e.g.,* a toll-like receptor (TLR) ligand including polyinosinic: polycytidylic acid (poly (I:C)) or CpG-ODN) that enhance DC maturation [142]. Xiang et al. showed that Gp120-Texo and also Gag-Texo (dexosomes derived from DCs transfected with an adenoviral vector expressing HIV gp120 or Gag protein) trigger the effective and long-term cytotoxic T cell (CTL) responses in mice [143,144]. The reports showed that the presence of PRRSV antigens in serum-derived exosomes (free of virus) isolated from both viremic (V) and non-viremic (NV) pigs was also suggested as a novel vaccine approach against PRRSV [145–147].

6. EVs as therapeutic tools in preclinical and clinical studies

Dendritic cells-/mesenchymal stem cells (MSCs)-based EVs have been further examined in clinical trials. The use of these EVs in

preclinical and clinical trials is described individually as follows.

6.1. MSCs-derived EVs

Stem/progenitor cells are the major source of EVs for therapeutic applications [148–151]. The therapeutic effects of MSCs-derived EVs were shown in different preclinical studies such as rodent tissue injury models [132]. Up to now, 79 trials were registered for "extracellular vesicles" and 208 for "exosomes". Among them, 45 "extracellular vesicles"-associated studies were related to the use of EVs as biomarkers, and 10 as therapeutic tools. About 128 studies were related to the use of "exosomes" as biomarkers and 19 as therapeutics [148]. Several preclinical studies indicated that MSCs or their exosomes (MSCs-Exo) can reduce lung inflammation and pathological impairment in various types of lung injury. In this context, delivery of MSCs-Exo was safer than MSCs due to aggregation of cells injected intravenously [152]. Moreover, the safety and efficiency of MSCs-derived exosomes were confirmed in severe patients with new coronavirus-associated pneumonia. Indeed, the patients treated with MSC-derived exosomes showed a lower level of C-reactive protein than that in the placebo group. Therefore, the inflammatory processes were decreased in exosome treatment [152].

Some clinical trials showed the efficiency of umbilical cord-derived MSCs in COVID-19 patients [122,153]. Treatment with MSCs improved the conditions of COVID-19 patients in comparison with the standard antiretroviral therapy [154–156]. In these studies, the injection of umbilical cord-derived MSCs as intravenously showed several side effects including aggregation in the microvasculature, oncogenicity and mutagenicity [157,158]. In contrast, MSC-derived EVs induced similar responses with high safety as compared to native MSCs [159].

By the end of 2020, seven clinical trials were registered using EVs to overcome SARS-CoV-2 infection. The exosomes-related experiments include a) Aerosol inhalation of the exosomes isolated from allogeneic adipose MSCs in the treatment of hospitalized COVID-19 patients (NCT04276987); b) Direct delivery of MSC-derived exosomes into the lungs through the atomization process (ChiCTR2000030261) along with conventional treatments; c) Human umbilical mesenchymal stem cells (HUMSCs)-derived exosomes for treatment of lung damage in COVID-19 patients (ChiCTR2000030484); d) Bone marrow MSC-derived exosomes for treatment of COVID-19 patients [122]. The improvement of laboratory tests and clinical symptoms without side effects were reported for these patients such as increased lymphocyte count, decreased acute phase markers (*e.g.,* C-reactive protein, and ferritin), and normalized neutrophil count [122]. More than sixty clinical trials are ongoing to study the effects of MSCs and EVs in COVID-19 patients.

6.2. DCs-derived EVs

The efficiency of dexosomes (DEX)-based vaccines was further studied in cancer patients such as intradermal vaccination with tumor antigen-loaded dexosomes in non-small cell lung cancer (NSCLC) patients which could elicit both innate and adaptive immunity [160]. Dexosomes were applied as cell-free anticancer vaccines in two phase I [115,161,162], and one phase II clinical trials [115,163,164]. A phase II clinical trial tested the efficiency of IFN-γ-DEX loaded with MHC-I-/II-restricted tumor antigens as an immunotherapeutic method after chemotherapy. The results showed that no T-cell response was found in patients bearing untreatable non-small cell lung cancer (NSCLC) [44,164]. The limited efficacy of DEX immunotherapy may be due to the presence of IFN-γ in DEX. This cytokine can upregulate PD-L1, an inhibitor of T-cell activation, in DCs and DEXs [165–167]. Thus, low expression of PD-L1 in DCs is a critical strategy against cancer. Indeed, combination of DC-based vaccines with the inhibitors of PD-L1 or CTLA-4 (anti-PD-1/PD-L1 or anti-CTLA-4 therapy) could elicit an effective immune response against cancer [168,169]. Regarding the published data, there are few studies on the efficiency of dexosomes in viral diseases. For instance, incorporation of a viral fusion protein (*e.g.,* ovalbumin and the G protein of Vesicular stomatitis virus) and targeting of antigens to DEXs were known as promising approaches to increase the immunogenicity of exosome-based vaccines [44,170]. However, the results of DEX immunotherapy against a variety of cancers (as mentioned above) can be used for treatment of virus-related cancers such as human papillomavirus.

7. Final remarks and outlook

In conclusion, understanding the EV-mediated immune mechanisms can develop future approaches to control and suppress viral and bacterial infections. For example, exosomes provide various strategies to combat respiratory viruses including SARS-CoV-2 infection. Thus, it is important to evaluate the efficiency of EVs harboring viral antigens in vaccine development. Among EV therapies, the use of dexosomes as therapeutic vaccines for malignant melanoma and non-small cell lung carcinoma patients was confirmed in three clinical trials (Phases I/II). These reports showed the safety and ability of dexosome-based vaccines for inducing both the adaptive (T lymphocytes) and the innate (natural killer cells) immune responses. Thus, antigen-presenting EVs can be considered as a novel vaccine strategy with low side effects against viral diseases, as well. On the other hand, EVs were developed as diagnostic and therapeutic agents by loading various agents into them. Generally, the methods of encapsulating cargo into EVs can be classified in two groups: cell-based loading methods, and non-cell-based loading methods. The potency of antiviral drug-loaded exosomes was proved as a cellular therapeutic approach against SARS-CoV-2, Influenza virus, and Enterovirus.

Some major gaps are proposed to use EVs in vaccine design such as a) isolation of antigenic EVs from different pathogens: EVs can be easily separated from parasites and bacteria while they are hard to isolate from viruses with the same size and density; b) large amounts of EVs are needed to quantify them in immunotherapy; c) Reproducible production of antigen-containing EVs and their quantification; d) Further and better studies on cell activation pathway mediated by EVs, and validation of the most suitable antigens for vaccine development; e) Effective and clear guidelines from the medical agencies are necessary for using EVs as vaccine platforms; f) Generation of EVs in large amounts is a barrier for human use; and h) Biological effects of EVs depend on the location of components of their cargo as internal and/or external (*i.e.,* present on the surface of the vesicles); thus resolving biological effects of EVs exactly is difficult.

Several preclinical and clinical studies showed that human cells-derived EVs are safe. However, EVs-mediated toxicity cannot be ignored; because the cargo of EVs may change due to the surrounding microenvironment or toxic stimulants, and thus affect the pathological processes. Indeed, the established guidelines are needed to determine the safety of EVs for each clinical trial. Moreover, storage conditions of EVs (*e.g.,* temperature, pH, freezing and thawing procedures) are important for their integrity. However, the use of extracellular vesicles has attracted a special interest in vaccine development and therapeutic approaches. In summary, general description of EVs in therapeutic approaches was shown in Fig. 4.

Contributors

PMP, AB, AM, MHP reviewed the current state of the art and prepared the tables. PMP and AB wrote the manuscript.

Disclosure statement

The authors declare no competing financial interests. All authors have approved the manuscript.

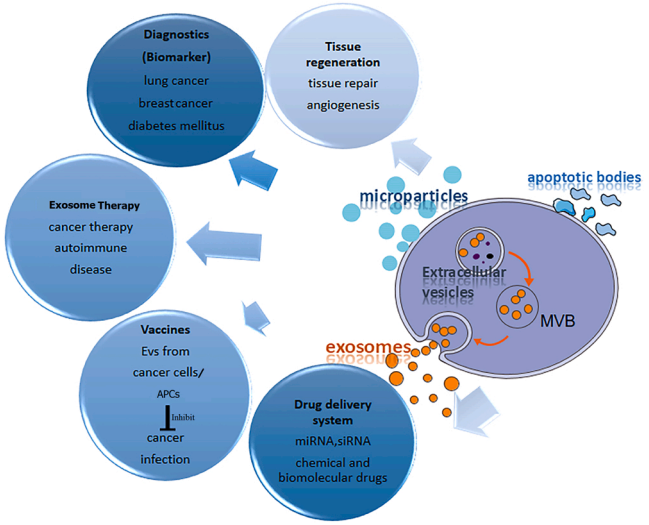

Fig. 4. General description of EVs in therapeutic approaches: MVB, multi-vesicular bodies; EVs, extracellular vesicles; APCs, antigen-presenting cells.

Data Availability

The data that has been used is confidential.

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