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Role of stem cell derivatives in inflammatory diseases

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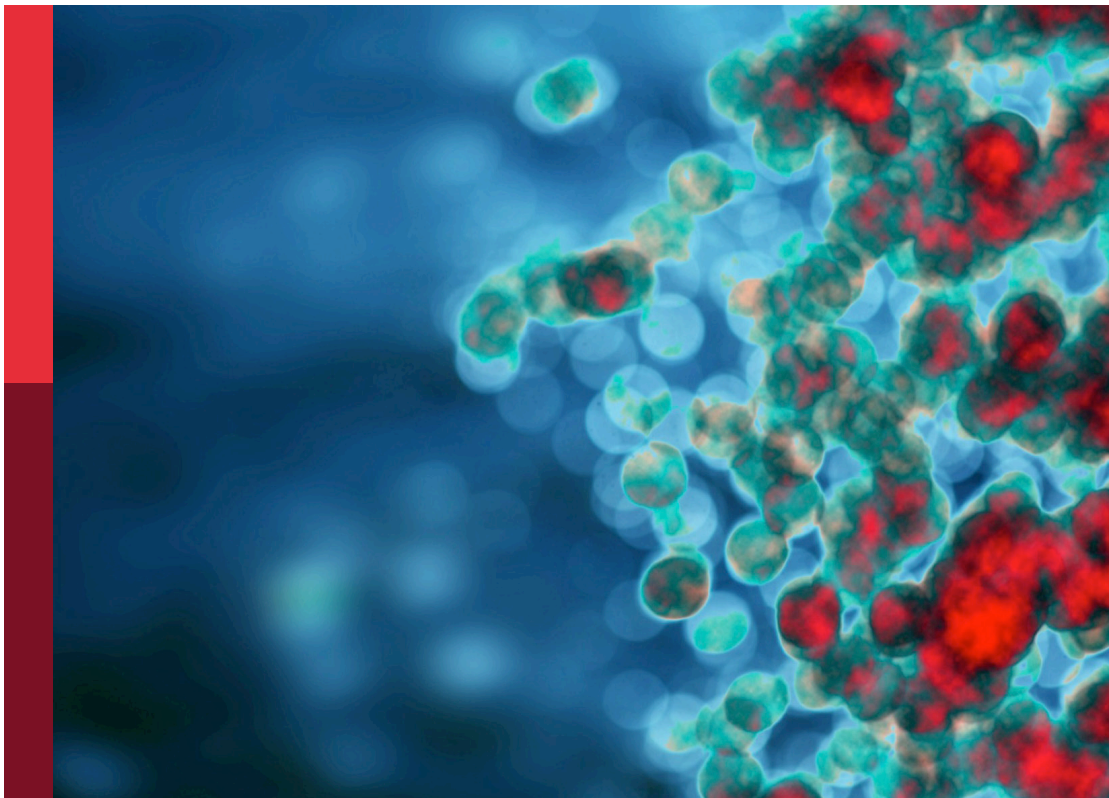
Published in
Frontiers in Immunology
Frontiers in Bioengineering and Biotechnology



ISSN 1664-8714
ISBN 978-2-8325-3479-3
DOI 10.3389/978-2-8325-3479-3

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Role of stem cell derivatives in inflammatory diseases

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Citation

Zhang, C., Li, H., Li, Z., Wang, J. H., Yao, Y. M., eds. (2023). *Role of stem cell derivatives in inflammatory diseases*. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-8325-3479-3

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frontiers | Frontiers in Immunology

TYPE Editorial
PUBLISHED 31 August 2023
DOI 10.3389/fimmu.2023.1275068

OPEN ACCESS

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RECEIVED 09 August 2023
ACCEPTED 22 August 2023
PUBLISHED 31 August 2023

CITATION
Zhang C, Li Z, Wang J, Yao Y, Li H and Fu X (2023) Editorial: Role of stem cell derivatives in inflammatory diseases. *Front. Immunol.* 14:1275068. doi: 10.3389/fimmu.2023.1275068

Editorial: Role of stem cell derivatives in inflammatory diseases

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KEYWORDS

stem cells, derivatives, inflammatory diseases, treatment, immune

Editorial on the Research Topic
Role of stem cell derivatives in inflammatory diseases

Inflammation is a complex and protective response of the immune system to pathogens. Appropriate inflammation response is beneficial to pathogen elimination and tissue repair, but uncontrolled inflammatory reactions often result in the damage of normal tissues (1). In recent years, there are more and more people troubled with inflammatory diseases including chronic wounds, asthma, osteoarthritis, and sepsis (2). The intractable inflammatory diseases were caused by disorders of immune systems, imposing a heavy burden on society and individuals. Innate and adaptive immune cells, such as neutrophils, macrophages, and lymphocytes, as well as their secreted cytokines contribute to the pathogenesis of inflammatory diseases. Recent research advances have enhanced our comprehension of the basic pathogenesis of the most common inflammatory diseases, which bring about the development of some innovative approaches.

In the past decades, stem cell therapy has been used as a potential therapeutic intervention for various human diseases due to their special characteristics. For example, mesenchymal stem cells (MSCs) are thought to be the most sought-after stem cells with immunomodulatory property for treating a variety of inflammatory diseases (3). However, safety concerns have limited the clinical application of stem cells. It is indispensable to promoting the development of safe and efficient therapeutic strategies based on stem cells. Recently, the performance of stem cells after transplantation has increasingly been attributed to their exocrine function. It is evident from the literature that stem cell derivatives including extracellular vesicles (EVs) can mimic the function of stem cells without the concerns of immune response and ethical issues (4).

EVs contain diverse cellular molecules including RNAs, DNAs, and proteins, promoting the information exchange between cells. Compared with their parental stem cells, EVs have several advantages including high safety, absence of immune reactions,

fewer ethical issues, and decreased potential for embolism formation and carcinogenicity. Recent research reported the pivotal role of stem cell-derived EVs (SC-EVs) in a wide spectrum of diseases such as cancer, myocardial infarction, and skin wounds (5–7). But the study on the role of SC-EVs in inflammatory diseases is still in the infancy.

The Research Topic of "*The Role of* Stem Cell Derivatives *in Inflammatory Diseases*" was designed to introduce the role of stem cell derivatives, especially EVs, in the pathogenesis, diagnosis, and treatment of inflammatory diseases. A total of 12 manuscripts were accepted in the topic, including 4 original articles, 7 reviews, 1 mini review, 8 from China. 2 from United States, 1 from Poland, and 1 from Vietnam.

Skin wound healing is a highly sophisticated process consisting of four distinct and overlapping phases: hemostasis, inflammation, proliferation, and remodeling (8). Chronic wounds usually arise owning to halt at one or more points in above phases, especially in inflammation phase. Wei et al. reviewed the effects of MSC-EVs on the function of skin repair cells including inflammatory cells, vascular endothelial cells, fibroblasts, and epidermal cells and application avenues of MSC-EVs on wounds such as local injection and combination with biomaterials. Jia et al. focused on the potential of EVs derived from adipose-derived stem cells (ADSC-EVs) in regulating wound inflammation and discuss the mechanisms underneath this phenomenon. Atopic dermatitis (AD) and psoriasis are systemic and immune-allergic inflammatory skin diseases (9). The review article by Yang et al. brought an extensive overview of the therapeutic effects of MSCs and their derivatives including EVs on AD and psoriasis.

Four original researches in this topic provide strong evidence for the therapeutic effects of SC-EVs on inflammatory diseases. Acute radiation syndrome (ARS) is associated with the exposure to high doses of radiation and featured by immune suppression and organ failure. Seim et al. identified EVs from the plasma of mice after whole body irradiation (WBIR) as important participants in ARS and for the first time demonstrated that MSC-EV administration prior to WBIR would decrease ARS. Asthma is a chronic respiratory disease featured by airway inflammation and remodeling. Xu et al. identified that Hypoxic EVs derived from human umbilical cord MSCs (Hypo-hUCMSC-EVs) can reduce allergic airway inflammation and remodeling by atomizing inhalation. Additionally, for treatment of osteoarthritis and chondrocyte-related disorders, Nguyen et al. investigated the effects of EVs released from hUCMSC primed by cytokines including transforming growth factor beta (TGFβ), interferon alpha (IFNα), or tumor necrosis factor alpha (TNFα) on osteoarthritic chondrocyte physiology. In an ovine pneumonia/sepsis model, Homma et al. reported the beneficial effects of MSCs (10×10^6 cells/kg) isolated from bone marrow (BM- MSCs) on sepsis-induced multiorgan dysfunctions, but EVs derived from the same amount of BM-MSCs failed to function. The possible reason may attribute to the small dose of the EVs and repeated treatment should be performed.

Treatments with SC-EVs for other inflammatory diseases such as periodontitis and inflammation-induced fibrosis were also reviewed by Cai et al. and Lv et al. The review written by Zhao et al. summarized the current researches on mechanism of MSC-EVs affecting inflammatory diseases by modulating epigenetic modification. Liu et al. and Karnas et al. provided reviews of immunomodulatory effects of SC-EVs on immune system and immune cells including macrophages, granulocytes, mast cells, natural killer cells, dendritic cells, T cells, and B cells in some inflammatory disease models.

These articles present studies in the treatment of inflammatory diseases with EVs derived from stem cells of various sources. Based on the given results, SC-EVs are suggested as a potential substitute for stem cell therapy. So, this topic provides a critical guide to clinic application of SC-EVs for the treatment of inflammatory diseases.

Author contributions

CZ: Writing – review & editing, Writing – original draft. ZL: Writing – review & editing. JW: Writing – review & editing, Validation. YY: Writing – review & editing, Writing – original draft. HL: Writing – original draft, Writing – review & editing. XF: Writing – original draft, Writing – review & editing.

Funding

The manuscript was supported in part by the National Natural Science Foundation of China (82172211, 92268206, 81830064, 82172231), National Key Research and Development Programs of China (2022YFA1104303), the CAMS Innovation Fund for Medical Sciences (CIFMS, 2019-I2M-5-059), Military Medical Science and Technology Youth Training Program (21QNPY128), and the 1·3·5 Project for Disciplines of Excellence, West China Hospital, Sichuan University (ZYGD22008).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 20 October 2022
DOI 10.3389/fimmu.2022.1042983

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Cytokines and Soluble Mediators in Immunity, a section of the journal Frontiers in Immunology

RECEIVED 13 September 2022
ACCEPTED 30 September 2022
PUBLISHED 20 October 2022

CITATION
Lv K, Wang Y, Lou P, Liu S, Zhou P, Yang L, Lu Y, Cheng J and Liu J (2022) Extracellular vesicles as advanced therapeutics for the resolution of organ fibrosis: Current progress and future perspectives.
Front. Immunol. 13:1042983.
doi: 10.3389/fimmu.2022.1042983

Extracellular vesicles as advanced therapeutics for the resolution of organ fibrosis: Current progress and future perspectives

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Organ fibrosis is a serious health challenge worldwide, and its global incidence and medical burden are increasing dramatically each year. Fibrosis can occur in nearly all major organs and ultimately lead to organ dysfunction. However, current clinical treatments cannot slow or reverse the progression of fibrosis to end-stage organ failure, and thus advanced anti-fibrotic therapeutics are urgently needed. As a type of naturally derived nanovesicle, native extracellular vesicles (EVs) from multiple cell types (*e.g.*, stem cells, immune cells, and tissue cells) have been shown to alleviate organ fibrosis in many preclinical models through multiple effective mechanisms, such as anti-inflammation, pro-angiogenesis, inactivation of myofibroblasts, and fibrinolysis of ECM components. Moreover, the therapeutic potency of native EVs can be further enhanced by multiple engineering strategies, such as genetic modifications, preconditionings, therapeutic reagent-loadings, and combination with functional biomaterials. In this review, we briefly introduce the pathology and current clinical treatments of organ fibrosis, discuss EV biology and production strategies, and particularly focus on important studies using native or engineered EVs as interventions to attenuate tissue fibrosis. This review provides insights into the development and translation of EV-based nanotherapies into clinical applications in the future.

KEYWORDS

fibrosis, extracellular vesicles, exosomes, nanomedicine, biomaterials

1 Introduction

Organ fibrosis is a serious and unsolved health problem worldwide. The main pathological feature of fibrosis is the abnormal formation and deposition of excessive extracellular matrix (ECM), which eventually results in disrupted architectural remodeling and progressive organ dysfunction. Fibrosis can occur in nearly all major organs (Figure 1), such as liver, heart, lung, and kidney, and it is attributed to ~45% of all deaths in the world (1). For example, more than 800 million patients were affected by chronic liver disease (liver fibrosis); about 1.5% of global deaths were caused by chronic kidney disease (CKD, renal fibrosis) in 2012 (2, 3). The mechanism of fibrosis is extremely complicated, and many pathological factors, such as infections, immune reactions, chemical insults, oxidative stress, and hazardous agents, have been proved to be involved with fibrosis (4). In recent decades, many single factor-targeted treatments have been developed and have shown certain beneficial effects in preclinical studies, but most of them fail to achieve clinical approval. Thus, novel and advanced antifibrotic therapeutics are desired in the clinic (5).

In recent years, extracellular vesicle (EV)-based therapies have emerged as a potent strategy for resolving organ fibrosis. EVs are nanoscale bilayer vesicles secreted by live cells that exert similar functions as parental cells by delivering various types of cargoes, such as lipids, proteins, nucleic acids, and metabolites (6). As a type of naturally derived nanomaterial, EVs have multiple advantages, such as abundant cell sources, intrinsic bioactive properties, low immunogenicity, rare toxicity, flexibility to modify, and ability to cross biological barriers compared to synthetic materials (7). In many preclinical studies, EV-based nanomedicines have been shown to prevent multiple types of organ fibrosis through complicated mechanisms, such as promoting the recovery of damaged tissues, resolution of inflammation, inactivation of myofibroblasts, and fibrinolysis of excess ECM components (5). Although the results are encouraging, some important problems, such as proper cell sources, EV engineering strategies, detailed mechanisms, and possible limitations, remain elusive and need to be comprehensively reviewed. This will be helpful for the improvement and clinical translation of EV-based antifibrotic therapies.

In this review, we briefly introduce the key pathology of organ fibrosis as well as EV biology and production and emphasize the important studies that are highly relevant to using native or engineered EVs for decreasing organ fibrosis. We also discuss the possible limitations in this field and provide insights into developing advanced EV therapeutics for the treatment of fibrotic diseases.

2 Pathology and current therapies of fibrosis

Fibrosis, characterized by the activation of myofibroblasts, excessive deposition of ECM, and inhibition of ECM degradation, is a common adverse outcome of many

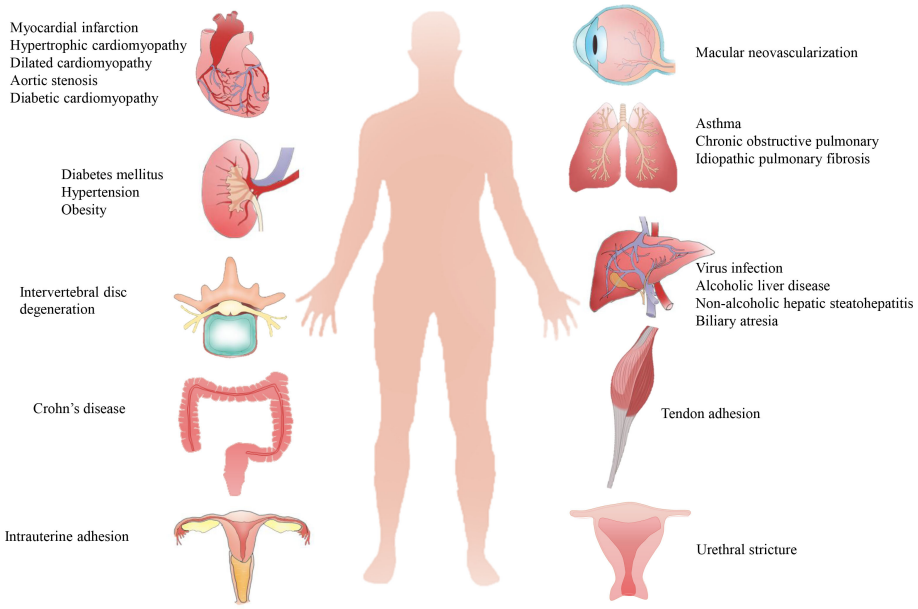

FIGURE 1
Pathological causes of organ fibrosis. Fibrosis can occur in most organs or tissues, such as the heart, liver, lung, kidney, tendon, intrauterine and intervertebral disc, due to complicated pathological causes.

etiological conditions after acute or chronic organ injury (8, 9). A variety of cell types and signaling pathways synergistically regulate the occurrence and progression of organ fibrosis (Figure 2). In this section, we briefly review the critical cellular events, signaling pathways and current clinical strategies for fibrotic disease treatment.

2.1 Key cellular events of fibrosis

In the early phases after organ injury, persistent stress may induce parenchymal cell death (*e.g.*, necrosis, pyroptosis, and ferroptosis) and trigger abnormal activation and infiltration of multiple types of immune cells (*e.g.*, macrophages) and inflammation (10). Subsequently, cytokines and chemokines secreted by these infiltrated immune cells, such as transforming growth factor-β (TGF-β), interleukins (ILs), and platelet-derived growth factor (PDGF), further amplify the severity of inflammation and organ damage (11). The activation of myofibroblasts is the central event mediating ECM synthesis and deposition in the later phases after organ injury. Activated myofibroblasts can be identified by several marker proteins, such as α-smooth muscle actin (α-SMA) and PDGFβR (12). However, the origins of myofibroblasts remain incompletely understood, but multiple cell sources have been reported (Figure 2A), such as resident fibroblasts, mesothelial cells, circulating fibroblasts, epithelial cells, endothelial cells, pericytes, vascular smooth muscle cells, and other specialized tissue cells (*e.g.*, hepatic stellate cells). Additionally, many possible mechanisms, such as cell proliferation, epithelial to mesenchymal transition (EMT), mesothelial to mesenchymal transition (MMT) and endothelial to mesenchymal transition (EndoMT), are involved in this process (5, 13).

The imbalance between ECM deposition and ECM degradation is another vital event during fibrosis. Briefly, the degradation of ECM proteins (*e.g.*, collagens) is mainly controlled by matrix metalloproteinase (MMP)-mediated proteolysis but is antagonized by multiple tissue inhibitors of matrix metalloproteinases (TIMPs), which are the endogenous inhibitors of MMPs (14, 15). MMPs can be classified into different subtypes, such as interstitial collagenases, gelatinases, metalloelastases and membrane-type MMPs, according to their enzyme-substrate specificity and subcellular locations (16). For example, interstitial collagenases (*e.g.*, MMP-1, MMP-13, and

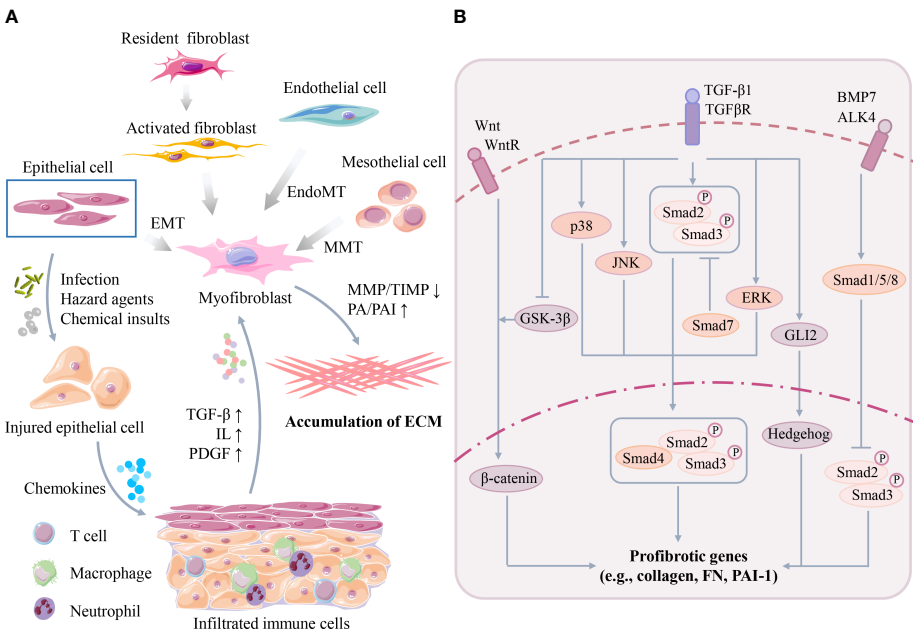

FIGURE 2
(A) Key cellular events of organ fibrosis. Organ damage can trigger infiltration of immune cells, followed by secretion of excessive cytokines/chemokines to activate myofibroblasts. Myofibroblasts are originated from many cell types, such as epithelial cells, mesothelial cells and endothelial cells, through EMT, MMT or EndoMT. Activated myofibroblasts promote organ ECM synthesis/deposition. **(B)** Key signaling pathway of organ fibrosis. TGF-β participates in the molecular mechanism of fibrosis in Smad-dependent and Smad-independent manner such as MAPK families. Moreover, TGF-β also interacts with other profibrotic pathways, such as the Wnt/β-catenin, Hedgehog and BMP-7 pathways. (TGF-β, transforming growth factor-β; ILs, interleukins; PDGF, platelet-derived growth factor; EMT, epithelial to mesenchymal transition; MMT, mesothelial to mesenchymal transition; EndoMT, endothelial to mesenchymal transition; MMP, matrix metalloproteinase; TIMPs, tissue inhibitors of matrix metalloproteinases; PA, plasminogen activator; PAI, plasminogen activator-inhibitor 1; MAPK, mitogen-activated protein kinase; JNK, JUN amino-terminal kinase; ERK, extracellular signal-regulated kinase; GSK-3β, glycogen synthase kinase-3β; FN, fibronectin).

MMP-18) can proteolyze type I, II, III interstitial collagens, while gelatinases (*e.g.*, MMP-2 and MMP-9) can cleave the denatured collagens and basement membrane ECM (16, 17). Conversely, TIMPs can blunt ECM proteolysis by inhibiting the activity of MMPs, and the upregulation of TIMPs is commonly associated with an accumulation of ECM (18). In addition, the dysregulation of serine proteases and their inhibitors, such as urokinase plasminogen activator (uPA) and plasminogen activator-inhibitor 1 (PAI-1), is also associated with abnormal ECM remodeling and fibrosis (14).

2.2 Key signaling pathways of fibrosis

Although many possible pathways have been revealed in the pathogenesis of organ fibrosis in recent decades, TGF-β has been recognized as a master regulator of myofibroblast activation and fibrotic processes (Figure 2B). TGF-β has three isoforms (TGFβ1-3) and can stimulate the biosynthesis and accumulation of ECM components, such as collagen I and fibronectin (FN), in epithelial cells and mesenchymal cells (19, 20). TGF-β1 can be secreted by many types of cells, such as macrophages, lymphocytes, epithelial cells, fibroblasts, pericytes, endothelial cells and platelets, at injured tissue sites (19). Once activated after secretion, TGF-β1 binds to its receptor (TGF-βR) and activates downstream profibrotic pathways in a Smad-dependent (classical) or Smad-independent (nonclassical) manner (21). In the Smad-dependent pathway, TGF-β1 induces phosphorylated Smad2/3 proteins, and then Smad4 binds to the Smad2/3 complex and translocates the complex into the nucleus to induce the transcription of many essential profibrotic genes, such as collagens, FN and PAI-1 (9). Smad7 is a negative regulator of this process, which can compete with Smad2/3 for binding to activated TGF-βR and thus block TGF-β/Smad signaling (22). Moreover, TGF-β1 can also interact with other Smad-independent pathways, such as the mitogen-activated protein kinase (MAPK) family p38 MAPK, JUN amino-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) pathways. MAPKs further phosphorylate the linker region of Smad2/3 and thus modulate Smad3 transcriptional activity (9).

Furthermore, TGF-β has potential crosstalks with other profibrotic pathways, such as the Wnt/β-catenin, Jagged1/Notch, Hedgehog, and bone morphogenic protein-7 (BMP-7) pathways (23). For example, TGF-β activates Wnt signaling by inhibiting glycogen synthase kinase-3β (GSK-3β), thereby disrupting the stabilization of β-catenin or suppressing Wnt inhibitors and enhancing the transcription of β-catenin-targeted profibrotic genes (*e.g.*, FN, PAI-1, Snail and MMP-7) in injured kidneys (24, 25). TGF-β can also induce the transcription of GLI2 (an activator of Hedgehog signaling), which subsequently upregulates Hedgehog-targeted profibrotic gene (*e.g.*, α-SMA) expression (26–28). In contrast, BMP-7 activates Smad1/5/8, which can block the nuclear translocation of phosphorylated receptor-Smad2/3 and thus inhibit TGF-β signaling (29). Due to the complicated mechanisms of organ fibrosis, it can be speculated that single pathway-targeted antifibrotic strategies may not be efficient.

2.3 Current antifibrotic strategies

Since fibrosis is a long-term outcome of persistent organ damage, it can be assumed that the resolution of fibrosis may be achieved when the pathological causes of injuries are eliminated. Based on previous studies of fibrogenesis, several therapeutic strategies (Table 1) are proposed (30), such as immunoregulation, degradation of ECM and elimination of myofibroblasts. Currently, some potential targets or medicines for fibrosis in different organs have been reported, and some of them have progressed to clinical trials or clinical phases (Table 1). For example, pirfenidone and nintedanib (NIN), two compounds with pleiotropic mechanisms of action, are approved for the management of idiopathic pulmonary fibrosis (IPF) due to their effects on slowing lung functional decline. Pirfenidone may inhibit TGF-β, inflammatory cytokines (*e.g.*, tumor necrosis factor-α, TNF-α) or oxidative stress, and NIN may inhibit tyrosine kinase receptors such as PDGFR, vascular endothelial growth factor receptor (VEGFR) and fibroblast growth factor receptor (FGFR) (31). However, the two drugs cannot prevent or reverse the existing organ fibrosis according to physiological, histological, and radiological examination results (31). Thus, more advanced antifibrotic treatments are urgently required in the clinic.

3 Biological properties of EVs

EVs are a group of nanoscale bilayer vesicles and are widely distributed in the cultured medium of almost all cell types and biofluids, such as blood, urine, saliva, and breast milk (32). Numerous evidence indicates that EVs are key mediators of cell-to-cell or organ-to-organ communication since they can deliver multiple types of bioactive cargoes to regulate the signaling of the recipient cells under physiological or pathophysiological conditions (33). In this section, we briefly introduce the biological properties of EVs, such as biogenesis, uptake, production, and stability.

3.1 EV biogenesis

The current classification of EVs is mainly based on their cellular origin and/or biological function. According to their sizes or biogenesis routes, EVs can be divided into many subtypes, such as exosomes (~30-200 nm), microvesicles (MVs, ~200-1000 nm) and apoptotic bodies (~800-5000 nm)

TABLE 1 Current anti-fibrotic strategies and drugs.

Strategies	Drugs	Classes	Targets/ mechanisms	Target diseases	Phase
Myofibroblasts and the TGF-β pathway	Pirfenidone	stress-activated kinases inhibitor	TGF-β, PDGF, SDF-1α	IPF, pulmonary fibrosis, hepatic fibrosis, renal fibrosis, systemic sclerosis, keloid	Clinic
	Nintedanib (BIBF 1120)	tyrosine kinase inhibitor	TGF-β, PDGF, VEGF, FGF	IPF, systemic sclerosis	Clinic
	Imatinib mesylate (Glivec)	tyrosine kinase inhibitor	PDGF	IPF, pulmonary fibrosis, hepatic fibrosis, nephrogenic systemic fibrosis, systemic sclerosis	1/2/3
	Pamrevlumab (FG-3019)	human recombinant monoclonal antibody	CTGF	IPF	2/3
	Macitentan	endothelin receptor antagonist	endothelin-1	IPF	2
	BMS-986263	etinoid-conjugated lipid nanoparticle containing HSP47 siRNA	HSP 47	hepatic fibrosis, cirrhosis, NASH	1/2
ECM	STX-100	specific monoclonal antibody	αvβ6 integrin	IPF, chronic allograft dysfunction	2
Immunomodulators	TD139	thio-digalactoside inhibitor	galectin-3	IPF	1/2
	Belapectin (GR-MD-02)	Galectin inhibitor	galectin-3	NASH, cirrhosis	2
	PRM-151	monocyte development inhibitor	Pentraxin 2	IPF, primary myelofibrosis, post-essential thrombocythemia myelofibrosis	1/2/3
	Spironolactone	aldosterone antagonists	Anti-inflammatory	myocardial fibrosis, endomyocardial fibrosis, hepatic fibrosis, cirrhosis, renal fibrosis, ESRD	2/3/4
	Lenabasum	cannabinoid type 2 receptor (CB2) agonist	Anti-inflammatory	cystic fibrosis	2
	Digitoxin	cardiac glycosides	IL-18/NF-κB	cystic fibrosis	2
	Pentoxifylline	anticytokine	TNF-α	head and neck fibrosis, radiation injuries, cirrhosis, NASH, CKD	1/2/3/4
Antioxidants	N-acetylcysteine (NAC)	antioxidant GSH prodrug	Oxidative stress	IPF, pulmonary fibrosis, cystic fibrosis, non-cystic fibrosis, cirrhosis, ESRD	1/2/3/4
Others	Aramchol	inhibitors of *de novo* lipogenesis	Hepatic SCD1	hepatic fibrosis, NASH	2/3
	Emricasan (IDN-6556)	caspase inhibitor	Apoptosis	hepatic fibrosis, cirrhosis, NASH	2

TGF-β, transforming growth factor-β; PDGF, platelet-derived growth factor; SDF-1α, stromal cell derived factor-1α; IPF, idiopathic pulmonary fibrosis; VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; CTGF, connective tissue growth factor; HSP47, heat shock protein 47; NASH, nonalcoholic steatohepatitis; ESRD, end stage renal disease; TNF-α, tumor necrosis factor-α; CKD, chronic kidney disease; SCD1, Stearoyl-CoA desaturase 1.

(34, 35). Among these EV subtypes, exosomes and MVs are frequently reported in most of the previous studies related to fibrosis. Thus, we will focus on these two types of EVs in the following sections (Figure 3). Briefly, the first step of exosome biogenesis is that the plasma membrane is endocytosed to form early endosomes, which then mature into late endosomes, also known as multivesicular bodies (MVBs). The delimiting membrane of MVBs can be invaginated to form intraluminal vesicles (ILVs), followed by the endosomal sorting complex required for transport (ESCRT)-dependent or ESCRT-independent (tetraspanins related) steps. In the ESCRT-dependent process, ESCRT-0 binds to the ubiquitination sites of early endosomes, ESCRT-I and ESCRT-II induce the formation of MVBs, and ESCRT-III promotes intraluminal budding of endosomal vesicles and scissors ILVs into the MVB lumen. The last step of exosome formation is the binding of some MVBs to the cell membrane to release ILVs, whereas other ILVs binds to lysosomes and are degraded (36). Unlike exosomes, MVs are mainly originated by outward budding at the cell plasma membrane, which is regulated by several rearrangements within the plasma membrane, such as altered lipid/protein components and Ca^{2+} levels. Although exosomes and MVs may have different biogenesis routes, they still share some common pathways, such as ESCRT and the conversion of ceramide from sphingomyelinas (37). However, the detailed mechanisms of other EV subpopulations remain elusive and need to be explored in future studies.

3.2 EV composition and uptake

EVs can participate in intracellular communication by delivering multiple types of bioactive contents into recipient cells (Figure 3). EVs are enriched in lipid contents, such as cholesterol, sphingomyelin, and hexosylceramides (38), which contribute to the *in vivo* stability of EVs. Due to their biogenesis

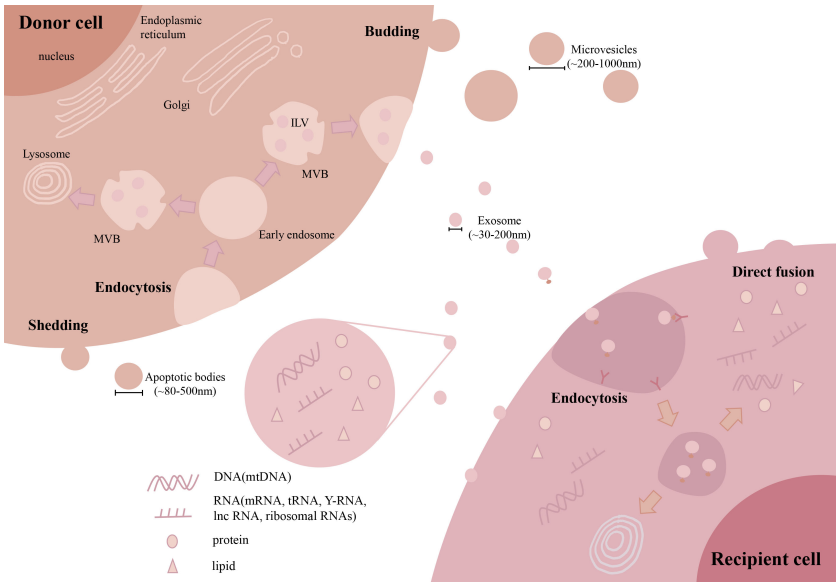

FIGURE 3
Biogenesis and uptake of EVs. During exosome biogenesis, the plasma membrane is endocytosed to form early endosomes and then matures into MVBs. The delimiting membrane of MVBs invaginates from the ILVs. Finally, some MVBs bind to the cell membrane to release ILVs. MVs are mainly originated by outward budding at the cell plasma membrane. For cellular uptake, EVs can be taken up by recipient cells *via* direct membrane fusion or endocytosis by interacting with receptors on the surface. (Extracellular vesicles: EVs, microvesicles: MVs, multivesicular body: MVB, intraluminal vesicles: ILV, microRNA: miRNAs, long noncoding RNA: lncRNA, mitochondrial DNA: mtDNA).

routes, EVs carry many proteins originated from the cell plasma membrane, cytoskeleton and cytoplasm, as well as other proteins involved in EV sorting and secretion, such as tetraspanins and proteins from the ESCRT-dependent pathway (39). In addition, EVs also contain large amounts of RNAs and DNAs, among which microRNAs (miRNAs) are the most abundantly studied to explain the effective mechanisms of EVs. Other types of RNAs, such as mRNAs, long noncoding RNAs (lncRNAs), tRNAs, Y-RNAs and ribosomal RNAs, have also been observed in EVs (40, 41). Interestingly, we and others had found the appearance of mitochondrial DNA (mtDNA), electron transport chain proteins and even fragmented mitochondria in EVs (42).

As a result, the composition of proteins and lipids on the surface of EVs may influence the efficiency of cell uptake (Figure 3). In brief, EVs can be taken up by target cells *via* direct membrane fusion or endocytosis, which has been well reviewed (43). Direct fusion relies on the lipid composition of EVs and target cells, and the fusion efficacy of EVs to the plasma membrane may be enhanced by acidic pH in the extracellular environment (44). In the case of endocytosis, EVs are docked by interacting with proteins, glycoproteins or lipids exposed on the cell membrane and then internalized by recipient cells (45). Overall, the surface signature of EVs can influence the pattern of EV uptake in target cells.

3.3 EV isolation and characterization

EVs can be isolated from various types of samples (*e.g.*, conditioned medium (CM) and body fluids) using different methods, such as ultracentrifugation (UC), density gradient ultracentrifugation, size-exclusive chromatography (SEC), immunoaffinity, ultrafiltration, coprecipitation (polyethylene glycol-based) and newly developed microfluidics (46), and each method has its advantages and shortcomings. For example, UC, including differential centrifugation and sucrose density gradient ultracentrifugation, remains the most widely used method which relies on the size and/or density of EVs. However, the production efficiency of UC is limited by long time consumption, operator dependence and rupture of EVs (47, 48). Methods based on EV size, such as SEC, can improve the purity and stability of EVs, while EV yield is limited (49, 50). The immunoaffinity method can selectively enrich EVs with high purity, but it is unsuitable for large volume samples due to its high cost (51). For future clinical applications, a large amount of therapeutic EVs with high purity is needed, and thus, a major issue in this field is how to overcome the current problems of high cost and low yield for larger-scale production of EVs.

According to the minimal information for studies of extracellular vesicles (MISEV) guidelines, isolated EVs should be characterized at least by morphology, particle size and marker

proteins (52, 53). The morphology of EVs is commonly observed using transmission electron microscopy or scanning electron microscopy (53). The particle sizes and numbers of EVs can be measured using nanoparticle analysis technology or dynamic light scattering (54, 55). Some key proteins involved in EV biogenesis or function, such as tetraspanins (e.g., CD9, CD63, CD81, CD82) or Alix, heat shock protein70 (HSP70), TSG101, and syntenin-1 associated with ESCRT, have been proposed as common EV markers (56, 57). However, the truth is that there are no specific markers for the identification of all EV subtypes until now (39). More importantly, for therapeutic purposes, the possible biosafety issues of EVs should be carefully considered, since it has been reported that some EVs from immortalized cell lines may carry oncogenic materials or endotoxins, while some EVs from infected cells may carry viral-derived proteins (34, 58). Thus, it is necessary to establish standardized and reproductive operating procedures, quality control criteria, and strictly sterile conditions during EV production (59).

3.4 Large-scale production and EV stability

For future clinical translation, a good manufacturing practice (GMP)-compliant protocol for EV production should be developed. The manufacturing of EV products can be briefly divided into upstream and downstream processings and subsequent quality control (60). In upstream processing, the large-scale expansion of EV-producing cells mainly depends on bioreactors, such as hollow fiber and stirred suspension bioreactors (using microcarriers) (61). Hollow fibers culture the cells in the hollow and semipermeable fibers and has less risk of contamination, while microcarriers are tiny beads with many varying features and require changing of culture media frequently (62). A recent study reported a turbulence strategy integrated into the cell culture in a stirred tank incompatible with a GMP bioreactor, which could obtain ~10^{13} EV particles from the supernatant of the one-liter bioreactor (63). In downstream processing, traditional isolation methods are not suitable for large-volume samples. Among the different methods, tangential flow filtration (TFF) may be one of the most powerful tools for industrial-scale EV manufacture (64) since it has a superior yield and bioactivity of isolated EVs compared to UC (65). Furthermore, TFF can be combined with other isolation approaches, such as SEC, to achieve higher scalability and reproducibility (66).

Stability is an important parameter for biological reagents, and the lipid bilayer structure of EVs provides unique protection to their bioactive cargoes (67). Some storage techniques that can long-term preserve EV bioactivities, such as freezing, lyophilization and spray-drying, have also been developed (68). For example, EVs suspended in buffered saline solution are stable for up to 2 years at -80°C without significant changes in properties (69), and the bioactivities of lyophilized EVs are similar to those of frozen EVs even stored at room temperature for 4 weeks (70). Therefore, the stability of EVs makes them ideal therapeutics in future clinical applications.

4 Therapeutic potential of native EVs

In recent decades, increasing numbers of studies have reported the potential of native EVs in preventing fibrosis *via* multiple mechanisms (Supplementary Table 1). Here, we briefly introduce the important studies regarding the anti-fibrotic effects (in major organs) of native EVs from various cell sources, such as stem cells, immune cells, tissue cells and blood (Figure 4).

4.1 Cardiac fibrosis

Cardiovascular disease (CVD) is one of the leading causes of death globally, and more than 17 million deaths worldwide are due to CVD per year (109, 110). Cardiac fibrosis can occur after almost all types of heart diseases, such as myocardial infarction (MI), hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM), aortic stenosis (AOS), and diabetic cardiomyopathy (111, 112). Although some potential medicines, such as angiotensin (AT)-converting enzyme and angiotensin II receptor antagonists, β-blockers, endothelin antagonists, and statins have been used in the clinic to protect cardiac function, their direct antifibrotic efficacy remains debated (113, 114). Thus, new and advanced antifibrotic therapies are urgently needed.

Increasing evidence shows that native EVs isolated from stem cells may have the potential to prevent cardiac fibrosis (Figure 4). Mesenchymal stem cells (MSCs) are a type of adult stem cell with immunoregulatory and tissue repair potentials that can be isolated from multiple tissue types, such as bone marrow (BMSCs), umbilical cord tissue (ucMSCs) and adipose tissue (ADMSCs) (115). The possible antifibrotic effects of MSC-derived EVs (MSC-EVs) have been reported in multiple models of cardiac damage (such as hypertension and MI), and the underlying protective mechanism is linked to the inhibition of EMT (miR-200, *etc.*), anti-apoptosis, induction of M2 macrophages or pro-angiogenesis (Supplementary Table 1) (71, 72, 116, 117). Induced pluripotent stem cells (iPSCs) are a new source of embryonic-like stem cells obtained by reprogramming of somatic cells (*e.g.*, skin fibroblasts), which

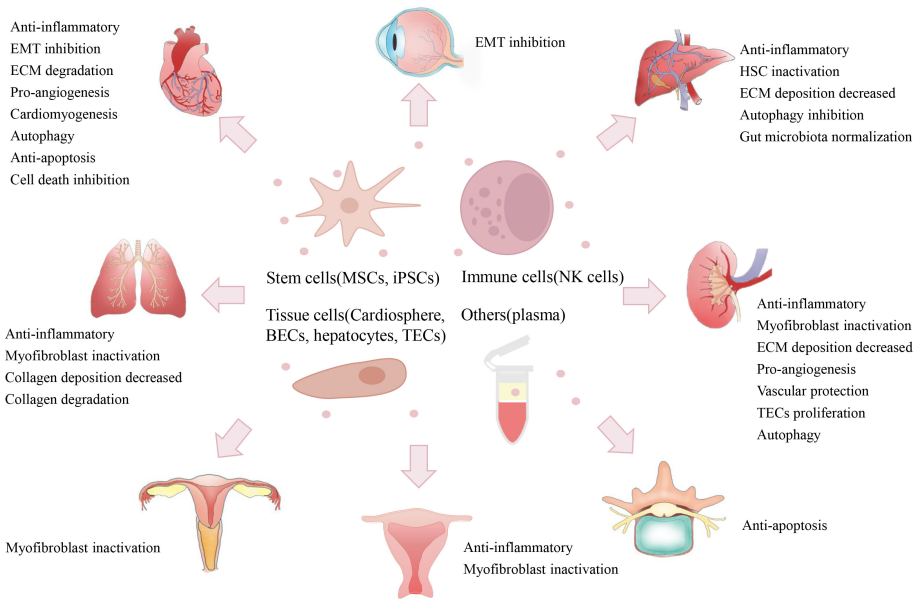

FIGURE 4
Therapeutic potentials of native EVs. Native EVs isolated from multiple cell sources (*e.g.*, stem cells, immune cells, tissue cells, and plasma) have exerted therapeutic efficacy on the alleviation of fibrosis in some major organs (*e.g.*, heart, lung, liver, and kidney) and other tissues (*e.g.*, IUA, IDD, and urethral stricture), and their therapeutic role may be due to multiple mechanisms, such as anti-inflammation, EMT inactivation, ECM degradation, and pro-angiogenesis. (Mesenchymal stem cells, MSCs; iPSCs, induced pluripotent stem cells; natural killer, NK; bronchial epithelial cell, BECs; tubular epithelial cell, TECs; epithelial to mesenchymal transition, EMT; extracellular matrix, ECM; intrauterine adhesion, IUA; intervertebral disc degeneration, IDD).

can further differentiate into many different cell types. iPSC-derived EVs reduced ECM deposition in the aortas of aged mice by inhibiting MMPs and elastase activity while enhancing the endothelial nitric oxide synthase (eNOS) pathway (73). Another therapeutic strategy is using EVs produced by iPSC-derived cardiac cells. For instance, iPSCs were first differentiated into contractile cardiomyocytes (iCMs), then iCM-derived EVs reduced the fibrotic areas of the left ventricle (LV) by enhancing autophagic flux after ischemic heart injury (74). EVs isolated from human embryonic stem cell (ESC)-derived cardiovascular progenitor cells recovered heart function (LV ejection fraction values/LV systolic dimensions) and reduced fibrotic areas after MI by promoting angiogenesis and inhibiting cardiomyocyte death (75).

In addition, native EVs from heart tissue cells also displayed certain antifibrotic effects in cardiac injury models (Figure 4; Supplementary Table 1) (118). The cardiosphere is a cluster of endogenous cardiac stem cells that forms when they are cloned in suspension, and cardiosphere-derived cells (CDCs) have been shown to have regenerative potential in cardiac injuries such as MI (119). CDC-EV treatment was shown to reduce the levels of cardiac hypertrophy, inflammation, and interstitial cardiac fibrosis in an Ang II-induced hypertension model by delivering Y RNA fragments to induce IL-10 expression (76). DCM is a serious pediatric cardiomyopathy, and ~50~60% of children with DCM will die in 5 years (120). In a swine model of DCM, CDC-EV treatment decreased myocardial fibrosis by shedding proangiogenic and cardioprotective miR-146a-5p to suppress inflammation (77). Regeneration-associated cells (RACs) are a group of heterogeneous cells (*e.g.*, endothelial progenitor cells, M2 macrophages and regulatory T cells) under vasculogenic conditions after heart injury (121). RACs-derived EVs could reduce interstitial fibrosis after myocardial ischemic injury by shedding functional miRNAs (*e.g.*, miR-150-5p, miR-195 and miR-142-3p) to promote angiogenesis and cardiomyogenesis while reducing the inflammatory response (78). Altogether, these findings indicate that native EVs from multiple cell types may mitigate cardiac fibrosis *via* immunological regulation, pro-angiogenesis or suppression of cell death.

4.2 Lung fibrosis

Chronic lung diseases are highly associated with progressive lung fibrosis, resulting in poor quality of life and high mortality of patients (122). For example, IPF, an aggressive lung disease with an uncertain cause, has a poor prognosis with a median

survival time of ~2-5 years after diagnosis (123) and ~3 million patients worldwide suffer from IPF (124). However, the therapeutic efficacies of current treatments are not ideal in the clinic (125). For example, IPF patients receiving pirfenidone still have a high mortality rate and uncertain survival times (126). Therefore, advanced treatments for the resolution of lung fibrosis are also needed.

Native EVs from stem cells (*e.g.*, MSCs) have shown therapeutic potency in preclinical models of lung injury, and immunoregulation may be one of the main mechanisms involved (Supplementary Table 1). For example, BMSC-EV treatment attenuated profibrotic factor (TGF-β1, α-SMA, collagen I/III) expressions as well as fibrotic areas after lipopolysaccharide-induced acute lung injury, and this effect may be due to the inhibitory role of miRNAs (miR-23a-3p and miR-182-5p) on the NF-κB/Hedgehog pathways (79). In a silicosis model, ADMSC-EV intervention reduced the collagen contents and F4/80$^+$ macrophage numbers and suppressed NF-κB/TLR activation in lungs by delivering functional miRNAs (*e.g.*, miR-146b) (80). In addition, the direct impact of MSC-EVs on profibrotic pathways had been reported. In a bleomycin (BLM)-induced IPF model, hucMSC-EV treatment inhibited myofibroblast differentiation and collagen deposition by delivering miR-21-5p/miR-23-3p to suppress the TGF-β pathway (81). BMSC-EV treatments also ameliorated fibroblast activation and α-SMA/collagen I expression in a BLM model, and miR-186 of EVs may inhibit SRY-related HMG box transcription factor 4 (SOX4), a regulator of lung development and monocyte infiltration (82, 83).

In addition, EVs from healthy lung tissue cells may be a possible therapy for the treatment of lung fibrosis (Supplementary Table 1). Airway epithelial cells (AECs) play regulatory roles in the development of lung fibrosis, and damaged bronchial epithelial cell (HBEC)-derived EVs can induce myofibroblast differentiation associated with airway remodeling (127). However, healthy AEC-EVs may have the opposite anti-fibrotic effects. A recent study found that EVs from healthy HBECs could reduce TGF-β-induced myofibroblast differentiation *in vitro*, and intratracheal administration of these EVs promoted collagen degradation in BLM-induced IPF by inhibiting the crosstalks between the TGF-β and Wnt pathways (84). Lung spheroid cells (LSCs), an intrinsic source of lung stem cells, have been shown to mitigate fibrosis development in a rat BLM model (128). Similarly, human LSC-derived EVs (hLSC-EVs) also inhibited myofibroblast proliferation and collagen production by shedding miRNAs (miR-30a, miR-99 and let-7), and the inhalation of these EVs decreased BLM- or silica-induced rat lung fibrosis (85), suggesting that inhalation of EVs is a promising route for treating lung fibrosis. Altogether, these reports suggest that stem cells and healthy lung tissue cells are potential EV sources for mitigating lung fibrosis with inflammation and myofibroblasts as the possible targets.

4.3 Liver fibrosis

Chronic liver diseases are also a major health issue globally, with more than 800 million patients affected and ~2 million deaths per year (2). Liver fibrosis can be triggered by many causes, such as alcohol, nonalcoholic hepatic steatohepatitis (NASH), and biliary atresia, and the fibrosis is associated with cirrhosis, liver failure and portal hypertension (11). Current clinical therapies for patients with chronic liver diseases are including the removal of the pathological cause, management of the complications, and final liver transplantation (11, 129). However, these strategies are not efficient due to the continued growth of cirrhosis and the paucity of available donor livers (130). Thus, the development of novel treatments to lighten the burden of patients with liver fibrosis is necessary.

Native EVs from MSCs and iPSCs are also major players in studies related to liver fibrosis therapy (Supplementary Table 1). For instance, human amnion MSC-derived EV treatment reduced α-SMA expression and fibrotic areas in a rat CCl_4-induced liver fibrosis model by inhibiting Kupffer cells and hepatic stellate cells (HSCs) activations (86). Chemokines, such as CXC chemokine ligands (CXCLs), are involved in the activation of HSCs through autocrine and fibrogenesis (131). Allogenic ADMSC-EVs reduced the CCl_4-induced collagen volume fraction and α-SMA/collagen I/III expression by transferring miR-150-5p to inhibit CXCL1 signaling in a mouse CCl_4 model (87). Chronic graft-versus-host disease (cGVHD) is a common complication of allogeneic hematopoietic stem cell transplantation and is highly associated with major organ damage, such as liver damage (132, 133). In cGVHD mice, hBMSC-EVs alleviated the degree of liver fibrosis and prolonged animal survival by inducing IL-10$^+$ regulatory cells and inhibiting IL-17$^+$ pathogenic T cells (88). In another study, human skin fibroblast-derived iPSC-secreted EVs decreased HSC activation and liver fibrotic areas by shuttling functional miRNAs (*e.g.*, miR-92a-3p) in a CCl_4- or bile duct ligation-induced mouse model (89).

In addition, EVs from liver or other tissue-derived stem cells also showed potential anti-fibrotic effects (Supplementary Table 1). Human liver stem cells (hLSCs) are a stem cell population derived from human adult liver cells that exhibit antifibrotic effects in a murine model of NASH (134). A recent study found that hLSC-derived EVs could attenuate plasma alanine aminotransferase (ALT) and liver fibrosis in a murine model of NASH by delivering miRNAs (miR-29a, miR-30a and let-7) to inhibit collagen I and Snail expression (90). Tonsils are an alternative source of MSCs since tonsil-derived MSCs (T-MSCs) can be readily obtained from surgically removed tonsil tissues. T-MSC-EV treatment reduced the expression of α-SMA, TGF-β, Vimentin and connective tissue growth factor (CTGF) in the liver of the CCl_4 model by delivering miR-486-5p to inhibit Hedgehog signaling (91).

Interestingly, EVs from immune cells or healthy liver cells may have a potential role in preventing liver fibrosis

(Supplementary Table 1). As a type of innate immune cell, natural killer (NK) cells are important regulators of HSC activation (135, 136). NK-cell line (NK-92MI)-derived EVs reduce TGF-β-induced HSC activation and α-SMA/collagen expression by transferring miR-223 to inhibit the autophagy-related (ATG7) pathway (92). EVs from primary human hepatocytes could also attenuate CCl_4-induced hepatocyte injury and profibrotic factor (α-SMA, collagen and CTGF) expression (93). In addition to eukaryotes, EVs isolated from prokaryotes may also have antifibrotic effects. *Akkermansia muciniphila* is a probiotic with beneficial effects on the host metabolic system and immune response. In a high-fat diet (HFD)- and CCl_4-induced mouse liver injury model, probiotic-derived EVs reduced liver functional damage and fibrotic factor (TGF-β, α-SMA and collagen I) expressions by normalizing gut microbiota composition disorders (94).

4.4 Renal fibrosis

Chronic kidney disease (CKD) is a huge public health issue that attributed to ~1.5% of deaths worldwide in 2012 (3). Renal fibrosis is the key feature of CKD and the leading cause of the end-stage renal disease (ESRD) and renal failure (137). Current clinical CKD therapeutic strategies mainly focus on controlling ongoing nephron injury, hyperfiltration and renal complications. Once CKD patients progress to ESRD, the only treatment is kidney replacement therapy or kidney transplantation (138). Thus, more efficient therapeutics to prevent renal fibrosis are needed.

As shown in Supplementary Table 1, EVs isolated from stem cells remain as a major candidate for antifibrotic therapy in kidneys (139). For example, hBMSC-EV treatment reduced α-SMA and ECM (collagen I) expressions in a mouse aristolochic acid nephropathy (AAN) model (95). Yes-associated protein (YAP) plays a vital role in fibrogenesis by retaining activated Smad2/3 in the cell nucleus (140). EVs from hucMSCs alleviated interstitial fibrosis by promoting YAP degradation in a rat UUO model (96). Autophagy is a conserved cellular process that removes unnecessary or damaged components, while it may be impaired in the diabetic nephropathy (DN) state (141). In a rat DN model, BMSC-EVs were found to reduce collagen accumulation by inducing mTOR-mediated autophagy (97). Importantly, the antifibrotic effects of MSC-EVs can be replicated in large animal models. In a porcine model of metabolic syndrome (MetS), the systemic administration of ADMSC-EVs restored kidney function and reduced tubulointerstitial fibrosis by IL-10-dependent immunoregulation (98). Additionally, intrarenal injection of ADMSC-EVs decreased ECM expression in another swine MetS model by inducing regulatory T cells (99). These findings suggest that *in situ* administration of EVs may be an efficient way to improve their therapeutic potency.

Renovascular disease (RVD), represented by the narrowing of one or both renal arteries, can cause high blood pressure and renal dysfunction in patients. In a swine atherosclerotic RVD model, intrarenal-injected ADMSC-EVs decreased interstitial fibrosis while improving the glomerular filtration rate (GFR) and peritubular capillary density by shedding some vasculo-protective genes (*e.g.*, HGF and VEGFR) (100). In addition, EVs isolated from other stem cells may also have therapeutic effects. For example, mouse ESC-derived EVs reduced renal dysfunction (creatinine and blood urea nitrogen) and α-SMA expression by promoting tubular epithelial cell (TEC) proliferation and angiogenesis in a mouse model of ischemic acute kidney injury (AKI) (101). In a mouse AAN, hLSC-derived EVs reduced renal interstitial fibrosis *via* miR-29b-mediated inhibition of the Wnt/β-catenin pathway (102).

In addition to stem cells, EVs from healthy kidney tissue cells may also have an antifibrotic role (Supplementary Table 1). For example, in a rat ischemic AKI model, renal tubular cell-derived EV treatment reduced collagen I/II/IV/V deposition, α-SMA/FN expression and neutrophil infiltration by shuttling mRNAs encoding cytoplasmic ribosomal proteins (Rps6 and Rps13) (103). Interestingly, EVs isolated from the plasma of MI could inhibit the apoptosis and autophagy of TECs (NRK-52E) *in vitro*, and decrease the fibrotic area in a contrast-induced nephropathy (CIN) model by delivering miR-1-3p to target ATG13 and activate the AKT pathway (104). These studies suggest the potential of native EVs in attenuating renal fibrosis through a variety of mechanisms.

4.5 Other organ fibrosis

In addition to these major organs, many other organs or tissues can also undergo fibrosis (Figure 4). For example, in the reproductive system, intrauterine adhesion (IUA) is a serious fibrotic disease due to disordered repair after endometrial basal layer damage. Rabbit BMSC-derived EV treatment inhibited endometrial fibrosis and the TGF-β1/Smad2 pathway in a rabbit model of endometrial injury (105). Subretinal fibrosis is a common complication of macular neovascularization and causes irreversible loss of central vision (142). hucMSC-EV treatment was found to suppress the EMT of retinal pigment epithelial cells *in vitro* and reduce laser-induced subretinal fibrosis in mice by shedding miR-27-3p to inhibit the homeobox protein Hox-C6 (HOXC6) (106). Intervertebral disc degeneration (IDD) is a major cause of chronic discogenic back pain/sciatica which results in poor quality of life in patients. Fibrosis progression of nucleus pulposus (NP) cells plays a vital

role in the pathology of IDD. Rat BMSC-EVs reduced TNF-α-induced ECM (collagen I) expression in NP cells by inhibiting proapoptotic RASSF5 signaling *via* miR-532-5p (107). Urethral stricture (abnormal narrowing of the urethra) is a complication of urological surgery and is caused by fibrosis of the urethral epithelium due to damage or infection. In a rat urethral injury model, hucMSC-EV treatment inhibited myofibroblast activation by transferring anti-inflammatory miR-146a (108). These reports indicate that MSC-EVs may exert antifibrotic effects in many types of organs by inhibiting EMT, inflammation or apoptosis.

5 Engineering strategies of EV-based anti-fibrotic therapies

Although native EVs isolated from multiple cell types have shown certain antifibrotic effects, their therapeutic efficiency may be restricted by some intrinsic limitations, such as short half-life, low specific organ retention, possible off-target effects, and insufficient cell sources. Therefore, the development of engineered EVs may resolve these issues and enhance their therapeutic potency (143). Abundant evidence shows that native EVs can be re-engineered by biological or chemical methods and serve as advanced nanomedicines for the resolution of organ fibrosis. In the following sections, we briefly discuss the current modification strategies and therapeutic outcomes of engineered EVs in different organ fibrosis models (Figure 5 and Table 2).

5.1 Engineered EVs as anti-fibrotic agents

5.1.1 Genetic modifications

Genetic engineering of parental cells is a common strategy to produce functional EVs. Engineered EVs with enhanced therapeutic properties or organ targeting efficacy can be obtained from donor cells with genetic modifications, such as gene or protein (*e.g.*, miRNAs and cytokines) overexpression or knockdown (7). Vector transfection and transduction are the main methods used for the genetic engineering of EV donor cells (66). For example, mouse ADMSC-derived EVs were engineered to overexpress miRNA-181-5p, a regulator of hepatic progenitor cell differentiation and autophagy (144). As a result, these engineered EVs had high potency to rescue liver function and reduce ECM (collagen I and FN) and vimentin expression in a CCl_4-induced liver fibrosis model (144). miRNA-21a-5p can synchronize with NF-κB activation and it is abundant in fibrotic tissues. Engineered hucMSC-EVs with antagomir-21a-5p showed higher potential in inhibiting fibroblast activation *in*

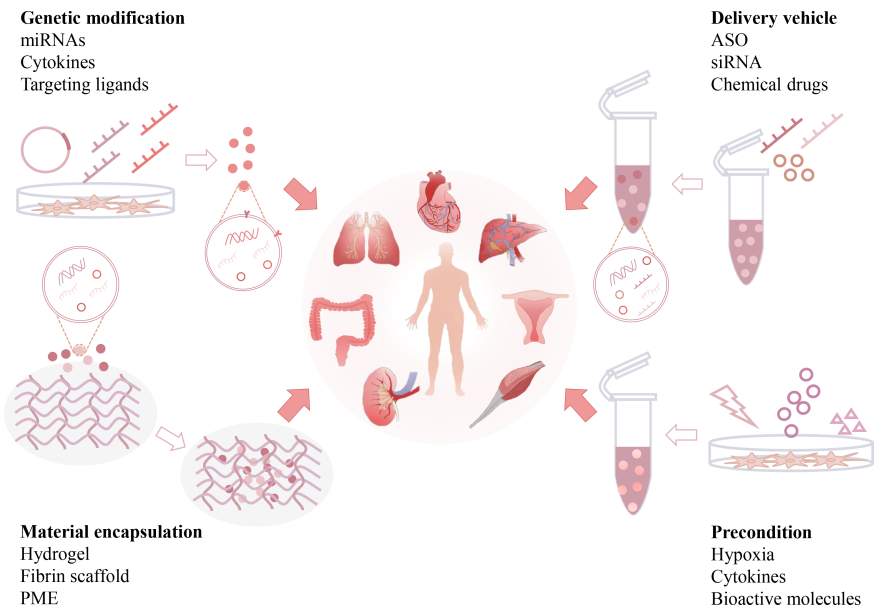

FIGURE 5
Engineering strategies of EV-based anti-fibrotic therapies. Native EVs can be re-engineered by multiple strategies, such as genetic modifications, preconditions, loading with therapeutic reagents, and incorporation with functional materials. Engineered EVs can exhibit additional beneficial effects, such as enhanced drug payload, longer half-life, better organ targeting capability and bioavailability, and serve as advanced nanomedicines for the resolution of organ fibrosis. (Antisense oligonucleotide: ASO, pneumatic microextrusion: PME).

TABLE 2 Therapeutic effects of engineered EVs.

Modifications	Fibrotic models	EV origins	Isolation methods	Reengineering routes	Therapeutic effects	Possible mechanisms	ref.
Genetic engineering	CCl_4	mice ADMSC	ExoQuick-TC Kit	Transfection to overexpress miRNA-181-5p	*In vivo*: AST, ALT, TB↓ COL I, Vimentin↓ Inflammation↓ *In vitro*: COL I, COL III, FN, α-SMA, Vimentin↓	Inhibiting the apoptosis pathway	(144)
	tendon adhesion	hucMSC	UC	Using antagonist to underexpress miR-21a-3p	*In vivo*: Tendon adhesion↓ α-SMA, COL III↓ *In vitro*: Fibroblast proliferation↓ α-SMA, COL III↓	Inhibiting NF-κB activity	(145)
	UUO	human ADMSC	UC	Lentiviral transfection to overexpress GDNF	*In vivo*: Interstitial area↓ Perfused capillaries↑ α-SMA↓ *In vitro*: Migration↑ Tube formation↑	Pro-angiogenesis	(146)
	MI	hucMSC	Total Exosome Isolation reagent	Using CRISPR/Cas9 to silence β-2 microglobulin	*In vivo*: Fibrotic area↓	Preventing the immune rejection	(147)
	UUO	Primary mouse satellite cells	UC	Using Lamp2b fused with RVG	*In vivo*: α-SMA, COL 1A1, COL 4A1, Vimentin, FN↓ myoD, myogenin, eMyHC↑	Downregulation of TGF-β pathway	(148)
Delivery vehicle	CCl_4	human BMSC	UC	Carrying siRNA or ASO targeting STAT3	*In vivo*: α-SMA, COL I, Vimentin, FN, Col 1a1↓	Anti-inflammatory	(149)
	Adriamycin	RAW 264.7	centrifugation	Delivery DEX	*In vivo*: Interstitial fibrosis↓	Anti-inflammation	(150)
Precondition	MI	mice BMSC	UC	Hypoxia condition (0.5% O_2) and culture for 24 h	*In vivo*: Cardiac function↑ Fibrotic scar size↓	Delivery of anti-apoptosis miR-210	(151)
	Urethral stricture	hucMSC	UC	Pretreatment with 10ng/mL TNF-α for 12h	*In vivo*: α-SMA↓ Collagen fibers↓ *In vitro*: Fibroblast activation↓ α-SMA, COL I, COL III↓ IL-6, IL-1β↓	Delivery anti-inflammatory miR-146a	(108)

(Continued)

TABLE 2 Continued

Modifications	Fibrotic models	EV origins	Isolation methods	Reengineering routes	Therapeutic effects	Possible mechanisms	ref.
	Sulfate-Induced Colitis	mice BMSC	Ultrafiltration	Pretreatment with IL-1β (25 ng/mL), IL-6(20 ng/mL) and TNF-α (20 ng/mL) for 24h	*In vivo:* Collagen deposition↓ Necrotic mucosal surface↓	Anti-inflammatory	(152)
	CCL_4	RAW264.7	sequential centrifugation	Pretreatment with 100ng/mL RLN for 24h	*In vivo:* Serum AST, ALT↓ Hepatic hydroxyproline↓ α-SMA↓ *In vitro:* α-SMA, COL Iβ↓	Anti-inflammatory	(153)
Material encapsulation	Ischemic AKI	mice BMSC	UC	KMP2 hydrogel to release EVs	*In vivo*: BUN, CREA↓ NGAL↓ α-SMA, FN↓ Inflammation↓ Renal microvascular injury↓	Decreasing cell apoptosis/ inflammation and improving microvascular endothelial cell regeneration	(154)
	Thioacetamide induce chronic liver injury	human ESC	UC	EVs embedded with a clickable PEG	*In vivo*: Hepatoprotective effects↑ COL I, α-SMA↓ MMP-9, MMP-13↑		(155)
	MI	MSC	ultrafiltration	Seeding EVs in fibrin scaffold	*In vivo*: Infarct size↓ LV wall↑ Viable cardiac tissue↑	Promoting endogenous angiomyogenesis	(156)
Combination	partial nephrectomy	hucMSC	UC	A hybrid scaffold of PME and PDRN combined with MSC-EVs primed with 20 ng/mL IFN-γ and TNF-α for 72h	*In vivo:* CREA, BUN↓ GFR↑ α-SMA, Vimentin, Snail1↓ IL-1RA, TNF-α↓ IL-10↑ *In vitro:* N-Cadherin, FN↓		(157)
	BLM	L-929	UC	Hybrid nanovesicles to delivery NIN	*In vivo:* Pulmonary function↑ Collagen deposition↓ α-SMA, MMP-7, TGF-β↓	Diminishing macrophage-induced inflammatory response	(158)
	Sulfate-Induced Colitis	Human dental pulp MSCs	UC	HIF-1α overexpression and TNF-α (10 ng/mL), IL-1β (10 ng/mL) and IFN-γ (50 ng/mL) pretreatment	*In vivo:* Fibrillar collagen proportion↓ *In vitro:* α-SMA, COL 1α↓		(159)

GDNF, glial cell line-derived neurotrophic factor; CRISPR/Cas9, clustered regularly interspaced short palindromic repeats/CRISPR-associated endonuclease; RVG, rabies viral glycoprotein peptide; ASO, antisense oligonucleotide; DEX, dexamethasone;TNF-α, tumor necrosis factor-α; RLN, relaxin; PEG, polyethylene glycol; PME, pneumatic microextrusion; PDRN, polydeoxyribonucleotide; IFN-γ, Interferon-γ; NIN, nintedanib.

vitro and α-SMA and collagen III expression in a rat tendon adhesion model than native EVs (145).

EVs can also be engineered with therapeutic proteins (*e.g.*, cytokines). Glial cell line-derived neurotrophic factor (GDNF) is a member of the TGF-β superfamily and plays a vital role in renal morphogenesis and angiogenesis (160). In a UUO renal fibrosis model, GDNF-overexpressing hADMSC-EVs exhibited higher potency to reduce peritubular capillary rarefaction and renal fibrosis score/α-SMA levels than native EVs (146). Disruption of the human leukocyte antigen (HLA) light chain β2-microglobulin (B2 M) gene may disable the function of HLA-I molecules and thus prevent hucMSC-mediated immune rejection (161). hucMSCs with B2 M deletion (B2 M^- hucMSCs) were generated using a CRISPR/Cas9 method, and these modified EVs were more efficient in reducing fibrotic areas and restoring cardiac function than native EVs in a rat model of MI (147).

Furthermore, the organ targeting capability of native EVs can be improved by genetic modifications. Fusion of targeting ligands (*e.g.*, peptides) into EV membrane proteins (*e.g.*, Lamp2b and CD63) is a common strategy to produce EVs with specific cell- or tissue-targeting ability (162). For example, a recent study showed that engineered mouse satellite cell-derived EVs using Lamp2b fused with a rabies viral glycoprotein peptide (RVG) had higher renal targeting efficacy. In a UUO model, delivery of miR-29 (a potent inhibitor of TGF-β3) by these modified EVs had higher antifibrotic potency (reducing renal α-SMA, collagen, vimentin and FN levels) than unmodified EVs (148). Altogether, these studies indicate that genetic engineering is a potent strategy to enhance the organ targeting and therapeutic effects of EVs.

5.1.2 Delivery of therapeutic reagents by EVs

Due to their bilayer structure and cargo transferring capacity, EVs can serve as natural carriers of therapeutic agents and protect them against *in vivo* degradation. The current techniques of loading cargoes into EVs can be divided into exogenous loading (coincubation, electroporation, extrusion or sonication of drugs with isolated EVs) and endogenous loading (genetic modification of EV donor cells) (66, 163). Antisense oligonucleotide (ASO)/siRNA is a type of nucleic acid drug that can silence targeted genes, but its therapeutic potency is largely limited by off-target effects and liver toxicity (164, 165). To overcome these limitations, ASO or siRNA targeting STAT3 (a key regulator of inflammation) was loaded into BMSC-EVs, and the engineered $iExo^{siRNA\text{-}STAT3}$ or $iExo^{mASO\text{-}STAT3}$ treatment showed higher potential to reduce collagen I deposition and α-SMA, vimentin and FN expression in a CCl_4-induced liver fibrosis model (149).

In addition, EVs can also be used to deliver chemical drugs to enhance their therapeutic index *in vivo* (Table 2). The anti-inflammatory drug, dexamethasone (DEX), has been used for fibrosis treatment (166). In a recent study, RAW 264.7 macrophage-derived DEX-packaging EVs exhibited a superior capacity to suppress renal inflammation and interstitial fibrosis without apparent glucocorticoid adverse effects in Adriamycin-induced nephropathy in mice (150). These studies suggest that engineered EVs are potent carriers for the delivery of therapeutic agents to alleviate fibrosis.

5.1.3 Preconditioned EVs

In addition to the mentioned EV modification methods, parental cells can also be primed/preconditioned with pathological stimuli to produce EVs with desirable profiles or contents useful for fibrosis resolution (Table 2). MSCs are usually preconditioned with hypoxia or cytokines to acquire and retain phenotypes relevant for therapeutic applications (167). For instance, EVs isolated from BMSCs under hypoxic conditions (0.5% O_2 for 24 h) had superior therapeutic ability in restoring cardiac function and ameliorating fibrotic scar area after MI, because those EVs had enriched miRNAs such as the anti-apoptotic miR-210 (151). Pretreatment of donor cells with cytokines can augment the immunomodulatory properties of the produced EVs. For example, TNF-α-preconditioned hucMSC-EVs reduced α-SMA and collagen expression in a rat urethral fibrosis model due to the enriched anti-inflammatory miR-146a in those EVs (108). EVs from mouse BMSCs primed with a cocktail of cytokines (IL-1β, IL-6 and TNF-α) had higher efficacy in reducing the necrotic mucosal surface and collagen deposition in a dextran sulfate sodium-induced colitis model (152).

In addition, pretreatment of donor cells with bioactive molecules may also enhance the therapeutic effects of EVs (Table 2). Relaxin (RLN) is an antifibrotic peptide hormone that has been shown to reduce liver fibrosis by reversing the activation of HSCs (168). In a mouse CCl_4 liver fibrosis model, EVs from RLN-preconditioned mouse macrophage cell lines (Raw264.7) showed higher potency to lower serum ALT/aspartate aminotransferase (AST) and reduced liver fibrosis (hydroxyproline and α-SMA levels) than native EVs (153). These studies suggest that the preconditioning of parental cells may be an efficient method to produce functionalized EVs.

5.2 Biomaterials for EV retention and delivery

Current evidence indicates that systemically administered EVs have a short half-life and can be rapidly cleared *in vivo*. To resolve this problem, an innovative strategy that encapsulates EVs with functional biomaterials has been proposed (Figure 5 and Table 2). EVs delivered by biomaterials such as hydrogels and scaffolds may have enhanced therapeutic efficacy due to prolonged EV release and improved bioavailability (169).

Hydrogels are three-dimensional hydrophilic networks with advanced properties, such as tunability, good biocompatibility and biodegradability, and high tissue retention, therefore, they have been widely used to encapsulate cells or drugs. This strategy can also be applied in the local delivery of EVs to improve their stability and half-life (170). Self-assembling peptide (SAP) is a type of biomaterial made of natural amino acids, and some of them can rapidly form a nanoscale hydrogel in ionic saline conditions (171). An injectable SAP hydrogel was used to encapsulate BMSC-EVs, which enabled a sustained release of EVs with preserved biofunction. In a mouse model of ischemic AKI, local delivery of BMSC-EVs by SAP hydrogels showed better efficacy in decreasing renal damage, inflammation and subsequent renal fibrosis (α-SMA and FN) than EVs alone (154).

Similarly, human ESC-EVs were encapsulated into a polyethylene glycol (PEG) hydrogel through a click reaction. The resulting EV-loaded hydrogel showed higher antifibrotic effects in a thioacetamide-induced liver fibrosis model, as indicated by lower expression of MMP-9/13, collagen I and α-SMA than EVs alone (155). The fibrin scaffold is a degradable biopolymer made of fibrinogen and can provide binding sites for cell migration and proliferation to promote tissue regeneration (172). In another study, an invasive EV spray was prepared by incorporating MSC-EVs with fibrin scaffold materials. In a mouse or a swine model of MI, EV spray treatment led to smaller infarct size, thicker left ventricular wall, and enhanced angiomyogenesis than EVs alone in the postinjury heart (156). Altogether, these findings suggest that biomaterial-based EV engineering is an efficient strategy for enhanced antifibrotic therapy.

5.3 Combined strategies for enhancing therapeutic potency

Moreover, the combination of multiple EV modifications may be an attractive strategy for advanced anti-fibrotic therapy (Table 2). For example, cytokine (TNF-α and IFN-γ)-preconditioned hucMSC-EVs were loaded into a hybrid scaffold made of pneumatic microextrusion (PME, consisting of PLGA, magnesium hydroxide and decellularized porcine kidney extracellular matrix) and polydeoxyribonucleotide (PDRN). This combined treatment showed a synergistic effect of reducing renal inflammation (IL-1RA and TNF-α) and fibrosis (α-SMA, vimentin and Snail) in a mouse model of partial nephrectomy (157). NIN is a tyrosine kinase receptor inhibitor for the treatment of lung fibrosis in the clinic, but its therapeutic efficiency is not ideal due to nonspecific organ distribution. In a recent study, NIN was loaded into hybrid nanovesicles made of clodronate disodium (CLD)-loaded liposomes and fibroblast cell line (L-929)-derived EVs. In a BLM-induced mouse lung fibrosis model, this engineered hybrid EVs showed higher lung retention than unmodified EVs and thus decreased macrophage-mediated inflammation and ECM deposition in the lungs (158). In addition, the combination of stimuli and genetic modification may enhance the therapeutic potency of native EVs. For example, EVs from human dental pulp MSCs with HIF-1α (a master regulator of hypoxia) overexpression and cytokine (IFN-γ, IL-1β and TNF-α) preconditioning were produced, and these EVs had higher potency to alleviate the fibrillar collagen proportion and colon length shortening in a mouse colitis model than native EVs (159). Altogether, the combination of multiple engineering strategies may further enhance the anti-fibrotic potency of EV-based therapies.

6 Clinical trials of EVs for fibrosis-related diseases

To date, few clinical trials have been conducted associated with direct organ fibrosis, and most of them are still in the early stages. For example, a randomized, placebo-controlled, phase 2/3 clinical pilot study was performed to investigate the safety and therapeutic efficacy of cord-blood MSC-EVs in preventing the progression of grade III and IV CKD. The results showed that intra-arterial and intravenous EV injections reduced the inflammatory immune reaction and improved kidney function (173). Recent studies suggest that severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection may cause severe lung damage and substantial lung fibrotic consequences in patients (174). To protect the damaged lungs, several EV-based clinical trials are already underway or seem to be underway. A multicenter, double-blind, randomized controlled trial (RCT) phase 2/3 trial is recruiting, and it aims to evaluate the efficacy and safety of MSC-EVs on reducing inflammation in moderate COVID-19 patients (NCT05216562). An open-label phase 1 study was conducted to evaluate the safety and immunoregulation of EVs carrying CD24 in patients with moderate/severe COVID-19 (NCT04747574). These modified EVs are produced from T-REx^{TM}-293 cells engineered to express CD24 (a vital immunomodulator), and its phase II trial is currently active, not recruiting (NCT04969172). Although the current state is still far from clinical applications, increasing evidence suggests that EVs are a potent cell-free, off-the-shelf antifibrotic strategy.

7 Future perspectives

Notably, a large number of studies indicate that EVs may be promising means to mitigate organ fibrosis. Moreover, as a type

of naturally derived nanomaterial, EVs have several advantages, such as intrinsic biological properties, the ability to cross biological barriers, and minimal immunogenicity or toxicity *in vivo* (7, 51). However, there are still some limitations that needed to be well resolved before further translation of EVs into clinical applications.

A primary challenge in this field is how to standardize the production of EVs on a large-scale during isolation, purification, and scalability. For research purposes, small amounts of EVs can be readily isolated by common techniques, such as UC, SEC and immunoaffinity (47), but the yields are far below the clinical requirement. For small animal models, such as rodents, the medium EV dosage is ~80-100 μg/mouse with systematic injection, while it dramatically increases to 75 mg/swine *in situ* in large animal models (175, 176). In this case, it can be assumed that the required EV amounts for human patients with fibrotic diseases are much larger. Thus, it is urgent to optimize the process of large-scale EV production with high yield and purity, as well as retain integrity and biofunction. More importantly, for clinical applications, it is strictly required to define the bioactive components, standard operating procedures, quality control criteria, virulence and sterility of EV products.

Native EVs usually exhibit a short half-life and insufficient organ retention *in vivo*. The distribution of EVs can be affected by many factors, such as parental cell types or administration routes (177). To improve the specific organ targeting efficiency of EVs, it is possible to select the appropriate cell source according to the therapeutic intent, such as bronchial epithelial cell-derived EVs for lung fibrosis and cardiosphere-derived EVs for cardiac fibrosis (77, 84). Another strategy is surface modification of EVs with targeting ligands, thereby enhancing the uptake of therapeutic EVs in targeted organs. The administration routes of EVs may also affect their therapeutic potency. For instance, i.v. injected EVs showed higher retention in the liver and spleen and lower retention in the pancreas compared to i.p. and subcutaneous injection (177). Thus, the selection of a proper delivery route should also be considered in the treatment of fibrosis in different organs.

Since the mechanism of fibrosis is complicated and many types of cells and pathways are involved, it seems that single-target therapy may not be efficient. In fact, many antifibrotic strategies, such as cessation of chronic tissue injury, resolution of local inflammation, deactivation or elimination of myofibroblasts, and degradation of ECM, have been evaluated individually or combined in preclinical studies or clinical trials (5). However, many current EV-based studies are still focusing on targeting a single cause (*e.g.*, inflammation and angiogenesis), and the direct antifibrotic potency of EVs needs to be further enhanced. To address this limitation, a better strategy is to develop engineered EVs that can target multiple essential cells or pathways involved in fibrosis, which may exhibit advanced therapeutic effects.

Another important limitation is that current findings related to the anti-fibrotic effects of EVs are mainly based on small animal (mouse and rat) models, while there have been only a small number of large animal (*e.g.*, swine) model-based studies (156). Moreover, these models may not fully mimic the pathology of human patients due to the large genetic and/or physiological differences between humans and small animals. For example, rodent models of liver fibrosis are commonly induced by toxic reagents (*e.g.*, CCl_4) or bile duct ligation, while virus (hepatitis B virus) infection, alcoholic liver disease, and nonalcoholic fatty liver disease are the main causes of liver fibrosis in the clinic (2, 178). Thus, standard and large animal models that can better mimic the fibrosis of patients are needed in this field.

8 Conclusion

In summary, EV-based therapeutics have shown promising effects in mitigating multiple types of organ fibrosis in many preclinical studies. Moreover, the therapeutic potential of EVs can be further improved using multiple modification strategies. To better translate EV-based therapies into clinical applications, more research is required to clarify the direct antifibrotic role of EVs and to establish large-scale production and efficient EV engineering strategies in the future.

Author contributions

KL wrote the manuscript and prepared for visualization. YW, PL, SL and PZ prepared for drawing elements, LY and YL played as supervision roles. JC provided financial support. JL is corresponding author and review the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version. All authors contributed to the article and approved the submitted version.

Funding

This study was partly supported by the National Natural Science Foundation of China (32071453, 31871001, 81571808, 32271438, 32001011), 1.3.5 Project for Disciplines of Excellence (ZYGD18014), the Fundamental Research Funds for the Central Universities (2020SCU12040), and the PostDoctor Research Project of West China Hospital of Sichuan University (2020HXBH047).

Acknowledgments

We would like to thank Wenshu Dai from Sichuan University for language editing.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1042983/full#supplementary-material

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 27 October 2022
DOI 10.3389/fimmu.2022.1041592

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Cytokines and Soluble Mediators in Immunity, a section of the journal Frontiers in Immunology

RECEIVED 11 September 2022
ACCEPTED 13 October 2022
PUBLISHED 27 October 2022

CITATION
Nguyen TH, Dao HH, Duong CM, Nguyen X-H, Hoang DH, Do X-H, Truong TQ, Nguyen TD, Nguyen LT and Than UTT (2022) Cytokine-primed umbilical cord mesenchymal stem cells enhanced therapeutic effects of extracellular vesicles on osteoarthritic chondrocytes. *Front. Immunol.* 13:1041592. doi: 10.3389/fimmu.2022.1041592

Cytokine-primed umbilical cord mesenchymal stem cells enhanced therapeutic effects of extracellular vesicles on osteoarthritic chondrocytes

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In recent years, extracellular vesicles (EVs) secreted by mesenchymal stem cells (MSCs) have emerged as a potential cell-free therapy against osteoarthritis (OA). Thus, we investigated the therapeutic effects of EVs released by cytokine-primed umbilical cord-derived MSCs (UCMSCs) on osteoarthritic chondrocyte physiology. Priming UCMSCs individually with transforming growth factor beta (TGFβ), interferon alpha (IFNα), or tumor necrosis factor alpha (TNFα) significantly reduced the sorting of miR-181b-3p but not miR-320a-3p; two negative regulators of chondrocyte regeneration, into EVs. However, the EV treatment did not show any significant effect on chondrocyte proliferation. Meanwhile, EVs from both non-priming and cytokine-primed UCMSCs induced migration at later time points of measurement. Moreover, TGFβ-primed UCMSCs secreted EVs that could upregulate the expression of chondrogenesis markers (*COL2* and *ACAN*) and downregulate fibrotic markers (*COL1* and *RUNX2*) in chondrocytes. Hence, priming UCMSCs with cytokines can deliver selective therapeutic effects of EV treatment in OA and chondrocyte-related disorders.

KEYWORDS

osteoarthritis, chondrocytes, mesenchymal stem cells, extracellular vesicles, cytokines, microRNA

10.3389/fimmu.2022.1041592

Introduction

Extracellular vesicles (EVs) are nano-sized, lipid membrane-enclosed particles that modulate the physiological conditions of the recipient cells (1). By effectively delivering a wide range of bioactive molecules involved in critical signaling pathways associated with apoptosis, proliferation, migration, extracellular matrix (ECM) synthesis, cartilage regeneration, and inflammation management, EVs have been studied for their therapeutic effects on several cartilage-related diseases (2). Recently, multiple approaches have been employed to enhance therapeutic effect, and targetable EV delivery, including engineering secreted cells, loading therapeutic molecules into naturally secreted vesicles (3), and conjugating the vesicles with targeting ligands (4). Additionally, the therapeutic cargo of EVs secreted by mesenchymal stem cells (MSCs) varies depending on MSC tissue sources (5). Since MSCs are very sensitive to environmental conditions, priming these cells with cytokines as supplements in the culture media can influence bioactive molecules packed in the derived EVs, thereby affecting the biological activities of the vesicles (6, 7).

In this study, we primed MSCs originated from the umbilical cord (UCMSCs) with anti-inflammatory cytokines (transforming growth factor beta - TGFβ and interferon alpha - IFNα) and inflammatory cytokines (including tumor necrosis factor alpha - TNFα), which are linked with osteoarthritis (OA) pathogenesis. In healthy cartilage, TGFβ stimulates chondrocyte proliferation while suppressing chondrocyte hypertrophy and maturation, as well as promoting chondrocytes to synthesize ECM components (8). Additionally, the inhibition of TGFβ signaling leads to chondrocyte terminal differentiation and the early onset of OA (9). Another stimulant in the anti-inflammatory, IFNα, plays a vital role in autoimmunity and inflammation and could effectively protect against antigen-induced arthritis by inhibiting pro-inflammatory cytokine (interleukin 1β (IL-1β), IL-6, IL-17, TNF, IL-12, and IFNγ) production while inducing TGFβ synthesis (10). Moreover, injection of IFNα into the synovial fluid promotes the generation of functional antagonists such as interleukin 1 receptor antagonist (IL-1Ra), soluble tumor necrosis factor receptors (sTNFR), and osteoprotegerin (OPG) for known OA-inducing factors of IL-1, TNF, and osteoprotegerin ligand (11). However, direct administration of these two cytokines to patients frequently harms the nearby tissues, such as a synovial membrane or subchondral bone, as well as general health, including headache, malaise, fever, and even depression (12). The inflammatory cytokine TNFα which is one crucial catabolic factor for cartilage, promotes synovial fibroblasts to release Matrix metalloproteinase (MMPs), resulting in cartilage destruction during OA progression (13). This cytokine is also able to signal chondrocyte apoptosis leading to a more severe OA phenotype (13). Moreover, priming UCMCS with cytokines enhances the anti-inflammatory and immunomodulatory potential of the secreted EVs (14–16). TNFα stimulation was shown to induce the expression of immunosuppressive factors in the parental MSCs, which produced exosomes that can modulate M2/M1 macrophage differentiation (14). The molecular content changes in EV derived from cytokine-stimulated MSCs can interfere with inflammation *via* PGE2/COX10 mechanism (15). Although the immunomodulatory effect of EVs from cytokines-primed cells in the context of OA has been reported before (14, 17), no publication was found indicating their influence on chondrocytes.

In OA, several miRNAs found in EVs have been demonstrated to regulate key signaling pathways involved in ECM maintenance, chondrocyte proliferation, migration, apoptosis, and inflammation (2). Thus, modulating miRNA composition might directly influence the EV therapeutic effect. In this study, we focused on two candidate miRNAs, miR-320a-3p and miR-181b-3p, which involve in cartilage homeostasis. Previous studies showed that miR-320a played essential roles in the secretion of matrix degradation factors (18) and chondrocyte proliferation (19) in OA models. Although not many studies focused on miR-181b-3p, it was described to inhibit proliferation as well as promote apoptosis of chondrocytes in OA (20).

As described, different EV sub-populations carry a different set of bioactive molecules (21), for instance, miRNAs content; thus, we assumed that they would have a distinct impact on chondrocytes. We further hypothesized that priming UCMSCs with the cytokines could alter the miR-181b-3p and miR-320a-3p levels in the secreted EVs, thereby modulating the effect of EVs in chondrocytes proliferation, migration, and their markers.

Results

Cytokine-primed UCMSCs and their EVs expressed regular morphology and molecular markers

We examined the morphology and cell surface markers of UCMSCs at passage 5 (P5), either non-priming or cytokine priming. We observed a typical UCMSC morphology with a spindle shape in both control (EV-depleted) and cytokine-primed (TGFβ, IFNα, TNFα) culture conditions (Figures 1A1-A4). Additionally, all cultured UCMSCs expressed MSC positive markers of CD90, CD105, and CD73 (> 95%). Meanwhile, MSC negative markers of CD45, CD34, CD11b, CD19, and HLA-DR were detected with low percentages (< 2%) (Figure 1B). Hence, cytokine priming did not alter the typical morphology and surface markers of UCMSCs.

EVs isolated from UCMSC culture medium were subjected to morphology analysis by transmission electron microscope (TEM). Three EV sub-populations including apoptotic bodies (ABs), microvesicles (MVs), and exosomes (EXs), were observed with distinguished shapes and sizes (Figures 1C1 - C3). ABs showed variable shapes with a diameter scale of approximately 500 nm to

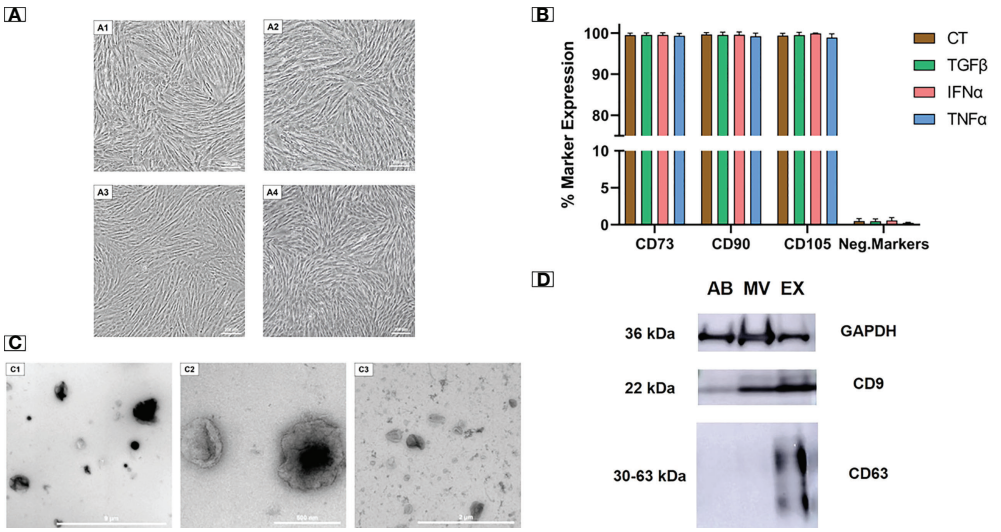

FIGURE 1
Cytokine-primed UCMSCs and their EVs expressed typical morphology and molecular markers. **(A1 - A4)** Typical morphology of **(A1)** non-priming UCMSCs; **(A2)** TGFβ-primed UCMSCs; **(A3)** IFNα-primed UCMSCs; **(A4)** TNFα-primed UCMSCs; were captured under Nikon Inverted Microscope Eclipse Ti-S at day 5 of P5. **(B)** Expression of MSC markers (n = 3) was analyzed using the flow cytometry approach. **(C1 - C3)** Morphology of different EV populations was observed under TEM. **(C1)** An AB representative with a diameter ranging from 500 nm to 2000 nm (scale bar 9 μm); **(C2)** A MV representative with irregular shapes and diameters from 200 nm to 400 nm (scale bar 500 nm); **(C3)** An EX representative that has cup-shaped morphology and sizes ranging from 40 to 200 nm (scale bar 2 μm). **(D)** A representative of EV markers (22). 15 μg total EV protein was loaded in each lane. Internal reference GAPDH and CD9 were detected in all three EV populations. CT, non-priming UCMSCs; TGFβ, TGFβ-primed UCMSCs; IFNα, IFNα-primed UCMSCs; TNFα, TNFα-primed UCMSCs. Apoptotic Bodies; MV, Microvesicles; EX, Exosomes. All representative images of EVs were obtained from EVs secreted by TGFβ-primed UCMSCs. Error bars indicate ± SD.

2000 nm, and they are packed within a rough membrane (Figure 1C1). MVs had variable membrane-bound morphologies with uneven surfaces and diameters ranging from 100 nm to 1 μm (Figure 1C2). EXs exhibited a typical cup-shaped morphology with their size ranging from 40 to 200 nm (Figure 1C3).

Additionally, the isolated EVs expressed standard EV protein markers (CD9 and CD63). As a control indicator, all three EV populations strongly expressed the internal reference protein of GAPDH. For general EV marker expression, CD9 was present abundantly in MVs and EXs and lightly in ABs. EXs also expressed a typical exosomal marker of CD63, which was absent in MVs and ABs (Figure 1D). The morphology and protein marker analysis confirmed the identity of three separated EV populations from UCMSC conditioned media.

Differential levels of microRNA 320a-3p and 181b-3p into EVs secreted by cytokine-primed UCMSCs

We measured the expression of selected miRNAs associated with OA pathogenesis present in UCMSCs and three secreted EV populations (ABs, MVs, and EXs) from normal and cytokine-primed conditions. Using qRT-PCR, we quantified the levels of two candidate miRNAs: miR-181b-3p and miR-320a-3p. Generally, both miR-181b-3p and miR-320a-3p were detected in all UCMSCs and isolated EV sub-populations of ABs, MVs, and EXs from different culture conditions (Figure 2 and Supplementary Figure 1).

In UCMSCs, cytokine treatments acted differentially on the expression of two candidate miRNAs. Particularly, TGFβ and TNFα significantly induced the levels of miR-320a-3p present in UCMSCs, indicated by lower delta Ct values (Supplementary Figure 1A). Besides, no significant impact was detected on the expression of miR-181b-3p (Supplementary Figure 1B).

In contrast, cytokine-priming significantly modulated the levels of miR-181b-3p while producing a little impact on the levels of miR-320-3p packed into EVs. Cytokine treatment suppressed the selective sorting of miR-181b-3p in all three EV sub-populations compared to the non-priming group, indicated by higher delta Ct values when normalizing to secreted cells, UCMSCs (Figure 2B). Comparing the effects among different cytokines in each EV sub-populations, IFNα and TNFα cytokine treatments further limited the miR-181b-3p content in ABs and MVs (Figure 2B). Indeed, we detected a greater relative expressionof miR-181b-3p in TGFβ-ABs compared to IFNα-ABs ($p = 0.0359$) and TNFα-ABs ($p = 0.0101$), as well as in TGFβ-MVs compared to TNFα-MVs ($p = 0.028$). miR-181b-3p also expressed stronger in MVs from IFNα-primed UCMSCs compared to TNFα-primed ones ($p = 0.038$) (Figure 2B). However, in the EX population, no significant difference in miR-

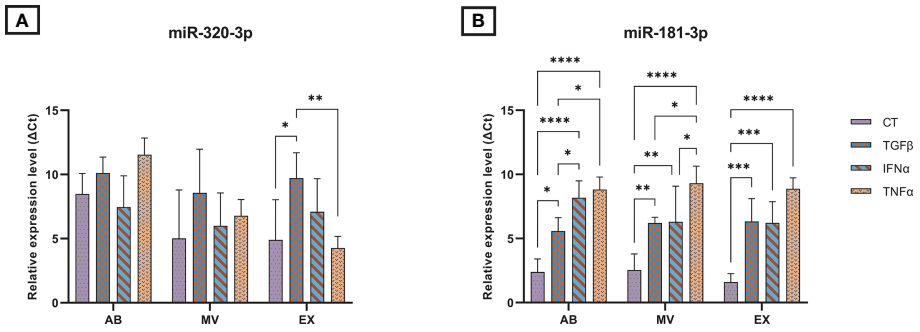

FIGURE 2
The relative expression of microRNAs in three EV populations in control and inducing groups. The miRNA expression levels in EVs are represented by ΔCt values with 7.5 ng cDNA input and were normalized to the secreted cells (UCMSCs). The relative expression level in different EV sub-populations of **(A)** miR-320a-3p; **(B)** miR-181b-3p. Cytokines priming suppressed the selective sorting of miR-181b-3p but not miR-320a-3p into UCMSC-derived EVs. AB: Apoptotic bodies; MV: Microvesicles; EX: Exosomes. CT-AB/MV/EX: AB/MV/EX secreted from non-priming UCMSCs; TGFβ-AB/MV/EX: AB/MV/EX secreted from TGFβ-primed UCMSCs; IFNα-AB/MV/EX: AB/MV/EX secreted from IFNα-primed UCMSCs; TNFα-AB/MV/EX: AB/MV/EX secreted from TNFα-primed UCMSCs. Results were averages of 3 biological samples (n = 3). Statistical significance was determined by Two-Way ANOVA and indicated by: * where $p < 0.05$; ** where $p < 0.01$; *** where $p < 0.001$; **** where $p < 0.0001$. Error bars indicate ± SD.

181b-3p inhibiting effects was observed among three different cytokine priming conditions (Figure 2B). Meanwhile, the amount of miR-320a-3p packed in ABs, MVs, and EXs was mostly stable in all non-priming and cytokine-priming cultures. Only a small change of miR-320a-3p content in the EX population was detected under the effect of TGFβ and TNFα, where this miRNA was relatively expressed higher in CT-EXs and TNFα-EXs compared to TGFβ-EXs ($p = 0.0299$ and $p = 0.0031$, respectively) (Figure 2A). Taken together, cytokine-primed UCMSCs selectively reduced the sorting of miR-181b-3p into EVs, whereas there were no effects on the miR-320a-3p.

Chondrocytes were successfully isolated from human articular cartilage

Human chondrocytes were isolated from the knee articular cartilage tissue digested with collagenase and were cultured in the DMEM/F12. As shown in Figure 3A, cells at P1 exhibited flattened and polygonal shape which is a typical morphology of chondrocytes. Additionally, isolated cells were positive with Alcian Blue staining dye, which is specific for chondrocyte cells and appears blue due to proteoglycans secretion (Figure 3B).

Cytokines affecting UCMSC-derived EV capacity in promoting chondrocyte proliferation

We performed an MTT assay on human chondrocytes to examine the effect of EVs derived from cytokine-priming UCMSCs on chondrocyte proliferation. In general, EVs generated from UCMSCs, either priming with cytokines or not, did not have any statistically significant effect on chondrocyte proliferation compared to EV-depleted media (No-EV) and among treatment groups (Figure 4).

EVs derived from TGFβ-primed UCMSCs promoted chondrocyte migration

We performed the wound scratch assay to access the capacity of EVs from cytokine-primed UCMSCs in regulating chondrocyte migration. In general, EVs from either non-priming or cytokine-primed UCMSCs significantly promoted chondrocyte migration starting from the 44-hour time point (later experimental time points) (CT-ABs, $p = 0.0445$ at 68 hours; and CT-MVs, $p = 0.0105$ at 44 hours; when compared to EV-depleted media) (Figure 5 and Supplementary Figure 2).

Among analyzed cytokines, EVs derived from TGFβ-primed UCMSCs significantly stimulated cell migration stronger than EV-depleted media at multiple time points. For details, TGFβ-ABs had higher migration induction at 52 hours ($p = 0.0169$) (Figure 5A). TGFβ-MVs enhanced migration at 48 hours ($p = 0.0329$), 52 hours ($p = 0.0108$), and 68 hours ($p = 0.0129$) (Figure 5B). Additionally, TGFβ-MVs exhibited a greater induction on cell migration than CT-MVs at 52 hours ($p = 0.0215$) and at 68 hours ($p = 0.0424$) and better than IFNα-MVs at 68 hours ($p = 0.0426$) (Figure 5B). At 68 hours, TGFβ-EXs significantly promoted migration stronger than No-EV ($p = 0.0351$) (Figure 5C).

Regarding IFNα cytokine priming, only IFNα-EXs induced cell migration faster than No-EV, but neither IFNα-ABs nor IFNα-MVs, at 44 hours ($p = 0.0319$), 52 hours ($p = 0.0376$), and 68 hours ($p = 0.0338$) (Figures 5A, B). Especially, IFNα-ABs and IFNα-MVs

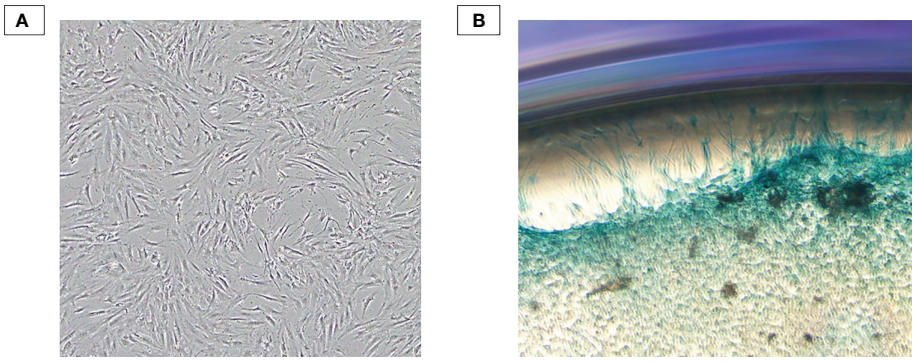

FIGURE 3
Chondrocyte characterization. **(A)** Human primary chondrocytes isolated from the human knee articular cartilage tissue at P1. Isolated cells exhibited typical morphology of primary chondrocytes: flattened and polygonal shape. **(B)** Alcian Blue staining result. Isolated cells were subjected to form colony and positive with Alcian Blue staining dye – an indicator of proteoglycan secretion.

showed suppression of chondrocyte migration at the 20-hour time point (compared to No-EV; $p = 0.0259$ and $p = 0.0190$, respectively) (Figures 5A, B).

For EVs secreted from TNFα-primed UCMSCs, TNFα-MVs stimulated migration more effectively than No-EV at 44 hours ($p = 0.0456$), whereas TNFα-EXs promoted efficiently at 44 hours ($p = 0.0492$) and 48 hours ($p = 0.0128$) (Figures 5B, C).

EVs derived from cytokine-primed UCMSCs alter the expression of chondrocyte markers by chondrocytes

To investigate the molecular alterations of chondrocytes in different EV-treated culture conditions, we isolated total RNA from cells after one-week culture under EV treatment and subjected them to qRT-PCR. The relative expression level of chondrocyte mRNAs, including normal chondrocyte markers of Collagen type II (COL2A1), Cartilage oligomeric matrix protein (COMP), Aggrecan (ACAN), and hypertrophic chondrocyte markers of Collagen type I (COL1A1), Runt-related transcription factor 2 (RUNX2), were calculated and represented as fold change.

In general, the treatment with either normal EVs or EVs associated with cytokine priming acted differentially on the expression of chondrocyte markers. We observed the highest expression of *COL2A1* in chondrocytes treated with TGFβ-MVs in all experimental groups (Figure 6A). Notably, non-priming EVs greatly enhanced the expression of *COMP* in chondrocytes, much stronger than any studied groups (Figure 6B). When

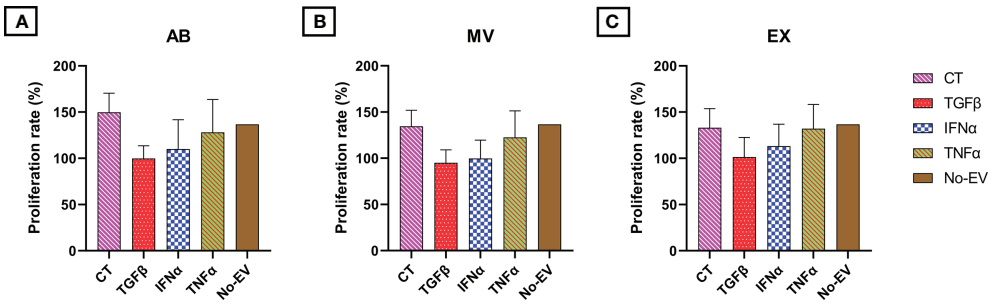

FIGURE 4
The influence of EV treatment on chondrocyte proliferation at 48h. Chondrocyte proliferation under the treatment of **(A)** AB populations; **(B)** MV populations; **(C)** EX populations. No statistically significant effect was observed among all EV treatment groups. CT-AB/MV/EX: chondrocytes treated with AB/MV/EX secreted from non-priming UCMSCs; TGFβ-AB/MV/EX: chondrocytes treated with AB/MV/EX secreted from TGFβ-primed UCMSCs; IFNα-AB/MV/EX: chondrocytes treated with AB/MV/EX secreted from IFNα-primed UCMSCs; TNFα-AB/MV/EX: chondrocytes treated with AB/MV/EX secreted from TNFα-primed UCMSCs; No-EV: chondrocytes cultured in DMEM/F12 5% EV-depleted FBS and no EV addition. The proliferation rate was acquired by normalizing absorbance measured at time point of 48 hours after incubation to absorbance measured at seeding time (0h), and the data were obtained from three independent biological replicates (n = 3). Statistical significance was determined by Two-Way ANOVA. Error bars indicate ± SD.

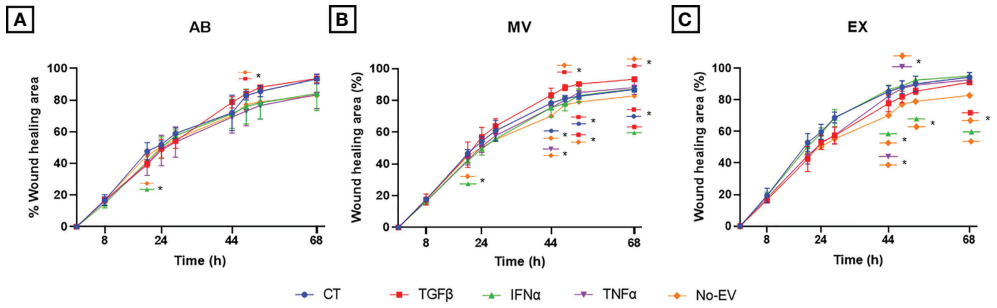

FIGURE 5
Priming UCMSCs with cytokines differentially affected the capacity of derived EVs to stimulate chondrocyte migration. Chondrocyte migration was analyzed using a wound-scratch assay under the treatment of **(A)** UCMSC-ABs **(B)** UCMSC-MVs; **(C)** UCMSC-EXs. TGFβ-EVs induced chondrocyte migration significantly at latter experimental time points. CT-AB/MV/EX: chondrocytes treated with AB/MV/EX secreted from non-priming UCMSCs; TGFβ-AB/MV/EX: chondrocytes treated with AB/MV/EX secreted from TGFβ-primed UCMSCs; IFNα-AB/MV/EX: chondrocytes treated with AB/MV/EX secreted from IFNα-primed UCMSCs; TNFα-AB/MV/EX: chondrocytes treated with AB/MV/EX secreted from TNFα-primed UCMSCs; No-EV: chondrocytes cultured in DMEM/F12 5% EV-depleted FBS and no EV addition. The images were captured at different time points in which chondrocytes migrated by time to close the wound and analyzed using ImageJ. Data are presented as the mean percent area of wound coverage in μm^2 ± SD (n = 3). Statistical significance was determined by Two-Way ANOVA and indicated by: * where $p < 0.05$. Error bars indicate ± SD.

analyzing the expression of *ACAN*, we observed that treatment with TGFβ-MVs significantly upregulated *ACAN* mRNA expression by chondrocytes compared to EV-depleted media ($p = 0.0156$) (Figure 6C).

Regarding hypertrophic markers, treating chondrocytes with EVs from both cytokine-primed and non-priming groups suppressed *COL1A1*. The downregulation of *COL1A1* was indicated by significantly lower expression levels in chondrocytes treated with CT-MVs and CT-EXs ($p = 0.0195$, and $p = 0.0185$, respectively); all three TGFβ-EV sub-populations (TGFβ-ABs, $p = 0.0135$; TGFβ-MVs, $p = 0.0373$; and TGFβ-EXs, $p = 0.0282$), IFNα-EXs ($p = 0.0411$), all three TNFα-EV sub-populations (TNFα-ABs, $p = 0.0019$, TNFα-MVs, $p = 0.0003$; and TNFα-EXs, $p = 0.0054$) (Figure 6D). Interestingly, the inhibition of *COL1A1* mRNA was further emphasized in the chondrocytes treated with TNFα-EVs, showed by a reduced expression of *COL1A1* by chondrocytes treated with TNFα-MVs compared to IFNα-MVs ($p = 0.0310$) (Figure 6D). In contrast with *COL1A1*, the expression of *RUNX2* was upregulated by chondrocyte treatment with EVs in general. Indeed, compared to cells cultured in EV-depleted media, *RUNX2* was expressed higher in chondrocytes treated with non-priming (CT) EVs (CT-ABs,
$p = 0.0498$; CT-MVs, $p = 0.0128$; and CT-EXs, $p = 0.0012$), TGFβ-MVs ($p = 0.01$) (Figure 6E). However, priming cells with cytokines seemed to diminish this undesirable effect with lower expression of *RUNX2* in chondrocytes treated with MVs and EXs from IFNα- and TNFα-primed cells compared to chondrocytes treated with CT-EVs and TGFβ-EVs (Figure 6E).

Taken together, treatment of chondrocytes with cytokine-primed EVs partially rescued the chondrocytes from hypertrophic phenotype and established the primary, normal physical state of the cells.

Discussion

In recent years, several techniques have been developed to optimize the therapeutic efficacy of EVs. Evidently, adding cytokines, such as IFNγ, TNFα, and IL1β, to the conventional MSC culture media affected the contents and biological activities of the derived EVs associated with OA (17, 23). Therefore, we investigated the influence of EVs derived from MSCs primed with anti-inflammatory (TGFβ and IFNα) and inflammatory (TNFα) cytokines on osteoarthritic chondrocytes. We found that cytokine priming did not affect the typical morphology and markers of UCMSCs. Additionally, the secreted EVs displayed distinguished sizes, morphologies, as well as surface markers (CD9 and CD63), which were in accordance with ISEV guidelines (24). These characteristics are also described in the previous studies of EVs from cytokine-primed UCMSCs (25, 26). This information allowed us to ensure the normal EV identity and further study the molecular profile and therapeutic effects of EVs under cytokine stimulations. Furthermore, stimulation of secreted cells with cytokines can also increase the amount of EVs produced by UCMSCs (14), which can potentially enrich therapeutic efficacy. Hence, the assessment of EV production from cytokine-stimulated UCMSCs should be considered in our future investigation.

As mentioned, the treatment of UCMSCs with various stimuli could affect the biological contents of EVs, including miRNAs, which have been reported for their potential roles in OA treatment (27, 28). Especially, it is emphasized that cytokine treatment can result in EVs with rich RNA profiles for inflammatory control (15), which can further reverse OA condition. This study reported the detection of miR-320a-3p and miR-181b-3p, which are involved in healthy cartilage

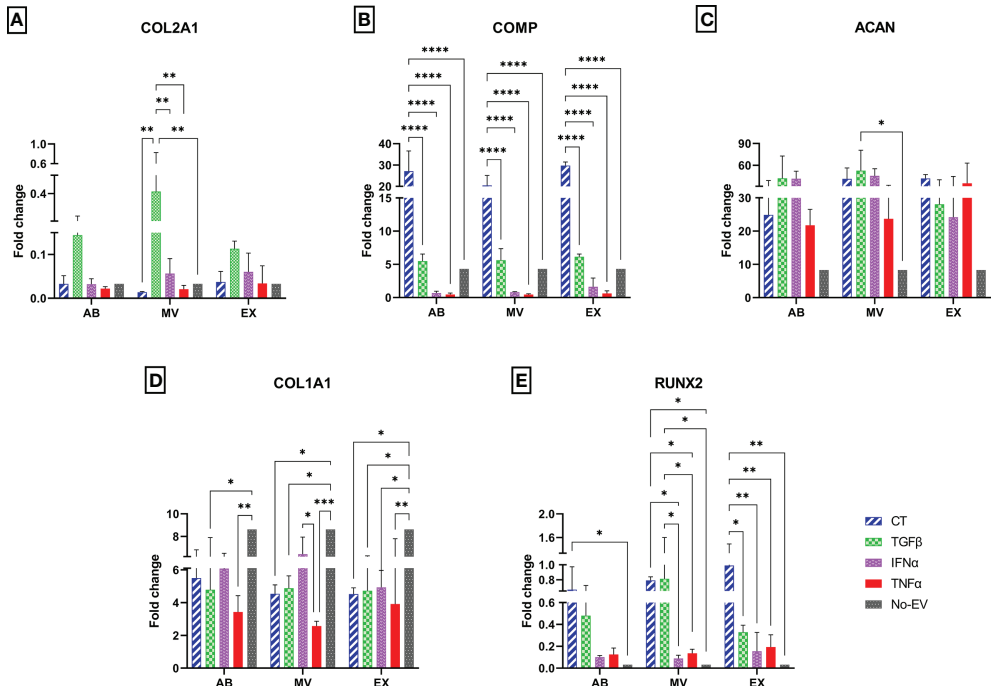

FIGURE 6
Different expression profiles of chondrocyte molecular markers. The mRNA relative expression levels in chondrocytes at passage 7 are represented by $2^{-\Delta\Delta Ct}$ values with 10 ng cDNA input and were normalized to the reference gene GAPDH and chondrocytes P3 cultured in DMEM 10% FBS. The relative expression level of **(A)** *COL2A1*; **(B)** *COMP*; **(C)** *ACAN*; **(D)** *COL1A1*; **(E)** *RUNX2*. Chondrocytes treated with EVs from TGFβ-primed UCMSCs expressed stronger *COL2A1* and lower *COL1A1*. AB: Apoptotic bodies; MV: Microvesicles; EX: Exosomes. CT-AB/MV/EX: chondrocytes treated with AB/MV/EX secreted from non-priming UCMSCs; TGFβ-AB/MV/EX: chondrocytes treated with AB/MV/EX secreted from TGFβ-primed UCMSCs; IFNα-AB/MV/EX: chondrocytes treated with AB/MV/EX secreted from IFNα-primed UCMSCs; TNFα-AB/MV/EX: chondrocytes treated with AB/MV/EX secreted from TNFα-primed UCMSCs; No-EV: chondrocytes cultured in DMEM/F12 5% EV-depleted FBS and no EV addition. Results were averaged of 3 biological replicates (n = 3). Statistical significance was determined by Two-Way ANOVA and indicated by: * where $p < 0.05$; ** where $p < 0.01$; *** where $p < 0.001$; **** where $p < 0.0001$. Error bars indicate ± SD.

maintenance and OA pathogenesis (18–20) in MSCs and three EV sub-populations released by UCMSCs. Our result indicates that miR-320a-3p was expressed higher in non-priming UCMSCs, while the expression level of miR-181b-3p was similar among groups. However, cytokines treatment diminished the packaging of miR-181b into EVs, shown by a significantly low relative expression of this EV miRNA associated with cytokine priming when normalized to the levels in UCMSCs. In literature, miR-181b promoted the NF-κB pathway, which leads to cartilage destruction and synovium membrane degradation (29–31). Blocking miR-181b activity reduced MMP13 expression but increased COL2 expression in articular chondrocytes (32). The attenuation of miR-181b activity can indirectly signal the FPR2- formyl peptide receptor and induce anti-inflammatory effects (33, 34). Thus, the reduction of miR-181b observed in EVs originating from cytokines-primed cells can be a positive marker for re-adjusting the appropriate EV components to produce more direct effects in cartilage regeneration. This exciting information requires further studies to validate whether two cytokines, IFNα and TNFα, could be the appropriate stimulus to enhance the therapeutic efficacy of UCMSC-EVs for OA treatment.

On the other hand, the level of miR-320a-3p remained stable across experimental EV treatments. Previous studies showed pieces of diverse evidence of miR-320a function in cartilage homeostasis (18, 19, 35). Peng et al. (19) also demonstrated the protective effects of miR-320a over cartilage degeneration by negatively regulating BMI-1 (19). However, miR-320a has also been shown as a potential OA marker as this miRNA promoted OA-induced matrix breakdown *via* the NF-κB pathway and interfered with osteoblast reformation (18, 35, 36). Thus, a future study is required to evaluate the roles of miR-320a-3p in OA pathogenesis and examine an alternative approach to adjusting this miRNA content in UCMSC-derived EVs.

Next, to examine our EVs' bioactivity *in vitro*, we isolated human primary chondrocytes from articular cartilage tissues obtained from a patient suffering from a knee injury and performed proliferation, migration and mRNA markers analysis assays. Our isolated cells showed typical chondrocyte

morphology and were positive with specific staining dye for proteoglycan. For functional analysis, in general, all EVs from either non-priming or cytokines-primed UCMSCs at the dose of 10 μg/mL did not promote chondrocyte proliferation significantly. This result may be due to an insufficient dose of EVs might be the issue, as higher doses (20, 40, 80 μg/mL) of BMMSC-EXs have been shown to increase the proliferation rate of chondrocytes (37). Additionally, the outcomes may be due to chondrocytes obtained from patients with knee injury reported herein instead of healthy chondrocyte cell lines. Hence, further experiments with chondrocytes induced with OA characteristics and higher EV dosage will be conducted to examine these possibilities. Notably, the miRNA distribution in EVs is an essential factor that might affect chondrocyte proliferation and migration; however, the regulation of miR-181b on these two biological processes remains unclear. A member of the miR-181 family, miR-181a, exerted adverse effects on chondrocyte proliferation by upregulating the expression of caspase-3, PARP, MMP-2, and MMP-9 to induce apoptosis and cartilage destruction (20). Thus, it is predicted that miR-181b can inhibit chondrocyte proliferation, and suppressing miR-181b expression can restore this ability. However, in this current study, the reduction in miR-181b might not contribute to proliferation results observed here, or other factors have surpassed its influence.

Contrary to cell proliferation, EVs from cytokines-primed UCMSCs expressed a higher capacity to promote chondrocyte migration compared to chondrocytes cultured in EV-depleted media, with the most significant effect belonging to TGFβ-EVs but only in later time points. In the previous study, TGFβ was shown to promote the PI3K-Akt signaling pathway, which was demonstrated to induce chondrocyte migration in a rat model (38, 39). Additionally, TGFβ stimulation can also regulate the integrin signaling pathway involving changes in integrin-ECM binding and the activation of FAK, which are critical factors in cell migration (40, 41). It is noted that all results obtained herein were in the comparison with chondrocytes cultured in EV-depleted media (DMEM/F12 supplemented with 5% EV-depleted FBS) but not as in most studies used PBS or chamber consisting of low serum media (upper) and PBS (lower) as the control group (42–44). Indeed, long-term cell storage with PBS increases cell death and thus cannot access cell functionality efficiently. Those factors may be reasons for the differences observed in this study compared to others.

In this study, we investigated the alteration in mRNA levels of chondrocyte markers under the treatment of EVs at high passage culture. The later culture passage exhibited an increase in *COL1* and a decrease in *COL2*. However, higher expression of healthy chondrocyte markers, including *COMP* and *ACAN*, was also detected, which supports cartilage regeneration and ECM synthesis at the later stage. Meanwhile, the expression of hypertrophic markers such as *RUNX2* diminished. Besides, we observed that EVs from cytokines-primed UCMSCs downregulated the expression of *COL1* and *RUNX2* and upregulated *COL2* and *ACAN* expression, but this effect was not consistent among EV populations. MiR320a was previously linked with low expression of hypertrophic marker RUNX2 (19). However, a stable level of miR-320a-3p in most of the isolated EVs from both non-priming and cytokine-primed UCMSCs hinders us from revealing the association between EV contents and chondrocyte markers. Notably, the increase in *COMP* expression was much more substantial in chondrocytes treated with EVs derived from non-priming UCMSCs. This means that EVs contribute to chondrocyte malfunctions or the recovery of damaged chondrocytes. However, further experiments, especially on miRNAs and target mRNAs, should be investigated to understand the mechanism of the effect of EV contents on chondrocyte mRNA expression.

In conclusion, cytokines influenced the miRNA composition of UCMSCs-derived EVs and their effects on chondrocyte physiology regarding cell proliferation and migration, as well as chondrocyte markers. However, it is noted that the results presented here are preliminary data that require more investigations on other miRNAs/proteins found in EVs in addition to the target genes and signaling pathways affecting the chondrocyte bioactivities. Additionally, for future perspectives, studies should be performed to examine the roles of different cytokines on UCMSC-derived EVs and their cargos in other aspects of OA, such as chondrocyte apoptosis and inflammation.

Experimental procedures

Ethical approval

Ethical approval for collecting and using human MSCs from the umbilical cord and human chondrocytes from articular cartilage was issued by the Vinmec International General Hospital Joint Stock Company's ethics committee (Ethical approval number: 311/2018/QĐ-VMEC). The umbilical cord tissues were collected from three healthy donors aged 20 to 40, and human cartilage tissues were acquired from three donors with knee arthroplasty. Donors signed written informed consent before donating their samples.

Umbilical cord-derived mesenchymal stem cell culture

UCMSCs were isolated from the umbilical cord following what was described in our previous study and stored for further experiments (45). UCMSCs at passage two (P2) were thawed and seeded at a density of 5,000 cells/cm^2 in DMEM/F12 (Gibco, Massachusetts, USA) with 10% (v/v) fetal bovine serum (FBS). Cells were incubated in 37°C/5% CO_2 condition and subcultured

with the same density until passage 5 (P5). The cells at P5 were cultured with EV-depleted media for three days prior to cytokine treatments (DMEM/F12 supplemented with 10% EV depleted-FBS, in which FBS was centrifuged at 120,000 × *g* for 18 hours at 4°C to eliminate EVs). Cells were maintained in EV-depleted media before exposing to cytokines individually for 48 hours with the following concentrations: 10 ng/mL TGFβ, 20 ng/mL IFNα, or 20 ng/mL TNFα. The conditioned media were harvested when cells reached 95% confluency for EV isolation (cell culture media were not renewed throughout incubation). After conditioned media collection, UCMSCs were characterized with Human MSC Analysis Kit (BD Biosciences) following the manufacturer's protocol, and flow cytometry data were analyzed with Navios Software 3.2.

Extracellular vesicle isolation

The conditioned media was centrifuged at 300 × *g* for 10 minutes at 4°C to remove cell debris. Sequential centrifugation steps were performed to separate three EV populations as follows: 2,000 × *g* for 20 minutes at 4°C to collect apoptotic bodies (ABs), 16,500 × *g* for 30 minutes at 4°C to pellet microvesicles (MVs), and 100,000 × *g* for 90 minutes at 4°C for isolation of exosomes (EXs) (Optima XPN-100 Ultracentrifuge, Beckman Coulter, California, USA). EV pellets were resuspended in DMEM/F12 or PBS and stored at − 80°C for further usage.

Extracellular vesicle marker analysis by western blot

Protein extraction and western blot were performed as described previously (45). Total EV protein concentrations were determined by Pierce™ BCA Protein Assay Kit (Thermo Scientific, Massachusetts, USA) and as equivalent to an optical density (OD) measured at 562 nm (SpectraMax M3, Molecular Devices, California, USA). Then, 15 µg of EV proteins were electrophoretically separated by 4 – 12% NuPAGE gels (Invitrogen, Massachusetts, USA) and probed with primary antibodies (Abcam, Cambridge, UK) against GAPDH, CD9, and CD63 overnight at 4°C, followed by the incubation with goat anti-Rabbit IgG secondary antibody (Invitrogen, Massachusetts, USA). Antibody binding was stained with ECL substrate and visualized on ImageQuant LAS 500 (GE Healthcare Life Sciences, Illinois, USA).

Extracellular vesicle morphology analysis by transmission electron microscopy

EV samples were fixed and stained following the protocol described in our previous study (45). Imaging was performed using a JEOL 1100 Transmission Electron Microscope (JEOL Ltd., Tokyo, Japan) at 80 kV at the National Institute of Hygiene and Epidemiology (NIHE).

Chondrocyte isolation and characterization

Human cartilage tissues were collected by the surgical doctors, stored in saline water at 4°C, and transferred to the laboratory. Before processing, the tissue was washed once with ethanol 70%, twice with PBS, and once with DMEM/F12; each solution was supplemented with 1% Pen/Strep (Thermo Fisher Scientific, USA) to ensure sterile and eliminate contaminants. The tissue was minced and digested in Hanks' Balanced Salt Solution (HBSS) (Thermo Fisher Scientific, USA) 0.2% collagenase type I 10000 U/mL solution (Gibco, Massachusetts, USA) (10 mL for every 1 gram of tissue) for 20 hours at 37°C. Cell culture media (DMEM/F12 supplemented with 10% FBS (v/v)) was added in a volume ratio of 1: 1 with HBSS. The harvested pellets were resuspended in DMEM/F12 supplemented with 1% Pen/Strep and 10% FBS (v/v), then seeded into a T25 cell culture flask and incubated at 37°C and 5% CO_2. The media were replaced by every three days during the cultures. After reaching 80% confluency, the cells were either stored or subcultured at the density of 10,000 cells/cm^2 to the next passage.

The images of chondrocytes were captured under Eclipse Ti-S Inverted Microscope (Nikon Instruments, Japan), and cells at P0 were processed to form the colony and stained with Alcian Blue to confirm cell type.

Total RNA extraction

Total RNA was extracted using Trizol™ reagent (Thermo Scientific, Massachusetts, USA) with a ratio of 9: 1 Trizol versus cell/particle suspension. The lysis mixture was added with $MgCl_2$ and chloroform and incubated at RT. The aqueous phase was collected and incubated in with isopropanol overnight at -20°C. Total RNA was then pelleted with centrifugation and then washed twice with RNase-free 75% ethanol before air-drying and resuspending in RNase-free water (volume based on pellet size).

Quantitative reverse transcription-PCR

Total RNA with sufficient quality was subjected to qRT-PCR to confirm the presence of EV miRNAs and chondrocyte mRNAs.

For EV miRNA analysis, extracted RNAs were used as templates to prepare cDNA using the miScript II RT kit (Qiagen,

TABLE 1 Experimental settings for chondrocyte functional analysis *in vitro*.

Experimental Groups	Description
CT-AB/ MV/ EX	Chondrocytes cultured in DMEM/F12 5 % EV-depleted FBS supplemented with 10 μg/mL AB/ MV/ EX from non-priming UCMSCs
TGFβ-AB/ MV/ EX	Chondrocytes cultured in DMEM/F12 5 % EV-depleted FBS supplemented with 10 μg/mL AB/ MV/ EX from TGFβ-primed UCMSCs
IFNα-AB/ MV/ EX	Chondrocytes cultured in DMEM/F12 5 % EV-depleted FBS supplemented with 10 μg/mL AB/ MV/ EX from IFNα-primed UCMSCs
TNFα-AB/ MV/ EX	Chondrocytes cultured in DMEM/F12 5 % EV-depleted FBS supplemented with 10 μg/mL AB/ MV/ EX from TNFα-primed UCMSCs
No-EV	Chondrocytes cultured in DMEM/F12 5 % EV-depleted FBS

The amount of EVs used in experiments was determined based on our previous study (45).

Hilden, Germany), following the manufacturer's instructions. Then, cDNA-containing mixtures (10 μL) were subjected to qPCR using the miScript SYBR Green PCR kit (Qiagen, Hilden, Germany) and two specific primers, miScript Primer Assay 10X (Qiagen, Hilden, Germany) designed to target miR-320a-3p and miR-181b-3p. The incubation was performed on Applied Biosystems 7500 Block (Applied Biosystems, Massachusetts, USA). The relative expression of miRNAs in UCMSCs was normalized to reference gene RNU6B (Qiagen, Hilden, Germany) and miRNAs in EVs was normalized to their secreted cells (UCMSCs) and represented by the ΔCt values, with a higher ΔCt value representing a less selective sorting of this miRNA into EVs and vice versa.

For chondrocyte mRNAs analysis, cells were cultured for one week under treatment as described in Table 1, and chondrocyte RNAs were isolated as above. cDNA was prepared using SuperScript™ IV Reverse Transcriptase (Thermo Scientific, Massachusetts, USA), and step by step was performed according to the manufacturer's protocol. cDNA products were then subjected to qPCR reaction, using specific-designed primers that targeted chondrocyte RNAs, including normal chondrocyte markers of Collagen type II (COL2A1), Cartilage oligomeric matrix protein (COMP), and Aggrecan (ACAN) and hypertrophic chondrocyte markers of Collagen type I (COL1A1) and Runt-related transcription factor 2 (RUNX2), and *GAPDH* as an internal control (primer sequences were listed in Supplementary Table 1). $2^{-\Delta\Delta Ct}$ method was applied to calculate the relative fold gene expression of samples.

Proliferation assay

Human articular chondrocytes were seeded (2,500 cells in each well of 96-well-plate) and incubated in media as listed in Table 1. No-EV was used as the control. Cells were incubated at 37°C and 5% CO_2 for 48 hours to proliferate. The cell proliferation rate was assessed by performing a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Abcam, Cambridge, UK) following the manufacturer's protocols. The proliferation rate was equivalent to the relative absorbance measured at 562 nm (SpectraMax M3, Molecular Devices, California, USA) at time points of 0 hours (as used for normalization) and 48 hours. The proliferation rate was calculated based on the OD values obtained from two time points.

Migration assay

Human articular chondrocytes were cultured in a 24-well plate with a density of 1.05×10^5 cells/well at 37°C and 5% CO_2 for attachment. After reaching 100% confluency, cells were then incubated with Mitomycin C (10 μg/mL) for 2 hours to inhibit cell proliferation. A physical scratch was created on the cell attachment layer, and detached cells were removed by washing with media. Treatments were established similarly to proliferation assay (Table 1). Cell migration to close the wound area was captured by an inverted microscope at multiple time points. The wound area was measured using ImageJ software (version 1.48) and calculated for the closure percentage over time, which represents the rate of cell migration.

Statistical analysis

The statistical analysis was performed on GraphPad Prism 9 (GraphPad Software, California, USA) using One-Way and Two-Way ANOVA, and Tukey HSD tests. The statistical significance was defined as a p-value < 0.05. All data were shown as means ± SD of three biological replicates.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributions

The conception and design of the study: UT, LN, ThN, HD, and XHN. Analysis and interpretation of data: UT, LN, ThN, HD, XHN, HD, TT, TN, CD, and H-XD. Manuscript drafting: ThN, HD and CD. Manuscript revising: UT, XHN. Final approval: UT. All authors contributed to the article and approved the submitted version.

Funding

This project was funded by Vietnam Ministry of Health with decision number 2575/QĐ-BYT (20/06/2019) and Vinmec Joint Stock Company (ISC.18.07). This source has no involvement in the study design, collection, analysis and interpretation of data in the writing of the manuscript and in the decision to submit the manuscript of publication.

Acknowledgments

We thank the doctors and nurses from Hanoi Medical Hospital for cartilage collection.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1041592/full#supplementary-material

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frontiers | Frontiers in Bioengineering and Biotechnology

TYPE Review
PUBLISHED 10 January 2023
DOI 10.3389/fbioe.2022.1083459

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Tissue Engineering and Regenerative Medicine, a section of the journal Frontiers in Bioengineering and Biotechnology

RECEIVED 01 November 2022
ACCEPTED 28 December 2022
PUBLISHED 10 January 2023

CITATION
Wei Q, Liu X, Su J-L, Wang Y-X, Chu Z-Q, Ma K, Huang Q-L, Li H-H, Fu X-B and Zhang C-P (2023), Small extracellular vesicles from mesenchymal stem cells: A potential Weapon for chronic non-healing wound treatment.
Front. Bioeng. Biotechnol. 10:1083459.
doi: 10.3389/fbioe.2022.1083459

Small extracellular vesicles from mesenchymal stem cells: A potential Weapon for chronic non-healing wound treatment

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Chronic non-healing wounds have posed a severe threat to patients mentally and physically. Behavior dysregulation of remaining cells at wound sites is recognized as the chief culprit to destroy healing process and hinders wound healing. Therefore, regulating and restoring normal cellular behavior is the core of chronic non-healing wound treatment. In recent years, the therapy with mesenchymal stem cells (MSCs) has become a promising option for chronic wound healing and the efficacy has increasingly been attributed to their exocrine functions. Small extracellular vesicles derived from MSCs (MSC-sEVs) are reported to benefit almost all stages of wound healing by regulating the cellular behavior to participate in the process of inflammatory response, angiogenesis, re-epithelization, and scarless healing. Here, we describe the characteristics of MSC-sEVs and discuss their therapeutic potential in chronic wound treatment. Additionally, we also provide an overview of the application avenues of MSC-sEVs in wound treatment. Finally, we summarize strategies for large-scale production and engineering of MSC-sEVs. This review may possibly provide meaningful guidance for chronic wound treatment with MSC-sEVs.

KEYWORDS

small extracellular vesicles, chronic non-healing wounds, cell dysfunction, mesenchymal stem cells, regenerative medicine

1 Introduction

Skin wound healing is a highly complex process participated by many kinds of cells including inflammatory cells, vascular endothelial cells, fibroblasts, epidermal cells, etc. This process can be divided into four distinct and overlapping phases: hemostasis, inflammation, proliferation, and remodeling (Rodrigues et al., 2019). Chronic wounds usually arise owning to halt at one or more points in above phases (Figure 1). With the advent of the global aging society, the number of patients with chronic wounds is increasing, which represent an economic burden worldwide and a heavy burden to patients. For example, in the United States, diabetic foot ulcers, one representative type of chronic non-healing wounds, brought about 130,000 lower-limb amputations in 2016 (Services UDoHaH, 2020). In China, the average hospitalization duration was reported to be 31 days with a

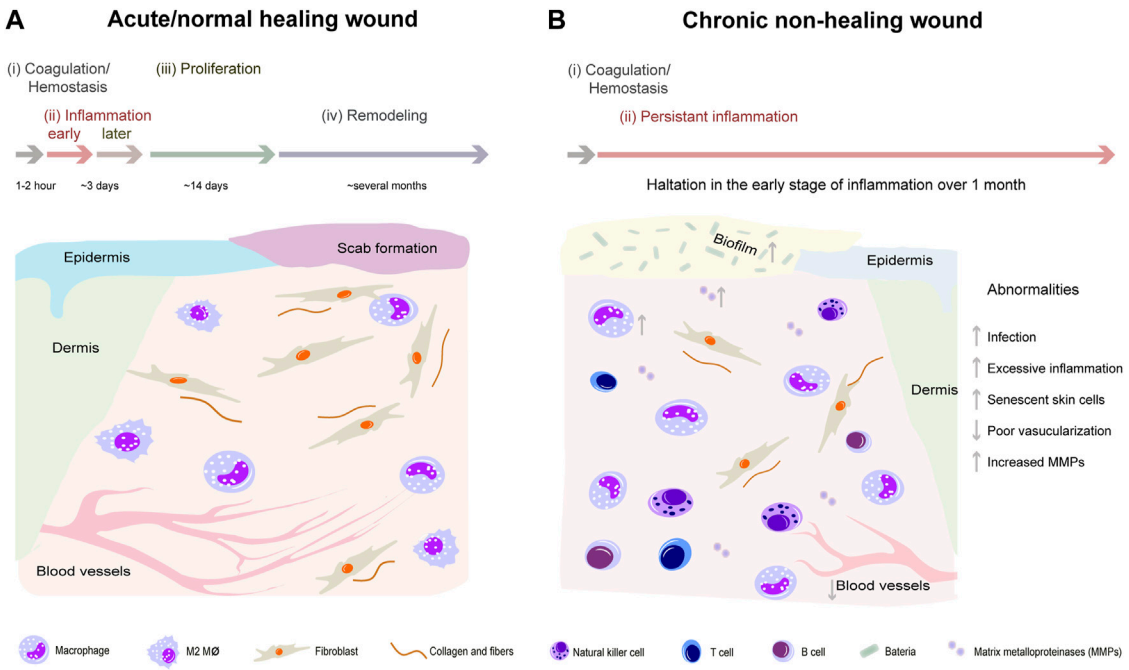

FIGURE 1
Comparison of representative pathophysiological processes between normal healing wounds and chronic non-healing wounds. Normal wound healing process can be divided into four distinct and overlapping phases: hemostasis, inflammation, proliferation, and remodeling **(A)**. However, chronic wounds usually halt at inflammation phase and are difficult to heal because of excessive inflammation, senescent skin cells, poor vascularization, and increased matrix metalloproteinases **(B)**.

medical cost of ¥17,182 (Jiang et al., 2011). Development of effective treatments for chronic non-healing wounds has been of great interest for many years. To date, there are numerous methods and strategies for treating chronic non-healing wounds (Han and Ceilley, 2017; Okur et al., 2020). Non-etheless, since chronic wound healing is a complex and long-term process involving inflammatory response, angiogenesis, re-epithelialization, and collagen deposition, the therapeutic effects of current treatments are limited and unsatisfactory (Table 1) (Zielins et al., 2014; Boateng and Catanzano, 2015). Therefore, novel curative therapies for chronic non-healing wounds need to be explored.

A large body of evidence has demonstrated that mesenchymal stem cells (MSCs) derived from a variety of tissues, possess great therapeutic potentials for chronic non-healing wound treatment by regulating multiple processes such as inflammatory response, angiogenesis, re-epithelization, extra cellular matrix (ECM) remodeling and scarless healing (Kosaric et al., 2019; Mazini et al., 2020; Fageeh, 2021). Nevertheless, there are many limitations and

TABLE 1 Current chronic wound treatments.

Current therapies	Advantages	Limitation	Ref
Debridement	Remove necrotic tissue, prevent infection	Lack of nutrition and blood supply, trauma	Jones et al. (2018)
Hyperbaric oxygen therapy	Prevent infection, boost angiogenesis	Lack positive effects on wound healing and amputation rates	Ruemenapf et al. (2022)
Antibiotics therapy	Prevent infection	Drug adverse effects, drug resistance	Bjarnsholt et al. (2008)
Flap transplantation	Re-vascularization	Multi-risk, trauma, low survival rate	Demmer et al. (2021)
Functional active dressing	Prevent infection, promote granulation forming, provide moist and suitable microenvironment	Lack of adequate clinical data, high costs, unsolved underlying pathophysiologic disorder around	Jones et al. (2018)
Vacuum sealing drainage technology	Prevent infection, increase blood supply, decrease wound size and discomforts		
Tissue engineering skin	Good histocompatibility, non-immunogenic, maintain normal morphology and function		

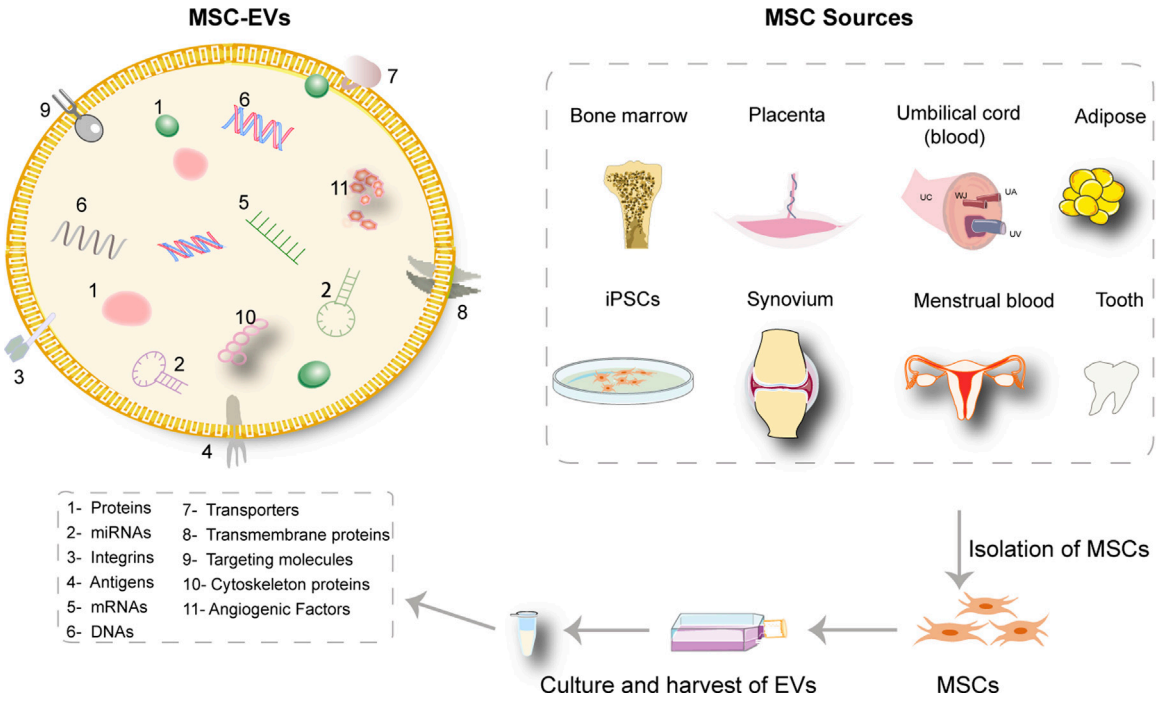

FIGURE 2
The content of MSC-sEVs and extensive sources of MSCs. The isolated sEVs from MSCs are enriched in a large variety of therapeutic molecules. The sources of MSCs are extensive including bone marrow, umbilical cord, placenta, adipose tissue, synovial membrane, exfoliated deciduous teeth, menstrual blood, and induced pluripotent stem cells (iPSCs).

obstacles in the direct application of MSCs, such as tumorigenicity (Jeong et al., 2011), immune rejection (Ankrum et al., 2014), and different cellular features (Conrad et al., 2009; Haga et al., 2015). Therefore, researchers still strive to develop a novel cell-free therapy for chronic non-healing wound treatment. Recently, several studies disclosed that MSCs act on chronic wound healing mainly through their paracrine function rather than their ability to differentiate into skin cells at wound sites (Cao et al., 2017). Small extracellular vesicles (sEVs) or exosomes are enriched in the secretome of MSCs. Thus lately, there is a rush to explore the role of MSC-sEVs in wound treatment (Yaghoubi et al., 2019; An et al., 2021; Vu et al., 2021) so as to optimize the application of sEVs as the substitute of cellular therapy with MSCs. sEVs were first described in the 1970s by Johnstone who separated them from sheep reticulocytes (Johnstone et al., 1987). In the past, sEVs were ignored and thought of as cellular dust. Today, scientists increasingly realize that sEVs carrying intercellular biological information are promising biological tools for treatment of a variety of diseases (Panfoli et al., 2018). Particularly, recent studies have reported that MSC-sEVs accelerate chronic wound healing by regulating and restoring normal cellular behavior at wound sites (Casado-Díaz et al., 2020).

In this review, we describe the characteristics of MSCs and MSC-sEVs and discuss their therapeutic potential and application avenues in chronic wound treatment. Additionally, strategies for large-scale production and engineering of MSC-sEVs were summarized, which contribute to obtaining large quantities and specific functionalized MSC-sEVs for clinical applications on chronic non-healing wound treatment.

2 Biological characteristics and clinical applications of MSCs

2.1 Biological characteristics of MSCs

MSCs are multi-potent adult stem cells possessing multi-lineage differentiation capacity and immunosuppressive properties. The sources of MSC are very extensive (Jo et al., 2021) and they can be harvested from bone marrow, umbilical cord, adipose tissue, synovial membrane, gingiva, and some unconventional sources including exfoliated deciduous teeth, menstrual blood, and fetal dermis of accidentally aborted fetuses (Figure 2) (Wang et al., 2019a; Dalirfardouei et al., 2019; Narbute et al., 2019). Accordingly, MSCs should express CD105, CD73, and CD90, with lack of expression of CD34, CD45, CD14 or CD19, CD79a or CD11b, and HLA-DR. Other markers including CD146, CD271, Stro-1, and SSEA-4 are reported to relate to the stemness and sources of MSCs (Lv et al., 2014). Criteria for isolation, culture, and identification of MSCs have been established by the International Society for Cellular Therapy (ISCT) (Galderisi and Giordano, 2014). The biological roles of MSCs in skin regeneration and repair have been reported widely (Kosaric et al., 2019; Zhang et al., 2020; Malhotra et al., 2021). Collectively, the biological roles of MSCs include controlling excessive immune response in skin wounds for their immunomodulatory capacity (Uchiyama et al., 2017), secreting paracrine factors such as EVs to contribute to the healing process (Qi et al., 2014; Bian et al., 2020), and differentiating into skin repair cells for their multiple differentiation potential (Zhang et al., 2020).

TABLE 2 Clinical trials of MSC therapy in skin non-healing defects.

NCT number	Conduct/ publication time	Human cell type	Disease	Phase	Object number	Time frame	Clinical parameters	Ref
03,267,784	2017–2020	Allogenic - ABCB5+ MSC	Diabetic neuropathic ulcer	I, II	23	12 weeks	Diminish wound surface area	Kerstan et al. (202); Kerstan et al. (2022)
Unreported	2007	Autologous-BM-MSC	Acute and chronic wounds (over 1 year)	—	13	20 weeks	Stimulate the healing process	Falanga et al. (2007)
Unreported	2009	Autologous-BM-MSC	Chronic wounds of lower limb	—	24	12 weeks	Reduce wound size, elongate pain-free walking distance, ameliorate blood perfusion of low limb	Dash et al. (2009)
00,955,669	2009–2010	Autologous-BM-MSC	Diabetic Critical Limb Ischemia and DFU	I	41	3 years	Boost leg blood perfusion and ulcerative healing, diminish amputation and ulcer recurrence	Chen et al. (2018a); Lu et al. (2019)
ChiCTR 2200055885	2009–2020	Allogenic UCB-MSC	PAD and incurable DFU	I	14	3 years	Quicken ulcer closure process, no relief of angiostenosis	Zhang et al. (2022a)
Unreported	2013	Allogenic UCB-MSC	DFU	—	15	12 weeks	Relieve pain, numbness and coldness, improve ABI and TcO2, reduce blood glucose level and amount of required insulin	Li et al. (2013)
Unreported	2013	Allogenic UCB-MSC	PAD	I	8	6 months	Ulceration healed in three of four, angiographic scores added in three of eight	Yang et al. (2013)
02,619,877	2015–2016	Allogenic AD-MSC	DFU	II	59	12 weeks	Accelerate wound closing and re-epithelialization	Moon et al. (2019)

BM, bone marrow; UCB, umbilical cord blood; DFU, diabetic foot ulcer; PAD, peripheral arterial disease.

2.2 Clinical applications of MSCs in wound treatment

MSCs have been frequently used and examined in clinic. For skin wound treatment, a series of clinical trials were performed to evaluate the efficacy of MSC application in serious skin burns (Falanga et al., 2007), non-healing ulcers (Dash et al., 2009; Li et al., 2013; Chen et al., 2018a; Lu et al., 2019; Moon et al., 2019; Kerstan et al., 2021; Zhang et al., 2022a; Kerstan et al., 2022) and advanced limb ischemia (Yang et al., 2013; Lu et al., 2019). For example, Sheng et al. first reported successful employment of MSCs to realize the regeneration of functional sweat glands in patients with deep burn injury (Sheng et al., 2009). Besides, Lu and colleagues showed that application of autologous BM-MSCs in forty-one patients with DFUs could lead to improved ulcerative healing rate and blood infusion, decreased ulcer relapse and amputation during a 3-year follow-up (Lu et al., 2019). In the light of registered trials on https://www.clinicaltrials.gov, more than three hundred clinical trials based on MSC therapy have been finished in patients with including but not only limited to autoimmune or degenerative diseases (Zhou et al., 2021). Here, we listed the clinical trials in patients with non-healing wounds in Table 2. On the whole, MSCs have been proven to have tolerable safety profile and effectiveness in certain clinical settings. However, a lack of verification on safety and therapeutic benefit from large-scale clinical trials barricades the transfer from bench to bedside, impelling researchers to find superior substitute such as MSC-sEVs.

3 Biological characteristics of MSC-sEVs

Extracellular vesicles (EVs) are defined as naturally released double-layered membrane particles or vesicles (Théry et al., 2018). Currently, there is still no consensus about the specific markers of EV subtypes. Herein, we endorsed the term small EVs (sEVs) with ranges defined no more than 200 nm based on minimal information for studies of extracellular vesicles 2018 (MISEV 2018) (Théry et al., 2018). Of note, a great deal of literature has endorsed the term "exosomes," which are defined as sEVs with a size range from 40–160 nm and exosomes have been the interest of their studies (Kalluri and LeBleu, 2020). Therefore, in the present review, the term of "sEVs" is employed to refer to small EVs and exosomes. In this section, will briefly introduce features of sEVs, advantages of MSC as the producers of sEVs and isolation and purification of sEVs. Biogenesis or uptake of sEVs is not covered here, but has been reviewed previously (Bian et al., 2019; Pegtel and Gould, 2019; Lv et al., 2020).

3.1 Characteristics and composition of sEVs

sEVs are predominately characterized by microstructures, sizes, and surface makers. Investigators mainly observe the vesicle-like or cup-like microstructure of sEVs with the help of transmission electron microscopy (Gurunathan et al., 2019; Jafari et al., 2020). Meanwhile, the size of sEVs could be examined by nanoparticle tracking analysis

(NTA) or tunable resistive pulse sensing (Sokolova et al., 2011). Together with typical structure and size, sEVs have exclusive markers on their surfaces. One type of the surface proteins are ESCRT-associated proteins, such as tumor suppressor genes (TSG101) and ALG-2 interacting protein X (Alix). While others are ESCRT-independent markers (CD9, CD63, CD81), flotillin, chaperones, and ubiquitinated proteins (de Gassart et al., 2003; Monguió-Tortajada et al., 2019). However, specificity of some markers (such as RACGAP1) and typical subtype markers to distinguish the subtypes of EVs are still debated, calling for more studies to unveil these doubts in the future (Xu et al., 2017).

sEVs are mainly composed of lipids, proteins, and nucleic acids including mRNAs, non-coding RNAs, and DNAs (Keerthikumar et al., 2016) and therefore are thought to be carriers of intercellular biological information. Furthermore, the biochemical content of sEVs varies according to their origin, and thus, the biological information they carry also differs. The heterogeneity of sEV composition is possibly reflective of their origins, sizes, functional impacts on acceptor cells. Reportedly, MSC-sEVs are also enriched in various bioactive molecules which have good therapeutic effects on diverse diseases (Simons and Raposo, 2009).

3.2 MSCs: Source of sEVs

Furthermore, employment of MSCs and their derivatives has gained momentum in the field of regenerative medicine (Malhotra et al., 2021). A line of basic and clinical trials have demonstrated that MSCs can create a beneficial microenvironment to regulate inflammatory response, facilitating the formation of a properly-vascularized granulation matrix, enhancing the proliferation and migration ability of skin cells and diminishing apoptosis, thus accelerating wound healing (Mazini et al., 2020). MSC-sEVs have also been verified to be therapeutic in preclinical studies and exert immune-modulating and regenerative effects (Bian et al., 2019). Of note, the establishment of immortalized MSCs impacts on neither the quality nor the quantity of MSC-sEVs. Besides, the immortalized treatment is beneficial for its sustainable and cloneable manufacture (Yeo et al., 2013). Compared with other stem cells, MSCs are safer and more favorable in allogenic application, which is evidenced by various clinical trials (Xiao et al., 2013; Squillaro et al., 2016; Bartolucci et al., 2017; Galipeau and Sensébé, 2018; Ban et al., 2021). Accordingly, we speculate that sEVs from MSCs are also safer than that of other stem cells for allogenic transplantation.

3.3 Isolation and purification of sEVs

Ultracentrifugation-based isolation has been most widely employed and considered to be the golden technique for sEVs separation on account of accessibility, simplicity, high yield, and harvest of comparatively homogenous size groups of sEVs, yet it is lengthy and labor-intensive and might lead to co-sedimentation of non-vesicular proteins (Pegtel and Gould, 2019; Tian et al., 2020). To fix the disadvantages of routine sEV isolation, multiple techniques based on different rationales have been used, such as ultrafiltration, polymer-based precipitation, immunoaffinity capture, size exclusion chromatography, and combined employment of the techniques (Tian et al., 2020). Ultrafiltration provides a faster substitute to ultracentrifugation. However, the particle yield and purity might be compromised because of absorption to cellulose and membranes and deformation of vesicles owing to pressure (Lobb et al., 2015; Konoshenko et al., 2018). Thus, ultrafiltration might be more suitable with limited liquid volume for clinical grade sEVs. As another suitable technique under clinical research conditions, polymer-based precipitation is also time-efficient, low-cost and leads to a high yield. The non-specific mechanism of it also brings about shortcomings including low quality of sEVs and co-sedimentation of non-vesicular contamination (Sidhom et al., 2020). Notably, immunoaffinity capture can harvest sEVs of higher purity but lower yield and is extensively applied as an adjuvant step with ultracentrifugation to enhance pureness. Drawbacks of this method include selecting one subtype of sEVs, not capturing all sEVs, being expensive, and confining to small sample size (Sidhom et al., 2020). Accumulating data have suggested that size exclusion chromatography is superior in purification of sEVs, which is exceptional in preservation of functionally and morphologically intact sEVs. But researchers should prevent biological target denaturation and take the sample volume into consideration when employing this method (Stulík et al., 2003; Sidhom et al., 2020).

Regrettably, different extraction and purification methods of sEVs may produce different populations of sEVs carrying various functional cargoes including proteins and nucleic acids. Additionally, some methods can also lead to the contamination of sEVs with non-vesicular "contaminants." Above technical limitations likely bring about contradictory or unrepeatable conclusions in the treatment of sEVs on diseases including non-healing wounds. Therefore, we believe that combined employment of the techniques is probably the best choice to obtain highly purified and intact sEVs which can perform in a replicable manner.

4 Therapeutic potential of MSC-sEVs for chronic non-healing wound treatment

For treatment of non-healing wounds, the severity of the wounds and overall health status will presumably affect the effects and outcomes of MSC-sEV treatment in clinical settings. Therefore, we might propose that effects of treatments on DFUs of different clinical grades of different groups should be evaluated respectively in clinical trials of MSC-sEV. It has been proved that MSC-sEVs can function on many stages of wounds (An et al., 2021). In this section, as briefly demonstrated in Figure 3, we will discuss the therapeutic potencies and underlying mechanisms of employment of multiple sourced MSC-sEVs to facilitate chronic non-healing wound by regulating and restoring cellular functions at wound sites.

4.1 Effects of MSC-sEVs on hemostasis stage

The wound healing cascade starts with hemostasis. The immediate response is vasoconstriction of the injured blood vessels to stop bleeding, followed by platelet aggregation, platelet plug formation and the coagulation cascade activation to form a fibrin clot which halts the blood flow and offers a scaffold for inflammatory cells (Rodrigues et al., 2019). Notably, data about the application of MSC-sEVs in hemostasis phase of the wound healing process is limited. It has been reported that MSC-sEVs possess procoagulant activity *in vitro*.

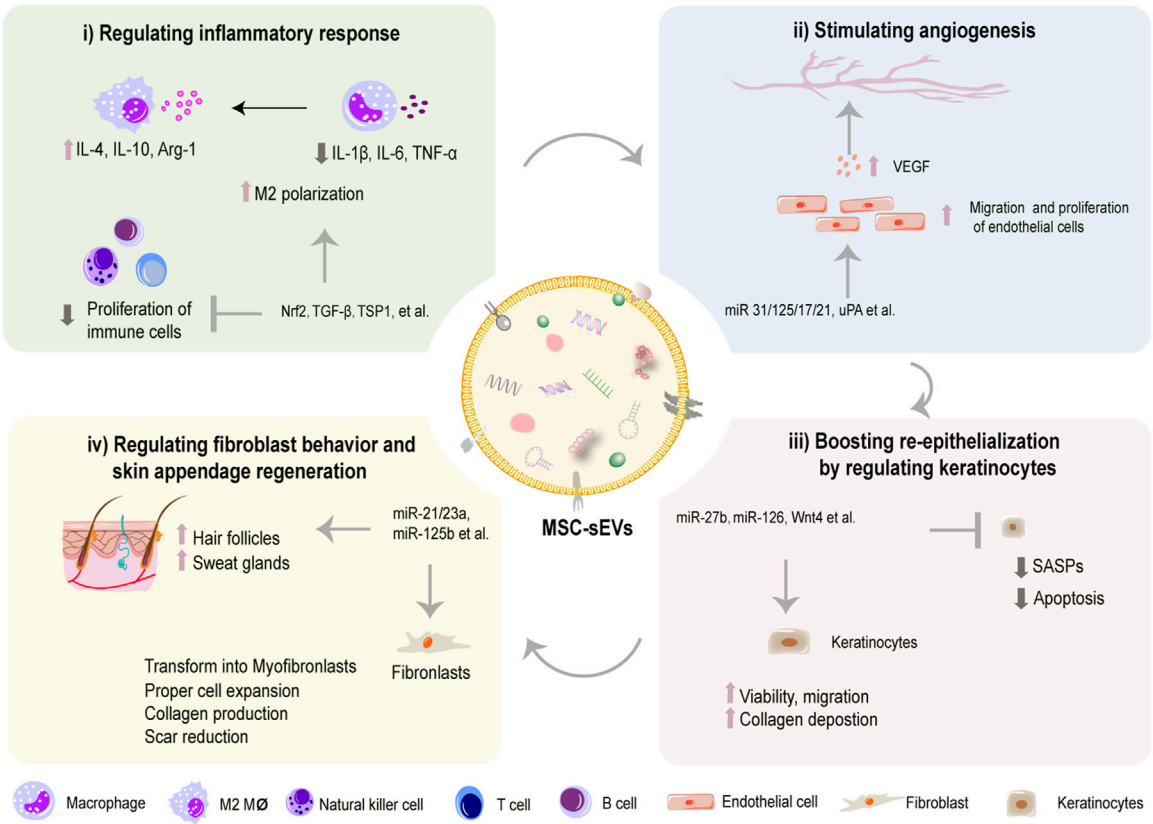

FIGURE 3
MSC-sEVs promote wound healing process by multiple mechanisms. MSC-sEVs can regulate inflammatory response by inhibiting proliferation of immune cells and shifting polarization of macrophages (i). MSC-sEVs stimulate angiogenesis by promoting VEGF-mediated migration and proliferation of endothelial cells (ii). MSC-sEVs boost re-epithelization by inhibiting SASPs and apoptosis and increasing the viability and migration of keratinocytes (iii). MSC-sEVs inhibit scar formation by modulating transformation of fibroblasts to myofibroblasts (iv).

Specifically, Tiffani et al. demonstrated that EVs, either from AD-MSCs or BM-MSCs, were functionally thrombogenic and likely to enhance clotting rates by expressing tissue factor and phosphatidylserine on their surfaces (Chance et al., 2019). So, we have reasons to expect potential effects of MSC-sEVs in hemostasis stage of wound healing process *in vivo* in the future.

4.2 Regulating inflammatory cells

Generally, inflammatory phase, which occurs at several minutes to hours after skin injury and lasts for several days, stands at the beginning stage of the healing cascade. A well-regulated inflammatory response is essential to trigger healing process of skin wounds. However, malfunction of immune cells, such as macrophages, T lymphocytes, and B lymphocytes, is regarded as the culprit to generate persistent excessive inflammation, which is the typical characteristic of chronic non-healing wounds (Li et al., 2021). Accumulating evidence has suggested that MSC-sEVs exert anti-inflammatory and immunomodulating effects on those cells thus facilitating wound healing (Ti et al., 2015; Monguió-Tortajada et al., 2017; He et al., 2019; Ha et al., 2020). They are able to convert pro-inflammatory M1 macrophages predominately into anti-inflammatory M2 phenotype (Figure 3i) (Ti et al., 2015). Besides, Khare et al. (2018) found that MSC-sEVs from bone marrow could regulate the activation and differentiation of B lymphocytes and inhibit proliferation of some types of immune cells, which may help pave the way for resolution of prolonged inflammation. Also, they could suppress the sensitization and proliferation of natural killer cells to alleviate excess inflammation (Fan et al., 2019). Meanwhile, over-abundant inflammatory cytokines are also common factors for extensive inflammation. Studies have demonstrated that sEVs harvested from bone marrow MSCs (BM-MSCs) pretreated with nuclear factor erythroid related factor 2 (Nrf-2) reduced wound inflammation in diabetic rats by downregulating pro-inflammation cytokines like tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) as well as upregulating anti-inflammation factors such as IL-4 and IL-10 (Wang et al., 2021a). Mechanically, functional cargos, such as non-coding RNAs (Fatima et al., 2017), are listed in Table 3 (Li et al., 2020). Therefore, MSC-sEVs may provide a clinically applicable method in alleviating overwhelmed inflammation in chronic non-healing wound.

4.3 Stimulating angiogenesis by regulating vascular endothelial cells

Angiogenesis is crucial in promoting wound healing and skin repair in terms of transportation of oxygen, nutrients, and immune cells to wounded sites, facilitating cell proliferation and ECM deposition (Wang et al., 2019b). Although healing process is

TABLE 3 MSC-sEVs play an active role in regulating inflammatory cells.

MSC source	Isolation	Target cells/ conditions	Functional cargo	Molecules/Pathways affected	Key functions/ Downstream genes	Reference
BM	precipitation	cutaneous wound in mice; human monocytes *in vitro*	miR-223	Pknox1↓	enhanced M2 polarization, TNF-α↓, IL-10↑, Arg-1↑	He et al. (2019)
Huc	Size-Exclusion Chromatography	T cell, PBMCs	—	—	reduced T cell proliferation	Monguió-Tortajada et al. (2017)
BM	Ultracentrifugation	PBMC, T and B cells	—	—	Decreased proliferation of PBMC, T and B cells and IgM levels	Khare et al. (2018)
Fetal liver	ultracentrifugation	NK cells	LAP, TGFβ and TSP1	p-SMAD2/3↓	Restrained proliferation, function and activity of NK cells	Fan et al. (2019)
LPS-pre-huc	ultracentrifugation	Diabetic cutaneous wound in rats; THP-1 *in vitro*	let-7b	TLR4↓, p-P65↓, NF-κB↓, p-STAT3↑, p-AKT↑	Skewed macrophage polarization to M2 and clearance of chronic inflammation	Ti et al. (2015)
Menstrual blood	Ultracentrifugation	cutaneous wound in diabetic mice	—	ARG/iNOS↑	Elevated M2/M1 ratio and resolved inflammation	Dalirfardouei et al. (2019)
Murine BM	ultracentrifugation	DFU model in mice; HDF	LncRNA H19	miR-152-3p↓ → PTEN↑ → p85PI3K↓→p-AKT↓→IL-1β↓, TNF-α↓, and IL-10↑	suppressed apoptosis and inflammation *in vitro* and *in vivo*	Li et al. (2020)
BM	Ultracentrifugation	EPC; cutaneous wound in diabetic rats	Nrf2	IL-1β↓, TNF-α↓and IL-10↑, IL-4↑	Reduced inflammation	Wang et al. (2021a)

Huc, human umbilical cord; BM, bone marrow; Pknox1, PBX/knotted 1 homeobox one; PBMCs, Peripheral Blood Mononuclear Cells; LAP, latency associated peptide; TGFβ, transforming growth factor β; TSP1, thrombospondin 1; ARG, arginase; THP-1, human myeloid leukemia mononuclear cells; TLR4, Toll-like receptors 4; DFU, diabetic foot ulcer; AT, adipose tissue; Nrf2, nuclear factor erythroid 2–related factor 2; EPC, endothelial progenitor cell; SMP30, senescence marker protein 30; NOX1/4, NAPDH, oxidase 1/4; TNF-α, Tumor Necrosis Factor α; hAAM, human acellular amniotic membrane.

influenced by many factors, poor vascularization may be the most principle cause for wound chronicity (Cheng and Fu, 2018). Furthermore, the depth causes for insufficient angiogenesis could be the undue inflammatory, excessive oxidative stress, DNA damage, and cell cycle arrest, leading to cell senescence and impairing pro-angiogenic ability of endothelial cells (Pulido et al., 2021). A line of evidence has suggested that MSC-sEVs can enhance proliferation and migration of vascular endothelial cells, thus promoting angiogenesis and blood vessel maturation in diabetic wounds (Figure 3ii) (Shabbir et al., 2015; Kang et al., 2016; McBride et al., 2017; Li et al., 2018; Shi et al., 2020; Guillamat-Prats, 2021; Wei et al., 2021). As concluded in Table 4, these desirable effects were associated with elevated expression of genes involved in proliferation (PCNA, cyclin D3), migration (Konoshenko et al., 2018), and angiogenesis (VEGF, Ang1, and Flk1) (Zhang et al., 2015a; Liang et al., 2016; Hu et al., 2018). At a molecular level, a group of non-coding RNAs are detected to enriched in MSC-sEVs, such as HOTAIR (HOX transcript antisense RNA), miR-221-3p, −21, −125a, −17, and −126, which have been proved to mediate favorable bioeffects by regulating above target genes (Tao et al., 2017; An et al., 2019; Yu et al., 2020; Born et al., 2021; Wei et al., 2021; Pi et al., 2022). For instance, we previously found that miR-17-5p in umbilical cord MSC-sEVs (hucMSC-sEVs) accelerated angiogenesis in diabetic mice *via* targeting PTEN/AKT pathway, thus exerting positive effects on wound healing (Wei et al., 2021). Besides, application of MSC-EVs has also been demonstrated to be efficacious in mitigating excessive oxidative stress and senescence-associated secretory phenotype of endothelial cells (Wang et al., 2020). To be specific, Zhang and coworkers reported that adipose sourced MSC-sEVs could alleviate oxidative stress, diminish reactive oxygen species (ROS) production and ameliorate mitochondrial function in endothelial cells under high glucose by regulating Sirtuin 3 (SIRT3)/superoxide dismutase 2 (SOD2) activity, thereby achieving better vascularization and diabetic wound healing (Sidhom et al., 2020). Likewise, Xiao et al. found that the treatment with MSC-EVs effectively improved the senescence of HG-treated endothelial cells in diabetic wounds *via* regulating miR-146a/Src pathway, leading to a decrease in aging-related proteins p21/p16/p53 and an enhanced angiogenesis in diabetic wounds (Xiao et al., 2021). Not only RNAs but also active proteins in MSC-sEVs can stimulate angiogenesis in wound restoration (Sung et al., 2019; Tutuianu et al., 2021). For instance, Angiopoietin-2 (Ang2) and deleted in malignant brain tumors 1 (DMBT1) have been reported to be abundant in MSC-sEVs to exert pro-angiogenic functions during wound healing (Chen et al., 2018b; Liu et al., 2021). Loaded with angiogenic components, MSC-sEVs are expected to be a prominent option to induce angiogenesis to treat poorly vascularized wounds in future clinical practice.

4.4 Boosting re-epithelization by regulating keratinocytes

Wound healing can be severely retarded by dysfunctional re-epithelization caused by impaired proliferative and migratory capacities of keratinocytes (Berlanga-Acosta et al., 2020). To restore efficient re-epithelialization is indispensable for successful wound healing. As summarized in Table 5, some studies have observed the potency of MSC-sEVs on boosting re-epithelialization in chronic wounds (Figure 3iii) (Zhang et al.,

TABLE 4 MSC-sEVs stimulate angiogenesis by regulating vascular endothelial cells in wound healing.

MSC source	Isolation	Target cells/ conditions	Functional cargo	Molecules/ Pathways affected	Key functions/Downstream genes	Ref
BM	Ultracentrifugation	HUVECs (normal and chronic wounds)	STAT3	p-ERK1/2↑, p-Akt↑, p-STAT3↑, HGF↑, IGF1↑, NGF↑, SDF1↑	enhancement of proliferation and migration HUVECs	Shabbir et al. (2015)
AT	Ultracentrifugation	HUVECs	MiR-31	FIH1↓, CD31↑	Enhanced angiogenic ability of HUVECs	Kang et al. (2016)
Nrf2-OE-AT	Co-Precipitation	EPC *in vitro*, diabetic foot ulcer in rats	Nrf2	SMP30↑, VEGF↑, P-VEGFR2/VEGFR2↑	Promoted cell viability, migration and angiogenesis both *in vitro* and *in vivo*	Li et al. (2018)
Urinary	Ultrafiltration	cutaneous wound in diabetic mice; HMECs	DMBT1	VEGFA↑, p-AKT↑, CD31↑	Elevated angiogenic responses *in vitro* and angiogenesis *in vivo*	Chen et al. (2018b)
BM	Polymer precipitation; Ultracentrifugation	HUVECs	Wnt3a	—	Enhanced proliferation, migration and angiogenesis *in vitro*	McBride et al. (2017)
AT	Ultracentrifugation	HUVECs immunodeficient mice	MiR-125a	DLL4↓, Ang1↑, Flk1↑	Activated angiogenesis *in vitro* and *in vivo*	Liang et al. (2016)
Modified-AT	Ultrafiltration Ultracentrifugation	Diabetic wounds in mice, EPC	mmu_circ_0000250	miR-128-3p↓, SIRT1↑	Activated autophagy and proangiogenic abilities and suppressed apoptosis *in vitro*; increased neovascularization *in vivo*	Shi et al. (2020)
Modified synovium	Ultracentrifugation	cutaneous wound in diabetic rats; HMECs	MiR-126	p-AKT↑, p-ERK1/2↑	Stimulated angiogenesis *in vivo*; activated proliferation, migration and tube formation of HMEC-1	Tao et al. (2017)
huc	Ultracentrifugation	deep second-degree burn injury in rats; HUVECs	Ang-2	CD31↑	Enhanced migration and tube formation of HUVECs and angiogenesis *in vivo*	Liu et al. (2021)
huc	Ultracentrifugation	cutaneous wound in diabetic mice; HUVECs	Wnt4	PCNA↑, cyclin D3↑, N-cadherin↑, β-catenin↑, E-cadherin↓	Elevated proliferation, migration and angiogenic abilities of HUVECs; activated neovascularization *in vivo*	Zhang et al. (2015a)
Modified AT	affinity chromatography	HUVECs	MiR-21	PTEN↓, p-AKT↑, p-ERK1/2↑, HIF-1α↑, SDF↑,VEGFA↑	stimulated vascularization	An et al. (2019)
Thrombin pretreated hucb	Ultracentrifugation	cutaneous wound in rats; HUVECs	angiogenin, angiopoietin-1, HGF, VEGF	p-ERK1/2↑, p-AKT↑	Enhanced proangiogenic activity *in vitro* and accelerated neovascularization and cutaneous wound healing *in vivo*	Sung et al. (2019)
Huc	Ultracentrifugation	cutaneous wound in diabetic mice; HUVECs	miR-17-5p	PTEN↓, p-AKT↑, HIF-1α↑, VEGF↑	Boosted proliferation and migration, tube formation of HUVECs and neovascularization *in vivo*	Wei et al. (2021)
ATV-pretreated BM	Ultracentrifugation	Skin wounds in diabetic rats, HUVECs	MiR-221	PTEN↓, p-AKT↑, p-eNOs↑, VEGF↑	Promoted proliferation and migration activity of HUVECs and neovascularization *in vivo*	Yu et al. (2020)
HOTAIR-OE-BM	Ultracentrifugation	Skin wound in rats and diabetic mice; HUVECs, HMECs	Lnc HOTAIR	VEGF↑	Improved angiogenesis and accelerated wound healing, boosted pro-angiogenic activities of endothelial cells *in vitro*	Born et al. (2021)
BM	Ultracentrifugation	3D human Skin Organotypic model; ECs	Ang2, ET-1, EG-VEGF/ PK1, Persephin, uPA		Promoted angiogenesis in model and enhanced angiogenic ability *in vitro*	Tutuianu et al. (2021)
AT	Ultracentrifugation	HUVECs, cutaneous wounds in aged and diabetic mice	MiR-146a	p-Src↓, p-VE-cadherin↓, p-caveolin-1↓, p21↓, p16↓, p53↓	Decreased SASP, rescued angiogenesis *in vitro*; promoted neovascularization in wound healing	Xiao et al. (2021)
BM	Ultracentrifugation	EPCs; ischemic hindlimb in aged mice	MiR-126a	Spred-1↓, p16Ink4a↓, CD31↑	Rejuvenation of aged EPCs, attenuated SA-β-Gal expression	Wang et al. (2020)

BM, bone marrow; HUVEC, human umbilical vein endothelial cell;,STAT3, signal transduction and activators of transcription 3; Erk1/2, extracellular regulated kinase 1/2; AKT, protein kinase B; HGF, hepatocyte growth factor; IGF-1, insulin like growth factor 1; NGF, nerve growth factor; SDF1, stromal cell-derived factor1; HiPSC, human induced pluripotent stem cell; FIH1, factor-inhibiting HIF-1; Nrf2, nuclear factor erythroid 2–related factor 2; EPC, endothelial progenitor cell; SMP30, senescence marker protein 30; HMEC, human microvascular endothelial cell; DMBT1, deleted in malignant brain tumors 1; VEGFA, vascular endothelial growth factor A; DLL4, delta-like 4; HGF, hepatic growth factor; VEGF, vascular endothelial growth factor; HOTAIR, HOX, transcript antisense RNA; EC, endothelial cells; Ang-2, angiopoietin-2; ET-1, endothelin; EG-VEGF/PK1, endocrine gland derived vascular endothelial growth factor; uPA, urokinase-type plasminogen activator; hAAM, human acellular amniotic membrane.

TABLE 5 MSC-sEVs exert therapeutic effects in boosting re-epithelialization during wound healing.

MSC source	Isolation	Target cells/ conditions	Functional cargo	Molecules/Pathways activated	Key functions/Downstream genes	Ref
AT	Ultracentrifugation	Skin wound in rats; HDFs, keratinocytes		p-AKT↑, p-histone H3↑	Stimulated proliferation and migration of skin cells *in vitro*	Ferreira et al. (2017)
Huc	Ultracentrifugation	Deep second-degree burn in rats; HaCaTs, RDFs	Wnt4	β-catenin↑, p-GSK3β↑, p-AKT↑→CK19↑, PCNA↑, Col I↑	Enhanced proliferation and migration of skin cells *in vitro*; promoted re-epithelialization *in vivo*	Zhang et al. (2015b)
Huc	Ultracentrifugation	HaCATs, HDFs *in vitro*; skin wounds in mice	MiR-27b	ITCH↓→JUNB↑→ IRE1α↑	improved proliferation and migration of skin cells *in vitro*; improved epidermal re-epithelialization and collagen proliferation	Cheng et al. (2020)
Huc-WJ	Ultracentrifugation	HaCaT, skin wounds in mice		N-AIF↓, M-AIF↑, N-PARP-1↓, PAR↓	Enhanced re-epithelialization and angiogenesis *in vivo*; inhibited apoptosis and increased proliferation and migration of HaCaT	Zhao et al. (2020)
BM	Ultracentrifugation	3D human Skin Organotypic model; HDFs, HaCaT	—	—	Faster re-epithelialization; enhanced proliferation and migratory capacity *in vitro*	Tutuianu et al. (2021)
BM	Differential centrifugation	Keratinocytes *in vitro*; diabetic wound in mice *in vivo*	MiR-155 Inhibitor	FGF-7↑, VEGF↑, MMP-2↓,MMP-9↓, TGF-β1↓, IL-1β↓, IL-6↓, and TNF-α↓	Accelerated re-epithelialization, angiogenesis and collagen deposition	Gondaliya et al. (2022)

AT, adipose tissue; HDFs, human dermal fibroblasts; AKT, protein kinase B; HaCaT, human immortal keratinocyte line; HSF, human skin fibroblast; RDFs, Rat dermal fibroblasts; GSK-3β, glycogen synthase kinase-3β; PCNA, proliferating cell nuclear antigen; PTEN, phosphatase and tensin homolog deleted on chromosome ten; ERK, extracellular regulated kinase; Bax, BCL2-associated X protein; Bcl-2, B-cell lymphoma-2; huc, human umbilical cord; ITCH, Itchy E3 ubiquitin protein ligase; JUNB, Recombinant Jun B Proto Oncogene; IRE1α, inositol-requiring enzyme 1α; HFFs, human foreskin fibroblasts; MMP, matrix metalloprotein; PDGFA, platelet derived growth factor A; AIF, apoptosis-inducing factor; PARP-1, poly ADP, ribose polymerase 1; PAR, poly ADP, ribose; HIF-1α, hypoxia inducible factor-1α; KGF, keratinocyte growth factor; Hes 1, hairy and enhancer of split-1; CXCR4, C-X-C chemokine receptor type 4.

2015b; Ferreira et al., 2017; Sung et al., 2019; Cheng et al., 2020). Specifically, Tutuianu et al. validated regenerative abilities of BMMSC-sEVs. Exposure to these sEVs enhanced the proliferation and migratory abilities of keratinocytes *in vitro* (Tutuianu et al., 2021). Consistently, Wang et al. applied adipose MSC-sEVs encapsuled in an antibacterial polypeptide-based F127/OHA-EPL hydrogel to treat diabetic wounds and observed remarkable acceleration of re-epithelialization and angiogenesis *in vivo* (Wang et al., 2019c). Besides, Zhao et al. found that hucMSC-sEV administration dramatically increased cell proliferation and inhibited apoptosis *via* restraining apoptosis-inducing factor nucleus translocation (Zhao et al., 2020). While some studies postulate that the miRNA related cargoes containing in MSC-sEVs may be responsible for the bioeffects. For example, Gondaliya et al. observed faster re-epithelialization and enhanced wound repair in diabetic mice treated with MSC-sEVs loaded with miR-155 inhibitor *via* accelerating keratinocyte migration and enhancing fibroblast growth factor-7 (FGF-7) level (Gondaliya et al., 2022). Additionally, miR-205 is reported to be involved in cell migration and proliferation. Lack of miR-205 leads to epidermal defects because of impaired cell proliferation (Wang et al., 2013). This microRNA regulates AKT activation and, therefore, promotes migration of keratinocytes and enhances wound healing (Yu et al., 2010). Moreover, the presence of miR-205 was found in sEV samples from adipose tissue MSCs through the Next-Generation Sequencing experiments. However, it was reported that an miR-205-independent activation of AKT was responsible for the migration and proliferation of keratinocytes in skin wound healing after exposure to adipose tissue MSC-derived EVs (Ferreira et al., 2017). Taken together, there is no doubt that beneficial effects of MSC-sEVs are potential and remarkable. But more studies are required to determine the essential functional constituents and to elucidate the complexity of MSC-sEVs from different tissues for further development of application.

4.5 Promoting regenerative healing through optimizing behaviors of fibroblasts

During normal wound healing, fibroblasts proliferate, migrate, and differentiate into myofibroblasts to participate in synthesizing the ECM, to secrete cytokines and growth factors (Dong et al., 2017), and to enhance wound contraction, thereby promoting wound closure (Darby and Hewitson, 2007). However, these capacities of fibroblasts are impaired in chronic wound microenvironments (Wertheimer et al., 2001), which brings about insufficient ECM production and collagen deposition principally in proliferation phase (within 1 month or longer). The mechanism for this impairment includes cellular senescence induced by excessive oxidative stress and advanced glycation end products in diabetic wounds (Bian et al., 2020). Encouragingly, MSC-sEVs are reported to rejuvenate senescent fibroblasts. For example, sEVs from human placental MSCs significantly improved the biological functions of senescent fibroblasts such as promoting their proliferation and migration, enhancing ECM synthesis, and decreasing the overexpression of matrix metalloproteinases (MMPs) (Bian et al., 2020; Zhao et al., 2021). Further study revealed the mechanism for these effects involving inhibiting the expression of receptor for AGEs (RAGE) and stimulating the activation of Smad signaling pathway in these cells (Bian et al., 2020; Zhao et al., 2021). Interestingly, Zhu and colleagues reported that hucMSC-sEVs could not only promote proliferation and migration of fibroblasts, but also remarkedly boost cutaneous nerve regeneration by stimulating fibroblasts to produce nerve growth factors (NGFs), which can stimulate nerve regeneration, re-

establish local sensory innervation homeostasis, and enhance angiogenesis, thereby achieving an ideally regenerative healing both morphologically and functionally (Zhu et al., 2022). Additionally, ADSC-Exos can transport miRNAs, lncRNA, and functional proteins (Choi et al., 2018; Cooper et al., 2018; Parvanian et al., 2020; Qian et al., 2021) to promote the migration, proliferation, ECM secretion of fibroblasts (Hu et al., 2016; Wang et al., 2021b).

Pathological scars including keloids and hypertrophic scars arise from excessive wound healing after chronic inflammatory stimulation. The pathological mechanism is the aberrant activation of fibroblasts and myofibroblasts, which synthesize more ECMs in scar tissue, mostly *via* the abnormal activation of transforming growth factor-β (TGF-β)/Smads pathway and Yes-associated protein predominantly during remodeling phase (at least over 14 days to 1 month) (Finnson et al., 2013; Zielins et al., 2014; Song et al., 2018; Clark, 2021). Recently, multiple sourced MSC-sEVs have been proven effective in achieving scarless and high-quality wound regeneration (Figure 3iv) (Wang et al., 2017; Dalirfardouei et al., 2019; Jiang et al., 2020a). Zhang et al. found hucMSC-sEVs treatment worked smartly not only as an activator of the Wnt/β-catenin signaling pathway to heal impaired skin but also an inhibitor of the signal *via* exosomal 14-3-3ζ mediated YAP regulation to avoid scar formation (Zhang et al., 2016). Similarly, in another study, Zhang et al. reported that miR-21-5p and miR-125b-5p in MSC-sEVs from umbilical cord blood played critical roles in suppressing myofibroblast differentiation from fibroblasts, thereby favoring scarless wound healing (Zhang et al., 2021). In line with the same idea, Fang et al. examined miRNA profiles in hucMSC-sEVs by high-throughput sequencing and verified that a couple of miRNAs (miR-21, −125b, −23a and −145) decreased myofibroblast formation *via* suppression of transforming growth factor β (TGF-β) Smad2 pathway (Fang et al., 2016). Encouragingly, similar therapeutic effects were also obtained by another team when they applied MSC-sEVs loaded with tumor necrosis factor-α stimulated gene-6 (TSG-6) or miR-138-5p (Jiang et al., 2020b; Zhao et al., 2022). Additionally, the wounds treated with ADSC-Exos also demonstrated faster wound healing and less collagen deposition (Li et al., 2022). So we conclude that MSC-sEVs exert multiple-mechanism therapeutic effects on wound regeneration (as demonstrated in Table 6).

4.6 Promoting regeneration of skin appendages

Most human skin wounds heal with scar and without skin appendages including hair follicles and sweat glands. This outcome seriously affects the appearance of patients and leads to the damage of skin physiological function. Recently, MSC-sEVs were reported to entice regeneration of hair follicles (Table 7). ADSC-EVs containing a variety of cytokines stimulated hair follicle growth by activating the Wnt signaling pathway which is essential to hair follicle induction (Zhang et al., 2022b). Wnt3a is enriched in sEVs from hBMMSCs and BMMSC-sEVs can activate the Wnt/β-catenin signaling in recipient dermal papilla (DP) cells (Rajendran et al., 2022). Our research also implied the hair follicle regeneration potential of sEVs from DP cells (Zhang et al., 2022c). Another group of researchers have successfully designed miR-218-5p abundant sEVs derived from dermal papilla cells and observed a notably promoted hair follicle development by up-regulating β-catenin in mice (Hu et al., 2020). Therefore, MSC-sEVs promote the regeneration of hair follicles mainly by the activation of Wnt/β-catenin pathway (Hu et al., 2020). Additionally, Chen et al. also observed that TGF-β1-enriched MSC-sEVs could realize a faster reconstruction of sweat gland function in wounded skin (Chen et al., 2022a).

5 Application avenues of MSC-sEVs on wounds

Because of their native advantages including anti-inflammatory, angiogenesis, repair promoting, and scar inhibiting mentioned in section 3, MSC-sEVs have been explored to boost wound healing in various forms. Indeed, use of different avenues may affect treatment outcomes of sEVs. In this section, we will focus on the application avenues of sEVs on wounds including injection of free sEVs directly or the combination with advanced biomaterials (Figure 4).

5.1 Injection of free sEVs

5.1.1 Local injection

Local injection refers to injecting free sEVs into or around the wounds subcutaneously. Generally, “subcutaneous injection” (Li et al., 2016), “peri-wound injection” (Ti et al., 2015), “intradermal injection at wound edge” (Dalirfardouei et al., 2019), “intra-dermally injection around wound with 4 sites” (Wei et al., 2021) and other terms are used in literatures to refer to the local injection of free sEVs. The injection site is usually in the layer of dermis. After injected locally, sEVs can directly regulate the cell behavior around the wound and improve the wound microenvironment to facilitate healing process. For example, Qiu et al. (2020) reported that injecting sEVs from adult MSCs (AS-MSC-sEVs, 100 μg in 100 μL PBS) pretreated with neonatal serum locally around the wounds of wide type mice with 4 sites every 3 days significantly promoted the cutaneous wound healing. Similarly, we injected hucMSC-sEVs around diabetic wounds of mice at 4 sites (12.5 μL per site) every other day. We observed that injecting locally around the wounds of mice can facilitate healing process of chronic diabetic wounds of db/db mouse through miR-17-5p-mediated enhancement of angiogenesis (Wei et al., 2021). Xia et al. injected sEVs that isolated from young mouse wound-edge fibroblasts (EV-young, 1 μg/μL) around the wounds once a day in an elderly rat and observed wound healing process. They found that sEV-young can induce fibroblasts of elderly rat to transit to myofibroblasts and increase the abundance of myoblasts around wounds (Xia et al., 2022).

Although a large number of literatures reported that local injection of sEVs can facilitate chronic wound healing process. Inconsistent and sketchy description of local injection in these literatures hinders us to analyze that what dose, what administration interval and what injection method of free sEVs can promote chronic wound healing. Additionally, local injection may disturb the wound and cause waste of sEVs.

5.1.2 Intravenous injection

Intravenous administrations including tail vein injection and epicanthus injection are common modes in animal experiments. Using intravenous injection, the bioactive components can enter blood circulation directly, realizing faster drug absorption rate than other methods of administration. Additionally, the therapeutic responses as well as associated toxicity are more predictable than other injection administration (Wong et al., 2008). Dou and the colleagues injected delusional apoptotic bodies (dABs) (100 μg) *via*

TABLE 6 MSC-sEVs exert curative effects by optimizing fibroblast behavior.

MSC source	Isolation	Target cells/ conditions	Functional cargo	Molecules/Pathways affected	Key functions/ Downstream genes	Ref
BM	Ultracentrifugation	cutaneous wound in rats; HaCaT, HDFs	—	TGF-β1↓→Smad2/3/4↓; TGF-β3↑, Smad7↑	Improved anti-fibrotic and scar-less wound healing	Jiang et al. (2020a)
Menstrual blood	Ultracentrifugation	cutaneous wound in diabetic mice	—	Col1/Col3↓	Reduced cellularity in granulation tissue and diminished scar formation	Dalirfardouei et al. (2019)
Modified synovium	Ultracentrifugation	cutaneous wound in diabetic rats; HDFs	MiR-126	p-AKT↑, p-ERK1/2↑	Accelerated re-epithelialization and collagen maturity *in vivo*; boosted migration and proliferation of HDFs	Tao et al. (2017)
AT	—	HDFs	miRs (-4484, -619-5p, -6879-5p)	Col I↑, Elastin↑, KGF↑, CD34↑, VEGF↑	Boosted proliferation and migration of HDFs	Choi et al. (2018)
BM	Polymer precipitation and ultracentrifugation	HDFs	Wnt3a	—	promoted proliferation and migration abilities	McBride et al. (2017)
Fetal dermal	Polymer precipitation	skin wounds in mice; HDFs	Jagged 1	Notch 1↑ → Hes 1↑→ PCNA↑, CK19↑	Promoted proliferation, migration and secretion abilities *in vitro* and *in vivo*	Wang et al. (2019a)
BM	Ultracentrifugation	3D human Skin Organotypic model; HDFs, HaCaT	—	—	Faster re-epithelialization; enhanced proliferation and migratory capacity *in vitro*	Tutuianu et al. (2021)
Murine BM	ultracentrifugation	DFU model in mice; HDF	LncRNA H19	miR-152-3p↓ → PTEN↑ → p85PI3K↓→p-AKT↓	Improved proliferation and migration and suppressed apoptosis *in vitro* and *in vivo*	Li et al. (2020)
Thrombin pretreated hucb	Ultracentrifugation	cutaneous wound in rats; HDFs	angiogenin, angiopoietin-1, HGF, VEGF	p-ERK1/2↑, p-AKT↑	elevated proliferation and migration activity of HDFs; faster wound healing *in vivo*	Sung et al. (2019)
Huc	Ultracentrifugation	HDFs; skin wounds in mice	miR-21, -23a, -125b, -145	TGF-β2↓, Smad2↓	Suppression of myofibroblasts transformation or scar formation	Fang et al. (2016)
Huc	Ultracentrifugation	HaCATs, MDF; second-degree burn in rats	14-3-3ζ	YAP + p-LAS → p-YAP↑, cytoplasmic retention of YAP↑, a-SMA↓, col I↓ and col III↓	Reduced skin cell proliferation and nuclear translocation of β-catenin under high cell density *in vitro*; restricted excessive cell expansion and collagen deposition during remodeling period *in vivo*	Zhang et al. (2016)
AT	Polymer precipitation	skin wounds in mice; HDF		ERK/MAPK↑→MMP3/TIMP1↑, TGF-β3/TGF-β1↑, Col III/Col I↑	Mitigated myofibroblast differentiation; optimized ECM remodeling and lessened scar formation *in vivo*	Wang et al. (2017)
TSG-6 modified BM	Polymer precipitation	skin wounds in mice	TSG-6	TGF-β1↓, p-SMAD2^{Ser467}/3$^{S423/S425}$↓→ col I↓, col III↓, α-SMA↓; MCP-1↓, TNF-α↓, IL-1β↓, IL-6	prevented inflammation and collagen deposition, restricted scar formation *in vivo*	Jiang et al. (2020b)
Huc	ultracentrifugation	Skin wound in rats; HDF	miR-21-5p, -125b-5p	TGFBR1↓, TGFBR2↓, α-SMA↓, collagen I↓	Suppressed myofibroblast differentiation and scar formation, improved regenerative healing	Zhang et al. (2021)

BM, bone marrow; HaCaT, human immortal keratinocyte line; HDF, human dermal fibroblast; TGF-β, transgenic growth factor β; Huc, human umbilical cord; MDFs, mouse dermal fibroblasts; YAP, Yes-associated protein; LAS, large tumor suppressor; AT, adipose tissue; Erk, extracellular regulated kinase; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; MCP, monocyte chemoattractant protein-1; TNF-α, tumor necrosis factor-α; TGFBR, TGF-β, receptor type II.

the tail vein every 2 days after wounding and observed cutaneous wound size every 2 days. The dABs were developed by combing the membrane of ABs and mesoporous silica nanoparticles (MSNs) preloaded with microRNA-21 or curcumin (Dou et al., 2020). Hu et al. (2016) compared the influence of local and intravenous injection of AMSC-sEVs on wound repair process for the first time. Using bioluminescence imaging, the migration and distribution of DIR-labeled AMSC-sEVs (200 μg/μL in PBS) were studied after they were injected into mice suffering a back wound. They found that the fluorescence of AMSC-sEVs gathered at wound site at day 7 and was still detected at day 21 after being injected intravenously. Surprisingly, AMSC-sEVs with intravenous injection showed faster wound closure than local injection group. This phenomenon may be attributed to following reasons. Firstly, local injection of sEVs around the wound will disturb the wound and inevitably destroy the wound healing process. Secondly, local injection may result in the loss of sEVs and reduce their availability. Thirdly, the homing effect of sEVs meditaed by the receptors or adhesion molecules on their membrane surface can regulate the recruiting of sEVs to wound sites.

TABLE 7 MSC-sEVs enhance regeneration of skin appendages.

MSC source	Isolation	Target cells/conditions	Functional cargo	Molecules/ Pathways affected	Key functions/Downstream genes	Ref
Human BM	ultracentrifugation	dermal papilla (DP) cells, outer root sheath (ORS) cells from patients with androgenic alopecia	Wnt3a	Wnt/β-catenin↑	Promoted proliferation and activation of DP cells, upregulated proliferation and migration of ORS cells, enhanced hair growth	Rajendran et al. (2022)
Dermal papilla	Ultrafiltration	Shaved skin in mice	miR-218-5p	β-catenin↑, CD133↑, Ki67↑	Promoted hair growth	Hu et al. (2020)
Huc	Ultracentrifugation	Wounded skin and paw in mice; keratinocytes	TGF-β1	E-cadherin↓, a-SMA↑, Slug↑, Cxcr4↑, Sox9↑, Lgr5↑,Oct4↑	Quickened wound healing and sweat gland restoration; enhanced migratory and stem cell properties of keratinocytes	Chen et al. (2022a)

MDFs, mouse dermal fibroblasts; Col I/III, collagen I/III; MMP, matrix metalloprotein; TIMP, tissue inhibitor of metalloproteinase; SA-β-Gal, senescence-associated-β-gal; RAGE, receptor for advanced glycation end products; SASP, senescence-associated secretory phenotype; AT, adipose tissue; HUVEC, human umbilical vein endothelial cell; Src, non-receptor tyrosine kinase C; EPC, endothelial progenitor cell; Spred-1, sprouty-related protein 1.

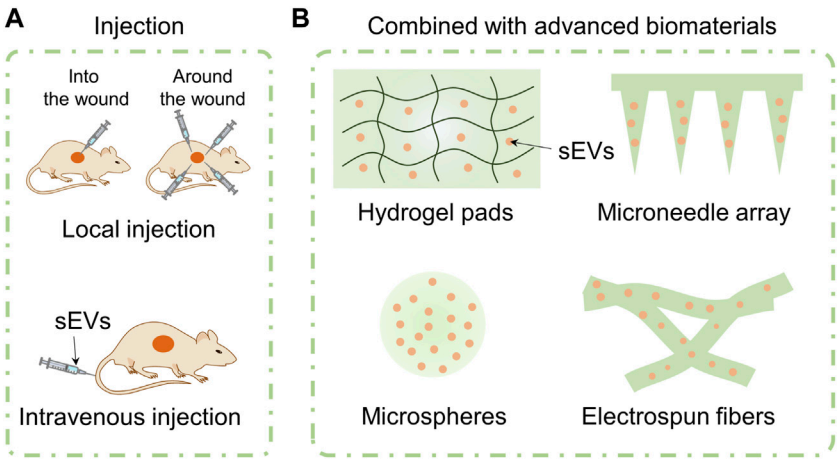

FIGURE 4
Application avenues of sEVs including injection of free sEVs and combination with advanced biomaterials. Free sEVs are applied by means of local injection or intravenous injection **(A)**. MSC-sEVs are loaded in hydrogel pads, microneedle array, microspheres, and electrospun fibers **(B)**.

In our opinions, it is difficult to compare the amount of sEVs and therapeutic effect between local injection and intravenous administration accurately because the content, concentration, and dosing interval of sEVs as well as weight and species of experimental animals used in reported literatures are different from one another. But we can still speculate that using intravenous injection may require fewer exosomes and achieve higher wound closure rate, theoretically. However, there are still application bottlenecks for both injection methods. The rapid clearance of sEVs applied by local injection leads to repeated administration which increases the patient's suffering. Despite relative long half-time of sEVs applied by intravenous injection, the higher dose (usually higher than 100 μg/μL) of and lower targeting efficacy of sEVs may cause unnecessary waste and potential organo-toxicity. Up to now, several kinds of biomaterials have been used to achieve on-demand release of sEVs, named functional active wound dressings. Therefore, developing new approaches by loading sEVs into ingenious biomaterials is expected to solve application bottlenecks of injection of free sEVs.

5.2 Combination with biomaterials

Short half-time and rapid clearance *in vivo* have been the challenges for injecting free sEVs in practical applications (Golchin et al., 2022). Due to high designability of biomaterials, researchers have combined sEVs with innovatively designed biomaterials to prolong the retention time to increase their utilization, and achieve on-demand release *in vivo* (Las Heras et al., 2020). In this section, we discussed and overviewed some ingenious symphonic designs between sEVs and advanced biomaterials like hydrogel pad, microneedle array, microspheres or electrospun nanofibers.

5.2.1 Hydrogel pad

Hydrogel pad with high biocompatibility has been explored widely to function as delivery system for cells or drugs in the treatment of wound healing. Furthermore, it can offer an appropriate microenvironment to facilitate migration and proliferation of cells at wound site (Peppas et al., 2006). In 2020, Akbari et al. reviewed the

application of biocompatible hydrogels for delivering natural and engineered exosomes (Akbari et al., 2020). Here, based on its high adjustability on composition and structure, we overviewed the applications of hydrogel pads with single layer or bi-layer in promoting wound healing process.

To prolong retention time of sEVs, we encapsuled VH298-EVs in 15% gelatin methacryloyl (GelMA) hydrogel which has attracted great attraction in regenerative medicine because of its injectable and UV-crosslinked properties. We compared the *in-vitro* and *in-vivo* release behavior of VH298-EVs from 15% GelMA hydrogel or in free form. For *in-vitro* result, PKH26 labeled VH298-EVs released from 15% GelMA hydrogel could still be detected after co-culturing with human vascular endothelial cells (HUVECs) for 72 h which is longer than that in free form (about 48 h). Additionally, the fluorescence signal of PKH26 used to label VH298-EVs from 15% GelMA distributed evenly around the wounds at day 4 by *in-vivo* imaging system, in comparison with free VH298-EVs with 4-point injection method (Wang et al., 2022). To improve retention time, Ma et al. incorporated engineering sEVs secreted by NR8383 cells stimulated with lipopolysaccharides (LPS) and bioglass (BG) ion extracts (LPS/BG-exos) into a macro-porous hydrogel composed of sodium alginate (SA) and hyaluronic acid (HA) (Ma et al., 2022a). The macro-porous hydrogel was crosslinked with calcium ion and UV consecutively. The release behavior of LPS/BG-exos from macro-porous hydrogel pad was studied by immersing LPS/BG-exo-loaded macro-porous hydrogel pad into 1 mL PBS at 37°C and the concentration of released LPS/BG-exos was measured by BCA Protein Assay Kit. About 50% of LPS/BG-exos showed burst release from macro-porous hydrogel within 6 h and they were released completely at day 8, indicating that the macro-porous hydrogel pad can control the release behavior of LPS/BG-exos to achieve better therapeutic effect. However, the physical adsorption of sEVs in hydrogel pads will result in a burst release of sEVs. To meet this challenge, Fan et al. (2022) encapsuled BMSC-sEVs into a dual-network electroconductive hydrogel composed of GelMA, polypyrene, and tannic acid (TA). The abundant polyphenol groups in TA facilitated the immobilization of BMSC-sEVs. About 80% of BMSC-sEVs were released from hydrogel without TA immediately and retention time was only 7 days. In sharp contrast, the retention time of BMSC-sEVs from hydrogel with TA was evaluated as 14 days, leaving enough time for them to exert their effects.

However, the hydrogel pad with single layer cannot meet the challenge of perfect skin repair, especially scarless wound healing, because the single layer of hydrogel pad cannot realize multi-step release of sEVs. Therefore, a hetero-structured hydrogel pad was developed in which the upper- and lower-layers were loaded with two kinds of sEVs. The different swelling rates between the upper- and lower-layers result in the sequential release of sEVs, performing different functions for promoting wound healing and inhibiting scar formation, respectively (Shen et al., 2021). To our knowledge, MSC-sEVs-loaded hydrogels with three or more layers for faster wound healing have not yet been reported. But we believe that loading sEVs with various functions in different layers of multi-layer hydrogel pads is expected to match each stage of wound healing accurately and achieve perfect skin repair.

5.2.2 Microneedle array

Although hydrogel pads can realize sustained release or multi-step release of sEVs, traditional hydrogel pads cannot pass skin barrier, discounting the therapeutic effect of drugs. As a promising delivery system, microneedle array can transport macromolecules, drugs or sEVs with their microscale needle arrays through skin's barrier in a minimally invasive manner, realizing long-term delivery of bioactive components (Chen et al., 2021). Yuan et al. encapsuled HUVECs-derived sEVs (H-EVs) and tazarotene into a microneedle patch composed of GelMA and polyethylene glycol diacrylate (PEGDA). *In-vitro* and *in-vivo* experiments demonstrated that this microneedle system realized sustained release of PKH26-labeled H-EVs and reached over 80% cumulative release amount after 10 days (Yuan et al., 2022). In another study, Ma et al. reported a core-shell-structured microneedle array in which ferrum-pretreated MSC-derived artificial nanovesicles (Fe-MSC-NVs) were packed into the inner hyaluronic acid (HA) core and polydopamine nanoparticles (PDA NPs) were encapsuled in the outer methacrylated hyaluronic acid (HAMA) shell. The height of 860 μm allowed this core-shell-structured microneedle array to penetrate the skin for the delivery of Fe-MSC-NVs and PDA NPs. Besides, the core-shell structure allowed PDA NPs and Fe-MSC-NVs to be released at different healing phase to scavenge ROS-mediated inflammation reaction and to accelerate the proliferation, as well as angiogenesis (Ma et al., 2022b). In future study, the spatiotemporal-controlled release of sEVs by the stimuli-responsive microneedle array like physiological signal stimuli (pH, glucose and enzymes) or physical signal stimuli (temperature, light or mechanical stress) used in the treatment of cancer or diabetes (Makvandi et al., 2021) may shed light on the precise regulation of the skin wound healing process.

5.2.3 Microspheres

Although extensive studies have been reported to encapsule sEVs in hydrogels or microneedle arrays, the uniform distribution and release rate of sEVs from those materials are usually uncontrollable and rapid (Chen et al., 2022b). Microspheres produced by microfluidic technology show uniform size and longer release time (release kartogenin for up to 5 weeks) (Yang et al., 2020a), arising great interest in delivering drugs, growth factors, and nanophase materials. Chen et al. fabricated a biofunctional microsphere through microfluidics technology in which Tβ4-sEVs were encapsuled by photocrosslinked GelMA and PEGDA. The continuously released Tβ4-sEVs (until about 21 days) improved the angiogenesis activity of coronary endothelial cells (CAECs) *via* the miR-17-5p/PHD3/Hif-1α pathway (Chen et al., 2022b). Recently, by electrostatic interaction, Cai et al. immobilized hypoxic sEVs (H-sEVs) on PDA-coated injectable porous poly (lactic acid-co-glycolic acid) (PLGA) microspheres, achieving sustained release of H-sEVs until 21 days without decayed bioactivity and promoting vascularized bone regeneration (Gao et al., 2022).

5.2.4 Electrospun nanofibers

In the last 2 decades, electrospun nanofibers have been explored widespread in a variety of biomedical applications owing to their tunable morphologies and biophysical chemistry properties including diameters, patterns, surface modification, and mechanical properties (Xue et al., 2019). In terms of regenerative medicine, the electrospun scaffolds composed of nanofibers showcased similar morphology and modulus to ECM, supporting migration and growth of cells (Xue et al., 2019). By employing electrostatic interaction between electropositive polyethyleneimine (Wu et al., 2018) and electronegative sEVs, Su et al. immobilized MSC-sEVs on the surface of PEI-coated

polycaprolactone (PCL) electrospun nanofibers (Su et al., 2021). This bio-system can modulate the response of macrophages and regulatory T cells around skin wounds in mice exquisitely, in which the PCL fibrous scaffold can act as the "recruiter," while the immobilized MSC-sEVs function as the "trainer" for immune cells respectively (Su et al., 2021). In another similar study, electronegative hADSCs-Exos were tethered to PLGA nanofibrous scaffold decorated with electropositive Mg-gallate metal organic framework (Mg-GA MOF) to accelerate bone regeneration. hADSCs-Exos released slowly from nanofibrous scaffold can stabilize bone growth microenvironment and promote angiogenic activity, while Mg^{2+} induced the osteogenic differentiation of MSCs and GA offers potent antioxidative and anti-inflammatory abilities (Kang et al., 2022).

Therefore, we have reasons to believe that the wonderful concerto between sEVs and advanced biomaterials will play wonderful movement in the treatment of chronic non-healing wound.

6 Scalable production and engineering of sEVs

Although sEVs have been recognized as the potential candidates in clinical applications and have been explored widely, the low yield, inadequate therapeutic effect, and targeting efficiency of sEVs are still stumbling blocks of native sEVs for large-scale clinical applications. Very recently, a workshop, named "massivEVs", organized by the International Society for Extracellular Vesicles (ISEV), "The Extracellular Vesicle Foundry" (evFOUNDRY), and "Extracellular vesicles from a natural source for tailor-made nanomaterials" (VES4US) also expressed serious concern on the large-scale production of sEVs from sources, upstream and downstream technologies, validation, standardization, and regulation (Paolini et al., 2022). Up to now, numerous ingenious strategies have been developed to conquer those bottlenecks, promoting the application of sEVs in clinical practice. In this section, we will focus on how to increase the yield and achieve the engineering of sEVs on demand for wound healing.

6.1 Improving the yield of sEVs

Changing culture environments of parent cells has been reported to be beneficial to increase the yield of sEVs. For example, a bioreactor composed of hollow fibers (Watson et al., 2016) can sustain larger numbers (more than 10^9 per mL) of cells and produce highly concentrated cell culture supernatants, thus leading to mass production of sEVs. Using this bioreactor, the yield of sEVs can be improved about 40 folds than conventional system. Additionally, oxygen environment like oxidative stress (Atienzar-Aroca et al., 2016) or hypoxic (King et al., 2012) stimulus was also reported to be responsible for the yield of sEVs. Sandra Atienzar-Aroca et al. observed that retinal pigment epithelium cells (RPEs) secreted larger amounts of sEVs when they were exposed under proper oxidative stress (achieved by treating cells with 40 or 80 nM ethanol) (Atienzar-Aroca et al., 2016). The yield of sEVs can be enhanced about 2 folds when the concentration of ethanol in the cell culture medium of PREs was 80 nM, in comparison with that without ethanol. In another study, it is reported that exposing tumor cell lines (MCF7, SKBR3, and MDAMB 231) to the moderate (1% O_2) and severe (.1% O_2) hypoxia environments significantly increased the number of sEVs present in the conditioned media (King et al., 2012).

Applying external chemical or physical stimulus has been reported to regulate cellular metabolic behavior of parent cells to improve the yield of sEVs. Up to now, several chemical agents have been investigated to affect and improve the secretion of sEVs including BG (Wu et al., 2021), metformin (Liao et al., 2021), cytochalasin B (Pick et al., 2005), monesin (Savina et al., 2003), and serotonin (Glebov et al., 2015) by adding them to the culture medium of parent cells. These reagents affect the production of sEVs through different signaling pathways in parent cells. For example, the ion products of BG can upregulate the expression of neutral sphingomyelinase-2 (nSMase2) and Rab27a to enhance nSMases and Rab GTPases pathways which is essential to regulate the vesicle formation and membrane traffic in human-derived MSCs (Wu et al., 2021). However, metformin was reported to activate autophagy-associated pathway in MSCs to enhance the production of sEVs (Liao et al., 2021). Monesin (a Na^+/H^+ exchanger) can induce exchanges in intracellular calcium, thus stimulating sEV release from K562 cells. Similarly, the involvement of cAMP- and Ca^{2+}- dependent signaling pathways was reported as the underlying mechanism of serotonin in the regulation of EV secretion from microglia cells (Glebov et al., 2015).

In addition to those chemical or bio-stimulation, applying high frequency acoustic (Ambattu et al., 2020), low level electricity (Fukuta et al., 2020), mechanical forces with the combination of 3D scaffold (Guo et al., 2021), lower pH (Parolini et al., 2009; Ban et al., 2015), and ionizing radiation (Jabbari et al., 2019) can also increase the yield of sEVs from various cells. For instance, Ambattu et al. reported that applying an AC electric field with high frequency acoustic of 10 MHz on U87-MG and A549 cells with 7 cycles over 280 min achieved about 8 to 10 folds enhancement in the production of sEVs (Ambattu et al., 2020). By the treatment of relative lower electric intensity (constant current, .34 mA/cm^2) for 60 min, the quantity of sEVs from 3T3-Swiss albino cells and B16F1 cells was enhanced by 1.7 and 1.26 folds, respectively (Fukuta et al., 2020). Guo et al. seeded dental pulp stem cells (DPSCs) or MSCs on Fibra-Cel scaffolds to fabricate a 3D bioreactor and explored the effect of mechanical stimulation with flow stimulation on the yield of sEVs. Surprisingly, the yield of sEVs from above 3D bioreactor under .5 mL/min flow stimulation was improved by about 40.7 and 3.4 folds than the 2D and 3D static counterparts, respectively (Guo et al., 2021).

Overexpressing related regulatory proteins involved in sEV biogenesis *via* gene transfection can also ensure high yields of sEV production. STEAP3, syndecan-4, and L-aspartate oxidase were identified as three sEV production boosters that were involved in sEV biogenesis, the formation of multivesicular bodies, or cellular metabolism to yield more sEVs with high quality (Kojima et al., 2018). In another similar study, activating actomyosin was believed to pull the actin-bound MVB toward the cellular plasma membrane to facilitate the secretion of sEVs (Mittelbrunn et al., 2015). Kai O. Böker et al. reported that overexpression of tetraspanin CD9 significantly augmented the amount of sEVs secreted by different human cell lines, like HEK293, HeLa, SH-SY5Y, as well as B and T lymphocytes (Boker et al., 2018). Other methods such as fusing with synthetic lipid (Jhan et al., 2020), changing cell culture parameters or collection frequency of sEVs (Patel et al., 2017) were also developed to achieve higher yield of sEVs. Although many studies have explored how to improve the production of sEVs, standard operations and unified understanding of underlying mechanisms need to be investigated in depth.

TABLE 8 Current methods for engineering sEVs.

Engineering on parent cells	Engineering sites	Components	Ref	Engineering on isolated sEVs	Engineering sites	Components	Ref
Genetic transfection	Membrane	Membrane proteins	Alvarez-Erviti et al. (2011)	Chemical modifications	Membrane	Cationic lipids, nanoparticles	Nakase and Futaki. (2015); Qi et al. (2016); Tian et al. (2018)
EXPLOR	Lumen	Proteins	(Yim et al., 2016; Choi et al., 2020)	Co-incubation	Lumen	Hydrophobic drugs	Pascucci et al. (2014); Wang et al. (2022)
Co-culturing	Lumen	Hydrophobic drugs	Pascucci et al. (2014)	Electroporation	Lumen	Antisense oligonucleotides, Cas9 mRNA, and guide RNAs, siRNAs, short hairpin RNAs	Kamerkar et al. (2017); Usman et al. (2018)
Cellular nanoporation	Lumen	Therapeutic mRNAs, Targeting peptides	Yang et al. (2020b)	Sonication	Lumen	Hydrophobic drugs	Kim et al. (2018)
Ischemic preconditioning	Lumen	MiRNAs	Li et al. (2015)	Extrusion	Lumen	Hydrophobic drugs, Enzymes	Jang et al. (2013); Haney et al. (2015)
Inflammatory cytokines	Lumen	MiRNAs	Domenis et al. (2018)	Saponin-assistant permeabilization	Lumen	Hydrophilic drugs	Fuhrmann et al. (2015)
				Lipid-assistant chemical transfection	Lumen	SiRNAs	Shtam et al. (2013)
				Hydrophobic modification	Lumen	SiRNAs	Didiot et al. (2016)

6.2 Engineering sEVs

One of the main reasons that sEVs have greater advantages over MSC therapy is that it can be customized to promote wound healing process through on-demand engineered approaches on the parent cells or the isolated sEVs summarized in Table 8. Generally, according to processed objects, developed engineering methods can be classified as engineering on parent cells or engineering on the isolated sEVs.

Up to now, several methods have been developed by researchers to customize the contents of sEVs by operating parent cells. Genetic engineering is recognized as a common strategy to enrich non-coding RNAs and proteins in the lumen or on the surface of sEVs. By virtue of the transfection of hBMSCs by miR-29b-3p lentiviral vector, the miR-29b-3p-enriched sEVs were generated to suppress excessive capillary proliferation and collagen deposition in the late proliferation and remodeling phases of wound healing. By transfecting HEK293 cells with H19-overexpressing (H19-OE) lentiviral vector and followed by a gradient extrusion method, long non-coding RNA (LncRNA)-H19 was encapsuled into sEV-mimetic nanovesicles (Tao et al., 2018). The obtained H19-sEV-mimetic nanovesicles were able to counteract the regeneration-inhibiting effect of hyperglycemia, thus accelerating the healing process of diabatic wounds. Similar method was employed to load SERPINA1, SERPINF2, and SERPING1 into sEVs respectively to promote healthy ECM and facilitate the formation of a beneficial fibrin scaffold and the resolution of the inflammation phase during wound repair (Park et al., 2022). Moderating culture environment of parent cells can also modulate the content or the therapeutic effect of sEVs. The angiogenesis efficacy of MSC-derived sEVs can be improved by applying an ischemic preconditioning on MSCs for 1 h (Li et al., 2015), which might be a useful solution to boost angiogenesis at wound site. Very recently, our colleagues reported TGF-β1-enriched sEVs by pretreating HUMSCs with isoproterenol (ISO) under anoxic environments. In this study, ISO was employed to promote the secretion of TGF-β1, while the hypoxic environment could boost the yield of sEVs. The engineered sEVs enhanced the migratory behavior and stem cell properties of epidermal keratinocytes and accelerated wound re-epithelization *in vivo*. To mimic inflammatory microenvironment, lipopolysaccharide (LPS) was employed to stimulate hUC-dMSCs to generate sEVs enriched with miRNA let-7b. They could mitigate inflammation and accelerate diabetic wound healing by upregulating the expression of anti-inflammatory factors and inducing macrophages to shift to anti-inflammatory M2 phenotype (Ti et al., 2015). Pretreating adipose MSCs with inflammatory cytokines like IFNγ or TNFα would enhance the anti-inflammatory efficacy of adipose MSCs-derived sEVs, shifting macrophages from M1 to M2 phenotype (Domenis et al., 2018), which provided a clue for the management of inflammatory process at wound site.

Moreover, several strategies were also proposed to augment the targeting efficacy or therapeutic efficiency of isolated sEVs for accelerating wound healing. To improve therapeutic efficiency, different kinds of cargoes like non-coding RNA, proteins, and drugs were loaded into sEVs. Co-incubation may be the simplest solution to encapsule cargoes into sEVs (Pascucci et al., 2014). Very recently, our group loaded VH298 (a stabilizer of HIF-1α) into sEVs successfully by co-incubating them at 37°C for 1 h (Wang et al., 2022). The VH298-loaded sEVs facilitated the migration and tube formation

of HUVECs by activating HIF-1α pathway, thus promoting angiogenesis during wound healing process. Unlike the soft phospholipid bilayer membrane of their parent cells, the high level of cholesterol and sphingomyelin on the membrane of sEVs makes them rigid and difficult to open at room temperature. Therefore, enhancing the permeability of phospholipid bilayer membrane of isolated sEVs is the prerequisite to regulate the contents of isolated sEVs. It has been reported that aggressive environments are needed to destroy membrane structures of sEVs and promote drug fusion. In general, the permeability of phospholipid bilayer membranes can be increased by electroporation (Kamerkar et al., 2017; Usman et al., 2018; Lv et al., 2020), freeze-thaw (Gondaliya et al., 2022) and so on. A modified calcium chloride ($CaCl_2$) transfection was used to load miR-155 inhibitor into the lumen of BM-MSC-sEVs by letting the mixture of miR-155 inhibitor, sEVs, and $CaCl_2$, as well as PBS go through a freeze-thaw cycle. The synergistic effects between miR-155 inhibitor and sEVs facilitated the migration of keratinocytes and rebalanced FGF-7 and MMPs levels, thereby accelerating wound healing (Gondaliya et al., 2022). In another study, electroporation was employed to encapsule miR-21-5p mimics into adipose MSC-sEVs. The engineered sEVs enhanced the proliferation and migration behaviors of keratinocytes by activating Wnt/β-catenin pathway and then facilitated diabetic wound healing *in vivo* (Lv et al., 2020). The great progress in increasing the yield of sEVs and in engineering sEVs will further promote the clinical applications of sEVs in chronic non-healing wounds.

Conclusion and prospects

As summarized here, findings from various investigations have implied that extensively sourced MSC-sEVs hold great therapeutic potency for treating chronic non-healing wounds (Lou et al., 2021). The curative benefits can be achieved by functional components in MSC-sEVs from the following main aspects. Firstly, MSC-sEVs could restrict overwhelmed inflammation by modulating immune cell portions and their behaviors and re-balancing cytokines toward an anti-inflammatory role in inflammatory wounds. Secondly, these MSC-sEVs can stimulate angiogenesis which guarantees adequate oxygen and nutrition supply to wound sites. Thirdly, they can also manage to suppress senescence-associated secretory phenotype (SASP) to rejuvenate skin cells and to achieve favorable healing process. Fourthly, re-epithelialization is significantly enhanced after MSC-sEV employment. Finally, application of MSC-sEVs can help to amend scarring and achieve a regenerative repair by priming fibroblasts and optimizing collagen distribution (Li et al., 2022).

Non-etheless, a series of concerns and flaws should be overcome before we bring MSC-sEVs into clinical setting. Firstly, MSCs derived from multiple sources and cultured under disunited conditions will release sEVs with diverse components. As a result, controversies arise about reliable functionality and reproducible results. Next, for now, there are no agreed or existing uniform criterions for isolation, identification or qualitative methods, lacking of industry standardization. In addition, native MSC-sEVs are sophisticated compartments and contain various molecules, such as RNAs, proteins, and chemokines. Hence, it's hard to determine the most effective constituent, impeding further MSC-sEVs clinical application. Another obstacle is the fact that the yield and efficiency of native or unmodified MSC-sEVs haven't yet been satisfactory. Although researchers have found ways to improve productivity, functionality, and targeting efficacy, how to develop a large-scale standardized method to enhance the throughput and therapeutic efficiency of MSC-sEVs is still a major constraint that limits clinical application of MSC-sEVs in the future. Additionally, the operating methods on the cellular behavior of parent cells or the effect on other content inside of sEVs needs further exploration. Finally, agreed requirements for producing and quality control should be a must to guarantee the safety and potency of MSC-sEVs. A bulk of data about MSC-sEV employment has accomplished from *in-vivo* preclinical experiments which may not inexorably mirror the clinical characteristics. Thus, further research is still imperative to unveil the advantages and disadvantages of clinical application of MSC-sEVs on chronic non-healing wounds. After above issues being resolved, it is believed that MSC-sEV therapy will be a potential and encouraging method for rapid and complete regeneration of chronic non-healing wounds.

Author contributions

QW and XL drafted the manuscript and prepared the figures. XL, J-LS, Y-XW, and Z-QC revised the manuscript. XL, KM, and Q-LH revised the manuscript and the figures. XL, H-HL, X-BF, and C-PZ conceptualized, reviewed and funded the manuscript.

Funding

This work was supported by the National Nature Science Foundation of China (82172211, 81830064, 82172231, 22205260), Beijing Municipal Natural Science Foundation (7202197), the CAMS Innovation Fund for Medical Sciences (CIFMS, 2019-I2M-5-059), the Military Medical Research and Development Projects (AWS17J005, 2019-126) and Military Medical Science and Technology Youth Training Program (21QNPY128).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 24 January 2023
DOI 10.3389/fimmu.2023.1120175

OPEN ACCESS

EDITED BY
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SPECIALTY SECTION
This article was submitted to Cytokines and Soluble Mediators in Immunity, a section of the journal Frontiers in Immunology

RECEIVED 09 December 2022
ACCEPTED 11 January 2023
PUBLISHED 24 January 2023

CITATION
Karnas E, Dudek P and Zuba-Surma EK (2023) Stem cell- derived extracellular vesicles as new tools in regenerative medicine - Immunomodulatory role and future perspectives.
Front. Immunol. 14:1120175.
doi: 10.3389/fimmu.2023.1120175

Stem cell- derived extracellular vesicles as new tools in regenerative medicine - Immunomodulatory role and future perspectives

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In the last few decades, the practical use of stem cells (SCs) in the clinic has attracted significant attention in the regenerative medicine due to the ability of these cells to proliferate and differentiate into other cell types. However, recent findings have demonstrated that the therapeutic capacity of SCs may also be mediated by their ability to secrete biologically active factors, including extracellular vesicles (EVs). Such submicron circular membrane-enveloped vesicles may be released from the cell surface and harbour bioactive cargo in the form of proteins, lipids, mRNA, miRNA, and other regulatory factors. Notably, growing evidence has indicated that EVs may transfer their bioactive content into recipient cells and greatly modulate their functional fate. Thus, they have been recently envisioned as a new class of paracrine factors in cell-to-cell communication. Importantly, EVs may modulate the activity of immune system, playing an important role in the regulation of inflammation, exhibiting broad spectrum of the immunomodulatory activity that promotes the transition from pro-inflammatory to pro-regenerative environment in the site of tissue injury. Consequently, growing interest is placed on attempts to utilize EVs in clinical applications of inflammatory-related dysfunctions as potential next-generation therapeutic factors, alternative to cell-based approaches. In this review we will discuss the current knowledge on the biological properties of SC-derived EVs, with special focus on their role in the regulation of inflammatory response. We will also address recent findings on the immunomodulatory and pro-regenerative activity of EVs in several disease models, including *in vitro* and *in vivo* preclinical, as well as clinical studies. Finally, we will highlight the current perspectives and future challenges of emerging EV-based therapeutic strategies of inflammation-related diseases treatment.

KEYWORDS

extracellular vesicles, stem cells, paracrine activity, immunomodulation, inflammation, regenerative medicine, tissue injury

1 Introduction

Inflammation is one of the essential reactions of the body for the tissue damage that triggers a cascade of events accompanying the recruitment of immune cells into the site of injury. However, dysregulation or overactivation of the immune system may lead to the several pathological conditions such as life-threatening cytokine storm, fibrosis, uncontrolled infections, autoimmune diseases or cancer (1).

Tissue regeneration is one of the most dynamically developing fields of the contemporary medical sciences, that also includes the development of strategies that would effectively modulate inflammatory response, reducing harmful pro-inflammatory phenotype and promoting reparatory mechanisms. The pivotal role in this area is played by the stem cell-based therapeutic strategies, that take an advantage from the unique features of those cells including self-renewal and differentiation capacity, that may be critical for their successful use in the translational medicine. However, recent years of studies have revealed that SCs may contribute to the tissue repair and immunomodulation of the local environment by several different pathways, mainly those mediated by their secretory activity that also includes release of the biologically active extracellular vesicles (EVs). Indeed, growing data demonstrate that SC-derived EVs (SCs-EVs) may serve as potential new-generation cell-free therapeutic agents that share similar biological features with their cells of origin (2). Many studies indicate, that EVs may not only regulate the crosstalk between innate and adaptive immune system, but most importantly, they may be important players in the treatment of inflammation-related disorders, exhibiting immunomodulatory and pro-regenerative activity, contributing to the restoration of homeostasis (3).

2 EVs as paracrine factors with diverse biological functions

2.1 Definition and classification of EVs

Extracellular vesicles (EVs) are a heterogeneous population of membrane-enclosed vesicles that are released from the cell surface and possess no ability to replicate (4). EVs are secreted by both normal cells, as well as neoplastic and apoptotic cells, and their presence has also been found in several body fluids, including saliva, urine, milk or amniotic fluid (5). For several years the classification of EVs was based on their size and the cellular compartment of their origin, which also influences their different molecular composition. Thus, three main groups of EVs have been initially recognized: exosomes, ectosomes, apoptotic bodies and oncosomes (6).

Exosomes are considered as a group of vesicles ranging in size from about 30 nm to 120 nm. They are secreted by exocytosis as a result of the fusion of multivesicular bodies (MVBs) with the cell membrane, which results in the release of cargo-containing exosomes into the extracellular area. As exosomes are formed in the late endosomal compartment, they are believed to be enriched in proteins from the tetraspanin (CD9, CD63, CD81) and heat shock family (HSP70 and HSP90), as well as proteins involved in sorting and endosomal transport, such as e.g. apoptosis-linked gene 2-interacting protein X (Alix) or TSG100 (7). Ectosomes, also called microvesicles, have a diameter of 50 nm to 1 μm and are released from the cell surface by the protrusion of a membrane fragment and disruption of the subcellular cytoskeleton, leading to vesicle formation and its budding from the cell surface. They were demonstrated to be enriched in selectins, integrins, CD40L, phosphatidylserine, and a number of other cell-membrane molecules characteristic for the cells which they are derived from (8). Apoptotic bodies are vesicles ranging in size from 50 nm to 2 μm, that are formed as a result of cell fragmentation during the process of programmed death (apoptosis). The mechanism of their formation leads to the enrichment in histones and phosphatidylserine, but they were also shown to contain DNA fragments as a consequence of their mechanism of formation (9).

Oncosomes are considered as a separate group of EVs that are vesicles secreted by the cancer cells. They are usually larger (1-10 μm) and have tumor markers on their surface. They can be classified as a cell-specific fraction of ectosomes secreted by cancer cells, playing an important role in the interaction with cells present in the tumor microenvironment, including cellular components of the immune system (10).

2.1.2 Challenges in EV nomenclature

Despite the fact that the indicated classification of EVs is still commonly used in the majority of papers, there has been a growing issue related to the collective definition of different vesicular entities that have been reported so far. EVs encompass rapidly developing, but still relatively new field of scientific interest, with constantly evolving knowledge on their biology, accompanied by emerging experimental approaches and newly developed methodologies. Thus, in 2014 International Society for Extracellular Vesicles (ISEV) in its first position paper has initially provided criteria of EV definition, as well as minimal set of methodological standards and appropriate experimental controls that should be taken into the consideration in EV-related studies, to provide accurate data that reliably supports the stated conclusions (11). Later on, following the progress in the field and further verification of previously established guidelines, ISEV released updated position paper in 2018, pointing out the need for further standardization of experimental approaches (4). Nevertheless, growing evidence demonstrates the lack of consensus and equivocal data on unique markers and subcellular origin of particular EV subsets, with several indications on morphological and phenotype characteristics to overlap between different vesicular fractions (12). Additionally, several new EV subtypes were recently reported, including exomeres, exophers, or migrasomes (13), which demonstrates the complexity of cellular secreting machinery. Moreover, ISEV points out growing overuse of term “exosomes” without clear experimental evidence on their identity, which leads to misunderstanding and misinterpretation of inaccurate data (14). It is also challenging to exclusively isolate homogenous fraction of exosomes without other EV subtypes (15). Furthermore, depending on the type and source of the starting material, as well as an isolation method, there may be a significant variation in the composition of obtained EV pools, additionally impacted by the heterogeneity of the reported protocols (16).

Thus, taking into account recent advances in the understanding of EV biology and the development of methodological approaches,

recently established new ISEV guidelines recommend to avoid direct categorisation on "exosome" or "microvesicle" terms and to use general term "EVs" instead. Eventually, some operational terms for EV subtypes, that relate to their biophysical properties that have been well characterized experimentally in particular study, such as "small/large EVs", "CD81+ EVs" etc. may also be applied (4). Thus, in current review we will use general term of "EVs" that collectively combines several types of vesicular particles reported in the cited literature.

2.2 Molecular composition of EVs

EVs are well known to contain several types of biomolecules that come from their parental cells. The molecular content of EVs is a consequence of their vesicular structure, where a small fragment of the cytoplasm is surrounded by a lipid bilayer. Thus, the bioactive composition of EVs is mainly determined by the type of cells from which they are derived from, as well as the mechanisms of their formation in the cell. It has been also shown that this content may also depend on the activation state of the cell (17). Currently, thousands of different RNA, proteins and lipids have been identified in EVs and were classified e.g. in the ExoCarta database (18). From a functional point of view, the rich molecular composition of EVs can be transferred from vesicle-producing cells to other target cells, affecting their functional status, which may be utilized to modulate the functions of various cells both *in vitro* and *in vivo*.

EVs contain a lipid components which are mainly a part of the biological membrane surrounding the cytoplasmic part of the vesicle. Despite the typical components of cell membrane that can be found in EV membrane, particular enrichment in a cholesterol, sphingomyelins, phosphatidylcholine and phosphatidylethanolamines has been also shown (19), indicating an important role of those molecules in the process of vesicle segregation in MVBs (20). Additionally, the role of the lipid content was also shown to take a part in the biological activity of EVs (21).

Among the key bioactive components of the cytoplasmic part of the EVs, two basic components can be distinguished, including proteins and nucleic acids. The protein content of EVs is enriched in proteins of the endosomal compartment, including Rab GTPases and SNAP (soluble NSF attachment protein) receptor (SNARE) proteins involved in the fusion of vesicles with the cell membrane, but also annexins, flotilllin, as well as proteins related to EVs biogenesis, e.g. Alix and Tsg101 (22). In addition, EVs are also enriched in the proteins that are a part of membrane microdomains and lipid rafts, including tetraspanins (23). It was also demonstrated that EVs may contain several other regulatory factors such as transcription factors (24), enzymes (25), growth factors (26), cytokines and signaling molecules (27).

EVs also contain nucleic acids, in particular RNA, found mainly in the form of mRNA and miRNA. Importantly, the presence of the latter RNA type, known as an important regulatory molecules, pays particular scientific attention in the context of potential bioactive compounds responsible for the functional activity of EVs (28). Currently, the presence of a mechanism for selective sorting and packing of miRNAs into EVs is postulated, as evidenced by numerous studies showing the enrichment of some miRNAs in vesicles, when compared to their donor cells (29). So far, the detailed mechanism of such selectivity is still not fully understood. Nevertheless, several concepts have been proposed, including the role of RNA-induced silencing (RISC) complex, involved in the binding of miRNA to proteins from the Argonaute family (30). Other studies have also demonstrated that heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1) may be responsible for the control of miRNA loading into EVs (31). Interestingly, recent research demonstrates that in addition to mRNA and miRNA, EVs may also contain other types of non-coding RNA, including transporting RNA (tRNA), small interfering RNA (siRNA) or vault RNA (vRNA) (32). However, the risk of non-EV-associated extracellular protein-RNA complexes that may be co-isolated with EV preparations must be always carefully considered in the data interpretation.

Recent studies also indicate the presence of genomic DNA in EVs, which enables its horizontal transfer between cells, resulting in a modulation of gene expression, and thus influencing the biological characteristics of cells. For example, nearly 350 chromosomal DNA sequences have been identified in EVs produced by cardiomyocytes (33). In addition, the presence of mitochondrial DNA has been also demonstrated (34). Similarly to other components of EVs, a certain selectivity of DNA fragments has been also observed as a result of both EV type and the activation state of the secreting cells (35).

2.3 EVs biogenesis and secretion

The mechanisms of biogenesis and secretion may vary depending on a type of EVs (Figure 1). Exosomes are considered to be initially formed in MVBs, which may be either degraded upon association with lysosomes or may be secreted by exocytosis (36). The two-way fate of the MVBs may be determined by their lipid content, where cholesterol-rich MVB populations have been shown to be secreted (37) and lysobisphosphatidic acid- enriched ones to bind to lysosomes and be degraded (38). The formation of MVBs involves the segregation of their contents at the endosome's boundary membrane and the subsequent budding of intraluminal vesicles into its interior. This process involves endosomal sorting complex responsible for transport (ESCRT) associated with Alix proteins and syntenin (39). However, some studies suggest that MVBs formation may also occur independently of ESCRT complexes, with the simultaneous involvement of sphingomyelinases that enrich exosomes in ceramides (40). The participation of tetraspanins in exosome formation was also demonstrated (41). In the case of ectosomes, the mechanism of their formation is less understood. Nevertheless, it has been shown that their formation is accompanied by oligomerization of cytoplasmic proteins and their anchoring in the cell membrane by myristoylation and palmitoylation (42). The participation of the actin cytoskeleton and proteins from the GTPase family in the ectosome formation process has also been reported (43).

The exact mechanism of EV release from the cell surface is still not fully revealed. However, it has been shown to be accompanied by the reorganization of the sub-membranous cytoskeleton and involvement of Rab GTPases and SNARE proteins, that are responsible for the fusion of vesicles with the cell membrane (44). Moreover, it is possible to externally stimulate cells to secrete EVs, e.g.

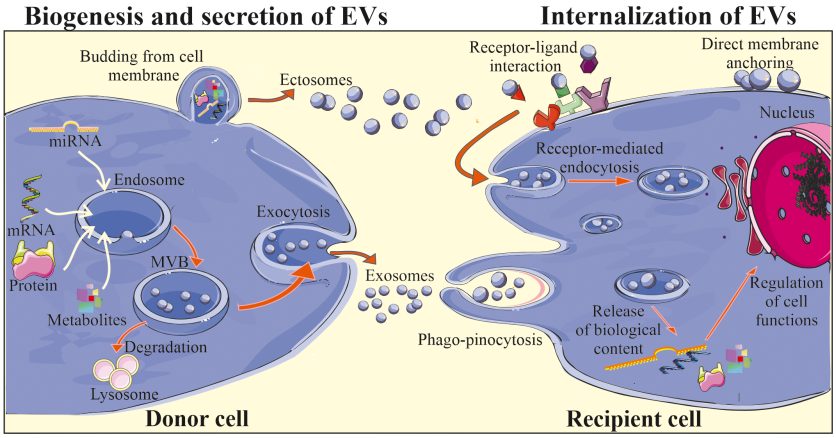

FIGURE 1
Biogenesis and biological activity of EVs. Two main subtypes of EVs are exosomes and ectosomes (microvesicles) that differ in terms of their biogenesis and secretion. Exosomes are initially formed in MVBs located in the cytoplasm, with the involvement of endosomal pathway and intracellular trafficking of MVBs, that may be either degraded in the lysosomes or may fuse with the plasma membrane, releasing exosomes into the extracellular milieu. Ectosomes are considered to be generally larger than exosomes and are formed through the outward plasma membrane budding and shedding. After release EVs may interact with the recipient cells, delivering their cargo *via* direct fusion with cell membrane, endocytosis, receptor-ligand interaction or phago/pinocytosis. Consequently, internalization of EV content may lead to the changes in the biological activity of the target cell.

by activating the thrombin receptor in the case of platelets (45), inducing an increase in the intracellular calcium ions concentration (46) or stimulation of dendritic cells (DCs) by the lipopolysaccharide treatment (47).

2.4 Biological activity of EVs

For several years EVs were considered as contaminants and debris lacking an essential biological function. Later on, EVs were envisioned as a waste disposal machinery, which allows cells to rapidly get rid of a molecules and metabolites that are not needed anymore (48). However, in last few decades remarkable advance in the understanding of EV biology have been done together with the growing number of scientific reports confirming an important role of EVs as part of the paracrine activity of cells (49). Indeed, subsequent studies have demonstrated the role of EVs in the process of information exchange between the cells. It has been widely postulated that EVs may contribute to the cell-to-cell communication, which includes the step of their interaction with the target cell, that may occur in several ways: by endocytosis, phagocytosis, or by direct fusion with the cell membrane including receptor-ligand interactions, subsequently leading to the release of bioactive cargo (Figure 1) (50). The exact mechanism of EV binding to the cell membrane of recipient cell is still not thoroughly investigated. However, it has been demonstrated that e.g. syncytin that binds to major facilitator superfamily domain 2a (MFSD2a) receptors present in the cell membrane may participate in this process (50). Adhesion molecules, including integrins, lipid rafts and proteins from SNARE and Rab families may also mediate the fusion of EVs with cell membrane (36). Interestingly, some selectivity of EV binding to specific types of target cells has also been demonstrated. For example, EVs secreted by neuroblastoma cells showed affinity to neurons and glial cells, while vesicles from stimulated cortical neurons were endocytosed only by neurons (51). One of the postulated mechanisms of selective binding of EV with recipient cell includes the influence of tetraspanins, which interact with integrins and other anchor proteins, modulating their functions (52). Moreover, ligand-receptor interplay may also be involved in the control of this process, as was shown for EVs secreted by endothelial progenitor cells (EPCs) that were reported to bind *via* the C-X-C motif chemokine receptor 4 (CXCR4) to its ligand stromal cell-derived factor 1 (SDF-1) present on the endothelial cells (53).

EVs can serve as paracrine mediators that target cells by transferring their bioactive content in the form of different types of nucleic acids, receptors, enzymes, transcription factors, immunomodulators and even morphogenic factors such as Wnt (54) and Notch (55) signaling proteins. Delivery of the EV cargo into the recipient cells opens several ways of potential regulation of cellular processes, including influence on gene and protein expression, as well as activity of intracellular signaling pathways. Depending on the cell origin and the type of the target cells, EVs were showed to either stimulate or inhibit cell proliferation and differentiation, act as cytoprotective agents reducing cell death (56), exert pro-angiogenic stimuli (57), regulate myelin formation (58) and modulate immune cells, as will be discussed below (Figure 2). Importantly, EVs may act not only as paracrine factors, transferring the biological information between different types of cells, but were also shown to play pivotal role in the autocrine signaling (59).

On the other hand, EVs may also participate in the pathogenesis of many diseases. As an example, EVs secreted by tumors may promote their progression by stimulation of pro-angiogenic processes and inhibition of the immune system (60). EVs have also been shown to contribute to the transmission of prions (61), α-synuclein responsible for the pathogenesis of Parkinson's disease (PD) (62), as well as β-amyloid, which contributes to the development of Alzheimer's disease (AD) (63). Moreover, EVs can transfer the drug resistance phenotype between cells, which is related to the transfer of drug-efflux membrane pumps (Figure 2) (64).

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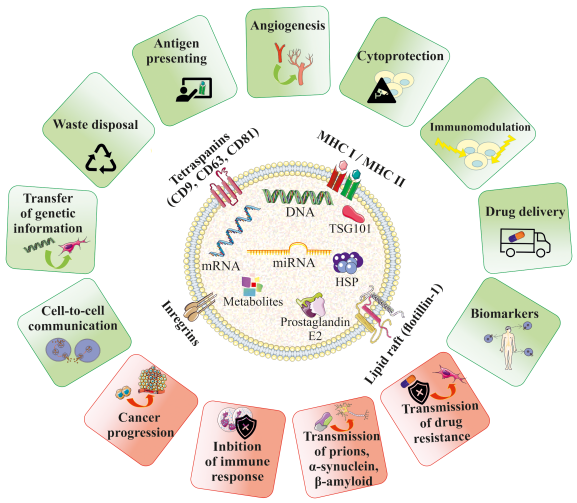

FIGURE 2
Biological role of EVs in homeostasis and pathophysiology. EVs contain bioactive cargo that is responsible for their multimodal activity. EVs may mimic the properties of their cells of origin and were shown to be paracrine factors that play role in cell-to-cell communication and influence the fate of the target cells in several ways, including e.g. stimulation of angiogenesis, cell survival or modulation of the immune response. EVs may also serve as waste disposal machinery, drug-delivery systems and biomarkers for the diagnostic purposes. An influence of EVs in the development of several diseases has been also reported.

2.5 Role of EVs in the regulation of immune system

Among the variety of reported functions, EVs are also envisioned as important factors modulating the function of the immune system, both as activators or inhibitors, depending on the biological context. Their role in the immunity relies both on the interaction of EVs from other cell types with immune cells, as well as on the secretion of EVs by the cellular components of immune system, regulating its fate in the paracrine or autocrine manner (Figure 3) (65). Thus, EVs mediate communication between immune cells, taking part in orchestrating an immune response. In particular, they are a part of interaction of innate and adaptive immunity, modulating cell response and release of cytokines, chemokines and other immune-active factors (65).

In the context of immune defence against pathogenic factors, EVs are involved in the communication between bacteria and host cells, playing either protective or pathogenic role in the infection. On one hand, bacteria-derived EVs may serve as a shuttle particles contributing to virulence spread. On contrary, secretion of EVs by the host cells may be a method to expel intracellular bacteria, neutralize bacterial toxins or stimulate both innate and adaptive immune response (66). As an example, EVs secreted by neutrophils infected by *Mycobacterium tuberculosis* stimulated autophagy, expression of costimulatory molecules and superoxide anion production in bacteria-containing macrophages, enhancing their clearance from this intracellular pathogen (67). In another study, EVs produced by DCs infected with *Listeria monocytogenes* stimulated immature DCs to pro-inflammatory state and anti-viral defense (68). An involvement of EVs in the fungal infections has also been demonstrated. For example, it was reported that neutrophils may secrete EVs that act as anti-fungal agents containing antimicrobial cargo such as neutrophil elastase, myeloperoxidase, cathepsin G, azurocidin, and defensin, that may inhibit growth of *Aspergillus fumigatus* (69).

Recent studies put novel insights into the mechanism of EV function in the immune system, which opens new possibilities in the control of immunological response for the therapeutic purposes. Immunoregulatory activity of EVs is related to their biological content, that consist of molecules known to be involved in the regulation of immune cells. As an example, heat shock proteins (HSP) that were shown to be present in EVs are known immunomodulants (70). Several lipid and lipid-related signaling mediators such as phospholipases, prostaglandin E2 or arachidonic acid were also reported to be a part of EV cargo (71). Additionally, presence of major histocompatibility complex (MHC) class II, and co-stimulatory CD86, as well other immunologically-active molecules such lymphocyte function-associated antigen 1 (LFA-1) and intercellular adhesion molecule 1 (ICAM-1) was also shown on EVs derived from antigen presenting cells (APCs), that were able to regulate the proliferation of B and T cells (72). In this context, EVs released by APCs such as macrophages or DCs may participate in the antigen-specific interaction between immune cells *via* the cross-dressing mechanism (65). EVs may bind to the surface of APCs, contributing to the antigen presentation to T cells or may be internalized by APCs, delivering their antigen peptide/MHC complexes, contributing to the antigen spread (73). This mechanism plays a pivotal role in the development of anti-tumor response, where tumor-derived EVs may be taken up by APCs, enhancing cross-presentation of tumor-specific antigens to cytotoxic T cells (74). It has been also shown that EVs secreted by the immune cells can transfer surface Fas ligand on their surface, thereby contributing to the control of cell death during the immune response (75).

Apart from the possible ways of EV-mediated activation of immune system, several findings demonstrate their immunosuppressive role in homeostasis and disease. However, despite growing evidence on multimodal immunomodulation of immune system through EVs, exact mechanism of their action, together with immunomodulatory cargo responsible for this effect still remain to be deeply determined. Nevertheless, several studies have shed light on the potential EV-related factors that may exert their suppressive activity. As an example, EVs secreted by tumor cells were shown to carry programmed death-ligand 1 (PD-L1) that suppresses cytotoxic T cells (76). Additionally, widely postulated immunomodulatory activity of EVs may be an essential mechanism that allows to control excessive or chronic activation of immune system, as well as autoimmunity, thus contributing to protection against several pathological conditions. For instance, neutrophil-derived EVs were shown to inhibit pro-inflammatory cytokine release by macrophages *via* modulation of Mer receptor tyrosine kinase (MerTK) and PI3K/Akt pathways (77), with the possible mechanism of their immunosuppressive action related to the presence of phosphatidylserine (78). miRNA content may be also involved in the immunomodulatory activity of EVs that leads to the anti-inflammatory phenotype of immune cells (79). As an example, EVs from endothelial cells were shown to harbour miR-10a that mediated inhibition of monocyte activation *via* NF-κB pathway, both *in vitro* and *in vivo* (80). The immunomodulatory activity of EVs has also been demonstrated in many other systems,

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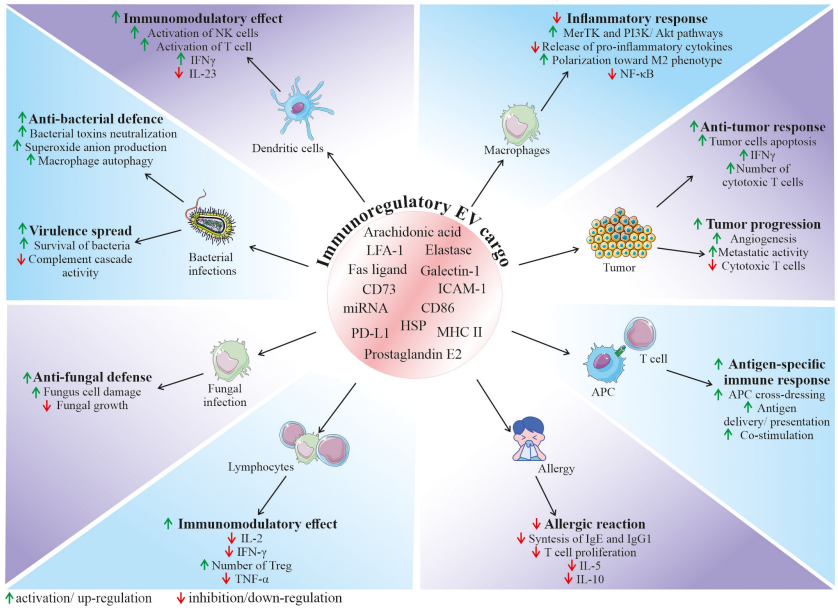

FIGURE 3
Role of EVs in the regulation of the immune response. Depending on the origin, EVs can contain and deliver a diverse bioactive cargo with immunoregulatory activity, that can influence various cell types and modulate their functional status. It has been demonstrated that EVs may have an impact on many immune-related processes, including regulation of immune system activation status, mediation of anti-bacterial and anti-fungal defence, modulation of anti-tumor response, as well as inhibition of harmful overactivation of the immune system. APC, antigen presenting cells; ICAM-1, intercellular adhesion molecule 1; HSP, heat shock protein; IFN-γ, interferon gamma; LFA-1, lymphocyte function-associated antigen 1; MHC II, major histocompatibility complex class II; MerTK, mer receptor tyrosine kinase; NK cells, natural killer cells; PD-L1, programmed death-ligand 1; TNF-α, tumor necrosis factor alpha; Treg, regulatory T cells.

including the respiratory tract, where they decreased allergic reaction (81). In another study, breast milk-derived EVs inhibited activation of peripheral blood mononuclear cells, increasing the number of regulatory T cells (82).

Most importantly, as EVs are natural carriers of several biomolecules that come from their parental cells, they might share functional similarities with their source cells. Thus, the unique biological properties of SCs, including ability to modulate immune system, arouses particular interest in the utilization of their EVs (SCs-EVs) in the context of interaction with the immune system. Indeed, based on the several recent findings, SC-EVs have been recognized and appreciated as a potential mediators inhibiting harmful overactivation of immune cells, accompanied by the simultaneous promotion of beneficial, pro-regenerative phenotype in the site of injury, followed by the restoration of homeostasis (3). Thus, these biological effects of SCs-EVs give a hope to develop new strategies of treatment of several diseases at their various stages. Additionally to the already discussed different types of cargo commonly present in vesicles from different cells, EVs derived from mesenchymal stem cells (MSCs) were shown to contain CD73, which is ecto-5′-nucleotidase capable to convert adenosine monophosphate (AMP) into adenosine, that may bind to A2 receptors present on the surface of immune cells, exerting immunosuppressive effect (83). MSCs-derived EVs (MSCs-EVs) may possess miR-21 that is involved in the activation of tolerogenic transforming growth factor β (TGF-β) signaling (84). Indoleamine 2,3-dioxygenase (IDO) known as a tryptophan-degrading enzyme, transferred in EVs from MSCs and DCs may also mediate their immunomodulatory effect (85, 86). Glycan-binding protein galectin-1 found in EVs from MSCs isolated from placenta is also known as immunomodulatory factor that promotes proliferation of regulatory T cells (Tregs) (87).

Taking together, EVs secreted not only by the immune cells, but also by the SCs may be promising immunoregulatory factors and thus promising candidates for the further development of therapeutic approaches.

3 SCs-EVs as an alternative option to cell-based therapies

Due to the increasing evidence that EVs are not only the waste elimination apparatus, but they possess multimodal biological potential, EV field encompass a rapidly growing scientific interest in terms of their possible use in the regenerative medicine. Importantly, they are envisioned as potential new-generation therapeutic tools that may overcome several limitations related to the whole cell-based therapies. Thus, there is growing hope for the use of SCs-EVs as an alternatives to cell therapy, as they may not only mimic the phenotype of the cells from which they originate, but also possess several advantageous features (88). For instance, the utilization of EVs minimizes the risk of developing a tumor resulting from transplanted cells, in particular pluripotent SCs. What is more, direct comparison of the influence of MSCs and their EVs on T-cell subsets proliferation *in vitro*, indicated that the co-culture with MSCs, but not with MSCs-EVs, reduced the proliferation of CD3+ cells. On contrary, EVs stimulated proliferation of Tregs, increased apoptosis of CD3+ cells and elevated level of IL-10. These results may indicate higher immunomodulatory activity of EVs, comparing to their parental cells, which may be

beneficial for the therapeutic purposes (89). Moreover, animal studies have shown the potential possibility of administration of EV preparations in the form of aerosols, which allows their local delivery to the respiratory system (endotracheal) or to the central nervous system (intranasally) (90, 91). Additionally, biocompatible lipid bilayer structure of EVs that encloses naturally or exogenously loaded genetic cargo, protecting it against degradation, opens a possibility to use EVs as vectors, that bypass the limitations of virus-based nucleic acid delivery, related to immunogenicity and packaging capacity (92). Importantly, small size of EVs facilitates their transfer throughout the body and enables them to cross blood-brain barrier (BBB) (93). Additionally, there has been an increased interest in the possibility to modify EVs by their engineering that includes either surface or cargo modification, to improve their biological activity or enhance stability and targeted delivery (94). Taking together, the recognition of SCs-EV ability to transfer biologically active molecules between cells and thus their involvement in the paracrine signaling has made them an attractive option for the therapeutic purposes in several experimental models. Importantly, EVs may have a tremendous potential as therapeutic agents for the treatment of several diseases with the inflammatory component (Figure 4).

3.1 SCs as a source of EVs for the therapeutic applications

The unique ability of SCs to self-renew and differentiate into other types of cells has made them well established and main type of cells for the use in the medicine. For many years the prevailing view was that their regenerative activity is mainly a consequence of the ability to directly rebuild damaged tissue by proliferating and differentiating in the site of injury. However, recent years of research clearly indicate that some of the observed therapeutic effects after SCs administration result from their paracrine activity, related to the secretion of a number of cytokines and growth factors, which stimulate cells residing at the site of damage to undertake reparatory processes (2). Consequently, growing number of reports indicates that SCs, in addition to soluble molecules, may also release bioactive EVs, which may play an essential role in the pro-regenerative activity of those cells (95). Thus, currently several different types of SCs are considered as sources of EVs for the therapeutic applications.

3.1.1 Mesenchymal stem/stromal cells (MSCs)

MSCs are multipotent SCs of mesodermal origin, that are able to differentiate into chondrogenic, osteogenic and adipogenic lineages. They may be isolated from various sources, including bone marrow (BM-MSCs), adipose tissue (AT-MSCs) and postnatal tissues such as umbilical cord (UC-MSCs) (96). MSCs are known for their high secretory activity, which includes release of extracellular matrix (ECM) proteins, cytokines, chemokines, growth factors, but also bioactive EVs that may play a role in mediating crosstalk to local and distant tissues (97). This paracrine activity of MSCs makes them also crucial players in immunomodulation, which may trigger mostly anti-inflammatory signaling and suppress excessive activation of immune system components (98). Importantly, MSCs-EVs were demonstrated to share biological activity with their parental cells, that are known to possess immunomodulatory properties. Several studies have demonstrated an impact of tissue origin on potential differences in the functional activity of MSCs, which may be also reflected in the distinct biological activity of their EVs (99).

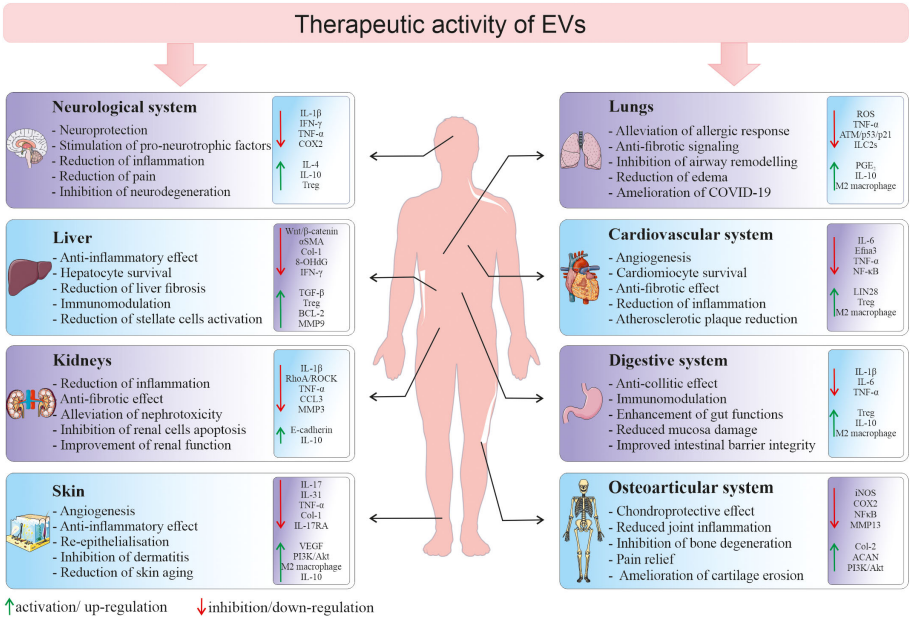

FIGURE 4
Therapeutic activity of EVs in different tissues and organs. Depending on a type of tissue/organ as a site of vesicle delivery, EVs they may modulate several cellular processes and signaling pathways in the local environment, leading to the tissue regeneration in the place of injury. 8-OHdG, 8-hydroxyguanosine; ACAN, aggrecan; BCL-2, B-cell CLL/lymphoma 2; CCL3, macrophage inflammatory protein-1 α; Col, collagen; COX2, cyclooxygenase-2; Efna3, ephrin A3; IFN-γ, interferon gamma; ILC2s, type 2 innate lymphoid cells; iNOS, inducible nitric oxide synthase; MMP, metalloproteinase; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PGE_2, prostaglandin E2; ROS, reactive oxygen species; TGF-β, transforming growth factor β; TNF-α, tumor necrosis factor alpha; Treg, regulatory T cells; VEGF, vascular endothelial growth factor; αSMA, alpha smooth muscle actin.

MSCs possess limited stemness and differentiation potential and thus reduced risk of teratoma formation when compared to pluripotent SCs. On the other hand, they also have relatively high proliferative capacity *in vitro* and do not require advanced and expensive culture reagents, which allows researchers to effectively reach the level of MSC expansion sufficient for the isolation of EV batches dedicated for the therapeutic applications (100). However, there are still several challenges of the effective use of MSCs as a source of EVs for the therapeutic purposes, including donor variability and need to optimize expansion methods in order to avoid cell senescence. Nevertheless, considering the lack of ethical concerns, ease of isolation from several sources potentially available in both autologous and allogeneic systems, biological safety and low immunogenicity, MSCs have become primary cells of choice for the purpose of the tissue regeneration. A natural consequence of this fact is that researchers are particularly interested in the application of EVs secreted by these cells (101). Thus, numerous studies show that MSC-EVs have significant cytoprotective, regenerative and immunomodulatory potential in several disease models.

3.1.2 Embryonic SCs (ESCs)

ESCs are pluripotent population of cells with unlimited proliferative capacity, capable to give rise into any type of cells within three germ layers. Consequently, ESCs were initially envisioned as potentially ideal type of SCs for the medical purposes (102). However, due to the ethical concerns regarding their sourcing, as well as the risk of teratoma formation, clinical application of ESCs has been highly limited (103). Nevertheless, due to the acellular nature, the use of EVs secreted by already available ESC lines (ESCs-EVs) still remains promising strategy for the regenerative therapies (104). Due to the potentially unlimited quantities of cells, ESCs are often used as starting cells that are differentiated toward more specified progenitors serving as a source of EVs for therapeutic approaches (105). Another interesting approach is to use ESC-EVs to boost the therapeutic efficacy of other SC populations. As an example, ESCs-EVs were demonstrated to reduce senescence and enhance pro-regenerative effects of MSCs in a mouse cutaneous wound model, by activating the PI3K/AKT pathway (106).

3.1.3 Induced pluripotent SCs (iPSCs)

iPSCs were initially obtained by the Prof. Yamanaka's group by genetic reprogramming of somatic cells through the forced expression of key transcription factors such as Oct3/4, Sox2, Klf4, and c-Myc (107). This achievement was awarded by Nobel Prize in 2012 and has opened new chapter not only in the field of stem cell biology, but also in the area of tissue regeneration. iPSCs display pluripotent properties similar to those of ESCs, allowing relatively easy accessibility to pluripotent cells without ethical problems related to the cells of embryonic origin. Consequently, due to their potentially unlimited proliferative and differentiation potential, iPSCs have been widely used for disease modelling, drug discovery and cell-based therapies, resulting in the substantial progress in the field (108).

Along with their paracrine activity, iPSCs have been also recognized as important donors of EVs for the basic research as well as the therapeutic applications. Similarly to ESCs, iPSCs are also differentiated into other cell types that are sources of EVs for the regenerative purposes (109). Interestingly, iPSCs were shown to be able to secrete EVs more abundantly and with higher capability to enter target cells, when compared to the MSCs (110), which may make these cells advantageous in the context of the donor cells for EV-based therapeutic approaches.

3.1.4 Other SCs types

Despite the special focus on the pluripotent and mesenchymal SC as main sources of EVs for the tissue regeneration, the pro-regenerative potential of EVs secreted by the several other SCs and progenitor cell was also investigated. As an example, therapeutic efficacy of EPCs- derived EVs was shown in different experimental setups (111). Similarly, protective effect of EVs from neural (112) and cardiac progenitors (113) was also demonstrated.

3.2 Toward therapeutic applications of SCs-EVs- preclinical studies

The ability of SCs-EVs to modulate immune response indicates that they may be used therapeutically for a broad spectrum of diseases. So far, EVs have been tested in several *in vitro* and *in vivo* preclinical studies that cover a broad range of experimental disease models. In this section we will provide an overview on the different approaches utilized to explore an emerging role of EVs as potential new-generation tools for the tissue and organ regeneration, including their immunoregulatory activity.

3.2.1 Cardiovascular diseases

Cardiovascular diseases (CVDs) are one of the most common causes of death, with limited efficacy of currently available therapeutic strategies. According to the data provided by the world health organization (WHO), CVDs are responsible for about one-third of all death cases worldwide, which corresponds to almost 18 million of human beings every year (114). Cardiac tissue has a limited regenerative capacity and endogenous systems are typically insufficient for the cardiac repair. Once injured, mammalian heart lacks the ability to replace damaged cardiomyocytes, which leads to the progressing loss of its function. Thus, the development of novel therapeutic approaches and identifying intrinsic and external factors together with new potential targets to improve cardiac performance are of special focus (115). CVDs encompass broad spectrum of disorders, but the two major representations of ischemic CVDs are acute myocardial infarction (AMI) and chronic myocardial disease (CMD), which differ in terms of their mechanisms of cause and clinical manifestation, with indispensable role of inflammatory response. Both conditions are life-threatening and lead to the subsequent cardiac remodelling and scar formation rather than regeneration, which can adversely affect function of the cardiovascular system (116).

AMI is a rapid event caused by the coronary artery occlusion by the ruptured plaque that blocks the blood flow, followed by the oxygen deprivation in the myocardium and death of cardiomyocytes. Consequently, due to the insufficient ability of heart to compensate the massive loss of cardiac cells following infarction, injured tissue becomes fibrotic and non-contractile, leading to the heart disfunction

such as dilatation, reduced ejection fraction, left ventricle stiffness and its remodelling (117). Current AMI therapeutic strategies include e.g. urgent reperfusion therapy, pharmacotherapy and surgical intervention, including heart transplantation (114). Despite advancement in the treatment, AMI still carries a high mortality rate, with increasing morbidity caused by the several risk factors that are a common part of contemporary, unhealthy lifestyle, such as smoking, obesity, hypertension, lack of physical activity and high exposure to the stress (118). Additionally, patients who survived AMI have a higher risk of recurrent AMI or other CVD-related complications (119). In a consequence, there is a great need for new, more effective therapeutic strategies, including those that would effectively support the natural reparatory mechanisms of the heart muscle and would reduce inflammatory response, minimizing subsequent cardiac tissue deterioration and adverse remodelling.

First attempts in this matter were focused on a cell-based therapies that relied on the administration of several types of stem and progenitors cells, including e.g. BM-MSCs, different populations of cardiac progenitor cells (CPCs) or cardiosphere-derived cells (120–122). However, despite indication on safety and some beneficial effects, the efficacy of cell-based therapies varied depending on a type of cells and route of administration, facing several limitations including low retention in the site of the delivery or a potential immunogenicity (123). Importantly, throughout the recent years there has been an accumulating evidence that the pro-regenerative effect of SCs in the AMI treatment is caused by their paracrine activity that triggers endogenous repair mechanisms and provides immunomodulatory signaling, rather than by their direct differentiation and proliferation in the site of administration (124). Indeed, recent years of studies have brought mounting evidence on protective and pro-regenerative capability of SC-derived EVs in the treatment of AMI and other CVD-related conditions. Thus, due to the unsatisfactory results of cell-based approaches, there has been an increased focus on the alternative solutions, including those related to the utilization of EVs that not only mimic several functional properties of cells of their origin, but also are non-tumorigenic, easy to be stored and may penetrate biological barriers more effectively than the whole cells (125).

It has been widely postulated that EVs from different cell sources may potentially modulate the local microenvironment in a heart tissue toward a regeneration, exhibiting beneficial potential in CVDs treatment (Table 1) (132). The mechanism of EV activity is related to their transfer of bioactive cargo, mainly miRNAs, that are known to be involved in the regulation of cellular processes within a cardiac tissue (133). As an example, pro-regenerative capacity of EVs secreted by iPSC-derived cardiomyocytes was demonstrated to be mediated by the miRNA, indicating the role of miR-106a-363 cluster that represses Notch3 signaling (134). EVs isolated from human iPSCs were also shown to be enriched in several different mRNA and miRNA that may be transferred into human heart-derived cells *in vitro*, improving their cardiac and endothelial differentiation potential, as well as exhibiting cytoprotective effects (135). Similarly, murine iPSCs-EVs were shown to exhibit anti-apoptotic effect in the murine ischemia/reperfusion (I/R) model *via* the delivery of their miR-21 and miR-210 (136). Important role of miR-210 was also reported for MSCs-EVs, that were able to enhance angiogenesis *in vitro*, as well as *in vivo* in murine AMI model. The mechanism of their action was related to the inhibition of Efna3 gene expression, that is known target of miR-210, acting as an angiogenic suppressor (57). As inflammatory process is indispensably related to the cardiac failure, immunomodulatory properties of MSCs-EVs that alleviate immunological response in the site of injury are of particular focus. The role of the miRNA transfer in the immunomodulatory activity of MSC-EVs was demonstrated, pointing a role of miR-182 that inhibited toll-like receptor 4 signaling and thus promoted macrophage polarization from pro-inflammatory to anti-inflammatory phenotype in the murine I/R injury model (137). Additionally, several papers have already indicated cytoprotective and pro-angiogenic effects of MSC-EVs, including murine (138) and rat model of myocardial infarction (127). In another study, administration of EVs secreted by the murine iPSCs improved heart function *in vivo* in the infarction-reperfusion model, without any signs of teratoma, in contrary to the injection of whole cells. Additionally, the therapeutic effect of those EVs was higher when compared to the group of animals treated with iPSCs, resulting in the greater improvement in left ventricle systolic function (128). Promising results of EV use in the small animal models encouraged scientists to follow attempts to test their efficacy also in a large animal models, which are an important step toward translating basic research into clinical practice. Porcine model seems to be the most optimal for the purpose of CVDs due to the several similarities in heart size and coronary circulation to the human heart (139). One of the first studies on the porcine model of AMI have demonstrated that the intracoronary injection of conditioned medium (CM) obtained from the MSCs culture significantly increased left ventricular ejection fraction (LVEF), decreasing the size of infarct zone and reducing the oxidative stress in the residual cells (140). Few years later similar results were also presented for the CM collected from porcine EPCs (141). MSCs-EVs were also used in the nonhuman primate AMI model, demonstrating improved cardiac functions and angiogenesis following vesicle administration, pointing out an important involvement of miR-486 signaling in those processes (142).

Therapeutic effects of EVs were also demonstrated in the CMD model studies, dedicated for an investigation of approaches that would primarily reduce chronic inflammatory state, scar fibrosis and cardiac tissue remodelling, which are a major hallmark of chronic cardiac disfunctions that lead to adverse clinical outcome (143). Cardiac fibrosis is a consequence of differentiation of cardiac fibroblasts into myofibroblasts and their excessive ECM deposition to replace dead cardiomyocytes following an acute injury and inflammatory signaling (144). However, fibrosis-related chronic disfunction of the cardiovascular system may be also a consequence of other factors, such as aging, diabetes mellitus or other metabolic disfunctions with an inflammatory background (145). In the context of EV-based CMD therapeutic approaches, EVs from cardiosphere-derived cells were shown to prevent cardiac remodelling and improve survival in murine non-ischaemic dilated cardiomyopathy model (146), as well as in the rat model of myocarditis (147).

Atherosclerosis is also one of common CVDs that has a strong inflammatory background. It results from the plaque formation inside the large arteries that narrow the vessel lumen. Chronic inflammation plays a pivotal role in the development and progression of atherosclerosis, starting from the activation of resident endothelial cells. Subsequently, it leads to the monocyte and leukocyte

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TABLE 1 Examples of EV use in preclinical studies related to CVDs treatment.

Source of EVs	Model	Major outcomes	References
Murine ESCs	*In vivo* murine I/R model	Augmented neovascularization Enhanced cardiomyocyte survival Reduced fibrosis	(126)
Human BM-MSCs	*In vitro*	Promoted proliferation, migration, and tube formation of HUVEC	(127)
	In vivo rat AMI model	Promoted angiogenesis Improved hemodynamic parameters Reduced infarct size	
Murine iPSCs	*In vitro*	Enhanced angiogenic capacity, migration, and survival of cardiac endothelial cells	(128)
	In vivo murine I/R model	Improved LV systolic function Induced vascularization Reduced apoptosis and hypertrophy	
Human ESC-CVPCs	*In vitro*	Improved cardiomyocyte cell viability and survival Promoted cell migration and tube formation of HUVEC	(105)
	In vivo murine AMI model	Promoted angiogenesis Improved cardiomyocyte survival Reduced scar size	
Human CDCs	*In vivo* porcine AMI model	Decreased infarct size Preserved LV function	(129)
	In vivo porcine CMD model	Attenuated adverse ventricular remodelling Reduced scar Increased proliferation of cardiomyocytes in the peri-infarct zone	
Murine BM-MSCs	*In vivo* murine model of atherosclerosis	Decreased area of atherosclerotic plaques Promoted M2 macrophage polarization	(130)
Murine AT-MSCs	*In vitro*	Decreased adhesion of monocytes to AoEC	(131)
	In vivo murine model of atherosclerosis	Reduced atherosclerotic plaque Decreased inflammatory activation of AoEC	

AMI, acute myocardial infarction; AoEC, aortic endothelial cells; AT-MSCs, adipose derived MSCs; BM-MSCs, bone marrow MSCs; CDCs, cardiosphere-derived cells; CMD, myocardial disease; CVPCs, ESC-derived cardiovascular progenitor cells; ESCs, embryonic stem cell; HUVEC, human umbilical vein endothelial cells; MSCs, mesenchymal stem/stromal cells; iPSCs, induced pluripotent stem cells; LV, left ventricle.

recruitment into atheroma, followed by the upregulation of pro-inflammatory cytokines, production of reactive oxygen species (ROS) and matrix metalloproteinases, consequently triggering thrombotic cascade which may lead to the AMI (148). SCs-EVs display a beneficial effect in the context of atherosclerosis treatment. As an example, administration of BM-MSCs-derived EVs into high-fat diet $ApoE^{-/-}$ mice stimulated M2 polarization of residual macrophages, which led to the decrease in the inflammation and reduction of atherosclerotic plaque area. The mechanism of EV action was possibly related to the transfer of miR-let7 family that regulated activity of downstream signaling pathways, such as NF-κB and PTEN (130). Similar immunomodulatory effect was also shown for AT-MSC-EVs, that diminished inflammatory activation of both aortic endothelial cells stimulated with tumor necrosis factor alpha (TNF-α), as well as LPS-stimulated macrophages *in vitro*, reducing atherosclerotic plaque *in vivo* in low-density lipoprotein (LDL) receptor deficient ($Ldlr^{-/-}$) mice fed with a high-fat diet (131). In another study, EVs from UC-MSCs inhibited activation of eosinophils treated with oxidized LDL and promoted their apoptosis. This effect was even greater for EVs secreted by UC-MSCs overexpressing miR-100, with indicated role of frizzled 5 (FZD5)/Wnt/β-catenin pathway downregulation involved in this process. Decreased inflammation and atherosclerotic plaque following EVs treatment was also confirmed in this study in the murine *in vivo* model (149).

Taking together, SCs-EVs may be a promising factors for CVDs treatment, relying on their immunomodulatory and pro-regenerative activity.

3.2.2 Neurological and neurodegenerative disorders

The central nervous system (CNS)- associated disorders are one of the leading causes of disability and death worldwide. Apart from malfunctions associated with either cancerous processes or acute injuries such as traumatic brain and spinal cord injury or ischemic stroke, neurodegenerative diseases are common feature among CNS pathologies, with prognosed rise in their frequency caused by the increasing life expectancy. They include the most commonly recognized malfunctions such as PD, AD, Huntington's disease or multiple sclerosis (150).

The molecular mechanisms underlying CNS-associated disorders are still poorly understood, but several studies indicate that inflammatory processes play an essential role in their development and progression (151). Thus, further studies are required to fully delineate and develop new approaches of their effective treatment. Among them, use of SCs and their EVs occurred to be a promising strategy (152), with the latter ones being of special focus due to their ability to overcome challenges associated with crossing the BBB. Thus, during a last decade EV-based treatments of CNS-associated

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malfunctions have emerged as potential therapeutic candidates, with several studies reporting neuroprotective effects of EVs secreted by the SCs (Table 2) (160). In *in vitro* models, MSCs-derived EVs were demonstrated to reduce apoptosis, promote proliferation and stimulate secretion of pro-neurotrophic factors by neuroblastoma cell lines (161). On the other hand, EVs produced by AT-MSCs were shown to stimulate differentiation of neural progenitors, influencing miRNA and cytokine expression in the target cells (162). Comparative study demonstrated the ability of EVs derived from both MSCs and iPSCs cells to enhance the astrocyte recovery after irradiation, however vesicles obtained from MSCs exerted superior immunomodulatory effects (163). In another study, EVs secreted by iPSCs-derived neural stem cells were reported to be enriched in miRNAs and proteins known to be involved in neuroprotection, synaptogenesis and cytoprotection, possessing anti-inflammatory activity *in vitro*, as well as *in vivo* in the murine model of status epilepticus, following their intranasal administration (156). Improved recovery and angiogenesis together with reduced neuroinflammation were also reported following injection of EVs from BM-MSCs in rat models of spinal cord injury (164) and traumatic brain injury (165). Similarly, in murine model of focal cerebral ischemia MSCs-derived EVs exerted neuroprotection and neovascularisation, resulting from the regulation of the immune response in the site of injury (166). Apart from the rodent models, neuroprotective activity of human neural stem cell-derived EVs was also reported in porcine model of ischemic brain stroke, where authors presented data confirming reduced edema and intracranial haemorrhage following intravenous administration of EVs (155).

Protective role of EVs was also shown in the several models of neurodegenerative diseases (167). As an example, administration of neuroblastoma-derived EVs lowered the level of amyloid-β peptide (Aβ) that is known to be elevated in AD (168). Similarly, intracerebral injection of MSC-derived EVs in the murine model of AD reduced the level of amyloid plaques, mediated by the transfer of neprilysin protein known as a endopeptidase able to degrade Aβ (154). EVs were also employed as a drug delivery system in murine model of PD, by their loading with antioxidative catalase followed by the EV intranasal delivery, exerting neuroprotective and anti-inflammatory effects *in vitro* and *in vivo* (91). Moreover, in rat model of PD animals treated intranasally with EVs secreted by human exfoliated deciduous teeth SCs exhibited improved gait parameters (169). In another study, MSCs-EVs were shown to cross BBB in rat PD model and lower dopaminergic neuron loss in substantia nigra, concomitantly with an increased level of striatum (153). Protective role of SCs-EVs was also reported for the treatment of multiple sclerosis (MS), as neurodegenerative disease of CNS with the inflammatory background related to the BBB dysfunction and chronic activation of lymphocytes against oligodendrocyte proteins, that leads to the demyelination and synaptopathy (170). As an example, EVs from placental MSCs improved motor function and spinal cord myelination in autoimmune encephalomyelitis murine MS model (158). In another approach, MSCs-EVs were combined with LJM-

TABLE 2 Examples of EV use in preclinical studies related to the therapy of neurological and neurodegenerative disorders.

Source of EVs	Model	Major outcomes	References
Human UC-MSCs	*In vivo* rat PD model	Promoted proliferation of SH-SY5Y cells Reduced dopaminergic neuron loss and apoptosis Increased level of the striatum Relief of an asymmetric rotation defect	(153)
Murine BM-MSCs	*In vivo* murine AD model	Reduced level of amyloid plaques Decreased number of dystrophic neurites	(154)
Human NSCs	*In vivo* porcine ischemic stroke model	Decreased relative swelling of the brain Eliminated intracranial haemorrhage Improved neural tissue preservation and functional levels	(155)
Human iPSCs-derived neural stem cells	*In vitro*	Decreased release of IL-6 from macrophages	(156)
	In vivo murine model of epilepticus status	Enhanced hippocampal neurogenesis Reduced epileptic state Enhanced neurogenesis in hippocampus Reduction of proinflammatory cytokines in hippocampus	
Human AT-MSCs	*In vitro* HD model	Reduced accumulation of mHtt aggregates Increased activation of mitochondria Reduced apoptosis of neural stem cells	(157)
Human PMSCs	*In vitro*	Promoted maturation of oligodendrocytes	(158)
	In vivo murine autoimmune EAE MS model	Improved motor function Increased spinal cord myelination	
Murine BM-MSCs combined with LJM-3064 aptamer	*In vivo* murine MS model	Reduced inflammatory cell infiltration into CNS Protected CNS demyelination Increased percentage of Tregs	(159)

AD, Alzheimer's disease; AT-MSCs, adipose derived MSCs; BM-MSCs, bone marrow MSCs; DCs, dendritic cells; CNS, central nervous system; EAE, encephalomyelitis; HD, Huntington's disease; iPSCs, induced pluripotent stem cells; MS, multiple sclerosis; MSCs, mesenchymal stem/stromal cells; NSCs, neural stem cells; PD, Parkinson's disease; PMSCs, placental derived MSCs; Tregs, regulatory T cells; UC-MSCs, umbilical cord Wharton's jelly MSCs.

3064 aptamer with previously demonstrated ability to induce remyelination. Such engineered hybrid particles exhibited anti-inflammatory activity and protected against CNS demyelination in murine MS model *in vivo* (159). Altogether, there has been accumulating evidence on the role of EVs in the treatment of different types of CNS-associated disorders.

3.2.3 Kidney injury

Proper functioning of kidneys is essential for the effective control of body fluids osmolarity, pH and removal of toxic metabolites. Thus, kidney injuries are life-threatening conditions resulting in the dysregulation of homeostasis (171). One of the most severe kidney disorders is acute kidney injury (AKI) that accompanied by the systemic inflammation leads to the rapid damage of organ structure followed by a loss of renal function, with the need of patient hospitalisation, high mortality rate and high risk of the development of chronic kidney dysfunction (172). Thus, the development of effective therapeutic approaches for the AKI treatment is an important challenge of the modern medicine. EVs play an important role not only as prognostic factors and biomarkers of renal disfunction, but have also been demonstrated as potential new-generation tools for the therapy of AKI (Table 3) (180). Importantly, an inflammatory response accompanying AKI has been widely reported to be significantly ameliorated by EVs from MSCs *via* their immunomodulatory stimuli. Meta-analysis study collecting the data from 31 preclinical studies on rodents have confirmed the therapeutic efficacy of MSC-EVs in AKI treatment (181). As an example, in the rat renal ischemia-reperfusion injury model, BM-MSCs-derived EVs inhibited apoptosis and stimulated tubular epithelial cell proliferation (182). In other study, EVs derived from native, but not from interferon gamma (IFN-γ)- stimulated UC-MSCs were able to alleviate the effect of hypoxia-induced AKI in the rat model (183). As nephrotoxicity is an important issue in oncological patients, being caused by the widely-used chemotherapeutic agents such as cisplatin, there is a need for the new therapeutic strategies that would reduce severe side effects related to the chemotherapy and improve clinical outcomes of patients. In the studies where rat cisplatin-induced AKI model was used, EVs secreted by UC-MSCs (184) and AT-MSCs (185) were able to exhibit cytoprotective activity, reducing cell death and inflammatory response. Additionally, in the murine model of cisplatin-induced AKI, EVs secreted by BM-MSCs improved renal function, but the effect was dependent on the route of EV administration, with multiple injections being beneficial over the single dose (186). The pro-regenerative activity in the context of renal function was also demonstrated for EVs from different types of cells. For instance, EVs from amniotic epithelial cells were shown to reduce nephrotoxicity in the murine model of cisplatin-induced AKI (187). ESC-EVs were also demonstrated to exhibit pro-regenerative effect in the murine model of ischemia-reperfusion AKI, by stimulating angiogenesis and proliferation of renal epithelial cells, as well as reducing renal fibrosis. These observations correlated with the activation of the resident Sox9+ cells that are known to be involved in the processes of formation and regeneration of renal tubular epithelium (104).

Apart from AKI, EVs were shown to exhibit immunoregulatory, cytoprotective and pro-regenerative activity in a treatment of chronic kidney disease (CKD) that leads to the progressive nephropathy. One

TABLE 3 Examples of EV use in preclinical studies related to the treatment of kidney diseases.

Source of EVs	Model	Major outcomes	References
K-MSCs	*In vivo* AKI murine model	Promoted angiogenesis Decreased cell apoptosis	(173)
Murine BM-MSCs	*In vitro*	Reversed changes in the morphology and expression of E-cadherin and α-SMA in HK2 cells	(174)
	In vivo murine CKD model	Protection against unilateral ureteral obstruction Enhancement of the expression of α-SMA and E-cadherin in kidney Reduced tubular damage	
	In vitro	Suppressed ER stress Protection of cells against damage and apoptosis Promoted proliferation of renal tubular epithelium	(175)
	In vivo murine kidney I/R model	Suppressed ER stress Protection against renal I/R injury	
	In vitro	Attenuated morphological changes and restored EMT in HK2 cells	(176)
	In vivo murine UUO model	Ameliorated renal function Decreased interstitial lymphocyte infiltration	
Murine AT-MSCs	*In vivo* murine AKI model	Promoted functional kidney recovery Decreased apoptosis of tubular epithelial cells	(177)
	In vivo rat CKD model	Reduced pathological changes and renal fibrosis Protection of kidneys against inflammation, mitochondrial dysfunction, and apoptosis	(178)
Rat BM-MSCs	*In vitro*	Prevented SMAD2/3 and ERK1/2 phosphorylation in HK2 cells	(179)
	In vivo rat CKD model	Inhibition of renal fibrosis Ameliorated renal function and morphology	

AKI, acute kidney injury; AT-MSCs, adipose derived MSCs; BM-MSCs, bone marrow MSCs; CKD, chronic kidney disease; EMT, epithelial-mesenchymal transition; ER, endoplasmic reticulum; HK2, human kidney 2 cells; I/R, ischaemia-reperfusion; K-MSCs, kidney-derived MSCs; UUO, unilateral ureteral obstruction; αSMA, alpha smooth muscle actin.

of the important causes of CKD is renal hypoxia and persistent inflammation, which lead to the kidney fibrosis (188). Due to the complexity of CKD pathogenesis, current pharmacological treatments are unsatisfactory (189). The therapeutic effect of EVs in CKD treatment was demonstrated in meta-analysis covering the results from 35 studies, that mostly based on the unilateral ureteral obstruction (UUO) model of this disease (190). Protective, anti-inflammatory and anti-fibrotic role of MSC-EVs in the chronic renal dysfunction was observed both *in vitro* and *in vivo* (176), with an indication on an important role of EV-based miRNA transfer involved in those processes (Table 3) (179).

3.2.4 Liver disfunctions

Liver disfunctions, including acute injuries and chronic diseases, are considered as a significant burden experienced by many individuals, that may consequently lead to the life-threatening conditions such as end-stage cirrhosis, fibrosis or liver malignancies. Still, one of the standard therapeutic approaches is a liver transplantation. However, due to the limited availability of donors, mortality from liver-related malfunctions continues to be a critical issue, that raises the urgent need for an effective, alternative therapies for the liver replacement (191).

Liver-related diseases may be caused by alcohol, drugs, metabolic diseases or viral hepatitis. In terms of the treatment of liver disfunctions, the major goal is to inhibit fibrosis related to the chronic liver disease, that causes hepatic dysfunction, activation of hepatic stellate cells, excessive deposition of ECM and immunological response to the local inflammation (192). Thus, anti-fibrotic and anti-inflammatory therapeutic strategies including treatment with EVs, are of current interest. Indeed, several experimental approaches have demonstrated the effectiveness of different types of SC-derived EVs in ameliorating liver disfunctions (Table 4). As an example, iPSC-derived EVs were shown to supress fibrosis in two murine models of liver injury, caused by either treatment with CCl_4 or by bile duct ligation (193). EVs secreted by MSCs differentiated from ESCs were also reported to alleviate thioacetamide-induced chronic liver injury, reducing cirrhosis and pro-fibrotic production of collagen I and α-smooth muscle actin (αSMA), with simultaneous decrease in the pro-apoptotic and pro-inflammatory factors (198). Similar antifibrotic effect was also demonstrated in CCl_4-induced liver fibrosis for EVs isolated from UC-MSCs and the mechanism of their action was related to the inhibition of epithelial-to-mesenchymal transition (EMT) of hepatic cells (194). In another study, UC-MSCs-derived EVs were also demonstrated to ameliorate acute liver injury due to the antioxidative and antiapoptotic effect (199). It was also shown that hepatocyte-derived EVs are able to alleviate inflammatory response and pro-fibrotic activation of hepatic stellate cells, as well enhance proliferation of hepatocytes, both *in vitro* and *in vivo* in the murine model of CCl_4 injury (195).

3.2.5 Respiratory system diseases

Involvement of EVs in the respiratory system is also well documented, with their role not only as potential biomarkers, but also as therapeutic agents, regulating the immune cell functions during airway inflammatory diseases (Table 5) (207). Particularly, MSCs-EVs hold a great promise as factors mimicking beneficial immunomodulatory properties of their parental cells, thus augmenting inflammatory response typically associated with the respiratory system malfunction and tissue damage (208). Additionally, possibility to administer EVs *via* inhalation facilitates their entry into pulmonary system and targeted delivery of their cargo into the site of interest.

Among the variety of lung diseases, one of the most severe conditions are related to the acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) that carry high morbidity and mortality rates, resulting from the rapid respiratory failure (209). In porcine model of influenza-induced ALI, intratracheal administration of porcine MSC-EVs resulted in diminishing lung injury, inhibition of virus replication in lung epithelial cells *in vitro* and *in vivo* and reduction of inflammation within the lung tissue (202). MSCs-EVs were also shown to alleviate alveolar inflammation and pulmonary edema in *E. coli* endotoxin-induced ALI (90). Similarly, in *ex vivo* perfused human model of *E. coli*-driven pneumonia, MSCs-EVs increased alveolar fluid clearance and

TABLE 4 Examples of EV use in preclinical studies related to the treatment of liver dysfunctions.

Source of EVs	Model	Major outcomes	References
Human iPSCs	*In vitro*	Modulation of the profibrogenic transcriptome profile in activated HSCs	(193)
	In vivo murine model of ameliorating liver disfunctions	Reduced development of fibrosis	
Human UC-MSCs	*In vivo* murine CCl4-induced liver fibrosis model	Reduced development of fibrosis Reduced expression of collagen I and III Inactivation of TGF-b1/Smad signaling pathway	(194)
Mouse hepatocytes	*In vivo* murine hepatic fibrogenesis model	Reduced inflammation Reduced development of fibrosis Suppressed monocyte/macrophage infiltration	(195)
LX-2	*In vitro*	Decreased proliferation and invasion of HCC	(196)
	In vivo murine model of HCC	Reduced tumor size Increased apoptosis of HCC	
Human AT-MSCs	*In vitro*	Increased chemosensitivity of HCC cells	(197)
	In vivo murine model of HCC	Increased sensitiveness of HCC to chemotherapeutic agents	

AT-MSCs, adipose derived MSCs; HCC, hepatocellular carcinoma; HSCs, hepatic stellate cells; iPSCs, induced pluripotent stem cells; UC-MSCs, umbilical cord Wharton's jelly MSCs.

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TABLE 5 Examples of EV use in preclinical studies related to the treatment of the respiratory system diseases.

Source of EVs	Model	Major outcomes	References
Human UC-MSCs	*In vivo* murine model of asthma	Reduced inflammatory response and airway remodelling Prevented lung remodelling Reduced inflammatory cell infiltration Decreased level of pro-inflammatory cytokines Inhibited TGF-β1-Smad2/3 signaling pathway	(200)
	In vivo murine model of lung ischemia-reperfusion injury	Attenuated inflammation and edema Attenuated activation of iNKT cells and macrophages Decreased level of pro-inflammatory cytokines Inhibition of macrophage and iNKT cells activation	(201)
Porcine BM-MSCs	*In vitro*	Inhibition of virus replication in lung epithelial cells Inhibition of virus-induced apoptosis replication in lymphatic endothelial cells	(202)
	In vivo porcine model of influenza-induced ALI	Inhibition of virus replication in lung epithelial cells Reduced lung injury Attenuated level of pro-inflammatory cytokines	
Human BM-MSCs	*In vivo* murine model of lung injury	Decreased lung vascular endothelial permeability	(203)
	Ex vivo human model of pneumonia	Improved alveolar fluid clearance in lungs Reduced level of bacteria	(204)
Human Amnion Epithelial Cells	*In vivo* murine model of idiopathic pulmonary fibrosis	Prevention against lung injury	(205)
Human iPSC-MSCs	*In vivo* murine model of asthma	Ameliorated allergic airway inflammation Alleviation of airway hyperresponsiveness Decrease of inflammatory cell infiltration	(206)

ALI, acute lung injury; BM-MSCs, bone marrow MSCs; iNKT, invariant natural killer T cells; iPSCs, induced pluripotent stem cells; MSCs, mesenchymal stem/stromal cells; UC-MSCs, umbilical cord Wharton's jelly MSCs.

antimicrobial activity of macrophages. This effect was even enhanced by the pre-treatment of parental MSCs with Toll-like receptor 3 agonist (204). In the murine model of lung injury EVs secreted by BM-MSCs decreased the lung vascular endothelial permeability caused by the of haemorrhagic shock, with possible involvement of the mechanism related to the reduction of cytoskeletal RhoA signaling activity (203). In addition, anti-inflammatory, protective and/or regenerative properties of MSC-EVs have also been observed in rodent models of pulmonary hypertension (210), radiation-induced injury (211), bronchopulmonary dysplasia (212) and idiopathic pulmonary fibrosis (213). In the last one the regenerative effect has been also demonstrated for EVs secreted by iPSCs (214). Additionally, in the murine model of lung ischemia-reperfusion injury, administration EVs derived from MSCs attenuated inflammation and edema (201). Similar outcome was also reported in the rat model, indicating an influence of EVs on the expression of genes regulating inflammation and oxidative stress (215).

Beneficial effect was also shown for MSCs-EVs in rodent models of asthma as one of the common manifestations of immune system overactivation. As an example, it was demonstrated that vesicles secreted by UC-MSCs were able to reduce inflammatory response and airway remodelling. Importantly, this effect was boosted for animals that received EVs from hypoxia-stimulated cells (200). Similarly, MSCs-EVs were shown to inhibit group 2 innate lymphoid cells (ILC2s) that are known to be involved in the pathogenesis of airway allergy. Additionally, those EVs were able to reduce the level of pro-inflammatory cytokines and mucus production in the murine model of asthma, with the suggested role of miR-146a transfer involved in this effect (206).

3.2.6 Digestive system dysfunctions

Anti-inflammatory and immunomodulatory properties of EVs make them a promising option for the treatment of diseases associated with the digestive system that are typically related to the multimodal gut inflammation (216). Indeed, several attempts were performed in this field so far (Table 6). As an example, in the murine *in vivo* model of ulcerative colitis induced by the dextran sulphate sodium treatment, EVs from BM-MSCs ameliorated disease symptoms, including colon mucosa damage, by stimulating polarization of macrophages into anti-inflammatory M2 phenotype through the modulation of JAK/STAT signaling pathway (217). In another study, a suppressive influence of BM-EVs on macrophage activity was also demonstrated in murine model of inflammatory bowel disease (IBD), resulting in an improved gut functions and decreased mucosal inflammation (222). From another point of view, EVs from M2 macrophages were also reported to attenuate colitis in mice and their mode of action was related to the stimulation of Tregs *via* CCL1 chemokine (218).

3.2.7 Skin damage

Skin as the largest organ in the body plays an important role in the maintenance of homeostasis and provides a protective barrier against external hazardous factors, thus, is constantly exposed to potential severe injuries, including thermal and chemical burns, chronic wounds or persistent microbial infections, that may lead to the fatal trauma (223). SCs-EVs were used for the treatment of inflammatory skin diseases (Table 7). As an example, EVs from AT-MSCs diminished symptoms of atopic dermatitis in the murine *in vivo* model of this disease, induced by the dust mite treatment of animals.

TABLE 6 Examples of EV use in preclinical studies related to the treatment of the digestive system disorders.

Source of EVs	Model	Major outcomes	References
Murine BM-MSCs	*In vivo* murine model of ulcerative colitis	Attenuated colon mucosa damage Promoted polarization of M1 macrophages to the M2 state Suppressed inflammatory response	(217)
Murine M2 macrophages	*In vivo* murine model of colitis	Attenuated colitis Alleviated colon damage Increased percentage of Tregs Decreased level of pro-inflammatory cytokines	(218)
Grapefruit pulp	*In vivo* murine model of DSS-induced colitis	Enhanced anti-inflammatory capacity of intestinal macrophages Maintained intestinal macrophage homeostasis Decreased level of pro-inflammatory cytokines	(219)
Murine blood serum	*In vivo* murine DSS-induced colitis	Decreased permeability in colon tissues	(220)
Murine Tregs	*In vitro*	Promoted proliferation and inhibited apoptosis of YAMC cells	(221)
	In vivo murine model of DSS-induced colitis	Alleviated IBD	
Human BM-MSCs	*In vivo* murine model of IBD	Suppressed inflammatory response Reduced development of fibrosis Promoted M2 polarization of macrophages Decreased permeability of colon tissue	(222)

BM-MSCs, bone marrow MSCs; DSS, dextran sulfate sodium; IBD, inflammatory bowel disease; Tregs, regulatory T cells; YAMC, conditionally immortalized mouse colon epithelial cell line.

Following administration of EVs the number of eosinophils and serum IgE decreased, together with the reduction of pro-inflammatory cytokines levels in the skin lesions (230). Similarly, in the *in vivo* model of oxazolone-induced dermatitis, AT-MSCs-EVs reduced inflammation, as well as improved ceramide production and epidermal barrier, preventing skin water loss (231). In another study, EVs from UC-MSCs reduced excessive proliferation of epidermis cells, decreased expression of interleukin IL-17 and IL-23, as well as inhibited activation of DCs is the murine model of psoriasis (227).

Recent studies have also shown beneficial effect of EVs in skin regeneration (232). For instance, subcutaneously injected EVs isolated from iPSCs-derived MSCs enhanced angiogenesis and re-epithelialisation, leading to the wound closure. Additionally, they also stimulated proliferation of skin fibroblasts and ECM production (225). In another study of murine full-thickness skin wound model, EVs from UC-MSCs promoted proliferation and migrative capacity of both endothelial cells and skin fibroblast, as well as improved angiogenesis *in vitro*, with improved re-epithelialisation demonstrated *in vivo* (226). Similarly, in the context of chronic wound treatment, UC-MSCs-derived EVs applied in the hydrogel formulation onto the wound accelerated skin healing and regeneration in the diabetic rat model (233). Interestingly, EVs from AT, but not from BM were able to enhance skin healing in murine model of diabetic murine model. These differences corresponded to the differential cargo in both types of EVs, with predominant role of BM-MSCs-EVs and AT-MSCs in promotion of skin cells proliferation and angiogenesis, respectively (228). On the other hand, in another study there was no significant difference in the pro-regenerative potential of MSCs from both BM and AT in such model, which may indicate the variance in the mechanism of action between cells and their secretory vesicles (234). An importance of immunomodulatory signaling mediated by MSCs-EVs was also demonstrated for the skin damage treatment. As an example, EVs from melatonin-preconditioned BM-MSCs triggered macrophage M2 polarization, resulting in the decrease of pro-inflammatory cytokines and increase in the expression of anti-inflammatory IL-10, enhancing angiogenesis and healing in rat diabetic wound model (229).

EVs derived from iPSCs may be also used for the purpose of skin regeneration. Importantly, due to the higher "stemness" potential of iPSCs when compared to MSCs, scientist attempt to utilize these properties in the context of antiaging skin treatment. As an example, dermal fibroblasts treated with hiPSCs-EVs possessed higher proliferative capability and thus lowered senescence. Additionally, UVB-stimulated photoaging process in those cells was also decreased following hiPSCs-EVs treatment (224). Similar results were obtained by another group, which demonstrated that "cell-engineered nanovesicles" obtained by the serial membrane extrusion of human iPSCs augmented senescent alterations in skin fibroblasts (235). Nevertheless, EVs from MSCs were also used in several studies related to the protection against skin aging. In one of studies, AT-MSCs-derived EVs attenuated UVB-triggered photoaging both *in vitro*, as well as in the murine *in vivo* model, and their mechanism of action was related to the inhibition of inflammatory-induced macrophage differentiation and ROS production, resulting in lower wrinkle scoring (236). Interestingly, direct comparison study have revealed higher antiaging effect of EVs derived from hiPSCs than MSCs (110).

3.2.8 Pain

Fighting the chronic pain that accompanies several inflammatory-related diseases is still a challenging aspect of medicine. There are several attempts reporting the possible usage of EVs in the pain treatment (Table 8) (242). In one of the studies, UC-MSCs-EVs were used as a therapeutic agents in the rat model of neuropathic pain caused by the nerve injury. Intrathecal administration of EVs resulted in the reduced symptoms of pain and lower hind paw hypersensitivity, decreasing the expression of pro-inflammatory factors in dorsal root ganglion in the site of injury (237). In another report, intra-articular administration of secretome obtained from BM-MSCs stimulated with TNF-α and IFN-γ and ameliorated pain

TABLE 7 Examples of EV use in preclinical studies related to the treatment of skin dysfunctions.

Source of EVs	Model	Major outcomes	References
Human iPSCs	*In vitro* model of skin aging	Increased proliferation and migration of skin fibroblasts Decline in UVB-stimulated photoaging Decreased level of matrix-degrading enzymes	(224)
Human iPSCs-derived MSCs	*In vivo* rat skin wound healing model	Enhanced angiogenesis Increased proliferation of the skin Improved reepithelialisation	(225)
Human UC-MSCs	*In vivo* murine full-thickness skin wound model	Promoted proliferation and migrative of endothelial cells and skin fibroblast Improved re-epithelialisation Reduced level of proliferation suppressor genes	(226)
	In vivo murine model of psoriasis	Reduction of excessive epidermis proliferation Decreased level of pro-inflammatory cytokines	(227)
Human BM-MSCs	*In vitro*	Promoted viability of fibroblast, keratinocyte, and endothelial cells Induced endothelial cell migration	(228)
	In vivo murine model of diabetic skin healing	Accelerated wound closure Increased epithelial thickness	
	In vivo rat diabetic wound healing model	Increased macrophage M2 polarization Enhanced angiogenesis and healing Suppressed level of pro-inflammatory factors	(229)
Human AT-MSCs	*In vivo* murine model of atopic dermatitis	Decreased number of eosinophils and serum IgE Decreased level of pro-inflammatory cytokines Reduced inflammation	(230)

AT-MSCs, adipose derived MSCs; BM-MSCs, bone marrow MSCs; iPSCs, induced pluripotent stem cells; MSCs, mesenchymal stem/stromal cells; UC-MSCs, umbilical cord Wharton's jelly MSCs.

in the murine model of osteoarthritis (238). Moreover, EVs secreted by iPSCs-derived MSCs decreased tendinopathy-related pain symptoms in rat model *in vivo*, alleviating inflammation and enhancing proliferation of tenocytes (239). Not only EVs from SCs, but also immune cells may have the ability to reduce inflammation-related pain symptoms. For example, in the murine inflammatory pain model EVs from M2 macrophages were able to transfer miR-23a to microglia, increasing threshold of mechanical allodynia and thermal hyperalgesia *via* regulation of NF-E2-related factor 2 (NRF2) (241). Altogether these reports indicate that EVs may serve as a potential factors for the anti-pain treatment approaches.

3.2.9 COVID-19

Coronavirus infectious disease 2019 (COVID-19) caused by the severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2), was first time reported in Wuhan, China in a late 2021 and has rapidly spread over the world, emerging as a global pandemic issue. Till August 2022, COVID-19 affected more than half billion of people worldwide, causing death of more than 6 million (243). SARS-CoV-2 infects host cells by interaction of its spike protein with angiotensin converting enzyme 2 (ACE2) receptor, present on several types of epithelial and endothelial cells (244). Main clinical manifestations of this disease are related to the respiratory system, including strong cough, hypoxia, pneumonia and ARDS. However, it may also manifest by multiorgan disfunction, including cardiovascular, nervous or gastrointestinal system. COVID-19 is typically accompanied by mild to moderate flu-like inflammatory symptoms such as fever, muscle ache and general weakness, but in many individuals may lead to the acute cytokine storm, sepsis and in a consequence death (245). Long-term post-COVID complications were also widely reported, with multiple health issues that may last for several months from the moment of infection (246). COVID-19 outbreak has not only caused a death of many people, but also dramatically affected international economy, impacted global healthcare and negatively influenced a social life (247). Thus, increasing number of cases has raised a global pressure to find effective ways of COVID-19 prevention and effective treatment. Despite the rapid development of emergency vaccination, still its accessibility is not uniform, with accompanied hesitancy of the part of the society against the common vaccination. Additionally, there's a lack of specific and highly effective treatment against COVID. One of the crucial issues is to inhibit uncontrolled hyperactivation of immune system that leads to the cytokine storm and consequently to the multiorgan damage (248).

It was shown that EVs may be considered not only as biomarkers of COVID-19 outcome (249), but also as immunomodulatory agents that may ameliorate inflammatory complications and improve the clinical outcome of patients (Table 9) (254). In this respect, MSCs-EVs are predominantly tested as cell-free alternatives mimicking immunosuppressive properties of their cells of their origin. As an example, the potential of EVs from UC-MSCs to decrease the release of pro-inflammatory cytokines was demonstrated *in vitro* on human lung adenocarcinoma epithelial cells stimulated with SARS-CoV-2 peptides (250). Another study has demonstrated safety and efficacy of intravenous administration of BM-MSCs-derived EVs to 24 COVID-19-positive patients with moderate or acute ARDS. Additionally,

TABLE 8 Examples of EV use in preclinical studies related to the pain treatment.

Source of EVs	Model	Major outcomes	References
Human UC-MSCs	*In vivo* rat model of neuropathic pain	Reduced pain symptoms Decreased the expression of pro-inflammatory factors	(237)
Human BM-MSCs	*In vivo* mouse model of osteoarthritis	Ameliorated pain Protective effect on cartilage damage	(238)
Human iPSCs-derived MSCs	*In vivo* rat model of tendinopathy-related pain	Ameliorated pain Enhanced proliferation of tenocytes Down-regulation of the gene expression-related to inflammation	(239)
Mouse NSCs	*In vivo* rat model spinal cord injury	Reduced neuronal apoptosis Decreased microglial activation Attenuated neuroinflammation	(240)
Macrophages	*In vivo* model of murine inflammatory pain	Alleviated inflammatory pain	(241)

BM-MSCs, bone marrow MSCs; iPSCs, induced pluripotent stem cells; MSCs, mesenchymal stem/stromal cells; NSCs, neural stem cells; UC-MSCs, umbilical cord Wharton's jelly MSCs.

following EV treatment an improved oxygenation ratio and decreased inflammatory status was also reported, which opened a possibility for the further studies, including clinical trials on the higher number of patients (251). Interestingly, elevated number of EVs possessing ACE2 receptor were found in the plasma of COVID-19 patients and were shown to inhibit binding of viruses and their spike protein to HEK cells *in vitro*, as well as to ameliorate severity of this disease in the rodent model (252). There are also attempts to use EVs as vaccines against SARS-CoV-2 infection. As an example, EVs derived from *Salmonella typhimurium* decorated by spike receptor-binding domain were used as immunization factors in syrian hamster COVID-19 model, exerting the effective production on neutralizing antibodies against few variants of SARS-CoV-2 (253). Altogether, use of EVs as immunoregulatory factors may open a new perspectives of COVID-19 treatment and prevention.

3.2.10 Osteoarthritis

Osteoarthritis (OA) is a type of chronic degenerative disease of an articular cartilage. Consequently, it leads to the progressive inflammation, pain and joint dysfunction, predominantly in the knees, but also hips and fingers. It has been indicated as one of the ten most disabling disorders in the developed countries, with about 10% of men and close to 20% of women aged over 60 years to have symptomatic OA. Apart from age, major risk factors associated with OA are joint injuries and obesity (255). Currently available therapeutic approaches are limited and concentrate mainly either on temporal, pharmacological pain relief and reduction of inflammation, or on the invasive surgical interventions and joint replacement (256).

SCs-EVs were proven to support OA treatment, with the special regard to those secreted by MSCs (Table 10). As an example, EVs from BM-MSCs were reported to increase the expression of type II collagen and aggrecan, with reduction of metalloproteinase 13 and iNOS, in OA-like chondrocytes *in vitro*. Additionally, they exhibited anti-inflammatory and cytoprotective effect *in vivo*, decreasing cartilage and bone degeneration in the knee joint in collagenase-induced murine OA model (257). In another study, BM-MSCs-EVs reduced expression of pro-inflammatory cyclooxygenase 2 (COX2) and NFκB signaling, with simultaneous enhancement of the proteoglycan and type II collagen level in TNF-α-stimulated chondrocytes derived from OA patients (258). Similarly, EVs isolated from AT-MSCs exhibited chondroprotective effect on IL-1β-stimulated OA chondrocytes *in vitro*, diminishing secretion of pro-inflammatory factors (TNF-α, IL-6, prostaglandin E2, nitric oxide, COX2) and increasing level of IL-10 and type II collagen (259). Furthermore, UC-MSCs-EVs had immunomodulatory effect in OA model *in vitro* and *in vivo*, promoting M2 macrophage polarization and secretion of anti-inflammatory IL-10, as well as inhibiting cartilage degradation. The mechanism of their action was related to miRNA cargo known to regulate PI3K pathway in targeted cells (260). Altogether, these data demonstrate the chondroprotective and immunomodulatory activity of EVs in the context of potential OA treatment.

TABLE 9 Examples of EV use in preclinical studies related to the COVID-19 treatment.

Source of EVs	Model	Major outcomes	References
Human UC-MSCs	*In vitro*	Reduced SARS-CoV2-induced inflammatory cytokines Decreased level of NF-κB-p65	(250)
Human BM-MSCs	*In vivo* SARS-CoV2 positive patent	Improved oxygenation ratio Decreased inflammatory status	(251)
Human HEK	*In vitro*	Inhibited binding of viruses to HEK cells	(252)
	In vivo murine SARS-CoV2 model	Ameliorated the symptoms of the disease	
Salmonella typhimurium	*In vivo* Syrian hamster SARS-CoV-2 model	Production on neutralizing antibodies Decreased size of inflammatory focal patches	(253)

BM-MSCs, bone marrow MSCs; HEK, human embryonic kidney cells; MSCs, mesenchymal stem/stromal cells; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; UC-MSCs, umbilical cord Wharton's jelly MSCs.

TABLE 10 Examples of EV use in preclinical studies related to the treatment of OA.

Source of EVs	Model	Major outcomes	References
Murine BM-MSCs	*In vitro* model of OA	Restored homeostasis in OA-like chondrocytes Decreased apoptosis of chondrocytes Decreased expression of pro-inflammatory factors	(257)
	In vivo murine model of OA	Reduced degradation of cartilage and bone	
Human BM-MSCs	*In vitro* model of OA	Decreased level of pro-inflammatory cytokines Decreased expression of NF-κB-p65 Promoted production proteoglycan by chondrocytes Enhanced proliferation of chondrocytes	(258)
Human AT-MSCs	*In vitro* model of OA	Reduced production of inflammatory mediators Decreased expression of iNOS	(259)
Human UC-MSCs	*In vitro*	Increase of macrophage M2 polarization	(260)
	In vivo rat model of OA	Inhibited cartilage degradation	

AT-MSCs, adipose derived MSCs; BM-MSCs, bone marrow MSCs; iNOS, inducible nitric oxide synthase OA, osteoarthritis; UC-MSCs, umbilical cord Wharton's jelly MSCs.

3.2.11 Cancer

Immunoregulatory capability of EVs makes them an attractive option for the treatment of cancer, as one of the leading causes of death worldwide. Oncological immunotherapy is one of the rapidly developing treatments, targeted to stimulate immune system toward anti-cancer defence, that includes checkpoint blockade therapies, use of chimeric antigen receptor (CAR) T-cells and cancer vaccines. Currently, there are attempts to use preparations containing EVs as anti-cancer vaccines (Table 11) (264). This strategy relies on the use of EVs secreted by the cancer cells or by APCs, with the special focus on DCs. The latter ones were shown to contain functional MHC class I and II antigens, as well as co-stimulatory molecules capable to activate the anti-tumor response of cytotoxic T cells (265). Moreover, utilization of autologous tumor-derived EVs harbouring cancer-specific antigens as nanovaccines opens new possibilities of the development of personalized anti-cancer treatment. However, due to the low immunogenicity of autologous EVs from cancer cells, there are attempts to combine them with other factors that would enhance anti-tumor response of immune system. As an example, researchers created hybrid nanoparticles by combining EVs of tumor and *E. coli* origin, that were able to stimulate maturation of DCs and trigger strong anti-tumor immune response in colon, melanoma and breast cancer murine models (262). In another study, cell membrane vesicles from melanoma cells were combined with CpG oligonucleotides, TLR-9 agonist and DCs-targeting aptamer, enabling specific activation of immune system against cancer, together with a long-term immune memory effect (263).

Additionally, SCs-EVs were also shown to exhibit anti-cancer activity. In particular, EVs isolated from BM-MSCs inhibited proliferation of HepG2 hepatoma, Kaposi's sarcoma, and ovarian tumor cell lines, inducing cancer cell death *in vitro*, as well as exhibiting anti-tumor activity following subcutaneous injection of EVs in the *in vivo* experiments (261). Similar results were also demonstrated for UC-MSCs-derived EVs in the model of bladder tumor (266). Thus, EV-based approaches may be a novel, promising strategy for the anti-cancer therapy.

3.3 Challenges and perspectives

Rapidly developing knowledge on EV biology and their functions result in the growing number of attempts to use EVs as new-generation tools in the regenerative medicine, as well as in several other biomedical fields. One on them is the attempt to use EVs as biological nanoparticles for the transport and targeted delivery of drugs and other biologically active particles, which relies on an intrinsic activity of EVs as mediators of cell-to-cell communication (267). As an example, in one study curcumin-loaded EVs were able to reduce pro-inflammatory signalling in macrophages *in vitro* more effectively, when compared to the curcumin itself, which demonstrates that EV-based strategy enhances bioavailability of this low-soluble compound. Additionally, survival rate of animals in the LPS-induced sepsis model was also significantly higher for EV-curcumin group, comparing to animals treated only with curcumin (268). Similarly, EVs from immature dendritic cells were also used to deliver anti-tumor agent-doxorubicin that was loaded to them *via* the electroporation. Such EVs were then demonstrated to specifically target tumor cells, inhibiting their growth both *in vitro* and *in vivo* (269). Interestingly, there are indications that EVs may be taken up by acceptor cells more effectively when compared to the liposomes, with the simultaneous high efficiency of EV "loading" with particular bioactive molecules (270). Additionally, due to their endogenous origin, EVs are envisioned as less immunogenic and cytotoxic when compared to the synthetic nanoparticles (271). Importantly, EVs have also been shown to be able to deliver siRNA to the murine brain *in vivo*, which opens new possibilities for the development of new, drug-carrying particles capable to cross BBB, which so far is an important factor limiting the effectiveness of the neurological diseases therapy (272).

EVs are promising therapeutic options that have additional potential to be engineered, both on the level of their parental cells and after their secretion. First approach includes cell preconditioning or genetic engineering, whereas second one bases e.g. on loading of EVs with particular therapeutic compounds. Such modification of "native" EVs may help to develop approaches to either overcome limitations related to EV use or to boost their therapeutic efficacy, targeted delivery or stability, which widens further possibilities of EV utilisation in the future biomedical applications (273).

3.3.1 Pitfalls and limitations of EV utilisation

Despite significant progress in the field, there are still several limitations of broader use of EVs in biomedical sciences. Translation

TABLE 11 Examples of EV use in preclinical studies related to the treatment of cancer.

Source of EVs	Model	Major outcomes	References
Human BM-MSCs	*In vitro*	Inhibited proliferation and viability of HepG2, Kaposi, and Skov-3 cell lines	(261)
	In vivo murine cancer model	Inhibition of tumor growth	
Escherichia coli combined with tumour cells	*In vivo* murine cancer model	Stimulated maturation of DCs Regression of tumor	(262)
Murine melanoma cells combined with CpG oligos, TLR-9 agonist, and DCs-targeting aptamer	*In vivo* murine melanoma model	Stimulated maturation of DCs Stimulated specific activation of immune system against cancer	(263)

BM-MSCs, bone marrow MSCs; DCs, dendritic cells; HepG2, human liver cancer cell line; TLR-9, Toll-like receptor 9.

of the basic science into the clinics encounters critical challenges and obtained EV preparations have to fulfil several stringent, but still not fully defined criteria, that include variety of quantitative and qualitative properties. Importantly, constantly increasing knowledge on EV biology raises new questions and doubts on their identity, optimal methods of isolation, as well as methodological barriers of their characterization (274). So far, several key aspects have been recognized as potential hindrances of EV utilization in pre-clinical and clinical studies.

One of the pitfalls is to obtain a pure EV fraction without accompanying non-vesicular entities such as protein complexes, lipoproteins or extracellular RNA, that are typically co-isolated by commonly used isolation methods such as ultracentrifugation (275). On the other hand, other methods that include elimination of concomitant impurities may cause significant reduction of EV yield, which is an important hindrance in terms of the medical use of EVs, where high amounts of EV preparations are required (276). Additionally, recent findings have demonstrated that the "protein corona" which surrounds EVs may be also needed for their biological activity and its removal by additional steps of EV purification may not be beneficial (277). Nevertheless, isolation method is one of the crucial factors that may influence functional properties of EVs and affect their downstream applications.

Another important difficulties to be overcome is a rapid macrophage-dependent clearance of EVs from the circulation (278) and their off-target biodistribution that lowers the level of EV accumulation in the site of interest (36). There are several factors influencing distribution of EVs after their *in vivo* uptake, including route of administration, dosing, cell source (279) and the size of EVs (280), that should be taken under the consideration during the design of EV-related studies.

Moreover, one of the critical bottlenecks in the clinical application of EVs is a lack of unified protocols of their isolation and characterization. Thus, there is also an urgent need for the development of reliable standarization and validation approaches, that would implement rigorous, complementary characterisation methods and would assure no batch-to-batch variation (271). However, due to the extreme complexity and variety of EV-related biological systems, it seems to be a huge challenge to find an optimal and universal experimental layout. As an example, based on the worldwide survey, there are several different isolation methods with ultracentrifugation being the most commonly used. However, the choice of EV isolation method will also vary depending on a type of the starting material, compromise between the purity and yield of obtained EV preparations, as well as their downstream application (281). Another difficulty is a standardized and controlled long-term storage of EV preparations, that would also allow to preserve their biological activity after thawing (282).

One of the critical hallmarks is also a scale-up production, that would not only ensure the sufficient quantity of EVs produced in a good manufacturing practice (GMP) standards, but would also not affect their quality (283). Several groups work on the development of bioreactor-based approaches for the bulk EV production (284). Additionally, scientists try to modify culture conditions of the donor cells, stimulating them physically or chemically in order to significantly increase the yield of secreted EVs (285). Despite existing challenges, several methodological approaches fulfilling GMP standard requirements were reported so far, including e.g. preparation of EVs from BM-MSCs (286) or UC-MSCs (287).

3.3.2 Clinical trials

Despite several encountered difficulties to be overcome to facilitate common use of EVs in the tissue regeneration, the promising results of preclinical studies have become the basis for the attempts on using EV preparations in a medical practice. Currently, there are several clinical trials conducted around the world with the use of EV preparations (288). According to the ClinicalTrials.gov website, on October 2022 there were 84 interventional clinical trials for "extracellular vesicles" inquiry, with 15 of them being already completed. Among the top ones, 25 studies were related to the respiratory tract diseases, 16 to graft versus host disease (GvHD) and 10 to CNS diseases, with majority of them being related to the biomarker studies. Still, the clinical use of EVs for the therapeutic purposes is limited to ongoing early-phase studies, but initial results indicate no significant side effects following EVs administration, indicating their safety and therapeutic potential (289). As an example, in a recently reported case study, EVs derived from UC-MSCs were used for the intracochlear administration in the 55-year old patient suffering from Menière's disease, who required an insertion of a cochlear implant, that typically causes inflammatory response and local fibrosis that may lead to the hearing loss. Obtained results demonstrated safety of EV injection, attenuation of inflammation and improvement of hearing capacity and speech perception parameter (290). Promising results have led to the preparation of the phase 1 clinical study. In another report, based on the previous data, including those obtained for the

nonrandomized open-label cohort study related to the effect of EVs from BM-MSCs in COVID-19 associated ARDS treatment (251), randomized phase 2 clinical study "EXIT-COVID19" has been also conducted, but without already published results. Several other trials are still on the "recruiting" or "not yet recruiting" stage. Thus, direct indication on the effectiveness of EVs in the clinical practice should be expected within the upcoming years, which will allow not only to confirm safety of EV administration, but also to compare efficacy of EVs with the currently available treatments. Based on that it will be possible to indicate the most promising areas of EV-based therapeutic applications as alternatives to the currently utilized approaches.

4 Conclusions

Last two decades have brought a significant advancement in the field of EV biology and their potential biomedical utilization. In this review, we have highlighted the recent knowledge on the understanding of the biological activity of EVs, especially those secreted by different types of SCs, in cell-to cell crosstalk, including their role in the regulation of the immune system. In this context, EVs have been widely reported as potential therapeutic factors exhibiting immunoregulatory and pro-regenerative properties. Discovery that EVs may harbour and transfer their bioactive content into the target cells, influencing their fate, opened a new possibilities of use of EV preparations as acellular therapeutic option in several diseases with the inflammatory background. However, despite the vast potential of EVs as drug-delivery systems, their wide utilization is associated with several challenges and limitations that have still to be addressed. Nevertheless, EVs offer a great promise as new-generation tools for an improved diagnostic and clinical purposes.

Author contributions

EK performed the literature search and wrote the manuscript. PD prepared figures and tables. EKZ-S revised the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This paper was funded by National Center for Research and Development STRATEGMED III grant (STRATEGMED3/303570/7/NCBR/2017) to EKZ-S, the National Science Centre MAESTRO 11 (NCN/2019/34/A/NZ3/00134) grant to EKZ-S and the National Science Centre MINIATURA 5 (NCN/2021/05/X/NZ3/00894) to EK.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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10.3389/fimmu.2023.1120175

Glossary

ACE2	angiotensin converting enzyme 2
AD	Alzheimer's disease
AFM	atomic force microscopy
AKI	acute kidney injury
ALI	acute lung injury
APCs	antigen presenting cells
AT-MSCs	adipose tissue-derived mesenchymal stem/stromal cells
ARDS	acute respiratory distress syndrome
BBB	blood–brain barrier
BM-MSCs	bone marrow-derived mesenchymal stem/stromal cells
ECM	extracellular matrix
CKD	chronic kidney disease
CM	conditioned medium
CNS	central nervous system
COVID-19	coronavirus infectious disease 2019
CVDs	cardiovascular diseases
CPCs	cardiac progenitor cells
DCs	dendritic cells
EPCs	endothelial progenitor cells
EVs	extracellular vesicles
ESCRT	endosomal sorting complex responsible for transport
ESCs	embryonic stem cells
GMP	good manufacturing practice
GvHD	graft-versus host disease
HCC	hepatocellular carcinoma
HLA	human leukocyte antigen
IBD	inflammatory bowel disease
ILC2s	group 2 innate lymphoid cells
iPSCs	induced pluripotent stem cells
I/R	ischemia/reperfusion
ISEV	International Society for Extracellular Vesicles
LVEF	left ventricular ejection fraction
SCs	stem cells
MHC	major histocompatibility complex
MVBs	multivesicular bodies
MS	multiple sclerosis
MSCs	mesenchymal stem/stromal cells
NTA	nanoparticle tracking analysis
OA	osteoarthritis
PD	Parkinson's disease
ROS	reactive oxygen species
SNARE	SNAP (soluble NSF attachment protein) receptor
TGF-β	transforming growth factor beta
TNF-α	tumor necrosis factor alpha
UC-MSCs	umbilical cord Wharton's jelly MSCs
WHO	world health organization.

frontiers | Frontiers in Immunology

TYPE Mini Review
PUBLISHED 13 February 2023
DOI 10.3389/fimmu.2023.1094685

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Cytokines and Soluble Mediators in Immunity, a section of the journal Frontiers in Immunology

RECEIVED 10 November 2022
ACCEPTED 01 February 2023
PUBLISHED 13 February 2023

CITATION
Liu X, Wei Q, Lu L, Cui S, Ma K, Zhang W, Ma F, Li H, Fu X and Zhang C (2023) Immunomodulatory potential of mesenchymal stem cell-derived extracellular vesicles: Targeting immune cells.
Front. Immunol. 14:1094685.
doi: 10.3389/fimmu.2023.1094685

Immunomodulatory potential of mesenchymal stem cell-derived extracellular vesicles: Targeting immune cells

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Various intractable inflammatory diseases caused by disorders of immune systems have pressed heavily on public health. Innate and adaptive immune cells as well as secreted cytokines and chemokines are commanders to mediate our immune systems. Therefore, restoring normal immunomodulatory responses of immune cells is crucial for the treatment of inflammatory diseases. Mesenchymal stem cell derived extracellular vesicles (MSC-EVs) are nano-sized double-membraned vesicles acting as paracrine effectors of MSCs. MSC-EVs, containing a variety of therapeutic agents, have shown great potential in immune modulation. Herein, we discuss the novel regulatory functions of MSC-EVs from different sources in the activities of innate and adaptive immune cells like macrophages, granulocytes, mast cells, natural killer (NK) cells, dendritic cells (DCs) and lymphocytes. Then, we summarize the latest clinical trials of MSC-EVs in inflammatory diseases. Furthermore, we prospect the research trend of MSC-EVs in the field of immune modulation. Despite the fact that the research on the role of MSC-EVs in regulating immune cells is in infancy, this cell-free therapy based on MSC-EVs still offers a promising solution for the treatment of inflammatory diseases.

KEYWORDS

extracellular vesicles, mesenchymal stem cells, immune cells, cell dysfunction, inflammatory diseases

1 Introduction

Orchestrated responses among variety of innate and adaptive immune cells, organs, cytokines, and chemokines constitute our body's immune systems to fight against external invasions, together (1). Generally, macrophages, granulocytes, natural killer (NK) cells, mast cells, and dendritic cells (DCs) are called innate immune cells, which can react quickly towards external invasions or injuries. Adaptive immune cells referring to B cells and T cells, are mainly responsible for mediating humoral immunity and cellular immunity, respectively (1). However, the chaotic responses of immune cells often lead to various inflammatory diseases like chronic wounds (2), rheumatoid arthritis (3), inflammatory bowel diseases (4), encephalomyelitis (5), and so on, imposing a heavy burden on the economy and society (6, 7). For example, the unbalanced ratio of anti-inflammatory M2 macrophages (M2φ)/pro-inflammatory M1 macrophages (M1φ) or delayed transition from M1φ to M2φ phenotype of macrophages leads to excessive inflammation, impairing healing process of chronic wounds (8). Therefore, accurately regulating and restoring the behaviors of immune cells are crucial for improving treatment outcomes of inflammatory diseases.

Mesenchymal stem cells (MSCs) are a kind of multipotent stem cells which exist in a wide range of tissues such as bone marrow (BM) (9), adipose tissue (AD) (10), umbilical cord (UC) (11), decidua (12), palatine tonsil (PT) (13), aborted fetal liver (FL) (14), etc. MSCs are believed to exert immunomodulatory or regenerative effects on the injured tissues in various diseases through secreting paracrine factors including extracellular vesicles (EVs) (15). Moreover, as a cell-free bio-entity, MSC-EVs have been recognized as a promising candidate with equal or better therapeutic effect than MSCs.

EVs are nanoscale bodies with cup-like lipid bilayer membranes, containing bioactive components such as lipid, protein, and nucleic acid molecules that are enriched in their parent cells. Based on their origin, size, and bio-genesis, EVs are presently classified into three main categories including exosomes (Exos, size from 50 nm to 100 nm), microvesicles (MVs, 20 nm to 1000 nm), and apoptotic bodies (Abs, 1000 nm to 5000 nm) (16). Proteomics analysis has showed that exclusive markers are expressed highly on their surfaces, such as ALG-2 interacting protein X (Alix), tumor suppressor genes (TSG101), CD9, and CD63 (17). Recently, researchers have investigated the modulation of immune cells by MSC-EVs and explored their clinical potential in the treatment of various inflammatory diseases. Some excellent reviews have discussed the immunomodulatory effects of MSC-EVs on one kind of immune cell like macrophages (18) or on the treatment of inflammatory diseases like liver immunity (19), autoimmune diseases (20), lung diseases (21), rheumatoid arthritis (22) and so on. The accelerated healing process of these inflammatory diseases by MSC-EVs is usually achieved by their immunomodulatory functions on various immune cells. Therefore, it is timely to summarize and discuss the immunomodulatory functions of MSC-EVs on these immune cells systematically.

In present mini-review, we mainly discuss the immunomodulatory potential and mechanisms of MSC-EVs from multiple sources by targeting innate and adaptive immune cells including macrophages, granulocytes, mast cells, NK cells, DCs and T cells as well as B cells (Figure 1 and Table 1). Additionally, the clinical trials of MSC-EVs administered in different forms for different inflammatory diseases are briefly summarized. Furthermore, the research trends and challenges of MSC-EV applications in inflammatory diseases are presented. Hence, based on the properties and effects on immune cells, MSC-EVs may be developed as a therapeutic strategy for inflammatory diseases.

2 Regulation of immune cell function by MSC-EVs

2.1 Innate immune cells

Traditionally, innate immune cells include macrophages, granulocytes, mast cells, NK cells and DCs, which respond quickly to external invasions either by releasing histamine and heparin or by their powerful phagocytosis (1). We mainly focus on the regulation of macrophages, granulocytes, mast cells, NK cells and DCs by MSC-EVs.

2.1.1 Modulation of macrophages or microglia by MSC-EVs

Macrophages exist in almost all tissues of our body and are differentiated from peripheral blood monocytes upon injured tissue recruitment (54). They can not only phagocytose intrusive pathogens but also induce inflammatory response by releasing chemokines (1). Furthermore, they are antigen-presenting cells (APCs) to process and present peptides of phagocytic pathogens on major histocompatibility complex class II (MHC-II) receptor, activating adaptive immune system (54). The macrophages can be reprogramed into two different polarization states to exert their Janus functions, that is classical activated M1φ phenotype (pro-inflammatory) and alternative activated M2φ phenotype (anti-inflammatory) upon the stimulus of cytokines (55). However, the unbalanced M2φ/M1φ ratio or delayed transition from M1φ to M2φ phenotype would lead to continual inflammation (8).

Li et al. observed that EVs derived from human UC-MSCs (hUC-MSC-EVs) reduced the number of macrophages (CD68) to suppress burn-induced inflammation (23). They claimed that miR-181c enriched in hUC-MSC-EVs decreased the expression of toll-like receptor 4 (TLR4), a receptor of lipopolysaccharide (LPS), and subsequently reduced the activation of nuclear factor kappa B (NF-κB)/p65. In another study, hUC-MSC-EVs were also reported to modulate PI3K-AKT signaling pathway to shift macrophage polarization from M1φ to M2φ phenotype by their abundant has-miR-122-5p, -148a-3p, -486-5p, -let-7a-5p, as well as 100-5p (24). Surprisingly, EVs derived from BM-MSCs (BM-MSC-EVs) were reported to achieve "One Stone Two Birds". They decreased the infiltration number of M1φ phenotype and promoted M2φ phenotype polarization in the plaque of atherosclerosis through miR-let7/IGF2BP1/PTEN and miR-let7/HMGA2/NF-kB signaling pathways, simultaneously (26).

Microglia are a special population of macrophages in the central nervous system. Microglia can not only phagocytose apoptotic neurons but also control synaptic pruning in the brain (56).

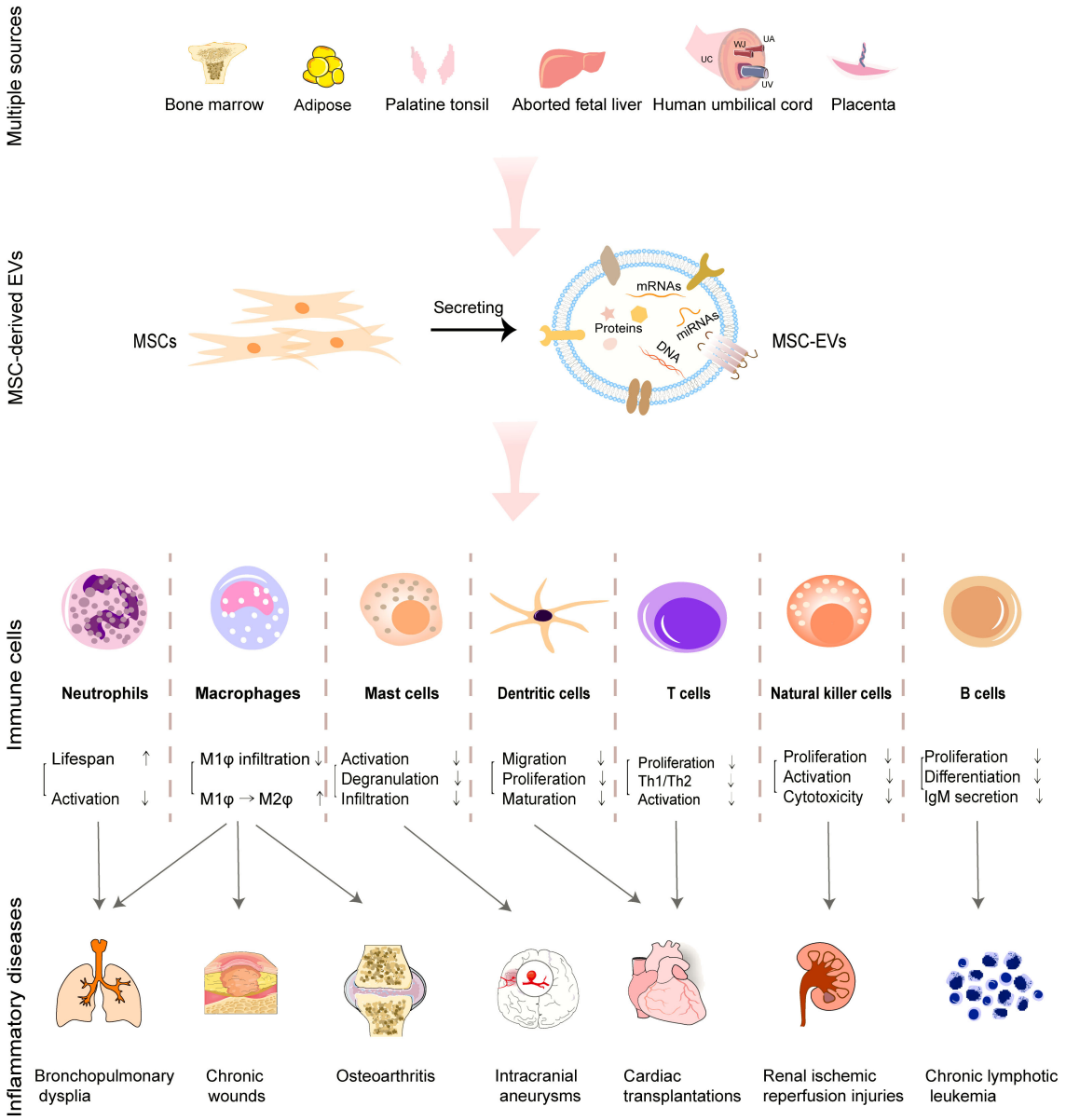

FIGURE 1
Roles of MSC-EVs derived from multiple tissues in regulating immune cells and facilitating the treatment of associated inflammatory diseases.

Microglia can be activated in a phagocytic state under the stimulus of nervous system injuries, releasing lots of cellular inflammatory transmitters (57). Like macrophages, activated microglia can be polarized into pro-inflammatory M1 and anti-inflammatory M2 subtypes, exerting their damage or protection function on neural network (58). Therefore, the proliferation, migration, and activation of microglia are crucial for nerve-related inflammatory responses.

Similarly, BM-MSC-EVs showcased analogical "One Stone Two Birds" modulation on microglia compared with macrophages. BM-MSC-EVs promoted microglial M2 polarization through activating NF-κB pathway and decreased the number of CD68+ microglia (29). In another study, human AD (hAD) derived MSC-EVs were reported to mitigate neuroinflammation mainly by suppressing M1 microglial activation through NF-κB and MAPK pathways and secondarily promoting M2 microglial polarization (30). Moreover, the EVs derived from human retinal progenitor cells were reported to stabilize microglia to avoid its excessive activation, migration, and proliferation, thereby decreasing the secretion of inflammatory cytokines like ionized calcium binding adapter molecule 1 (Iba1) and increasing the anti-inflammatory gene expressions of IL-4, IL-10, and TGF-β, thus mitigating neuroinflammation (59).

In short, the modulation of macrophages or microglia by MSC-EVs is mainly based on two aspects: decreasing their infiltration number and shifting their polarization from M1φ to M2φ subtype, but their potential mechanism still needs to be further explored.

2.1.2 Preconditioning of MSC-EVs

To improve the immunomodulatory functions of MSC-EVs on macrophages, some preconditioning methods, like the stimulus of hypoxia (27, 60), pharmacological agents (25, 61, 62) or pro-inflammatory cytokines (28, 63, 64), have been developed. For example, Sicco et al. pre-exposed hAD-MSCs to hypoxic condition

10.3389/fimmu.2023.1094685

TABLE 1 Specific pathways and components of MSC-EVs in regulating immune cells.

Targeting cells	Sources of MSCs	Active components	Targeting site/ pathways	Key functions	References
Macrophages	hUC	miR-181c	NF-κB	Reduce the number of CD68+ macrophages	(23)
	hUC	has-miR-122-5p, -148a-3p, -486-5p, -let-7a-5p, and 100-5p;	PI3K-AKT signaling pathway	Shift M1φ to M2φ	(24)
	hUC	let-7b	TLR4/NF-κB/STAT3/AKT	Increase macrophage plasticity	(25)
	mBM	miR-let7	miR-let7/IGF2BP1/PTEN and miR-let7/HMGA2/NF-kB	Decrease the infiltration of M1φ and shift M1φ to M2φ	(26)
	hAD	miR-223 and miR-146b	/	Shift M1φ to M2φ	(27)
	cAD	TGF-β and HGF	/	Shift M1φ to M2φ	(28)
Microglia	mBM	/	NF-κB pathway	Promote M2φ polarization and decrease the number of CD68+ macrophages	(29)
	hAD	/	NF-κB and MAPK pathway	Suppress M1φ activation and promote M2φ polarization	(30)
Granulocytes	hWJ	/	/	Enhance the phagocytosis and ROS production	(31)
	hAD	/	/	Recover respiratory burst and prolong the lifespan	(32)
	hESC	/	C5b-9	Downregulate NETs and IL-17	(33)
Mast cells	hAD	/	Serum IgE	Decrease the infiltration of mast cells	(34)
	hBM	/	PGE2 and EP4 receptor	Suppress activation of mast cells	(35)
	hPT	has-miR-214-3p and has-miR-424-5p	/	Suppress activation of mast cells	(13)
	hUC	/	STAT5 phosphorylation	Inhibit the degranulation of mast cells	(36)
NK cells	hUC	/	CX3CL1 and TLR-2	Inhibit the infiltration of NK cells	(37)
	hFL	TSP1	TGF-β/Smad2/3	Inhibit the proliferation, activation, and cytotoxicity of NK cells	(14)
DCs	mBM	/	/	Induce the maturation DCs	(38)
	mBM	/	/	Increase the number of tolerogenic DCs	(39)
	mBM	miR-146a	Fas	Impair the maturation of iDCs and the ability of mDCs to produce IL-12	(40)
	hBM	miR-21-5p	CCR7 gene	Impair the maturation and antigen uptake capacity of iDCs	(41)
	IOD-overexpressing BM	miR-540-3p and FHL-1 protein	JAK3 and AKT	Inhibit the maturation and functions of DCs	(42)
T cells	mBM	PD-L1 and TGF-β	/	Suppress the activation and proliferation of CD4+ T cells	(5)
	cWJ	TGF-β and adenosine	TGF-β and adenosine signaling	Inhibit the mitogen-induced proliferation of CD4+ T cells	(43)
	hEND	TGF-β	TGF-β signaling	Inhibit the activation of CD4+ T cells	(44)
	BM	PGE2 and TGF-β	/	Decrease the number of Th17 cells and increase the number of FoxP3+ Tregs	(45)
	cAD	TSG-6	FOXP3 protein	Increase the number of Tregs	(46)
	mESC	GM-CSF expressing	/	Enhance the migration of CD8+ Teffs and restrict the migration of Tregs	(47)
	hUC/hBM	CD73	adenosinergic signaling	Suppress the proliferation of T cells and promote the apoptosis of CD4+ Th1 cells	(48, 49)

(Continued)

TABLE 1 Continued

Targeting cells	Sources of MSCs	Active components	Targeting site/ pathways	Key functions	References
	mAD	β-catenin	Wnt/β-catenin signaling	Promote the migration and circulation of natural killer T cells	(50)
	hBM	miR-125a-3p	T cell receptor signaling	Suppress the differentiation of T cells to the effector phenotype	(51)
	IOD-overexpressing BM	miR-540-3p and FHL-1 protein	JAK3 and AKT	Inhibit the maturation and functions of DCs, and regulate T cell immune response in an indirect way	(42)
B cells	hBM	/	IgM	Inhibit the proliferation and differentiation of B cells	(52)
	hBM	/	MZB1 and BCR	Decrease their proliferation of B cells	(53)

hUC, human umbilical cord; mBM, murine bone marrow; hAD, human adipose tissue; cAD, canine adipose tissue; hWJ, human Wharton's jelly; hESC, human embryonic stem cell; hPT, human palatine tonsil; hFL, human fetal liver; cWJ, canine adipose tissue; hEND, human endometrium; mESC, murine embryonic stem cell, TGF-β, transforming growth factor beta; HGF, hepatocyte growth factor; TSP1, thrombospondin 1; FHL-1, four-and-a-half LIM domain protein 1; PD-L1, programmed death ligand-1; PGE2, prostaglandin E2; TSG-6, tumor necrosis factor-α-stimulated gene/ protein 6; GM-CSF, granulocyte-macrophage colonystimulating factor; NF-κB, nuclear factor-κB; PI3K, phosphatidylinositol 3 kinase; PTEN, phosphatase and tensin homolog; MAPK, mitogen-activated protein kinase; C5b-9, terminal complement activation complex; IgE, immunoglobulin E; EP4, E-prostanoid 4; STAT5, signal transducers and activators of transcription5; CX3CL1, C-X3-C motif chemokine ligand-1; TLR-2, toll-like receptor-2; CCR7, C-C chemokine receptor type 7; FOXP3, forkhead box P3; JAK3, Janus kinase 3; IgM, immunoglobulin M; MZB1, marginal zone B1; BCR, B cell receptor; M1φ, M1 macrophages; M2φ, M2 macrophages; ROS, reactive oxygen species; NET, neutrophil extracellular traps; IL-17, interleukin-17; NK cells, natural killer cells, iDCs, immature dendritic cells; mDCs, mature dendritic cells; Tregs, regulatory T cells; Teffs, T effector cells.
Symbol "/" means that the data is not applicable from published references.

(1% O_2) and collected the secreted EVs (27). The pretreatment of 1% O_2 elevated the contents of miR-223 and miR-146b in hAD-MSC-EVs. The hypoxia-changed hAD-MSC-EVs promoted the macrophage polarization from M1φ to M2φ phenotype, and then downregulated the production of interleukin (IL)-6 and Nos2, followed by upregulating the expression of Arg1 and Ym1 *in vivo* and *in vitro*. As for pharmacological agents, Ti et al. claimed that the pretreating of hUC-MSCs with LPS (100 ng/mL) for 48 h enhanced the expression of let-7b inside hUC-MSC-EVs. Let-7b is reported to regulate TLR4/NF-κB/STAT3/Akt pathway which is the potential controller for the macrophage plasticity (25). The precondition of parent cells with pro-inflammatory cytokines was also observed to increase the shifting effect of MSC-EVs on macrophages by improving the contents of the immunoregulatory microRNAs (miRNAs) (63–65) and proteins (28, 66). For example, IL-1β-primed (10 ng/mL, 12 h) hUC-MSCs were observed to secret miR-146a-enriched EVs, which promoted M2φ polarization, efficiently (63). In an interesting study, hUC-MSCs pretreated with tumor necrosis factor alpha (TNFα, 1 ng/mL, 3 d) generated the EVs containing upregulated miRNA-299-3p which accounted for the inhibiting effect of hUC-MSC-EVs on the activation of NLRP3 in macrophages, partially (65). Additionally, preconditioning hAD-MSCs with the mixture of interferon gamma (IFNγ)/TNFα (40 ng/mL, 48 h) upregulated the contents of miR-34a-5p, miR-21 as well as miR-146a-5p inside hAD-MSC-EVs. The obtained hAD-MSC-EVs switched macrophages from M1φ to M2φ phenotype (64). Similarly, in the treatment of experimental murine colitis, preconditioning cAD-MSCs with the mixture of IFNγ/TNFα (20 ng/mL, 24 h) upregulated the expression of immunosuppressive proteins such as hepatocyte growth factor (HGF) and transforming growth factor beta (TGF-β), thus dominating the polarization of macrophages (28).

Collectively, the existing pretreatment methods can efficiently enrich the immunomodulatory components in MSC-EVs. We believe that the exploration to obtain MSC-EVs with more specific components and greater immunomodulation potential *via* milder preconditioning may be the research direction in the future.

2.1.3 Granulocytes

As the main type of phagocytic granulocytes, short-lived neutrophils are recruited to injury sites firstly (1). They phagocytize and destroy pathogens accurately and rapidly by releasing lytic enzymes and producing reactive oxygen species (ROS), further undergoing neutrophil extracellular traps (NETs) (67). Furthermore, the phagocytosis of apoptotic neutrophils can promote anti-inflammatory responses of M2φ subtype and tissue repairing (9).

However, insufficient function and short lifespan of neutrophils often occurred on those patients with severe congenital neutropenia (SCN) (68) or chronic granulomatous disease (CGD) (69), thus leading to serious pathogen infection. Scientists observed the effects of MSC-EVs on the lifespan and biological behaviors of neutrophils from healthy donors, SCN or CGD patients *in vitro* systematically (10, 31, 32, 70). In their studies, the apoptosis and biological behaviors like respiratory burst and phagocytosis of neutrophils were characterized by annexin V-propidium iodide, nitro blue tetrazolium assay, and Giemsa staining. MSCs were isolated from Wharton's jelly (WJ, a mucosal connective tissue of UC) or from hAD. hAD-MSC-EVs could enhance the phagocytosis and ROS production in neutrophils from both CGD patients and healthy volunteers, and decrease the lifespan of neutrophils from CGD patients (70). However, the influence of hAD-MSC-EVs on neutrophils from SCN patients seemed different. In their observation, hAD-MSC-EVs recovered and prolonged the respiratory burst and lifespan of neutrophils from SCN patients or healthy volunteers significantly, while showed limited influence on the phagocytosis percentage of neutrophils from both SCN patients and healthy volunteers (32). For EVs derived from WJ-MSCs (WJ-MSC-EVs), the lifespan and phagocytosis of neutrophils from healthy volunteers were significantly augmented in comparison with their influence on respiratory burst (31). In-depth studies from aspects of genomics and proteomics should be conducted to explain why those two MSC-EVs showed different regulation behaviors on neutrophils from SCN or CGD patients, or from healthy donors. Very recently,

Loh et al. reported that EVs from human embryonic stem cell (hESC, cell line of E1-MYC 16.3)-derived MSCs (hESC-MSC-EVs) inhibited terminal complement activation complex C5b-9-mediated neutrophil activation, thus suppressing the release of NETs and IL-17 *via* a CD59-dependent mechanism (33). This study revealed bright application prospect of hESC-MSC-EVs on treating immune dysregulation in COVID-19 patients.

2.1.4 Other innate immune cells

Mast cells are the first responders of long-lived innate immune cells, that release heparin as well as histamine rapidly in response to an external infection (1). Excessive accumulation and activation of mast cells by immunoglobulin E (IgE) lead to allergy, interstitial cystitis, and other inflammatory diseases (71). Mast cells can be stabilized by MSC-EVs through different molecular mechanisms. Cho et al. observed that treatment with hAD-MSC-EVs (injected either intravenously or subcutaneously) ameliorated the infiltration of mast cells in atopic dermatitis *in vivo* by reducing the level of serum IgE (34). Liu et al. reported that hBM-MSC-EVs suppressed the activation of mast cells (cell line of LAD2) through upregulating the production of prostaglandin E2 (PGE2) and E-prostanoid 4 (EP4) receptors (35). EVs derived from hPT-MSCs (hPT-MSC-EVs) could attenuate TLR7-mediated activation of mast cells. In this study, imiquimod (IMQ, the agonist for TLR7) was employed to activate human mast cell line (HMC-1) (13). The introduction of hPT-MSC-EVs inhibited IMQ-induced HMC-1 activation and the expression of inflammatory cytokines in HMC-1 cells *via* transferring miRNAs like has-miR-214-3p and has-miR-424-5p. Very recently, a study launched by Lin et al. reported that hUC-MSC-EVs suppressed the activation of IgE-stimulated mast cells (cell line of KU812) and downregulated the expression level of NF-κB, thus inhibiting the degranulation of mast cells and release of IL-1β, TNF-α, and IL-6 (36). Furthermore, hUC-MSC-EVs attenuated IgE-induced STAT5 phosphorylation inside KU812 cells in a dose-dependent manner. Collectively, MSC-EVs can stabilize mast cells to relieve allergies by reducing their infiltration and degranulation through different mechanisms.

NK cells are lymphocytes that can detect and kill neighboring infected cells that don't express a certain number of MHC molecules on cell surface through the specialized receptors on NK cells (NKG2D, KIR, etc.) without prior sensitization (1). MSC-EVs can exert their modulative function on NK cells by regulating their behaviors such as proliferation, activation, and releasing cytotoxic substances. hUC-MSC-EVs were found to relieve the renal ischemic reperfusion injury (IRI) by decreasing the number of NK cells at injury site. hUC-MSC-EVs downregulated the expression of C-X3-C motif chemokine ligand-1 (CX3CL1) and TLR-2, thus inhibiting the infiltration of CD3-CD161+NK cells (37). In another study, hBM-MSC-EVs were also reported to inhibit the secretion of IFN-γ and TNF-α by activating NK cells, showing potential in treating therapy-refractory graft-versus-host diseases (72). EVs derived from human FL (hFL)-MSCs (hFL-MSC-EVs) showed efficient inhibition on the proliferation, activation, and cytotoxicity of NK cells by transferring thrombospondin 1 (TSP1, a regulatory molecule for TGF-β) to downregulate TGF-β/Smad2/3 signaling pathway in NK cells (14). In short, these reports showcased the therapeutic roles of MSC-EVs in inhibiting the lethality of NK cells.

DCs are recognized as the most efficient and professional APCs. They ingest antigens by internalizing invaders and process antigens, followed by presenting antigens to T cells (1). During this process, the antigen-processing immature DCs (iDCs) are transformed to mature DCs (mDCs, antigen-presenting cells) and migrate to secondary lymphoid organs, activating adaptive immune system. Therefore, the immunomodulation on DCs can be achieved by regulating their maturation and migration behaviors. HBM-MSC-EVs induced the hypoactive phenotype of DCs with repressed allorecognition and downregulated expression of costimulatory molecules and MHC-II, subsequently inhibiting the development of Th1 and Th17 cells in the *in-vivo* mouse models of type 1 diabetes and experimental autoimmune uveoretinitis (73). In another study, hBM-MSC-EVs were observed to induce the generation of iDCs, which were characterized by the reduced expression of IL-6 and IL-10 (74). However, the regulated expression of costimulatory markers and MHC-II seemed different in the researches of mAD-MSC-EVs. Cho et al. observed that mAD-MSC-EVs induced the maturation of DCs, characterized by the increased expression of co-stimulatory molecules (38). While, Shahir et al. claimed that the treatment of immature or LPS-induced mature DCs with mAD-MSC-EVs led to the tolerogenic DC population with downregulated expression of costimulatory markers (39). As for underlying molecular mechanisms, several key miRNAs or proteins in MSC-EVs may be involved in the regulation of DC behavior. MBM-MSC-EVs were found to impair the maturation of iDCs and the ability of mDCs to produce IL-12 by transferring miR-146a (the potential miRNA controlling the survival and maturation of human DCs) to iDCs (40). MiR-21-5p was another miRNA that regulated the maturation and function of DCs (41). HBM-MSC-EVs enriched with miR-21-5p degraded the C-C chemokine receptor type 7 gene (CCR7 gene, modulating the homing of DCs to the lymph nodes) and hampered the migration toward the CCR7-ligand CCL21. Furthermore, the treatment with hBM-MSC-EVs restricted the antigen uptake capacity of iDCs and downregulated the secretion of IL-6 and IL-12p70 as well as upregulated the secretion of TGF-β. In another study, the upregulated miR-540-3p and immunoregulatory four-and-a-half LIM domain protein 1 (FHL-1) in EVs derived from indoleamine 2,3-dioxygenase (IDO)-pretreated BM-MSCs were reported to regulate Janus kinase 3 (JAK3, an immune activator) protein negatively and inhibit activation of AKT, respectively, inhibiting the maturation and functions of DCs (42).

2.2 Adaptive immune cells

Adaptive immunity usually responds and forms immunological memory by binding with specific pathogens within a few days of disease onset, mainly depending on T or B cells.

2.2.1 T cells

T cells showcase multi-biofunctions including directly killing target cells, regulating antibody production of B cells and secreting lymphokines after the adaptive immunity system is activated (1). Generally, T cells are classified into two subpopulations based on the CD4 and CD8 receptors expressed on their surfaces, called helper and killer T cells respectively. Furthermore, in the presence of IL-12 and

IFN-γ, CD4+ T cells are activated into Th1 subtype to induce inflammation and kill pathogens. In the presence of IL-4, CD4+ T cells are activated into Th2 subtype to support the antibody production of B cells (1).

According to literatures, MSC-EVs exerted their immunoregulation functions on T cells with the assistance of APCs. Zhang et al. found that EVs derived from hESC (cell line of huES9.E1)-MSCs (hESC-MSC-EVs) mediated the polarization of Tregs from CD4+ T cells with the assistance of monocytes (75). In their observation, the differentiation of CD4+CD25+FoxP3+ Tregs was observed distinctly by co-incubating hESC-MSC-EVs with CD4+ T cells and human macrophages (THP-1) for 24 h. Furthermore, they found that hESC-MSC-EVs increased the generation of CD4+CD25+Foxp3+ Tregs from CD4+ T cells (activated by APC-enriched spleen cells) through a dose–dependent manner, indicating that MSC-EVs enhanced the production of Tregs through an APC-mediated pathway (76).

A lot of active proteins including tumor necrosis factor-α-stimulated gene/protein 6 (TSG-6) (46), TGF-β (44, 45), adenosine (45, 48), CD73 (48, 49), programmed death ligand-1 (PD-L1) (5), granulocyte-macrophage colony stimulating factor (GM-CSF) (47), and β-catenin (50) loaded in or expressed on the surface of MSC-EVs were involved in the modulation on T cells. As for proteins, TSG-6 in cAD-MSC-EVs was the key protein to increase Tregs by upregulating forkhead box P3 (FOXP3) protein (stabilizes precursor cells of Tregs) (46). TGF-β displayed on the surface of BM-MSC-EVs decreased the number of Th17 cells and increased FoxP3+ Tregs in GAD65-stimulated peripheral blood mononuclear cells (PBMCs) (45). Additionally, TGF-β surface-bounded or encapsulated in hEND-MSC-EVs exhibited significant inhibition on the activation of CD4+ T cells (44). Mokarizadeh et al. claimed that PD-L1 and TGF-β in mBM-MSC-EVs were responsible for suppressing the activation and proliferation of CD4+ T cells and promoting the generation of Tregs (5). Adenosine and adenosinergic signaling are efficient immunosuppressor and pathway employed by immunosuppressive Tregs by neutralizing pro-inflammatory adenosine 5′-triphosphate (ATP) in the extracellular environment, especially in injured tissues (77). CD73 expressed on the surface of hUC-MSC-EVs (48) or hBM-MSC-EVs (49) catalyzed the production of adenosine from adenosine 5′-monophosphate (AMP), suppressing the proliferation of T cells or promoting the apoptosis of CD39+ Th1 cells. Additionally, Crain et al. claimed that cWJ-MSC-EVs inhibited the mitogen-induced proliferation of CD4+ T cells in a dose-dependent manner with the synergistic effect between TGF-β and adenosine (43). GM-CSF (47) and β-catenin (50) inside MSC-EVs play an important role in immunoregulating T cells associated with tumor therapies. A prophylactic anticancer vaccine composed of GM-CSF-overexpressing mESC-MSC-EVs were reported to enhance the migration of CD8+ T effector cells to rise the expression of pro-inflammatory TNF-α and IFN-γ and restrict the migration of immunosuppressive Tregs (47). Besides, β-catenin inside mAD-MSC-EVs showed significant promotion effect on the migration and circulation of natural killer T cells (50). Furthermore, the stimulus of pro-inflammatory TNF-α and IFN-γ on cAD-MSCs increased the expression of immunosuppressive proteins such as TSG-6, PGE2, and TGF-β inside the derived EVs (28).

Abundant miRNAs inside MSC-EVs were also involved in the regulation process. MiR-125a-3p, which suppresses the proliferation of several cells (78), is the most highly upregulated miRNA inside hBM-MSC-EVs and is recognized to account for the suppression effect of hBM-MSC-EVs on the functional differentiation of T cells (51). MiR-540-3p is another involved miRNA responsible for the regulatory functions of MSC-EVs. He et al. engineered mBM-MSCs by gene transfection to get IDO1-overexpressing mBM-MSC-EVs with upregulated miR-540-3p and FHL-1 protein (42). MiR-540-3p could regulate JAK3, an immune activator, negatively. While, FHL-1 protein was found to suppress IGF/PI3K signaling and activate endoplasmic reticulum (ER) signaling. They cooperated with each other to mediate immunotolerance associated with APCs and T cells after organ transplantation (42).

The roles of T cells and the cooperation between T cells and other immune cells are elaborate and complex. The regulations of the proliferation, migration, activation, apoptosis, and homeostasis of T cells by MSC-EVs should be carefully considered.

2.2.2 B cells

Upon activation and proliferation, B cells can produce large quantities of highly-specific antibodies and secrete them into blood or tissue fluid. Analogously, B cells also include two subtypes, B1 cells (primary producer of natural antibodies, like immunoglobulin M (IgM)) and B2 cells. Furthermore, B2 cells have two subsets including marginal zone B (MZB) cells and follicular B (FOB) cells, which participate in innate and adaptive immune responses, respectively (1).

Controversial results exist about how MSC-EVs regulate B cells. Budoni et al. explored the role of BM-MSC secreted membrane vesicles (MVs, BM-MSC-MVs) in the inhibition of B cells (52). They observed that BM-MSC-MVs specifically inhibited proliferation and differentiation of B cells and suppressed IgM secretion in a dose-dependent manner, compared with T lymphocytes or NK cells. In another study, BM-MSC-EVs were found to decrease the proliferation of B cells *in vitro*, which might be attributed to the upregulated expression level of marginal zone B1 (MZB1) and B cell receptor (BCR)-mediated Ca mobilization in certain subsets of B-lymphocytes (53, 79). Additionally, BM-MSC-EVs promoted migration and chemoresistance of chronic lymphocytic leukemia (CLL) B cells, decreasing their apoptosis in a contact-independent manner by inducing BCR-like activation (80). However, a study launched in 2019 claimed that AD-MSC-EVs isolated by size-exclusion chromatography showed minimal effects on activated B cells, compared with the effects of AD-MSCs, AD-MSC-conditioned medium and AD-MSC derived soluble protein-enriched fractions. AD-MSC-EVs just induced the production of similar $CD24^{hi}CD38^{hi}$ B cells, but not real ones because these cells could not produce IL-10 (81). These controversial results may be attributed to different isolation techniques and origins for MSC-EVs in literatures. A standard isolation technology and culturing approach should be established to explore the impacts of MSC-EVs on B cells.

2.3 The effect of sources on therapeutic potentials of MSC-EVs

The different sources of MSCs affect the cargoes in EVs like proteins and RNAs (82, 83), thus influencing therapeutic potentials of MSC-EVs (84, 85) in many diseases such as Alzheimer's disease (86),

osteoarthritis (OA) (87), inflammatory response (88), and wound healing (89–91).

However, the comparative studies of MSC-EVs with different origins are relatively limited. HAD-MSC-EVs were reported to cause decreased Aβ peptide level in N2a cells than hBM-MSC-EVs, because hAD-MSC-EVs carried the larger amount of enzymatically active neprilysin (an Aβ-degrading enzyme), showing promising potential in the treatment of Alzheimer's diseases (86). Zhu et al. compared the treatment efficacy of EVs derived from synovial membrane MSCs (SM-MSC-EVs) with EVs derived from induced pluripotent stem cells (iMSC-EVs) in experimental OA (87). IMSC-EVs showed better therapeutic effects on collagenase-induced OA in mice by promoting the migration and proliferation of chondrocytes than SM-MSC-EVs. The different efficacy was also observed in another study which demonstrated that hBM-MSC-EVs had better therapeutic effects on OA treatment than hAD-MSC-EVs (92). A proteomics analysis showed that differentially expressed proteins (DEPs) between hAD-MSC-EVs and hUC-MSC-EVs were involved in immunity, complement activation, and protein activation cascade regulation in gene ontology (GO) items (93). This is in line with the previous report in which hAD-MSC-EVs, hBM-MSC-EVs, and hUC-MSC-EVs showed prominent immune modulation, regeneration ability, and tissue repair, respectively (94). In aspect of wound healing, the effects of hAD-MSC-EVs and mAD-MSC-EVs were better than that of hBM-MSC-EVs and mBM-MSC-EVs in a diabetic murine model (89, 90). The analysis of cargoes in EVs including proteins and miRNAs explained why hAD-MSC-EVs were closely related to angiogenesis, while hBM-MSC-EVs had more potential to facilitate cellular proliferation. Besides different tissues, age is another influence factor. For example, mBM-MSC-EVs from pre-pubertal group were enriched in miR-21-5p, which was a negative regulator for inflammatory response in macrophages, compared with that of adult groups (88). As for separate studies, the immunomodulation effects of hAD-MSC-EVs, hBM-MSC-EVs, hUC-MSC-EVs on T cells seemed different. HUC-MSC-EVs decreased the migration of CD4+T cells and reduced the percentage of Th1 cells without inducing their apoptosis (95), while BM-MSC-EVs induced the apoptosis of T cells and increased the ratio of Treg/Teff (96). HAD-MSC-EVs were reported to inhibit the proliferation of CD4+ and CD8+ T cells and suppressed the differentiation of CD4+ and CD8+ T cells (97).

In short, in-depth comparative studies are needed to compare the therapeutic potentials of MSC-EVs from different sources. Especially, the culture conditions of MSCs and the isolation methods as well as the administrations and doses of MSC-EVs should be considered carefully in *in-vitro* and *in-vivo* researches to claim which one is more effective than the other.

3 Clinical applications of MSC-EVs in inflammatory diseases

Currently, the therapeutic potentials of MSC-EVs have received intense attention in immune therapies, especially during COVID-19 pandemic. According to registered trials on https://www.clinicaltrials.gov, many clinical trials have been launched by different research teams to evaluate the safety and efficacy of MSC-EVs in the treatment of COVID-19 syndrome, SARS-CoV-2 infection, acute respiratory distress syndrome, organ grafting, irritable bowel diseases, burn wounds, osteoarthritis, Alzheimer's Disease, periodontitis, Type 1 diabetes mellitus and so on (Table S1). In a complected study (NCT04493242), one single 15 mL intravenous dose of BM-MSC-EVs increased oxygenation (PaO_2/FiO_2) by 192%, reduced about 32% absolute neutrophil count, and increased the number of CD3+, CD4+, and CD8+ lymphocyte by 46%, 45% and 46%, respectively in patients with severe COVID-19 or moderate-to-severe acute respiratory distress syndrome. This result showcased the abilities of MSC-EVs to restore oxygenation, downregulate cytokine storm, and reconstruct immunity system in COVID-19 patients (98). Recently, Shi et al. studied the biodistribution and efficacy of nebulized hAD-MSC-EVs in *Pseudomonas aeruginosa*-induced murine lung injury model and explored the safety of nebulized hAD-MSC-EVs in 24 healthy volunteers (NCT04313647) (99). They found that nebulized hAD-MSC-EVs mitigated lung inflammation and did not cause serious adverse effects on healthy volunteers after the 7th day. These results indicated that MSC-EVs administered in different forms are promising therapeutic candidates in practical treatment of inflammatory diseases.

4 Conclusion and perspectives

The regulation of various immune cells by MSC-EVs from multiple sources summarized in this mini-review showcases the great potential of MSC-EVs for the treatment of inflammatory diseases including chronic wounds, osteoarthritis, intestinal diseases, and so on. However, some unrevealed questions still stay in researchers' minds. The heterogeneity of MSC-EVs is reflective of size, content profile, cellular source, and phenotypic effects on recipient cells. But the exact mechanisms to determine the size, cargo sorting and fate in recipient cells remain elusive. Besides, even for the same group of MSC-EVs, different isolation methods may lead to discrepant net production and purity of MSC-EVs, along with unexpected cell debris or protein deposit, which may bring about deviated effects from genuine impacts mediated by MSC-EVs themselves. Thus, developing a standard and widely-accepted isolation technology of MSC-EVs is very imperative for the scientific community. Furthermore, can we build a research database about the contents in MSC-EVs and their targeting immune cells to realize on-demand design and construction of engineered MSC-EVs by determining the exact components of each MSC-EVs that exert immune regulatory functions? Convincedly, the time has come for MSC-EVs as a novel therapeutic approach for various inflammatory diseases.

Author contributions

XL, QW, and LL drafted the manuscript. XL and QW prepared the figures. XL, QW, SC, KM, WZ, HL, and FM revised the manuscript. QW and LL revised the figures. XL, HL, XF, and CZ conceptualized, reviewed and funded the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the National Nature Science Foundation of China (22205260, 82172211, 81830064, 82172231), National Key Research and Development Programs of China (2022YFA1104303), the CAMS Innovation Fund for Medical Sciences (CIFMS, 2019-I2M-5-059), the Military Medical Research and Development Projects (AWS17J005, 2019-126), and Military Medical Science and Technology Youth Training Program (21QNPY128).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1094685/full#supplementary-material

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frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 20 February 2023
DOI 10.3389/fimmu.2023.1092668

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Cytokines and Soluble Mediators in Immunity, a section of the journal Frontiers in Immunology

RECEIVED 08 November 2022
ACCEPTED 31 January 2023
PUBLISHED 20 February 2023

CITATION
Yang J, Xiao M, Ma K, Li H, Ran M, Yang S, Yang Y, Fu X and Yang S (2023) Therapeutic effects of mesenchymal stem cells and their derivatives in common skin inflammatory diseases: Atopic dermatitis and psoriasis.
Front. Immunol. 14:1092668.
doi: 10.3389/fimmu.2023.1092668

Therapeutic effects of mesenchymal stem cells and their derivatives in common skin inflammatory diseases: Atopic dermatitis and psoriasis

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Chronic skin inflammatory diseases including atopic dermatitis (AD) and psoriasis have been considered uncontrolled inflammatory responses, which have usually troubled patients around the world. Moreover, the recent method to treat AD and psoriasis has been based on the inhibition, not regulation, of the abnormal inflammatory response, which can induce a number of side effects and drug resistance in long-term treatment. Mesenchymal stem/stromal cells (MSCs) and their derivatives have been widely used in immune diseases based on their regeneration, differentiation, and immunomodulation with few adverse effects, which makes MSCs a promising treatment for chronic skin inflammatory diseases. As a result, in this review, we aim to systematically discuss the therapeutic effects of various resources of MSCs, the application of preconditioning MSCs and engineering extracellular vesicles (EVs) in AD and psoriasis, and the clinical evaluation of the administration of MSCs and their derivatives, which can provide a comprehensive vision for the application of MSCs and their derivatives in future research and clinical treatment.

KEYWORDS

MSCs (mesenchymal stem cells), MSCs derivatives, skin inflammatory diseases, atopic dermatitis, psoriasis, extracellular vesicles

1 Introduction

Skin inflammatory diseases, mainly including atopic dermatitis (AD) and psoriasis, are considered an uncontrolled response to systemic inflammation, the main symptoms and pathological features of which are manifested in the skin (1, 2). The problems caused by inflammatory skin diseases plague people all over the world and bring a huge economic burden. The incidence of AD accounts for higher than 20% of children and approximately 10% of adults in some countries including both developing and developed counties and

continues to increase (3, 4). Unlike AD, psoriasis accounts for approximately 1% of children and 11% of adults in an epidemiological study of 20 countries (5). Moreover, the age distribution of psoriasis has a bimodal onset including before the age of 40 years accounting for 75% of cases and after the age of 40 years, according to two different subtypes of its pathological features (6). The reasons we choose AD and psoriasis to represent the skin inflammatory diseases are that a) AD and psoriasis are kinds of skin inflammatory diseases that affect the largest number of patients and the quality of patients' life around the world, b) AD and psoriasis are difficult to cure and the treatments for them have a series of side effects, and c) among skin inflammatory diseases, the treatment and pathogenesis of AD and psoriasis are the hottest research topic nowadays.

As mentioned before, skin inflammatory diseases are mainly caused by the imbalance between pro- and anti-inflammatory factors. The pathogenesis of AD is known as the abnormal activation of T helper 2 (Th2) lymphocyte, which can subsequently secrete a series of pro-inflammatory cytokines including immunoglobulin E (IgE), interleukin-4 (IL-4), IL-5, IL-13, IL-17, IL-22, IL-31, and thymic stromal lymphopoietin (TSLP), leading to epidermal barrier defect and increased skin inflammation (7–9), whereas psoriasis is mainly considered the abnormal activation of Th1 and Th17 lymphocytes which secrete pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interferon-γ (IFN-γ), IL-17, and IL-23 (10). However, although the current therapeutic methods vary from phototherapy to immunosuppressant drugs and biological agents, the curing mechanism is based on the inhibition, not regulation, of the abnormal inflammatory response to suppress the symptoms. The traditional administration of drugs, such as corticosteroids and calcineurin inhibitors, can induce a series of side effects, including absorption and hypothalamic–pituitary–adrenal axis suppression, growth suppression, atrophy, cataracts, and drug resistance in long-term treatment (11, 12). Although recent research indicates that biological agents can effectively improve the symptoms of skin inflammatory diseases, they can also induce serious side effects. For example, the JAK inhibitors including abrocitinib and dupilumab can induce a series of adverse events such as upper respiratory tract infection, conjunctivitis, asthma, and nasopharyngitis (13). The TNFα inhibitor such as adalimumab can induce adverse events such as serious infection, tuberculosis, and tumor (14).

Mesenchymal stem/stromal cell (MSC) derivatives have been widely used in clinical treatments in virtue of their abilities of regeneration, differentiation, and immunoregulation (15, 16). The resources of MSCs can be harvested from various tissues including umbilical cord (UC-MSCs) (17) or its blood (UCB-MSCs) (18), bone marrow (BM-MSCs) (19) or adipose tissue (AD-MSCs) (20), and gingiva (GMSCs) (21), which may have different therapeutic effects on skin inflammatory diseases. In addition to those resources, there are also some important issues to be considered in stem cell-based therapy, such as the number of cells transplanted, preconditioning of the cell preparation, relevant targets of the therapy, and route and frequency of administration (22–24). In addition, the MSCs of patients with skin inflammatory diseases show abnormal biological abilities to regulate inflammation, differentiation, and regeneration compared to the healthy population. The target to improving those MSCs from the patients of skin inflammatory diseases may become another cured target for clinical treatments (25–27). However, there are still mild adverse effects such as headache, fever, and the risk of embolism, but there have been no documented cases of embolism during treatment of AD and psoriasis patients (28, 29). It is worth noting that various administrations of MSCs and their derivatives may have obviously different effects on treating common chronic skin inflammatory diseases, AD, and psoriasis. As a result, in this review, we provide an overview of current strategies regarding the use of MSC derivatives including the therapeutic effects of different resources, preconditioning of the cell preparation, extracellular vesicles (EVs), and the improvements of MSCs in the lesion skin; a clinical evaluation of patients treated who have MSCs in inflammatory skin diseases; and future directions needed to develop this field.

2 The therapeutic target aiming at lesional MSCs in AD and psoriasis

AD and psoriasis are systemic and immune-allergic inflammatory skin diseases, the mechanism of which is the dysregulation of immunology (30–32), whereas MSCs play a role on regeneration and more importantly on immunomodulation (33–35). As a result, it could be the new clinical target whether the biological function of MSCs in skin lesion of AD and psoriasis changed and evolved in the pathogenesis of skin inflammatory diseases. Recent studies reveal that skin-derived MSCs in patients show an obviously differential function compared with common MSCs. Orciani et al. found that MSCs isolated from the skin lesion of patients in AD can enhance the activation of Th1 and Th17 cells and promote the production of their pro-inflammatory cytokines including IL-6, IL-13, IL-17A, IL-17F, transforming growth factor-beta (TGF-β), and IFN-γ, whereas they decrease the number of Th2 cells and their production including IL-2, IL-4, IL-5, and IL-23A. Interestingly, some proinflammatory factors are not changed including chemokine (C–C motif) ligand 1 (CCL1), IL-17C, and TNF-α (36). Campanati et al. also found that MSCs derived from patients of AD can overexpress the levels of IL-6 and IL-13 whereas there is no significance with the level of IL-4 compared with healthy MSCs (27). In psoriasis, recent research found that MSCs from skin lesion performed an abnormal role in two ways compared with normal MSCs. Firstly, compared with healthy donors, MSCs from skin lesion of psoriasis patients decreased the level of TGF-β and its receptor and thus increased the ratio of Th17/Treg and their inflammatory cytokines including IFN-γ and TNF-α (37–40). Secondly, pathological MSCs from psoriasis patients expressed high levels of vascular endothelial growth factor (VEGF) and inducible nitric oxide synthase (iNOS), which was different from the MSCs of the normal population and AD patients. The increasing levels of VEGF and iNOS would be more vulnerable to recruit a number of immune cells, proinflammatory cytokines, and chemokines into skin lesion (41–43). Moreover, as AD and psoriasis are a kind of immune diseases, the hematopoietic microenvironment is altered in those chronic inflammatory diseases. Compared with BM-MSCs from healthy people, BM-MSCs show an abnormal secretion of inflammatory cytokines and chemokines in patients with psoriasis, which showed a different hematopoietic microenvironment (26). In addition, Zhang et al. found that bone marrow hematopoietic stem cells (BMHSCs) from psoriasis patients have a different cell

phenotype and an increased expression of CD45, which may account for the activation of T cells and be closely associated with disease severity (44) (Figure 1). Considering that the inflammatory cascade in AD and psoriasis begins at the mesenchymal level, an upstream therapeutic intervention to treat the abnormal MSCs can potentially improve the pathogenesis of those inflammatory diseases. However, the therapeutic methods to treat lesional MSCs have been still unrevealed and how to use the improved MSCs to treat the skin inflammatory diseases needs to be further studied.

3 The effects of MSCs from different resources on AD and psoriasis

With advancing technology for administering MSCs, the application of MSCs has emerged as a promising strategy for the treatment of skin inflammatory disease due to their capability of regeneration, immunomodulation, and differentiation (45). Recent research found that MSCs have efficacy in the reduction of disease severity and epidermal thickness, arranging layers of epidermal layers, and keeping an intact basement membrane through its powerful capability of immunoregulation (46, 47). MSCs can be harvested from different tissues as mentioned before, whereas from different resources MSCs may have various therapeutic effects on skin inflammatory diseases.

3.1 AD-MSCs

Among various resources of MSCs, AD-MSCs have become one of the most attractive therapies because of their easy way to harvest, few ethical concerns, and most importantly their secreting capacity of numerous growth factors and adipokines to assist tissue survival (48). In AD, intravenous administration of human AD-MSC in mice (2×10^5 or 2×10^6 cells/200 µL normal saline) can alleviate allergic inflammation which includes decreasing the number of degranulated mast cells (MCs), IgE level, amount of histamine released, and prostaglandin E2 level; inhibiting the secretion of pro-inflammatory cytokines and chemokines; increasing the expression of Th1 and Th2 cells; and promoting the expression of regulatory T (Treg) cells (49). Kim et al. found that intravenous administration of AD-MSCs in mice [1×10^6 cells in 100 µl phosphate-buffered saline (PBS)] can decrease the macrophage inflammatory protein-2 (MIP-2) level to overexpress the miR-122a-5p level, regulating the level of cytokine signaling 1 (SOCS1), to decrease the

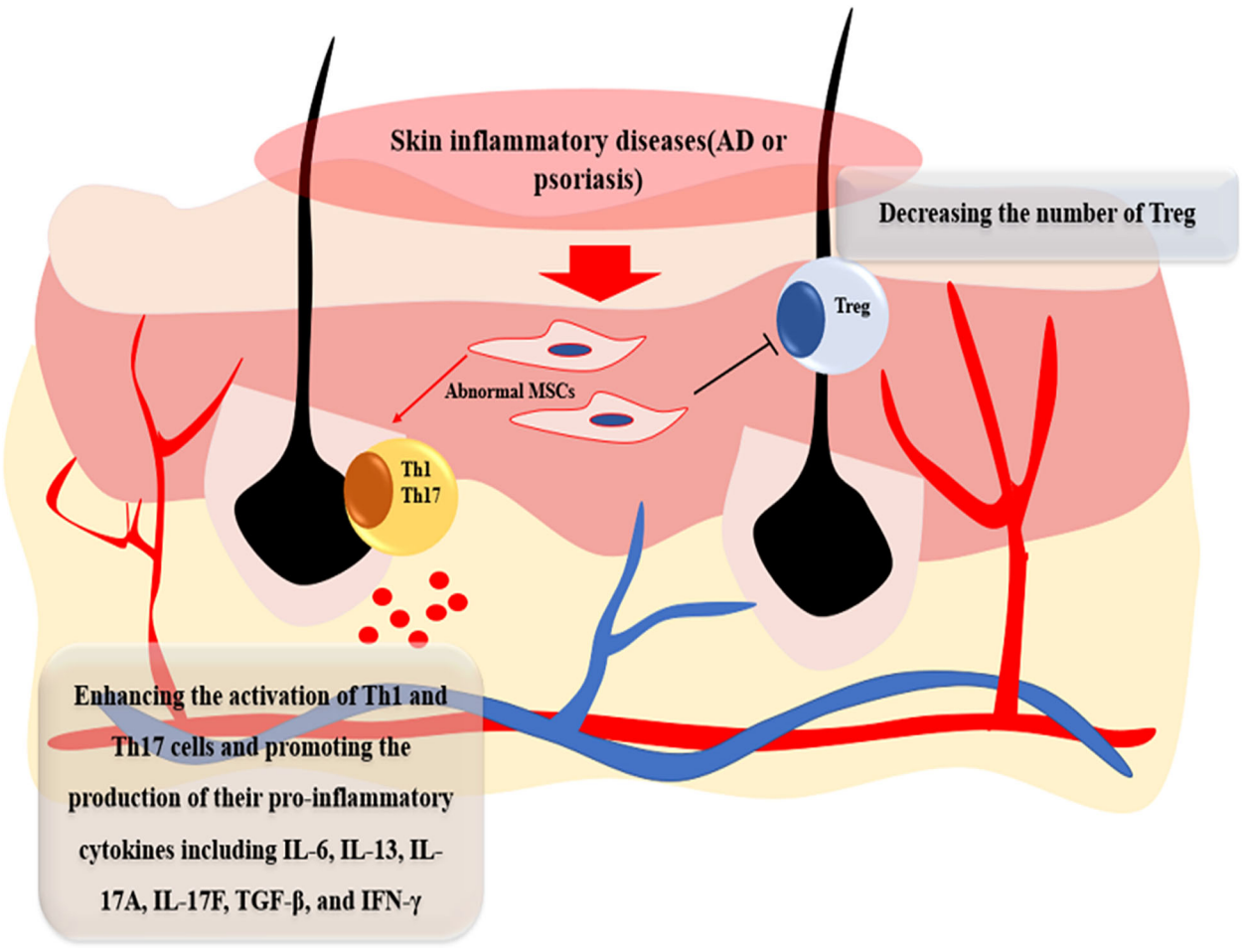

FIGURE 1
The effects of lesional MSCs in AD and psoriasis patients. Compared with MSCs from healthy donor, MSCs in skin lesion of AD and psoriasis patients can enhance the activation of Th1 and Th17 cells and promote the production of their pro-inflammatory cytokines including IL-4, IL-6, IL-13, IL-17A, IL-17F, IL-17C, CCL, TNF-α, and IFN-γ and suppress the activation of Treg.

internal inflammation and clinical symptoms (20). Except for the beneficial effects mentioned previously, Guan et al. also found that subcutaneous injection of mouse AD-MSCs in mice (1×10^6 cells in 1ml PBS) can especially inhibit the expression of Th17 and its relative pro-inflammatory products including IL-17A, CCL20, and matrix metalloproteinase 12 (MMP12) in AD (50) (Table 1).

3.2 UCB-MSCs

Another type of MSCs only found in treating AD is from umbilical cord blood. UCB-MSCs have the advantage of having an easy way to harvest and a low probability of pathophoresis (59). In AD, three ways are found in immunomodulatory effects functioned by UCB-MSCs in recent research. The first one is that subcutaneous administration of UCB-MSCs with 2×10^6 cells in mice can decrease the level of TNFα to inhibit the infiltration of mast cells and decrease the level of IgE into skin lesions by secreting TGF-β (18). Second is that subcutaneous administration of UCB-MSCs in mice (2×10^6 cells) can reduce allergic inflammatory symptoms by inhibiting Th2 cell differentiation and mast cell activation through the cyclooxygenase-2 (COX2)–prostaglandin E2 (PGE2) pathway (60). The last is that subcutaneous administration of UCB-MSCs in mice (2×10^6 cells) can decrease the level of pro-inflammatory cytokines including IL-4, TNF-α, thymus, activation-regulated chemokine (TARC), and IL-22 through secreting the epidermal growth factor (EGF) in skin lesion (61) (Table 1).

3.3 UC-MSCs

On the contrary, research on the effects of UC-MSCs in psoriasis has attracted extensive attention, whereas little attention is given to those in AD. In psoriasis, subcutaneous or intravenous administration of UC-MSCs (2×10^6 cells) can effectively reduce the severity of psoriasis-like dermatitis, delay the appearance of skin lesions, and accelerate the recovery of skin lesions by reducing the number of Th1 and Th17 cells and their secreted pro-inflammatory products and increasing the number of Treg cells (17). Other research found that intravenous administration of UC-MSCs in mice (1×10^6 cells) can inhibit the infiltration of immune cells into the dermal layer and suppress the secretion of IFN-γ from plasmacytoid dendritic cells (pDCs) (52).

3.4 MSCs from other resources

Despite fewer applications of other resources of MSCs in recent research, they indeed play an important role in treating skin inflammatory diseases. Unlike AD-MSCs or BM-MSCs, which include invasive procedures to harvest the cells, inadequate numbers for production, and the most worrying problem, that is, the sources from the mesoderm may have barriers to differentiation into ectodermal and endodermal tissues (55, 62), tonsil-derived mesenchymal stem cells (TMSCs) can be easily isolated from surgically removed tonsil and expanded in cultures, which have been proposed as an alternative source of adult stem cells (63). In AD, subcutaneous administration of TMSCs in mice (2×10^4 cells) can decrease the levels of pro-inflammatory cytokines including IL-6, IL-1β, TNF-α, and IL-4 secreted by Th1 and Th2 cells and the level of IgE secreted by B cells and mast cells (56). Bone marrow is the classical resource to harvest MSCs, but it is limited in production—BM-MSCs have been to some extent in AD. Na et al. found that intravenous administration of BM-MSCs in mice (2×10^5 cells) can suppress the activation of T cells and B cells. The T cell and its inflammatory products including IFN-γ and IL-4 have been suppressed by nitric oxide (NO)-dependent pathways to increase the level of transcription factors including T-bet, GATA-3, and c-Maf. B cells and IgE have been suppressed by the downregulation of AID and BLIMP-1 (19). Interestingly, some sources from the oral cavity to harvest MSCs have been found in treating AD and psoriasis. Xiong et al. found that subcutaneous or intravenous administration of MSCs isolated from human exfoliated deciduous teeth (SHEDs), a special type of MSCs with superior capability of immunoregulation, in mice (2×10^6 cells) can effectively improve the disruption of skin barrier function and enlarged spleens, decrease the levels of IgE and TLSP, and inhibit the activation of Th1, Th2, and Th17 cells in skin lesion (57). Moreover, Ye et al. found that intravenous administration of human gingiva-derived MSCs in mice (2×10^6 cells) can reduce the levels of pro-inflammatory cytokines including IFN-γ, TNF-α, IL-6, IL-17A, and IL-21 secreted by Th1 and Th17 cells and promote the increasing number of Treg cells in mouse psoriasis-like models (21). However, studies on the application and comparison of different types of MSCs in skin inflammatory diseases are still lacking. As a result, different types of MSCs in therapeutic effects of MSCs from the various resources in AD and psoriasis need to be further elucidated (Tables 1, 2).

4 The therapeutic effects of preconditioning MSCs on AD and psoriasis

With the technology advancing, recent research generally indicates that preconditioning MSCs can effectively improve the immunoregulation capability in treating diverse immune diseases (51, 64, 69). Superoxide dismutase (SOD), an antioxidant enzyme, plays an essential role in inflammatory diseases, which can convert the superoxide to hydrogen peroxide and oxygen and exert an anti-inflammatory role (70). SOD3, an extracellular isoform of SOD, can be transduced into MSCs, which can increase the therapeutic potency of MSCs in antioxidant response and immunomodulation (53). In AD, Sah et al. found that subcutaneous administration of SOD3-transduced UCB-MSCs (SOD-MSCs) in mice (2×10^6 cells) can improve the therapeutic effects of MSCs in two pathways. Firstly, SOD-MSCs can alleviate the allergic inflammation in keratinocytes through competitively interacting with the histamine H4 receptor (H4R) and IL-4Rα. Secondly, SOD-MSCs can reduce the inflammation in the skin through the JAK-STAT pathway (54). In psoriasis, another research found that subcutaneous administration of SOD-MSCs (UC-MSCs) in mice (2×10^6 cells) can ameliorate the symptoms of skin lesion by regulating the inflammatory pathways including toll-like receptor-7, nuclear factor-kappa B(NFκB), p38 mitogen-activated kinase (MAPK), and JAK-STAT pathways (71) (Figure 2). Other studies indicate that hepatocyte growth factor-transduced MSCs (HGF-MSCs) also exert a better capability of antioxidant response in various acute/chronic

TABLE 1 The therapeutic effects of MSCs and their derivatives from different sources in AD.

	Sources of MSCs	Animal model	Route of administration	Dose	Main outcome
Shin et al., 2017 (49)	Human AD-MSCs	Mouse model induced by *Dermatophagoides farinae*	Intravenous	$2 \times 10^5/2 \times 10^6$ cells	hAD-MSCs reduced epidermal thickness, lymphocyte infiltration, and MC degranulation
Kim et al., 2018 (22)	Human AD-MSCs	Mouse model induced by using DNCB	Intravenous	1×10^6 cells on 12 and 23 days	Decreasing MIP-2 to overexpress the level of miR-122a-5p, regulating the level of cytokine signaling 1 (SOCS1), to decrease the internal inflammation and clinical symptoms
Guan et al., 2022 (50)	Mouse AD-MSCs	Mouse model induced by ovalbumin	Subcutaneous	1×10^6 cells	Inhibiting the expression of Th17 and its relative pro-inflammatory products
Park et al., 2020 (18)	Human UCB-MSCs	Mouse model induced by *Dermatophagoides farinae*	Subcutaneous	2×10^6 cells	Decreasing the level of TNFα to inhibit the infiltration of MC and decrease the level of IgE into skin lesions by secreting transforming growth factor-beta (TGF-β)
Shin et al., 2021 (28)	Human UCB-MSCs	Mouse model induced by *Dermatophagoides farinae*	Subcutaneous	2×10^6 cells	Reducing allergic inflammatory symptoms by inhibiting Th2 cell differentiation and MC activation through the COX2–PGE2 pathway
Jung et al., 2022 (51)	Human UCB-MSCs	Mouse model induced by *Dermatophagoides farinae*	Subcutaneous	2×10^6 cells	Decreasing IL-4, TNF-α, TARC, and IL-22 through EGF in skin lesion
Jung et al., 2021 (52)	Human TMSCs	Mouse model induced by using DNCB	Subcutaneous	2×10^4 cells	Decreasing IL-6, IL-1β, TNF-α, and IL-4 secreted by Th1 and Th2 cells, respectively, and IgE secreted by B cells and MC
Na et al., 2014 (19)	Mouse BM-MSCs	Mouse model induced by ovalbumin	Intravenous	2×10^5 cells	Suppressing T cells and its inflammatory products by NO-dependent pathways. Suppressing B cells and IgE by the downregulation of AID and BLIMP-1.
Xiong et al., 2022 (53)	Human sheds	Mouse model induced by using DNCB	Intravenous/ subcutaneous	2×10^7 cells/mL, and 2×10^6 cells on days 17, 24, and 31	Improving the disruption of skin barrier function and enlarged spleens. Decreasing IgE and TLSP Inhibiting the activation of Th1, Th2, and Th17 cells in skin lesion
Sah et al., 2018 (54)	Human UCB-MSCs (SOD3-tranduced)	Mouse model induced by ovalbumin	Subcutaneous	2×10^6 cells on days 20, 28, and 42	Alleviating the allergic inflammation in keratinocytes through competitively interacting with H4R and IL-4Rα. Reducing the inflammation in the skin through the JAK-STAT pathway
Park et al., 2019 (55)	Human WJ-MSCs (preconditioned with the TLR3 agonist poly I:C or IFN-γ)	Mouse model induced by Af extract	Subcutaneous	\	Decreasing proinflammatory cytokines. Ameliorating epidermal thickness and inflammatory cell infiltration in skin lesions.
Cho et al., 2018 (56)	Human AD-MSCs (EVs)	Mouse model induced by *Dermatophagoides farinae*	Intravenous/ subcutaneous	0.14, 1.4, and 10 μg/ head	Reducing pathological symptoms, serum IgE, the number of eosinophils in blood, and the infiltration of MC, CD86+, and CD206+ cells in skin lesions. Decreasing IL-4, IL-23, IL-31, and TNF-α in AD skin lesions
Shin et al., 2020 (57)	Human AD-MSCs (EVs)	Mouse model induced by oxazolone (Ox)	Subcutaneous/ topical	1, 3, and 10 μg/head	Restoring epidermal barrier functions in AD by facilitating the *de novo* synthesis of ceramides

(Continued)

TABLE 1 Continued

	Sources of MSCs	Animal model	Route of administration	Dose	Main outcome
Kim et al., 2022 (58)	Canine AD-MSCs (EVs)	Mouse model induced by using DNCB	Subcutaneous	2×10^{10} particles/head	Decreasing serum IgE, epidermal inflammatory cytokines, such as IL-4, IL-13, IL-31, RANTES, and TARC. Repairing skin barrier by restoring transepidermal water loss, enhancing stratum corneum hydration, and upregulating the expression levels of epidermal differentiation proteins. Reducing IL-31/TRPA1-mediated pruritus and activation of JAK/STAT signaling pathway

disease (58). Meng et al. found that in psoriatic skin lesions, intravenous administration of HGF-transduced dental pulp stem cells (HGF-DPSCs) in mice (2×10^6 cells) can ameliorate psoriasis-like erythema, scaling, and thickening and the expression of cytokeratin 6 (CK6) and cytokeratin 17 (CK17). In addition, in inflammatory respect, HGF-DPSCs can decrease the levels of inflammatory cytokines such as IFN-γ, IL-6, and TNF-α; reduce the number of Th1; and increase the number of Th2. Moreover, HGF-DPSCs exerts more efficacy compared with pure DPSCs (72) (Figure 2). Finally, preconditioning MSCs with inflammatory factors can more effectively treat skin inflammatory diseases. Park et al. revealed that subcutaneous administration of human Wharton's jelly-derived MSCs (WJ-MSCs) preconditioned with the Toll-like receptor 3 agonist poly I:C or IFN-γ can decrease the levels of proinflammatory cytokines in a murine model of AD. Moreover, they can ameliorate more epidermal thickness and inflammatory cell infiltration in skin lesions than non-preconditioned MSCs (73). Zhang et al. found that intradermal administration of EVs derived from UC-MSCs stimulated by IFN-γ (IFNγ-sEVs) (150 μg) in mice can effectively reduce the symptom of psoriasis through decreasing the levels of pro-inflammatory cytokines including IL-17A, IFN-γ, IL-6, and TNF-α and Th17 cells and increasing the population of Th2 cells in both spleen and skin in psoriasis (74) (Figure 2). Up to now, there are still few research focusing on preconditioning MSCs in AD and psoriasis, which need to be further studied.

5 The therapeutic effects of EVs derived from MSCs on AD and psoriasis

Although MSCs have been widely used for treating skin inflammatory diseases due to their immunomodulation capability, but an increasing concern about their adverse effects such as embolism and inefficient homing to the target limits their application. Recent research

TABLE 2 The therapeutic effects of MSCs and their derivatives from different sources in psoriasis.

	Sources of MSCs	Animal model	Route of administration	Dose	Main outcome
Chen et al., 2022 (17)	Human UC-MSCs	Mouse model induced by IMQ cream	Intravenous/subcutaneous	2×10^6 cells	Reducing the severity of psoriasis-like dermatitis. Delaying the appearance of skin lesions. Accelerating the recovery of skin lesions by reducing the number of Th1 and Th17 cells and their secreted pro-inflammatory products. Increasing the number of Treg cells.
Chen et al., 2019 (64)	Human UC-MSCs	Mouse model induced by IMQ cream	Intravenous	1×10^6 cells	Inhibiting the infiltration of immune cells into the dermal layer Suppressing the secretion of IFN-γ from pDCs
Ye et al., 2022 (21)	Human gingiva-derived MSCs	Mouse model induced by IMQ cream	Intravenous	2×10^6 cells on 1 and 4 days	Reducing pro-inflammatory cytokines including IFN-γ, TNF-α, IL-6, IL-17A, IL-21 secreted by Th1 and Th17 cells Promoting the number of Treg cells
Sah et al., 2016 (65)	Human UC-MSCs (SOD3-tranduced)	Mouse model induced by IMQ cream	Subcutaneous	2×10^6 cells	Ameliorating the symptoms of skin lesion by regulating the inflammatory pathways including TLR-7, NFκB, MAPK, and JAK-STAT pathways
Meng et al., 2021 (66)	Human DPSCs (HGF-transduced)	Mouse model induced by IMQ cream	Intravenous	2×10^6 cells	Ameliorating the psoriasis-like erythema, scaling, and thickening and the expression of CK6 and CK17. Decreasing inflammatory cytokines such as IFN-γ, IL-6, and TNF-α. Reducing the number of Th1. Increasing the number of Th2.i90op.
Zhang et al., 2022 (67)	Human UC-MSCs (IFNγ-sEVs)	Mouse model induced by IMQ cream	Intradermal	150 μg	Reducing the symptom of psoriasis through decreasing the levels of pro-inflammatory cytokines including IL-17A, IFN-γ, IL-6, and TNF-α and Th17 cells. Increasing the population of Th2 cells in both spleen and skin.
Zhang et al., 2022 (68)	Human UC-MSCs (EVs)	Mouse model induced by IMQ cream	Subcutaneous	50 μg	Reducing proinflammatory cytokines and chemokines including IL-17, IL-23, TNFα, and CCL20 suppressing the activation of DCs through inhibiting the JAK-STAT pathway

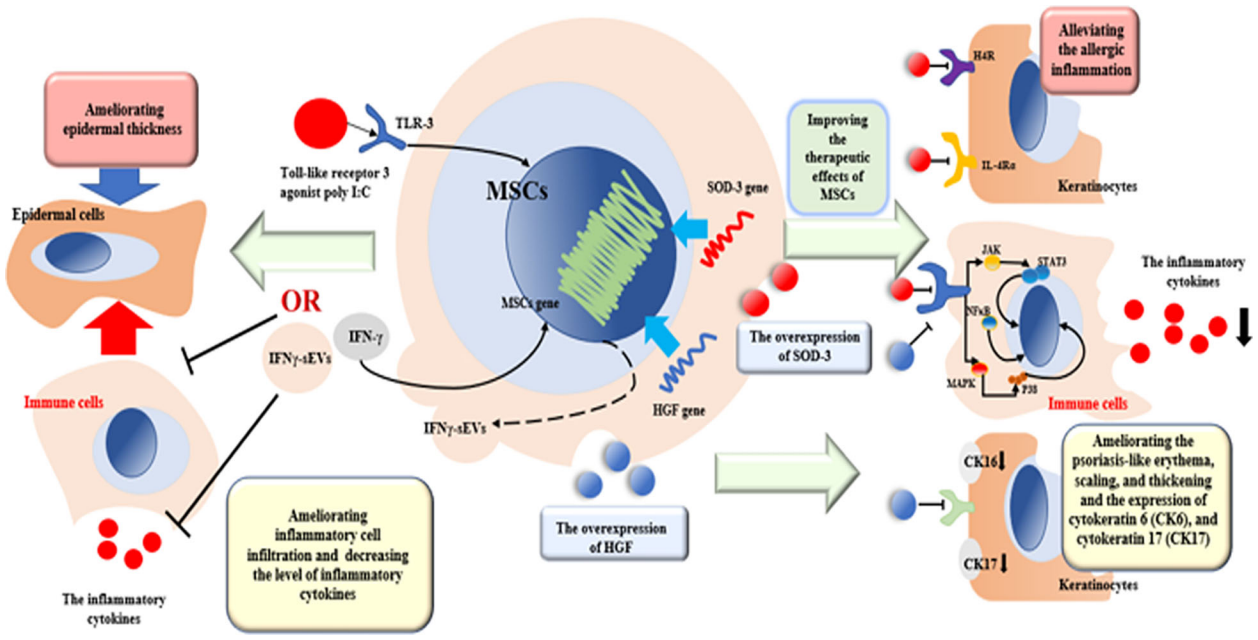

FIGURE 2
The potential mechanism of preconditioned MSCs and EVs in treating AD and psoriasis. SOD3-transduced MSCs can alleviate the allergic inflammation in keratinocytes through competitively interacting with H4R and IL-4Rα. SOD-MSCs can reduce the inflammation in the skin through the NFκB, MAPK, and JAK-STAT pathways. HGF-transduced MSCs can ameliorate psoriasis-like erythema, scaling, and thickening and the expression of CK6 and CK17 and decrease pro-inflammatory cytokines such as IFN-γ, IL-6, and TNF-α. Preconditioned with the TLR3 agonist poly I:C or IFN-γ can ameliorate more epidermal thickness and inflammatory cell infiltration in skin lesions. IFNγ-sEVs can decrease pro-inflammatory cytokines including IL-17A, IFN-γ, IL-6, and TNF-α and Th17 cells and increase the population of Th2.

revealed that EVs derived from MSCs cannot only effectively be instead of the functions of MSCs but also easy to be engineered to better exert their functions (17, 65, 74, 75). In AD, EVs are mainly from AD-MSCs and function in three ways. Firstly, in the AD mouse model, subcutaneous administration of EVs derived from AD-MSCs in mice can reduce the levels of inflammatory cytokines and IgE including IL-4, IL-5, IL-13, TNF-α, IFN-γ, IL-17, and TSLP in skin lesion. Secondly, they can reduce trans-epidermal water loss and enhance stratum corneum (SC) hydration. At last, EVs can restore the skin barrier and lipid metabolism in skin lesion (66, 67, 76). Moreover, in psoriasis, Zhang et al. found that subcutaneous administration of EVs derived from UC-MSCs in mouse psoriasis-like models (50 μg) can effectively reduce the level of proinflammatory cytokines and chemokines including IL-17, IL-23, TNFα, and CCL20 and suppress the activation of DCs through inhibiting the JAK-STAT pathway (68). Another research found that intradermal administration of IFNγ-preconditioned EVs from UC-MSCs in mice (150 μg) can also reduce the symptoms of psoriasis described previously (74) (Figure 2). However, the different resources and mechanism of EVs are still under revealed and the engineered EVs are little applied in treating skin inflammatory diseases, which needs to be further studied in future.

6 The clinical efficacy of MSCs and their derivatives applied in AD and psoriasis

The administration of MSCs in AD and psoriasis has been tested in clinical treatment; the efficacy and side effects are detailed in the following part. Shin et al. intravenously administered BM-MSCs to five patients with AD (1.0×10^6 cells/kg three times every 2 weeks) and observed for 16 weeks (treatment for 4 weeks and follow-up for 12 weeks). After 16 weeks, the follow-up was to identify the period during which the patient's improved symptoms are maintained by using medications without additional use of systemic steroids and immunomodulators. They found that the Eczema Area and Severity Index (EASI) improved significantly at 16 weeks, which had a long-term efficacy for an average of 38 weeks (range, 16–86), whereas it showed no serious side effects in the patients. Moreover, the pro-inflammatory cytokines in their blood significantly decreased at the end point (28). Similarly, Kim et al. recruited 34 patients with moderate-to-severe AD with a follow-up for 1 and 3 months revealed that subcutaneously administering a high dose of UCB-MSCs (5.0×10^7 cells) could effectively reduce the symptom of AD and with little adverse effects and no relapse (29). In psoriasis, Ahn et al. found a clinical case that a 47-year-old man, diagnosed with psoriasis in 1995, has received various treatments for 25 years but with no improved psoriatic condition. After both intravenous and local administration of UC-MSCs, his erythema gradually disappeared (10 ml for intravenous administration with 3×10^6 cells/ml in 0.9% physiological saline and 2–4 ml for local administration with 1×10^6 cells/ml in 0.9% physiological saline). The follow-up was 122 days, and the symptom was gradually becoming better without any side effects (77). In addition, in a 17-patient clinical trial, Cheng et al. also found that intravenous administration of UC-MSCs (1.5×10^6 cells/kg once time every 2 weeks, four times as a course of treatment) can effectively reduce the symptoms of psoriasis with no severe adverse effects. The follow-up

was at 15 days, 30 days, 45 days, 2 months, 3 months, and 6 months after treatment and there are no relapse and severe adverse effects observed (78). However, the number of clinical studies of using MSCs in AD and psoriasis is still inadequate. Moreover, more detailed information such as the choosing of safe dose, resources, and delivery way of MSCs should be cleared, which needs to be further studied.

7 Comparing alternatives of MSCs and their derivatives applied in AD and psoriasis

There are some limitations in the application of MSCs and their derivatives: a) there is a need for the conduction of double-blinded, placebo-controlled studies to indicate the potential clinical application of MSCs in AD and psoriasis, and b) the production and cost of MSCs cannot reach the standard, which makes it difficult to translate into clinical treatment. However, the application of those treatments is still more effective in treating AD and psoriasis at the present compared with other treatments. In the application of different resources of MSCs in both AD and psoriasis, we have summarized the mechanism of different types of MSCs in the diseases. Interestingly, we found that UC-MSCs performed in the present studies were only used in psoriasis (17, 52, 78), UCB-MSCs only in AD (18, 59–61). It may be attributed to the mechanism of both kinds of MSCs to regulate inflammation, in which UC-MSCs were more likely to regulate the activation of Th1 and Th17 cells and their production, whereas UCB-MSCs were more likely to regulate the Th2 cells as present studies have mentioned. However, there is no research to compare both kinds of MSCs in the same skin inflammatory diseases. Moreover, various resources of MSCs were applied to administer into AD such as AD-MSCs, UCB-MSCs, TMSCs, BM-MSCs, and MSCs from the oral cavity, but only UC-MSCs were directly used in psoriasis in the present studies as mentioned above. Firstly, it may be attributed to the different pathogenesis of AD and psoriasis (79), whereas both the two diseases show signs of dysregulation of inflammation (80). Secondly, the chosen resources of MSCs may be primarily biased on the local storage facilities and policies of MSCs (the application of UC-MSCs in psoriasis mainly from Chinese). However, the comprehensive and systematic comparison of MSCs lines is still urgently needed in AD and psoriasis at the present.

Despite many studies associated with the application of adult mesenchymal stem cells such as AD-MSCs in treating AD and psoriasis, there are still limitations. Firstly, the adult-MSCs can just be harvested from the patients, which may limit the production of MSCs. Secondly, the therapeutic effects of MSCs can be seriously influenced by the age of the patients. Lastly, as we mentioned in the manuscript, the MSCs from patients may have lower effects compared with healthy donors, whereas compared with adult MSCs, MSCs from the fetus such as UC-MSCs have lower immunogenicity and more powerful therapeutic effects. Most importantly, they can be extracted from oneself or non-relative donators, thus enhancing the production of MCSs. As a result, we believe that MSCs from the fetus such as UC-MSCs may be the best resource to be employed to treat AD and psoriasis.

Furthermore, based on the present studies in which the accurate target is still unrevealed, the preconditioned MSCs showed more healing capability than normal MSCs as mentioned in the previous paragraphs, which makes the preconditioning technology a more promising method in dealing with MSCs *in vitro*—the next step in choosing the resource of MSCs. Above all, the two skin inflammatory diseases have also been accompanied by other immune diseases, aside from skin symptoms (81–84). Based on the condition, only subcutaneous injection of MSCs to improve the skin symptoms cannot be enough to cure the AD and psoriasis completely. Moreover, intravenous injection of MSCs increases the risk of embolism. As a result, administration of a small molecule, EVs from MSCs, through the vein may be the best method to avoid the side effects. Moreover, the present studies uncovered that EVs from MSCs can be almost a substitute of MSCs in treating diseases, but EVs alone cannot accurately home to the target of the diseases. As mentioned above, engineered EVs with the targeted ligand may be perfect to resolve the problem. However, the technology applied to engineer EVs has been little studied and there is still a need to find out the key targets of AD and psoriasis. Moreover, despite the advancing technology and that the application of MSCs has been widely used in clinical treatment, the price for the administration is still high beyond the expectation of patients, not to mention using biological programming techniques in engineering EVs from MSCs to treat skin problems. Another strategy for the application of MSCs is subcutaneous administration of MSC-CM (conditioned medium), which may dissolve the high cost of MSCs (85). However, as the content of MSC-CM may not be ensured, the effects including therapeutic efficacy and adverse effects and its mechanism need more studies to elucidate. Above all, the techniques to improve the production of MSCs and thus decrease the cost will not only stimulate more and more studies on MSCs in treating diseases but also allow more patients around the world to use MSCs and their derivatives to improve their refractory disease.

8 Conclusion

Chronic skin inflammatory diseases such as AD and psoriasis are mainly caused by unregulated immune response, which not only can induce the symptoms of skin lesion but also are accompanied by other immune diseases. Evidence of therapeutic effects and mechanisms found by current studies indicates that biological therapy based on MSCs and their derivatives is a promising approach for the treatment of skin inflammatory diseases. As a result, additional studies aiming at uncovering the mechanisms of the therapeutic effects of MSCs in AD and psoriasis may help define better therapeutic strategies for these diseases.

Author contributions

SY, JY, KM, and XF conceived and designed the review. JY, MX, HL, MR, ShY and YY prepared the figures. JY, HL, SY, and XF wrote the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This study was supported in part by the National Nature Science Foundation of China (81801909, 81830064, 81721092) and the Military Medical Research and Development Projects (18-JCJQ-QT-020, AWS17J005, 2019-126).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer ML declared a shared affiliation, with no collaboration, with the authors to the handling editor at the time of the review.

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 05 April 2023
DOI 10.3389/fimmu.2023.1150971

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Cytokines and Soluble Mediators in Immunity, a section of the journal Frontiers in Immunology

RECEIVED 25 January 2023
ACCEPTED 23 March 2023
PUBLISHED 05 April 2023

CITATION
Xu X, Wang Y, Luo X, Gao X, Gu W, Ma Y, Xu L, Yu M, Liu X, Liu J, Wang X, Zheng T, Mao C and Dong L (2023) A non-invasive strategy for suppressing asthmatic airway inflammation and remodeling: Inhalation of nebulized hypoxic hUCMSC-derived extracellular vesicles.
Front. Immunol. 14:1150971.
doi: 10.3389/fimmu.2023.1150971

A non-invasive strategy for suppressing asthmatic airway inflammation and remodeling: Inhalation of nebulized hypoxic hUCMSC-derived extracellular vesicles

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Mesenchymal stromal cell-derived extracellular vesicles (MSC-EVs) are extremely promising nanoscale cell-free therapeutic agents. We previously identified that intravenous administration (IV) of human umbilical cord MSC-EVs (hUCMSC-EVs), especially hypoxic hUCMSC-EVs (Hypo-EVs), could suppress allergic airway inflammation and remodeling. Here, we further investigated the therapeutic effects of Hypo-EVs administration by atomizing inhalation (INH), which is a non-invasive and efficient drug delivery method for lung diseases. We found that nebulized Hypo-EVs produced by the atomization system (medical/household air compressor and nebulizer) maintained excellent structural integrity. Nebulized Dir-labeled Hypo-EVs inhaled by mice were mainly restricted to lungs. INH administration of Hypo-EVs significantly reduced the airway inflammatory infiltration, decreased the levels of IL-4, IL-5 and IL-13 in bronchoalveolar lavage fluid (BALF), declined the content of OVA-specific IgE in serum, attenuated the goblet cell metaplasia, and the expressions of subepithelial collagen-1 and α-smooth muscle actin (α-SMA). Notably, Hypo-EV INH administration was generally more potent than Hypo-EV IV in suppressing IL-13 levels and collagen-1 and α-SMA expressions. RNA sequencing revealed that various biological processes, such as cell adhesion, innate immune response, B cell activation, and extracellular space, were associated with the activity of Hypo-EV INH against asthma mice. In addition, Hypo-EVs could load exogenous miR-146a-5p (miR-146a-5p-EVs). Furthermore, INH administration of miR-146a-5p-EVs resulted in a significantly increased expression of miR-146a-5p mostly in lungs, and offered greater protection against the OVA-induced increase in airway inflammation,

subepithelial collagen accumulation and myofibroblast compared with nebulized Hypo-EVs. Overall, nebulized Hypo-EVs effectively attenuated allergic airway inflammation and remodeling, potentially creating a non-invasive route for the use of MSC-EVs in asthma treatment.

KEYWORDS

inhalation device, nebulized administration, mesenchymal stem cells, extracellular vesicles, asthma, lung injury

Introduction

Asthma is a chronic respiratory disease characterized by airway inflammation and airway remodeling (1). This disease affects more than 350 million people worldwide and has become a serious global public health concern (2). Current asthma therapy, including corticosteroids and long-acting β2-adrenoceptor agonists, focuses on symptom management rather than disease regression (3). Moreover, high doses of corticosteroids have side effects (4). New treatment strategies are still needed.

An increasing amount of evidence showed that exogenous mesenchymal stem cells (MSCs) effectively elicited inhibitory effects on airway inflammation and airway remodeling in asthmatic mice through the secretion of paracrine factors (5, 6). Extracellular vesicles (EVs) are nano-scale membrane vesicles released by almost all cell types (7). Transplantation of MSC-derived EVs (MSC-EVs) and MSC exhibits similar therapeutic effects on the alleviation of lung inflammation and reduction of collagen fiber content in chronic asthma mice (8), confirming MSC-EVs are a major kind of functional forms of MSCs (9). Strikingly, MSC-EVs possess conspicuous advantages over cell-therapy, such as high biosafety, low immunogenicity, easy storage, and can even be considered as an off-the-shelf product (10). Thus, MSC-EVs might represent an extremely promising cell-free therapeutic strategy for asthma, as confirmed in various experimental asthma models (11–14).

To data, most published articles on the use of MSC-EVs in the treatment of asthma (mice model) discussed their administration by tail vein. It should be noted that large amount of MSC-EVs administered intravenously (IV) will converge in the liver (15–19), which not only increased the metabolic burden of body but also caused the MSC-EV waste [especially the yield of MSC-EVs is insufficient today (20)]. Moreover, most patients with asthma might not accept this invasive administration route. The atomizing inhalation (INH) has been gaining immense attention in the treatment of lung damage, because it offers the advantages of rapid onset of action, reduced dosage amount, localized action, and avoidance of first-pass effect. We speculated that inhalation of nebulized MSC-EVs might be effective for the treatment of asthma, which has never been reported before.

In this study, we first created a nose-only and high-effective inhalation exposure system to mouse by simulating the process of human clinical atomization administration. We previously found that hypoxic environment (5% O_2) could promote hUCMSCs to release more EVs (called Hypo-EVs), and these Hypo-EVs (IV administration) were generally more potent than normoxic hUCMSC-EVs (21% O_2) in suppressing airway inflammation and remodeling in chronic asthmatic mice (14). Thus, we selected hypoxic hUCMSCs as the source of MSC-EVs and further explored the anti-asthma potential of Hypo-EVs when therapeutically INH administered to established disease pathology.

Materials and methods

Instrument, inhalation and mouse holding chambers

Medical/household compression atomizer, including air compressor (DM-YWH 01L) and nebulizer, was procured from Demi medical equipment Co.,ltd (Guangzhou, China). Inhalation chamber was made using silica glass, and consisted of 3 major parts (1) inlet pipe (height 5 cm, inner diameter 2cm); (2) six-way pipe (inner pipe:length 4cm, inner diameter 2cm; outer pipe: length 6 cm, inner diameter 3 cm; vent: diameter 0.4 cm); (3) base fixing device.

Centrifuge tubes (diameter 2.8 cm) routinely used in the laboratories were designed as mice holding chambers. The tips of the centrifuge tubes were removed to make a hole of around 0.9 cm diameter.

Cell culture

HUCMSCs used in this study were generated from fresh umbilical cord samples as we reported previously (21), and maintained in stem cell culture medium (Cyagen, Guangzhou, China) at 37°C with 5% CO_2. HUCMSCs between passages 3–7 were used for all experiments.

Extraction and characterization of hypo-EVs

HUCMSCs were cultured in serum-free culture medium for 24 h under hypoxic (5% O_2) conditions (Hypo-MSCs) (14). The cell

supernatants were collected and centrifuged at 300×g for 10 min, 2000×g for 20 min to discard cell debris. Then, Hypo-EVs were isolated by ultracentrifugation (Beckman Coulter Optima L-100 XP ultracentrifuge, Miami, FL) at 100,000×g for 90 min as previously described (21). After that, the pellets were collected, washed, and resuspensed in PBS (Hypo-EVs solution). All centrifugations were performed at 4°C and the Hypo-EVs solution was stored at -80°C. For the isolation of Hypo-EVs engineered by miR-146a-5p (miR-146a-5p-EVs), Hypo-MSCs were transfected with 50 nM miR-146a-5p mimic or mimic NC (GenePharma, Shanghai, China) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), followed by culturing with conditioned medium and ultracentrifugation.

The protein concentration of EVs was determined by using BCA protein assay kit (Beyotime, Nantong, China). EV surface markers TSG101 (ab133586, Abcam, Cambridge, MA), and HSP70 (ab181606, Abcam) were detected by Western blot (WB) as our previous description (22). Shape and ultrastructure of EVs were observed by transmission electron microscopy (JEM-1200EX; JEOL Ltd., Tokyo, Japan). The particle size distribution of EVs was determined by nanoparticle trafficking analysis using ZetaView PMX 110 (Particel Metrix, Meerbusch, Germany) according to the manufacturer's protocols.

Nebulized hypo-EVs tracking in mice

Hypo-EVs were labeled with Dir (Invitrogen) as our previous report (17). BALB/c mouse was nebulized with Dir-labeled Hypo-EVs (40 μg diluted in 0.5 mL PBS). The mice were sacrificed at each time point (day 1 and 7) respectively post-administration. The ex vivo fluorescence images of brain, heart, liver, spleen, lung, kidney, stomach and intestines were visualized using Xtreme II (BRUKER, Bremen, Germany) according to the manufacturer's protocol.

Mouse model of chronic asthma with nebulized hypo-EVs

Six-week-old female BALB/c mice were purchased from the Comparative Medicine Centre of Jiangsu University (Zhenjiang, China). The OVA-induced chronic asthma model has been previously described by our team (14). Briefly, apart from the control group, the mice were sensitized on day 0, 7, and 14 with 40 μg OVA (Sigma, Poole, UK) and 2 mg 10% aluminum hydroxide (Sigma) in PBS by intraperitoneal injection. Then, the sensitized mice were challenged with aerosol OVA (5%) in a plastic chamber (30 × 20 × 15 cm) three times per week from days 21 to 53. Aerosol OVA particles were created from a compression atomizer (403 M; YUWELL, Zhenjiang, China), directed into the plastic chamber, and vented to a fume hood.

A therapeutic regimen was instigated by inhaling 40 μg Hypo-EVs (suspended in 0.5 mL PBS, EVs-INH group) on day 26. Mice from EVs-IV group were treated with Hypo-EVs (40 μg suspended in 0.1 mL PBS) by intravenous injection. After four times treatment (day 26, 33, 40 and 47), the mice were sacrificed on day 55.

Analysis of cells and inflammatory cytokines in bronchoalveolar lavage fluid

The BALF was collected as described in our previous study (23), and centrifuged (1500 rpm for 5 min) to separate the cells and supernatants. Cell pellets were resuspended in PBS (1 mL), and total inflammatory cells was counted using Neubauer hemocytometer, and eosinophils count was performed using Wright and Giemsa staining (BASO, Zhuhai, China). The supernatants were kept at -80° C until they were used for cytokine analysis. The concentrations of IL-4 IL-5, and IL-13 in the BALF were determined by using commercial enzyme-linked immune sorbent assay (ELISA) kits (Multi Sciences, Hangzhou, China) following the manufacturer's instructions. The absorbance of the final reactant was measured at 450 and 630 nm using an ELISA plate reader (BioTek, Biotek Winooski, Vermont).

Measurement of serum OVA-specific IgE

The serum was prepared from mouse whole blood (mouse orbits). OVA-specific IgE in serum was measured using ELISA. Briefly, the 96-well plate was coated with 100 μL of OVA (100 μg/mL) per well and blocked with 5% skim milk. After washing, 1:250 dilution of goat anti-mouse IgE (Abcam) and HRP-conjugated rabbit anti-goat secondary IgG (1:5000, Multisciences) were used for detection. It was read at 450 nm in an ELISA plate reader (BioTek).

Lung histopathology

Lung tissues were collected, fixed with 10% neutral buffered formalin (48 h) and embedded in paraffin fixation. Then, 4-μm thick sections (3 sections per animal) were cut and stained with hematoxylin and eosin (HE), periodic acid–Schiff (PAS), and Masson trichrome. All pictures were captured using a Nikon microscope (Tokyo, Japan). Peribronchial inflammation score (grades 0-4) was evaluated in a blind-way (24). Goblet cell hyperplasia (grades 0-4) was determined using the method described by Padrid et al. (25). Image-Pro Plus software (Version X; Adobe, San Jose, CA) was used to quantify the areas occupied by collagen (blue, Masson trichrome staining), which were subsequently divided by the total area examined (as the percentage of collagen fibers) (8). At least 6 bronchioles were counted in each slide, and then, the mean inflammation score, goblet cell hyperplasia score, and percentage of collagen fibers were calculated for each mouse.

Immunohistochemistry

IHC were performed as described in previous study (26). Briefly, mouse lung tissue sections were incubated with an antibody collagen-1 (GB111364, diluted 1: 500; Servicebio,

Whhan, China) or a-SMA (GB11022-3, 1:1000 dilution; Servicebio) overnight at 4°C, followed incubating by HPR-conjugated secondary antibody (GB23303 or GB23301, 1:1000 dilution; Servicebio). Diaminobenzidine was used as the substrate. Integrated optical density of collagen-1 and a-SMA were detected by using Image-Pro Plus software.

RNA sequencing

Total RNA was extracted from OVA and EVs-INH mouse lungs using RNAiso Plus reagent (Takara Bio Inc., Japan). The RNA integrity was evaluated using Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA). RNA-Seq experiment was carried out by LC-BIO Bio Technology (Hangzhou, China). After sequencing, the data (|log FC|>1 and adjusted $P< 0.05$) were further analyzed using LC-Bio Cloud Platform (https://www.omicstudio.cn/) for heat map, and using DAVID Bioinformatics Tesources (https://david.ncifcrf.gov/home.js) for gene ontology enrichment.

RNA isolation and quantitative real-time PCR

Total RNA was extracted using RNAiso Plus (Takara) or mirVana RNA isolation kit (Ambion, Austin, TX) according to the manufacturer's manual. All of the primers for real-time PCR (Pank3, Zfp59, Arhgef9, Tubb1, Trpv4, Casc1, Gapdh, miR-146a-5p and U48) were purchased from Genecopoeia (Germantown, MD). Real-time PCR was performed using All-in-oneTM qPCR Mix (Genecopoeia) on a QuantStudio 5 Real-Time system (Thermo Fisher Scientific, Waltham, MA). Date was calculated by $2^{-\Delta\Delta Ct}$ method based on our previous description (27). The threshold cycle (CT) indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold. ΔCT (test) = CT (target, test) – CT (ref, test), ΔCT (calibrator) = CT (target, calibrator) – CT (ref, calibrator), ΔΔCT = ΔCT (test) – ΔCT (calibrator). The levels of mRNA and miR-146a-5p were normalized to Gapdh and U48 respectively.

WB

WB analysis of total protein from the lung tissues was performed as previously described (22). Equal amounts of proteins (50 μg) were electrophoresed in 10% sodium dodecyl SDS-PAGE and transferred onto polyvinylidene difluoride (PDVF) membranes. After blocking in a nonfat milk solution, the PDVF membranes were incubated with primary antibodies specific for TRAF6 (E-AB-18251, Elabscience, Wuhan, China), TIRAP (ab17218, Abcam) and GAPDH (60,004–1-lg, Proteintech, Rosemont, IL) overnight. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (ab97051 or ab6728, Abcam) at room temperature for 1 h. Next, the PVDF membranes were incubated with enhanced chemiluminescence reagent (Merck Millipore, Billerica, MA) before detection using a ChemiScope series 4300 (CLINX Science Instruments, Shanghai, China).

Safety assessment of nebulized hypo-EVs

Mice were nebulized with Hypo-EVs (40 μg diluted in 0.5 mL PBS) at day 0, 7, 14, 21, 28, 35, 42, and 49 (total eight times, 40 μg/time). Healthy mice were used as controls. Mice survival and weight was recorded once a week. At 56 days, mice serum was collected and the serum biochemistry, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea nitrogen (UREA) and creatinine (CREA), were detected using Beckman Coulter AU2700 automatic biochemical analyzer (Beckman). After mice were sacrificed, the major organs (heart, liver, spleen, lung, kidney, stomach, and intestines) were harvested for HE staining to assess the histological changes.

Statistical analysis

The statistical analyses were performed with GraphPad Prism (Version 5.0; La Jolla, CA). Data are expressed as mean ± SD. The groups were compared using the one-way analysis of variance (Tukey Kramer *post hoc* tests) or Student's t-test. A value of $P < 0.05$ was considered significant.

Results

Design of inhalation device for mice

To better simulate the process of human clinical atomization administration, we first created an aerosol inhalation device for mice by imitating the human atomization mask (nose-only exposure, Figure 1A). This device includes an inhalation chamber and a mouse-holding chamber. The inhalation chamber has a six-way pipe with small cylindrical vial, and a small hole was made in the wall of each cylindrical vial, in order to minimize any pressure buildup inside the aerosol chamber. Six-furcations of the inhalation chamber were placed at the equidistant levels from the inlet to ensure uniformity in the dose delivered to each mouthpiece and ultimately to the delivery ports of the animal restrainers (Figure 1B). Centrifuge tubes (50 mL) were used as the mouse-holding chambers. The animals were restrained with the medical cotton ball, which could prevent any plausible change in the direction of the mouse's movement. In addition, the tips of the centrifuge tubes were removed to make a smooth edge hole, so that nose of the mouse can be easily inserted (Figure 1C).

The medical air compressor attached to the nebulizer provides a positive pressure for the generation of aerosol mist (Figure 1D). Hypo-EVs were placed in the nebulizer. Plastic tubing was employed to connect the mouth of the nebulizer with the inlet pipe of inhalation chamber. Thus, the mist generated from the nebulizer could be transfer to the delivery port and be inhaled by the mouse restricted in the mouse-holding chamber (Figure 1E).

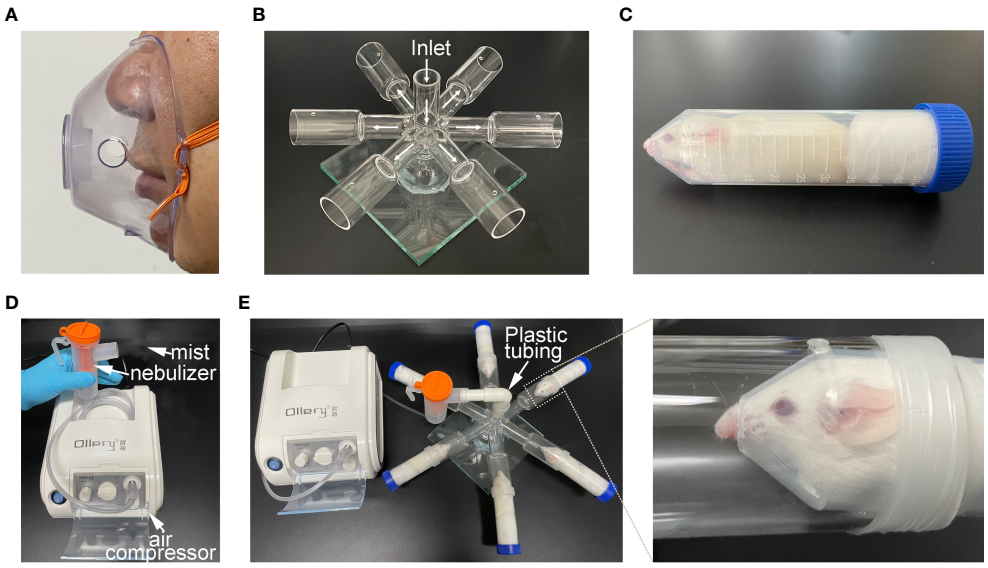

FIGURE 1
Construction of atomization inhalation system for mice. **(A)** atomization inhalation mask commonly used by human beings. **(B)** Six-way pipe inhalation chamber for mice depicting the flow pattern of the aerosol. This chamber consisted of three parts (inlet pipe, six-way pipe and base fixing device). **(C)** Holding chamber for mouse. **(D)** medical/household air compressor and nebulizer set-up. **(E)** Working diagram of mouse atomization inhalation system.

Identification of nebulized hypo-EVs

To allow the analysis of the characteristics of nebulized Hypo-EVs, nebulized Hypo-EVs ejected from nebulizer were liquefied by using an ice-cold centrifuge tube (Figure 2A). Subsequently, the collected liquid containing Hypo-EVs were subjected to ultracentrifugation. Then, the deposit was examined by using WB, nanoparticle tracking analysis (NTA), and transmission electron microscope (TEM). Hypo-EVs freshly extracted from hypoxic hUCMSC culture medium were used as control. WB revealed that several EV markers including tumor susceptibility gene 101 (TSG101) and heat shock protein 70 (HSP70) (28) were detected in these EVs (Figure 2B). NTA exhibited that the mean sizes of Hypo-EVs and nebulized Hypo-EVs were 128 and 122.5 nm, respectively (Figure 2C). TEM showed that nebulized Hypo-EVs and Hypo-EVs were the same in terms of the round nanoparticles and complete membranous structure (Figure 2C), indicating that nebulized Hypo-EVs produced by the atomization system maintain excellent structural integrity.

The biodistribution of nebulized hypo-EVs in mice

To determine the biodistribution of nebulized Hypo-EVs in BABL/C mice, Hypo-EVs were labeled with DiR first. Then, the DiR-labeled Hypo-EVs were administered through the nebulized route, and subsequently the organs of mice were dissected and the fluorescence intensity was detected. Mice synchronously inhaled PBS as a control. As shown in Figure 3, at 1 day after the DiR-labeled Hypo-EV inhalation, the strongest fluorescence intensity was observed in the lungs, with low intensity in the stomach and no accumulation in the brain, heart, liver, spleen, kidney, or intestines. At 7 day after nebulization, the fluorescence intensity was only enriched in the lungs, and decreased compared with 1 day.

Inhalation of nebulized hypo-EVs attenuated OVA-induced chronic airway inflammation in mice

To investigate the possible anti-inflammatory effects of nebulized Hypo-EVs on allergic airway reactivity, asthmatic mice were established, and the nebulized Hypo-EVs were inhaled (EVs-INH). Meanwhile, intravenous injection of Hypo-EVs (EVs-IV) was used as a positive control according to our previous report (14). The treatment regimen is illustrated in Figure 4A.

Lung histopathologic staining using HE showed that the asthmatic mice (OVA group) presented abundant infiltrates of peribronchial inflammatory cells compared with the control group, which was further identified by the increased inflammatory scores. Compared with OVA group, EVs-INH treatment significantly reduced the peribronchial inflammatory cell infiltration (Figure 4B). In addition, a differential cell count of bronchoalveolar lavage fluid (BALF) showed a significant decrease in total cells and eosinophil infiltrates in EVs-INH-treated mice (Figure 4C). Then, the type-2 cytokines IL-4, IL-5 and IL-13 in the BALF were determined. Our results showed that EVs-INH treatment dramatically decreased the protein levels of these three cytokines (Figures 4D–F). Compared with the EVs-IV gourp, significant lower levels of IL-13 were observed in EVs-INH-treated mice (Figure 4F). Serum levels of OVA-specific IgE were determined using ELISA, and as shown in Figure 4G, marked elevation of OVA-specific IgE were observed in OVA mice, which

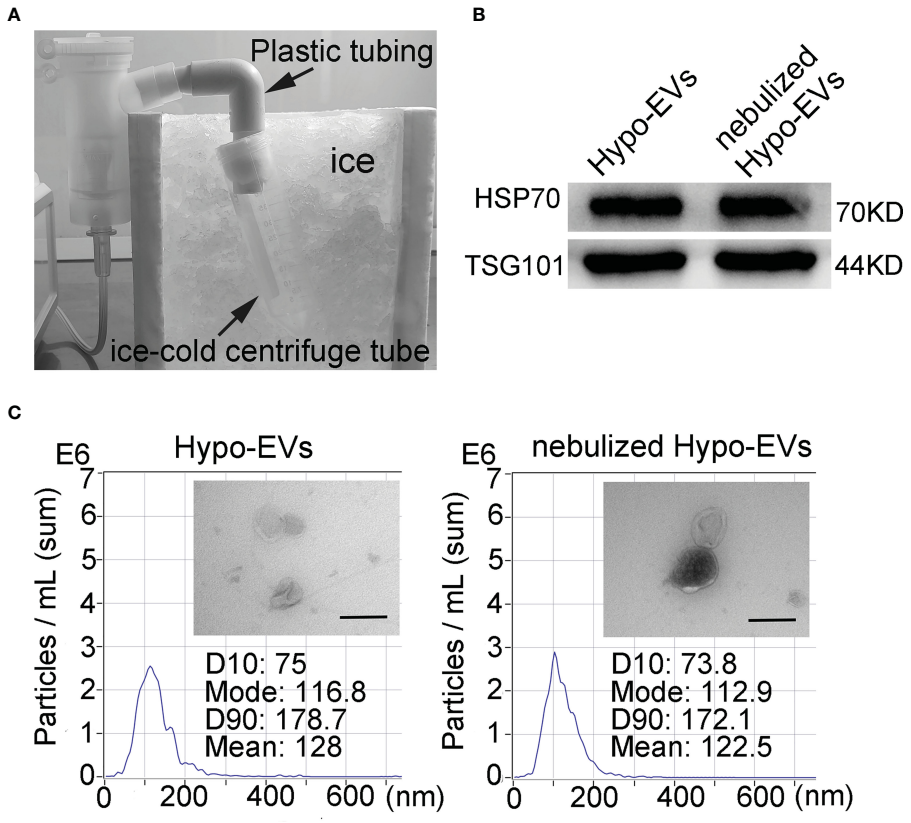

FIGURE 2
Characterization of Hypo-EVs. **(A)** The flowchart shows the process that nebulized Hypo-EVs ejected from nebulizer were liquefied by using an ice-cold centrifuge tube. **(B)** Western blot analysis of TSG101 and HSP70 expression in nebulized Hypo-EVs and fresh extracted Hypo-EVs. **(C)** EVs were observed under a transmission electron microscope (Scale bars = 200 nm), the images are shown at ×60000, and the size distributions were measured using the Nanoparticle Tracking Analysis.

strongly decreased with EVs-INH treatment. Taken together, these findings implied that inhalation of nebulized Hypo-EVs attenuated chronic airway inflammation and suppressed Type-2 predominant immune activity in the OVA-induced murine asthmatic model.

Inhalation of nebulized hypo-EVs prevented airway remodeling in chronic OVA mice

We further evaluated goblet cell hyperplasia in mouse lung tissues by PAS staining. As shown in Figure 5A, compared with control group, goblet cell numbers were significantly elevated in OVA-treated mice. EVs-INH treatment significantly reduced the aberrant OVA-induced promotion of goblet cell numbers. The subepithelial collagen deposition was investigated by using Masson trichrome and collagen-1 Immunohistochemical staining (IHC). As shown in Figures 5B, C, compared with OVA group or even EVs-IV group, less airway collagen fiber content or collagen-1 expression were observed in the lung tissues from EVs-INH-treated mice. The expression of α-SMA in lung tissues was further investigated as a detection of myofibroblast by using IHC. We found that EVs-INH inhibited the expression of α-SMA compared with that in the OVA or EVs-IV group (Figure 5D). These data suggested that EVs-INH treatment was effective in preventing airway remodeling in chronic asthma mouse.

Identification of genes regulated by EVs-INH in chronic OVA mouse lung

To better understand the molecular processes of nebulized Hypo-EVs, a global RNA sequencing of mouse lung from the OVA and EVs-INH groups was performed. Data were analyzed using conventional approaches based on Fragments Per Kilobase per Million mapped reads (FPKM), and the comparison generated a heat map of differentially expressed genes ($|\log FC| > 1$ and adjusted P-value < 0.05, Figure 6A). Compared with OVA group, EVs-INH mice presented 969 upregulated mRNAs and 856 downregulated mRNAs (Additional file 1), which were validated by real-time PCR through the determination of the expression of the three most lowly overexpressed (*Pank3*, *Zfp59* and *Arhgef9*) or downregulated (*Tubb1*, *Trpv4* and *Casc1*) genes (Figure 6B). Then, respective gene ontology enrichment analysis was conducted using DAVID bioinformatics resources. To increase the confidence levels of the analyses, we only presented the results of the 10 most relevant terms for the analyses of biological process (BP), cellular component (CC), and molecular function (MF) (Figure 6C and Additional file 2).

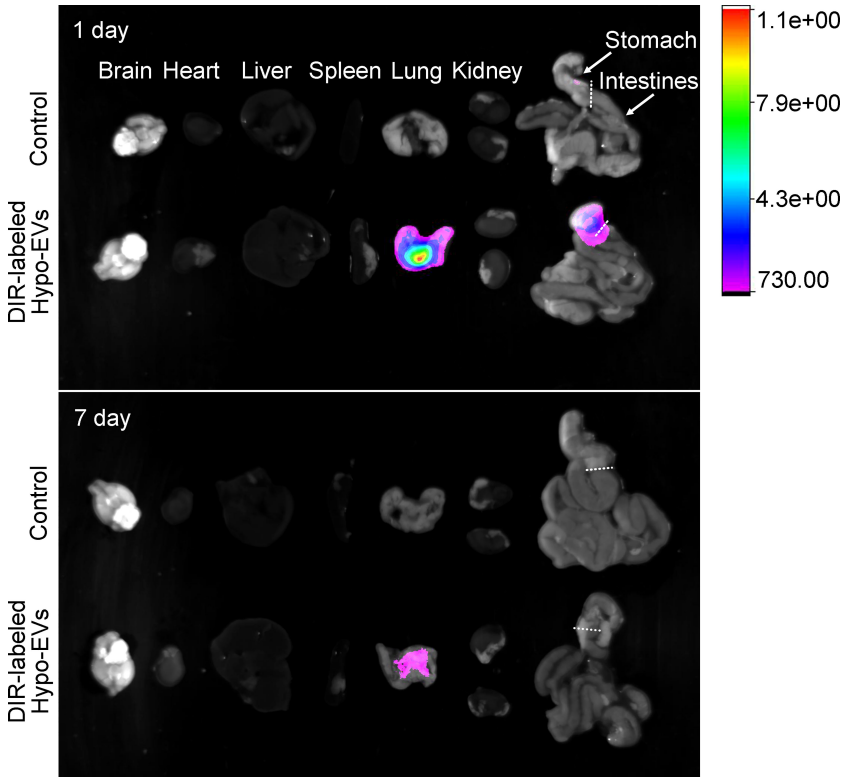

FIGURE 3
Biodistribution of Dir-labeled Hypo-EVs in major organs (brain, heart, liver, spleen, lung, kidney, stomach, and intestines) was visualized at 1 and 7 day after nebulized inhalation administration.

BP showed that upregulated mRNAs were largely involved in regulation of transcription form RNA polymerase II promoter (11.3%), cell adhesion (6.3%), and nervous system development (5.5%). The downregulated mRNAs were involved in innate immune response (8.2%), defense response to bacterium (6.9%), complement activation (6.5%), and positive regulation B cell activation (6.1%).

CC revealed that most upregulated mRNAs were involved in membrane (37.7%), cell junction (8.7%), and synapse (8.5%). Downregulated mRNAs were involved in extracellular space (14.8%), extracellular region (13.8%), and cytoskeleton (11%).

MF analysis suggested that the upregulated mRNAs showed extensive binding capacities to many components, such as, protein binding (29.2%) and metal ion binding (21.7%). Downregulated mRNAs were involved in ATP binding (9%), antigen binding (6.2%) and immunoglobulin receptor binding (6.1%).

Aerosol delivery of hypo-EVs carrying miR-146a-5p (miR-146a-5p-EVs) more inhibited airway inflammation and remodeling in chronic asthma mice

Recently, MSC-EVs are not only regarded as a next generation cell-free therapeutic tool (29), but also as a nano-scale gene delivery platform, especially miRNAs (30, 31). MiRNAs are potential candidates for asthma therapy. For example, miR-146a-5p was reported to efficiently protect mice against OVA-induced allergic asthma (32, 33), and also modulate anti-fibrosis responses (34). Thus, the anti-asthma effects of miR-146a-5p-EVs (Hypo-EVs carrying miR-146a-5p) might be more profound than those of Hypo-EVs. As shown in Figure 7A, compared with NC-EVs (Hypo-EVs carrying miR-146a-5p mimic control), a significant increase in miR-146a-5p levels was observed in miR-146a-5p-EVs. The accumulation of nebulized Hypo-EVs-delivered miR-146a-5p in the asthma mice was investigated. Compared with nebulized NC-EVs, nebulized miR-146a-5p-EVs resulted in significantly higher levels of miR-146a-5p in the lungs (190 fold) and stomach (3.3 fold), but not in brain, heart, liver, spleen, kidney, and intestines (Figure 7B). Notably, miR-146a-5p-EV treatment exhibited more inhibitory effects on airway inflammation (Figure 7C), goblet cell hyperplasia (Figure 7D), and collagen fiber content (Figure 7E) in chronic asthma mice. In addition, the downstream effector of miR-146a-5p, including TNF receptor-associated factor 6 (TRAF6) (35) and TIR domain-containing adaptor protein (TIRAP) (36), which are related to airway epithelial cell injury and airway inflammatory response in asthma, were decreased (Figure 7F).

Toxicity of inhalation of nebulized hypo-EVs

The safety of nebulized Hypo-EV treatment was examined during *in vivo* studies (Figure 8A). No animal died in 56 days

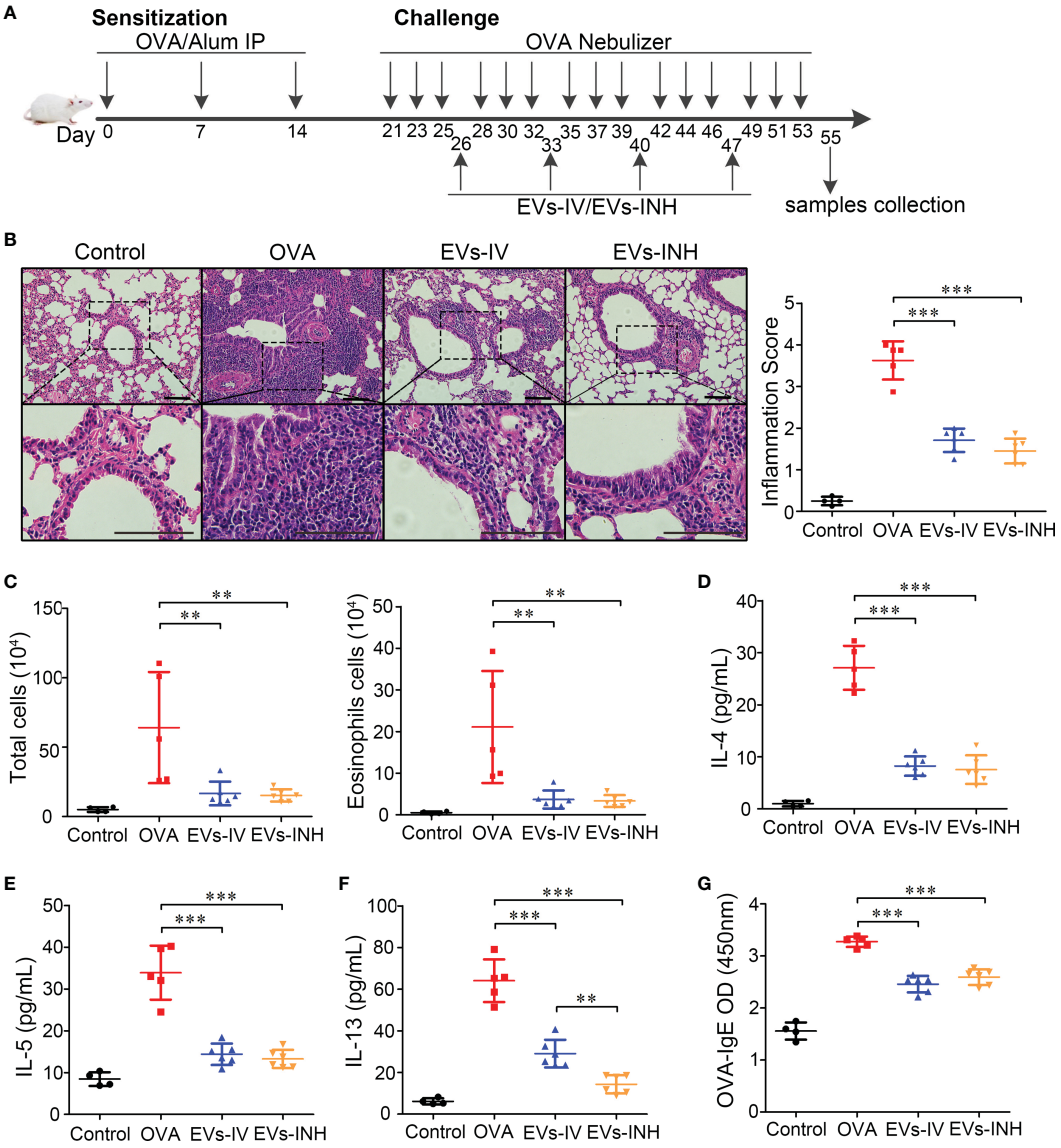

FIGURE 4
Inhalation of nebulized Hypo-EVs attenuated OVA-induced chronic airway inflammation in mice. **(A)** Experimental protocol for the development of chronic allergic asthma and treatment with Hypo-EVs. **(B)** Representative photographs of HE stained lung sections from each group (black bar =100 μm), the images are shown at ×200 (up panel) and ×400 (down panel), and the inflammatory infiltration was quantified by inflammation score. **(C)** Statistical analysis of the total inflammatory cells and eosinophils in the BALF. **(D–F)** IL-4, IL-5 and IL-13 levels in the BALF were measured by using ELISA. **(G)** The levels of OVA-specific IgE levels in serum were analyzed using ELISA. EVs-INH, Hypo-EV nebulization inhalation; EVs-IV, Hypo-EV intravenous injection. Each dot represents data from one animal and n = 4-6 per group. One-way analysis of variance (Tukey Kramer *post hoc* tests): $^{**}P < 0.01$, $^{***}P < 0.001$.

(date not shown). Compared with the health mice (Control group), no obvious tissue damages and inflammatory infiltration happened after EVs-INH treatment in the HE staining of major organs (heart, liver, spleen, lung, kidney, stomach, and intestines) (Figure 8B). EVs-INH had no impact on mouse body weight (Figure 8C). The liver and kidney function-related blood biochemical values (ALT, AST, UREA, and CREA) in the serum of mice on Day 56 were assayed. Compared with the healthy mice, no significant differences have been found in the mice after EVs-INH treatment (Figures 8D, E). All these results indicated that the EVs-INH possessed excellent biosafety.

Discussion

In recent years, MSC-EVs, a kind of nano-scale membranous vesicles secreted by MSC, are considered to be a best and market-promising substitute for MSC, science they are better defined, less complicated, easy storage, tiny, and seedless (9, 10). Numerous studies confirmed that intravenous administration (IV) of MSC-EVs exerted therapeutic potential to respiratory diseases in animal models (37), while, the investigation of nebulized inhalation (INH), a superior no-invasive drug delivery method for the treatment of pulmonary disease, are precious few. Dinh et al.

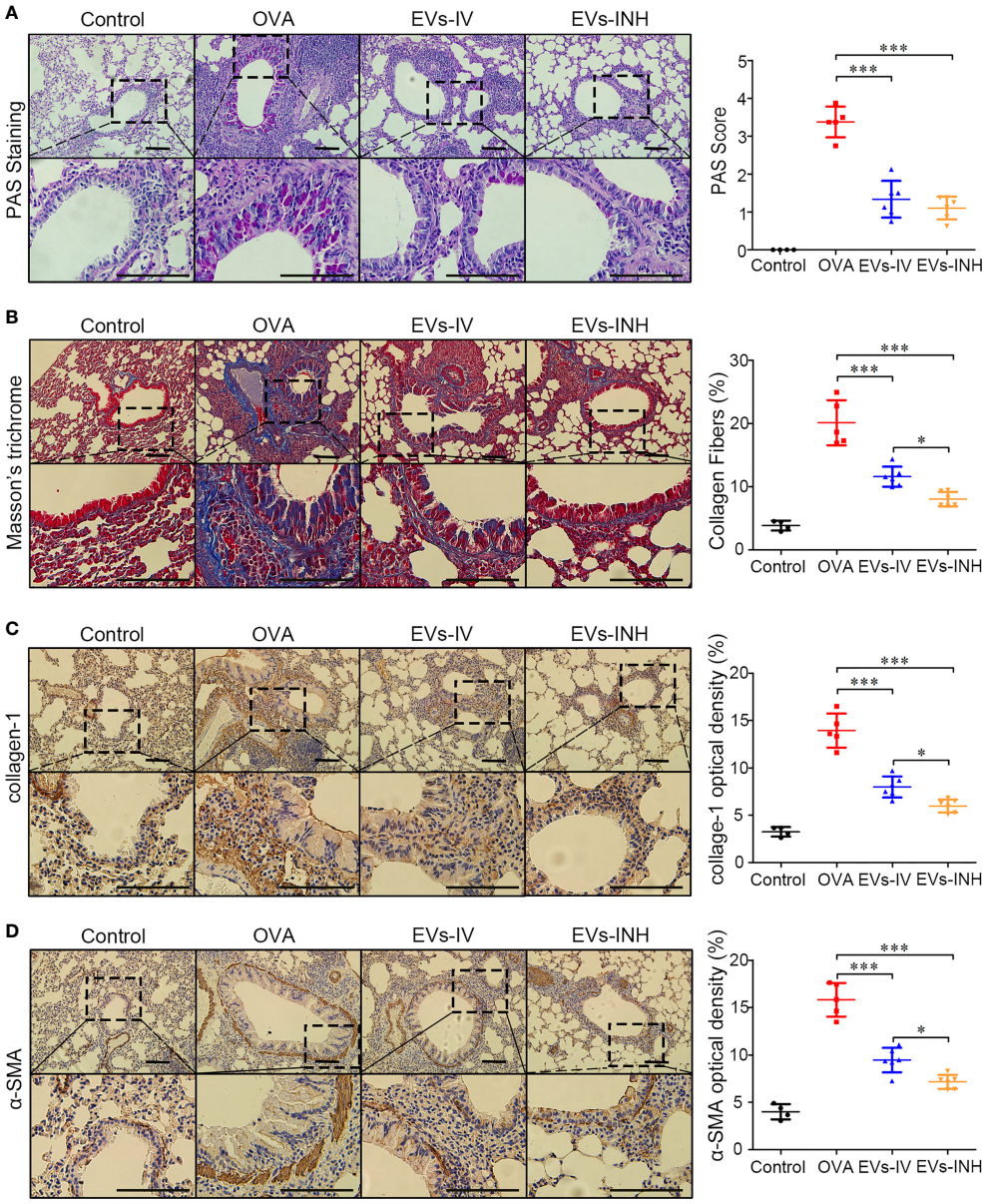

FIGURE 5
EVs-INH treatment prevented the airway remodeling in chronic asthma mice. **(A)** periodic acid-schiff (PAS) stained lung sections from each group (black bar = 100 μm), the images are shown at ×200 (up panel) and ×400 (down panel), and the goblet cell hyperplasia was quantified by PAS score. **(B)** Representative photomicrographs of airway stained with Masson trichrome (black bar = 100 μm), the images are shown at ×200 (up panel) and ×400 (down panel), and the percentage of collagen fiber content in airway was measured. Collagen-1 **(C)** and α-SMA **(D)** levels in airway were determined by immunohistochemistry staining, the images are shown at ×200 (up panel) and ×400 (down panel), and the percentage of immunostained area was quantified. Each dot represents data from one animal and n = 4-6 per group. One-way analysis of variance (Tukey Kramer *post hoc* tests): $^{*}P < 0.05$, $^{***}P < 0.001$.

(38) used air compressor and nebulizer to produce nebulized human bone marrow-derived MSC-EVs and presented a series of studies utilizing these EVs through the whole-body exposure to rat in a box to treat different models of lung injury and fibrosis. Shi et al. (39) used vibrating mesh nebulizers to produce nebulized human adipose-derived MSC-EVs (hADMSC-EVs). Then they placed the mouse head into the nebulizer nozzle and investigated the effect of hADMSC-EVs in the P. aeruginosa-induced murine lung injury. Zhao et al. (40) used commercially available rodent inhalers to produce nebulized hUCMSC-EVs. After intratracheal administration, the therapeutic effects of these EVs on lipopolysaccharide (LPS)-induce animal models were explored. In this study, we used medical/household air compressor and nebulizer to produce nebulized Hypo-EVs owing to their low prices, wide application, tiny smoke particles (1-5 μm), and adjustable fog. The atomized Hypo-EVs could still maintain excellent structural integrity, which further verified the feasibility of MSC-EV INH administration. More importantly, we created an available mouse inhalation device that consisted of six-way mouse inhalation chamber and mouse-holding chamber.

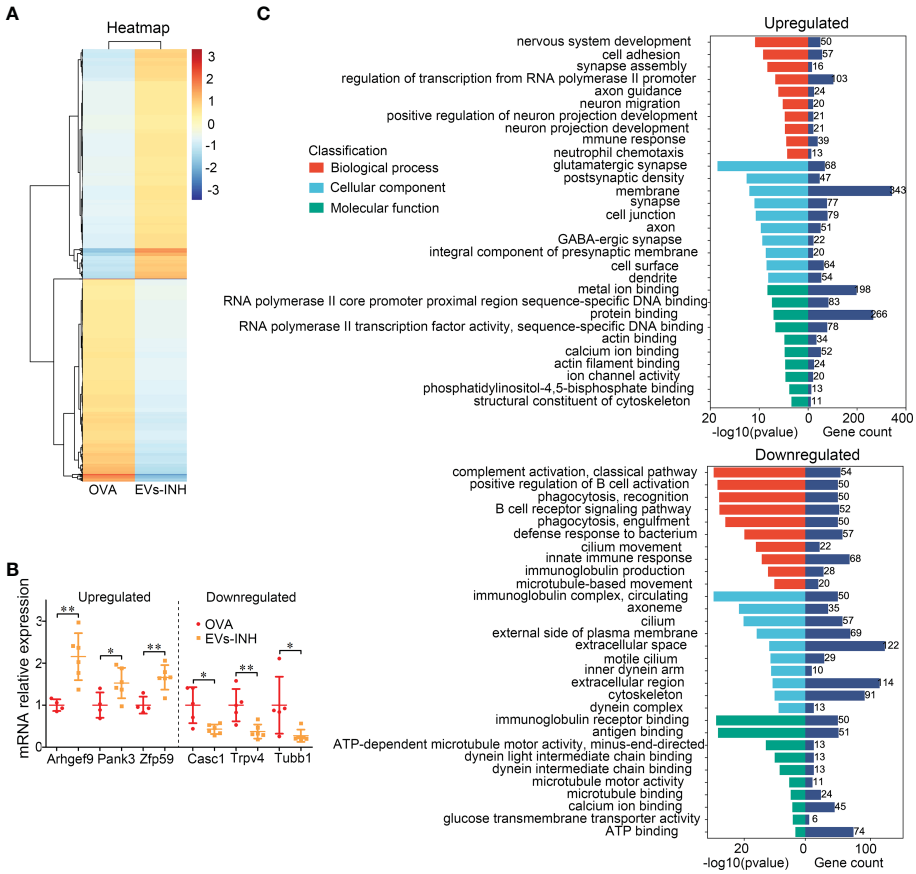

FIGURE 6
The differential expressed (DE) mRNAs in lungs between EVs-INH-treated mice and OVA-induced asthma mice. **(A)** Heat-map was constructed for DE miRNAs in EVs-INH vs OVA. **(B)** Real-time PCR verification of several of the lowlist differentially expressed genes. **(C)** GO analysis of the differentially up and down expressed genes in lungs between EVs-INH-treated mice and OVA-induced asthma mice. The top-ten statistically significant results identified including biological process (BP), cellular component (CC), and molecular function (MF) are listed. Each dot represents data from one animal and n = 4-6 per group. Student's t-test: $^{*}P < 0.05$, $^{**}P < 0.01$.

Compared with the above mentioned animal inhalation device, our device separately (1) simulated the atomization inhalation process of human beings and realized the nose-only EV exposure; (2) could be administered to multiple mice in parallel; (3) was cost-effective, which is particularly practical for scientists working in low-income countries. In short, we established a feasible, nose-only, high-efficient, and cheap atomization system for mice, which might promote the research progress of preclinical testing of MSC-EVs (or other EVs) administered through INH at laboratory scale.

Biodistribution of Dir-labelled Hypo-EVs in mouse major organs after INH administration revealed that fluorescence intensity mostly accumulated in the lungs. A low intensity was detected within the stomach at 1 day, which might be explained by the possibility of EVs being swallowed during the nebulization process, and this result was consistent with Shi's report (39). Generally, INH route has the potential for lung targeting and avoids the trapping of EVs in the liver, which is commonly reported during IV administration (15–19).

Asthma is a very common Type-2 immune mediated chronic respiratory disease that lacks effective treatment strategy. Here, we found that Hypo-EVs INH treatment significantly decreased airway inflammatory cell infiltration, numbers of total and eosinophils cells, protein levels of Type-2 inflammatory mediators (IL-4, IL-5, IL-13), and OVA-specific IgE level. It also prevented airway remodeling, concomitant with the reduced number of goblet cell metaplasia, content of subepithelial collagen, and expressions of collagen-1 and pro-fibrogenic markers α-SMA. More importantly, the direct delivery of Hypo-EVs into the lungs *via* the INH route offered greater protection against the OVA-induced increase in IL-13 levels, subepithelial collagen and myofibroblast accumulation compared with IV delivery of these EVs. Obviously, INH route might be a more effective delivery strategy than IV for the treatment of asthma using MSC-EVs (such as Hypo-EVs).

MSC-EVs participate in many biological processes, such as tissue regeneration (41), immune responses (42), and anti-fibrosis (43). However, little is known about their regulatory role in asthma, especially when administered *via* INH. In this study, we investigated the lung mRNA profile in OVA-induced and EVs-INH-treated mice through RNA-Seq, and the reliability of the RNA-seq results was later confirmed by real-time PCR. GO analysis revealed that EVs-INH showed positive regulation on cell adhesion, nervous system development, cell junction, and so on; while had negative regulation on innate immune response, positive regulation B cell

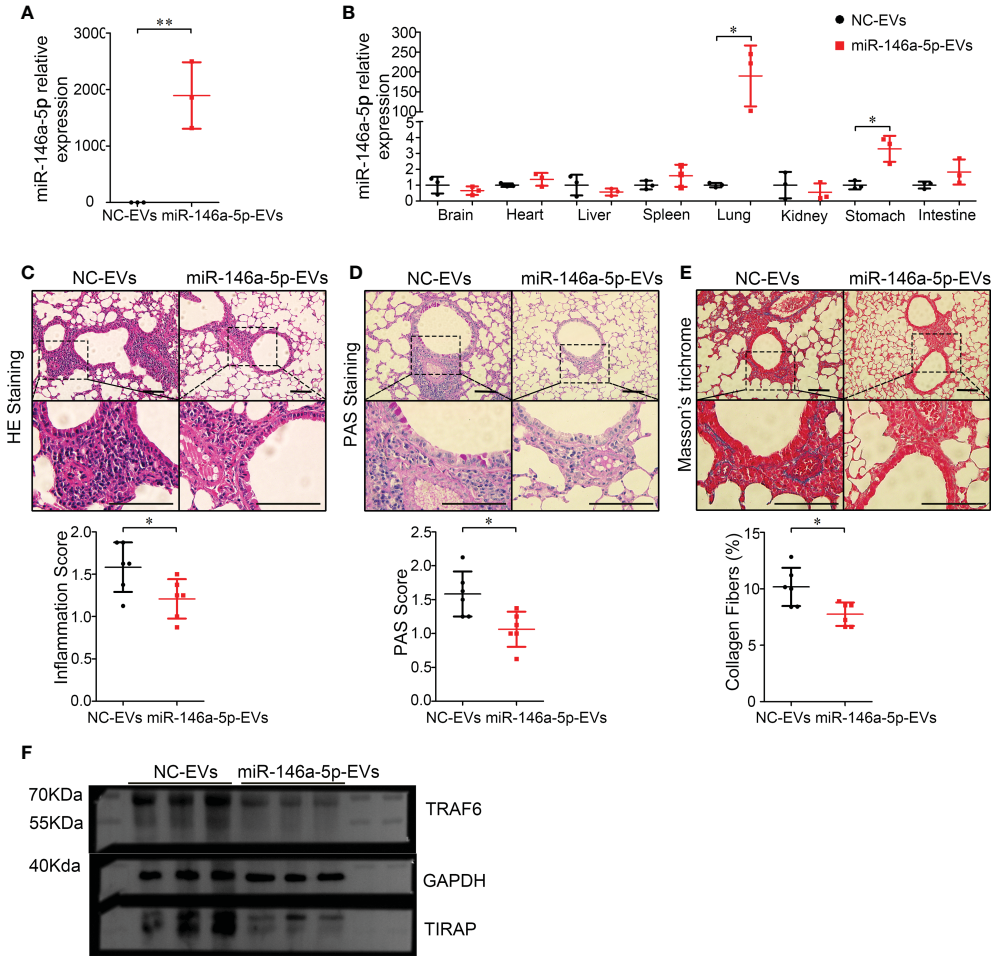

FIGURE 7
Inhalation of Hypo-EVs carrying miR-146a-5p (miR-146a-5p-EVs) more inhibited airway inflammation and remodeling in chronic asthma mice. **(A)** Relative miR-146a-5p levels in miR-146a-5p-EVs and NC-EVs were detected by real-time PCR (n=3). **(B)** Levels of miR-146a-5p in major organs (brain, heart, liver, spleen, lung, kidney, stomach, and intestines) following administration by aerosol miR-146a-5p-EVs and NC-EVs (n=3). Representative photographs of HE **(C)**, PAS **(D)**, and Masson trichrome **(E)** stained lung sections from each group (black bar = 100 μm), the images are shown at ×200 (up panel) and ×400 (down panel), and respectively inflammatory score, PAS score and and the percentage of collagen fiber content in airway were quantified (n=6). **(F)** The protein levels of TRAF6 and TIRAP in the lung samples were analyzed by western blot, and the GAPDH was as a loading control (n=3). Each dot represents data from one animal. Student's t-test: $^{*}P < 0.05$, $^{**}P < 0.01$.

activation, extracellular space, extracellular region, and antigen binding, which indicated that EVs-INH might have multiple targets and multiple effects in asthma.

In this study, we packed miR-146a-5p, a famous anti-inflammatory and anti-fibrotic miRNA (32–34), into Hypo-EVs (miR-146a-5p-EVs) and further investigated the therapeutic effect of miR-146a-5p-EVs on asthma mice. INH administration of miR-146a-5p-EVs resulted in significantly higher levels of miR-146a-5p in the lungs, little in stomach, no in other tissues, indicating that miR-146a-5p-EV INH treatment could deliver miR-146a-5p relatively specifically to mouse lungs. Although, miRNAs are potential candidates for treating respiratory diseases, including asthma (44), an efficient delivery system to directly deliver them to lung still lack. Thus, INH administration of MSC-EVs-carrying miRNAs might provide a good reference. More importantly, we found that miR-146a-5p-EVs were generally more potent than Hypo-EVs in suppressing airway inflammation and remodeling in asthmatic mice, which further supported the notion that engineering MSC-EVs is an effective way to improve the therapeutic effect of MSC-EVs (45, 46).

In present study, we first investigated the effects of Hypo-EV INH administration on health mice. Hypo-EV INH treatment had no impact on mouse survival, body weight, pathology of major organs, liver and kidney functions, partly confirming that INH route was safe.

This study had a number of limitations. (1) We only used a single dosage of Hypo-EVs in our animal model. (2) The specific target cells and molecular mechanism of EVs-INH were not further explored. (3) Due to the lack of relevant equipment, airway hyperresponsiveness, another main component to asthma, was not detected. (4) The effectiveness and the safety of normoxic MSC-EVs on asthmatic or normal mice were not investigated.

In conclusions, we created a nose-only, high-efficient, and cheap inhalation device for rodents and confirmed that inhalation of nebulized Hypo-EVs could effectively reduce airway inflammation and reverse markers of airway

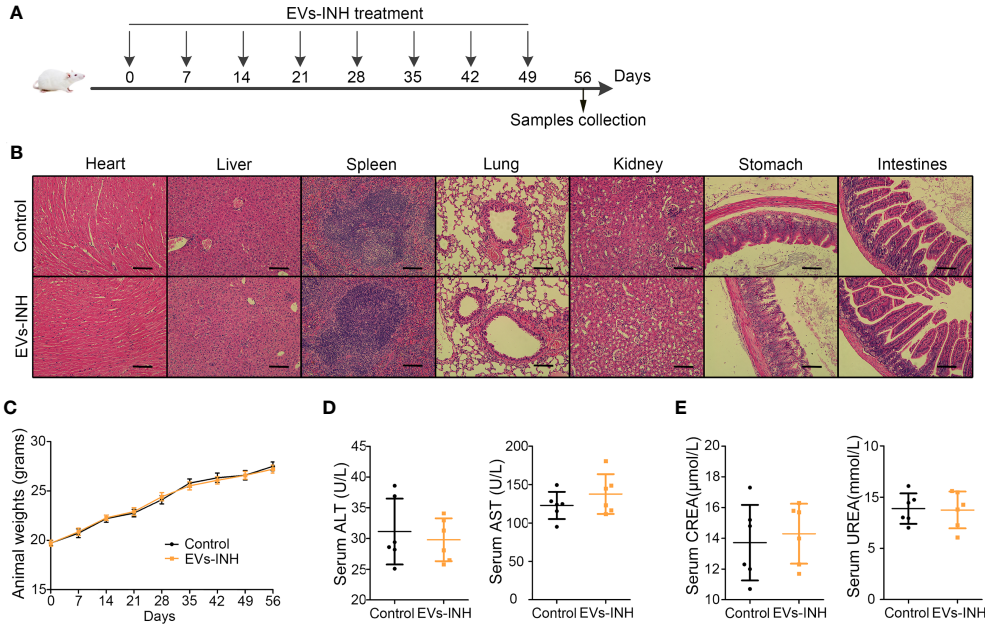

FIGURE 8
Biocompatibility evaluation of EVs-INH (n=6). **(A)** Schematic diagram for EVs-INH treatment. **(B)** HE staining, scale bar=100 μm, and the images are shown at ×200. **(C)** Mouse body weights. Indicators reflected the physiological function of the **(D)** liver (ALT and AST) and **(E)** kidney (CREA and UREA) were detected. ALT, glutamic pyruvic transaminase; AST, glutamic oxaloacetic transaminase; CREA, Creatinine. Each dot represents data from one animal and n = 6 per group. Student's t-test.

remodeling in asthma mice. These findings provided ideas for the determination of the equipment to use in the atomization research of MSC-EVs and a noninvasive strategy for ameliorating asthma.

Data availability statement

The datasets presented in this study can be found in online repositories. The name of the repository and accession number(s) can be found below: NCBI Gene Expression Omnibus; accession number GSE226639.

Ethics statement

Human umbilical cord samples were obtained from informed, consenting mothers at Affiliated Hospital of Jiangsu University, and the study was approved by the Ethics Committee of Affiliated Hospital of Jiangsu University. The animal study was reviewed and approved by Institutional Animal Care and Use Committee of Jiangsu University.

Author contributions

XX, YW, XinL, CM, TZ, LD conceived and designed the experiments. XX, YW, XinL, XG, YM, LX, MY, LD analyzed the data. XX, YW, XinL, XG, WG, XiL, JL, XW, LD performed the experiments. The manuscript was written by TZ, CM, LD. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the grants from the National Natural Science Foundation of China (81900562), and key research and development plan of Zhenjiang city (SH2021050).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1150971/full#supplementary-material

ADDITIONAL FILE 1

The DE mRNAs in lungs between EVs-INH-treated mice and OVA-induced asthma mice (|log FC| > 1 and adjusted *P*-value < 0.05).

ADDITIONAL FILE 2

The top-ten statistically signifificant results of biological process (BP), cellular component (CC), and molecular function (MF).

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frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 11 April 2023
DOI 10.3389/fimmu.2023.1151322

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Cytokines and Soluble Mediators in Immunity, a section of the journal Frontiers in Immunology

RECEIVED 26 January 2023
ACCEPTED 28 March 2023
PUBLISHED 11 April 2023

CITATION
Cai R, Wang L, Zhang W, Liu B, Wu Y, Pang J and Ma C (2023) The role of extracellular vesicles in periodontitis: pathogenesis, diagnosis, and therapy. *Front. Immunol.* 14:1151322. doi: 10.3389/fimmu.2023.1151322

The role of extracellular vesicles in periodontitis: pathogenesis, diagnosis, and therapy

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Periodontitis is a prevalent disease and one of the leading causes of tooth loss. Biofilms are initiating factor of periodontitis, which can destroy periodontal tissue by producing virulence factors. The overactivated host immune response is the primary cause of periodontitis. The clinical examination of periodontal tissues and the patient's medical history are the mainstays of periodontitis diagnosis. However, there is a lack of molecular biomarkers that can be used to identify and predict periodontitis activity precisely. Non-surgical and surgical treatments are currently available for periodontitis, although both have drawbacks. In clinical practice, achieving the ideal therapeutic effect remains a challenge. Studies have revealed that bacteria produce extracellular vesicles (EVs) to export virulence proteins to host cells. Meanwhile, periodontal tissue cells and immune cells produce EVs that have pro- or anti-inflammatory effects. Accordingly, EVs play a critical role in the pathogenesis of periodontitis. Recent studies have also presented that the content and composition of EVs in saliva and gingival crevicular fluid (GCF) can serve as possible periodontitis diagnostic indicators. In addition, studies have indicated that stem cell EVs may encourage periodontal regeneration. In this article, we mainly review the role of EVs in the pathogenesis of periodontitis and discuss their diagnostic and therapeutic potential.

KEYWORDS

periodontitis, extracellular vesicles, stem cell, pathogenesis, diagnosis

1 Introduction

Periodontal disease is the sixth most prevalent disease in the world (1, 2). According to a 2017 report, periodontitis affects 796 million people worldwide (3), places a substantial financial and health burden on those affected, and drastically lowers their quality of life (4, 5).

Abbreviations: MSCs, mesenchymal stem cells; EVs, extracellular vesicles; Th, helper T cells; Treg, regulatory T cell; LPS, lipopolysaccharide; BMSCs, bone mesenchymal stem cells; ADSCs, adipose-derived mesenchymal stem cell; DFSCs, dental follicle stem cells; SHED, stem cells of human exfoliated deciduous teeth; PDLSCs, periodontal ligament stem cells; GMSCs, gingiva mesenchymal stem cells; PRRs, pattern recognition receptors; MMP, matrix metallopeptidase; IL, interleukin; COX-2, cyclooxygenase 2; TNFa, tumor necrosis factor-a.

In recent years, risk factors of periodontitis have been grouped into three main categories: biofilms, host, and environment (6). When local biofilms and the mild host immune are in balance, the immune surveillance and appropriate immune response predominate (7). When exposed to persistent microbial challenges or when the pathogenicity of the local microbiome increases, the balance between biofilms and the host is lost, and the host's immune reactivity is excessive. This results in a highly inflammatory state with immune cell infiltration, pro-inflammatory and inflammatory cytokines up-regulation, excessive osteoclasts activation, ligament fiber degradation, granulation tissue formation, and final periodontal destruction (8–16).

Clinical features of periodontitis include red, swollen, and receding gums, bleeding on periodontal probing, a deeper pocket, the destruction of periodontal tissue, tooth displacement, and eventually tooth loss (17, 18). Unfortunately, due to the low sensitivity and low positive predictive value of these tests, the parameters can only evaluate historical data on periodontal tissue loss and cannot forecast future disease activity (19–21). Furthermore, these parameters vary among dentists, which impacts the accuracy of diagnosing periodontitis (22). To prevent and diagnose periodontitis early and effectively refer patients to specialized therapy, it is crucial to investigate more repeatable, sensitive, and specific methods of periodontal diagnosis that provide current and future disease information (23, 24).

Periodontitis treatment comprises non-surgical treatment, surgical treatment, and adjuvant medicine treatment (25–27). The treatment objectives are to control inflammation, halt disease progression, and help patients reconstruct a healthy and functional dentition (28). Surgical intervention is required when it is necessary to rebuild a bone defect to establish a good bone structure or when regeneration is needed to restore lost periodontal structures (29). Periodontal regeneration is a complex process due to the unique anatomical structure and composition of periodontal tissue, including periodontal ligament, cementum, and alveolar bone (30). Osteogenesis, inflammation control, and angiogenesis play important roles in periodontal regeneration (31, 32). At present, guided tissue regeneration techniques, including the transplantation of soft and hard tissues, the use of growth factors and host regulatory factors, and the use of biomaterials, are the mainstays of periodontal regeneration (33–35). Nevertheless, there are just a few materials with high potential for periodontal regeneration, and present technologies have limitations in attaining periodontal regeneration. Thus, looking for more durable and potent therapies and materials is critical to improving periodontal regeneration (36–38).

EVs are a group of bilayered lipid membrane-structured vesicles secreted by multiple kinds of cells. They carry a variety of substances from the parental cells, like DNA, RNA, lipids, and proteins (23, 39). EVs play a role in various pathological and physiological processes, including immunological regulation, inflammatory response, and tissue healing and regeneration. EVs have been discovered valuable to study physiological processes, pathologies, as well as regeneration (40).

We outlined the function of EVs in the pathogenesis and diagnosis of periodontitis and discussed methods used to isolate and characterize salivary and GCF EVs in this paper. We also reviewed recent research on stem cell-derived EVs in periodontitis therapy and addressed the flaws and future directions.

2 Extracellular vesicles

2.1 Definition of EVs

EVs can be secreted by humans, plants, animals, and microbial origins (40). Only a few nonmammalian sources have been explored in preclinical or clinical settings, and most studies and reviews have concentrated on EVs derived from mammalian cells and body fluids (40). EVs can be classified as endosome-derived exosomes (Exo), plasma membrane-derived microvesicles (MVs), and apoptotic bodies (ApoEVs) based on their secretion processes and characteristics (41, 42). Exosomes (30-100 nm) germinate inward from the endosome membrane, forming multivesicular bodies (MVBs) in the cytoplasm. Some MVBs are degraded by lysosomes, while others fuse with the cell membrane and are discharged into the extracellular environment by exocytosis. MVs (50 nm-2μm) and ApoEVs (50 nm-5 μm) were derived from outward budding. Exosomes and MVs are secreted during normal cellular processes, whereas ApoEVs are only produced during programmed cell death (43–46). In 2018, the International Extracellular Vesicle Society recommended researchers characterize EVs by size: "small EVs" (sEVs < 200 nm) and "medium and/or large EVs" (m/lEVs > 200 nm) unless specific EVs markers are available (47).

EVs can participate in physiological and pathological processes by directly binding to receptors on recipient cells, fusing with the plasma membrane of recipient cells and the membrane of the endosome following endocytosis (48, 49). Various endocytosis-related pathways, including clathrin-dependent endocytosis and clathrin-independent pathways such as caveolin-mediated uptake, macropinocytosis, phagocytosis, and lipid raft-mediated internalization, are thought to be the primary mechanisms of EVs uptake by recipient cells (48, 50).

2.2 Functions of EVs

In physiological and pathological processes, including cancer treatment, early diagnosis, tissue regeneration, and medication, the released EVs can remove metabolic proteins during cell maturation and regulate cell-to-cell communication (39, 51–54).

The RNA composition of EVs varies with pathological situations, and as a result, they have become a source of biomarkers for diagnosing human diseases (55). Proteins, genetic material, and lipids in EVs extracted from oral biofluids (saliva and GCF) have recently emerged as potential sources of biomarkers for periodontal diseases (23).

On the other hand, EVs are used in disease therapy in that the biological properties of EVs can modulate the phenotype and behavior of recipient cells (56, 57). Due to their innate ability to promote tissue regeneration, mesenchymal stem cells (MSCs) have

been employed as a source of regenerative EVs. Besides, MSCs-EVs have the following advantages (1): MSCs-EVs have the innate capacity to cross physiological barriers, such as the blood-brain barrier, due to their nanoscale size (58, 2). The risk of immune rejection and tumorigenicity induced by cell transplantation can be decreased with MSCs-EVs therapy (59, 3). MSCs-EVs are highly stable and biocompatible, and recipient cells may quickly absorb them (60, 4). MSCs-EVs are more convenient to store and transport since they can be kept stable at low temperatures (61, 5). Appropriate modification can enhance the targeting and repair abilities of MSCs-EVs (50, 62, 6). Studies have also demonstrated that MSCs-EVs have no adverse effects in toxicology tests (63, 7). MSCs-EVs are comparable to MSCs in their capacity to repair injured tissues, resist inflammation, inhibit cell apoptosis, and regulate immune responses (64–67). *In vitro* and animal studies have shown the potential of MSCs-derived EVs for treating periodontitis (68, 69). MSCs-EVs have demonstrated potential in the prevention and treatment of periodontal disease as well as periodontal regeneration due to their ability to regulate inflammation and promote osteogenesis (70, 71).

2.3 Extraction and characterization of EVs

Five basic extraction techniques are frequently employed based on the physical (density, size, and solubility) and biological (surface antigen) properties of EVs: precipitation, membrane affinity, size-exclusion chromatography, iodixanol gradient, and phosphatidylserine affinity (72). Additional methods have been developed to improve the specificity of EVs, including tangential flow filtration (73), field-flow fractionation (74), asymmetric flow field-flow fractionation (75), field-free viscoelastic flow (76), alternating current electrophoretic (77), acoustics (78), ion exchange chromatography (75), microfiltration (79), fluorescence-activated sorting (80), etc.

According to the latest MISEV 2018 guidelines for EVs characterization, scientists should include at least three distinct characteristics, such as EVs particle quantity, morphology, and protein markers (47). Dynamic light scattering (81) and nanoparticle tracking analysis (82) are frequently applied to estimate the quantity and size of EVs particles. Transmission electron microscopy (83), scanning electron microscopy (84), and atomic force microscopy (85) can be used to examine the morphology of EVs. Bicinchoninic acid assay (86), fluorimetric assays (87), or the global protein stain on SDS-PAGE (88) were used for EVs protein quantification. Western blot (89), enzyme-linked immunosorbent assay (90), bead-based flow cytometry (91), aptamer- and carbon nanotube-based colorimetric assays (92), and surface plasmon resonance (93) were employed to detect protein markers. Generally, at least one protein from each of the following groups must be assessed (47): (1) Transmembrane or GPI-anchored proteins connected to the plasma membrane or endosomes (Tetraspanins, integrins, etc.). (2) Cytosolic proteins (membrane binding proteins, etc.). (3) Major non-EVs co-isolated structural constituents (lipoproteins, Apolipoproteins, ribosomal proteins, etc.). When claiming specific analysis of sEVs, analysis of transmembrane, lipid-bound and soluble proteins associated with other intracellular compartments (histones, cytochrome C, etc.) is required. Secreted proteins recovered with EVs (cytokines and growth factors, adhesion, extracellular matrix proteins, etc.) are needed to document the functional activities of sEVs. To further indicate particle per volume and particle size distribution, the guidelines also suggested the addition of EVs purity metrics, such as protein/particle ratio, protein/lipid ratio, or RNA/particle ratio.

3 EVs and the pathogenesis of periodontitis

3.1 Direct pathogenic role of outer-membrane vesicles in periodontitis

The Gram-negative bacteria that are closely related to the progression of periodontitis, including Porphyromonas *gingivalis (P. gingivalis), Treponema denticola (T. denticola), Tannerella forsythia (T. forsythia), Actinomyces reticulata (A. reticulata), Fusobacterium nucleatum (F. nucleatum) and Prevotella intermedia (P. intermedia)* have been isolated from the periodontal pocket (94–96). *P. gingivalis* is the primary pathogen responsible for chronic periodontitis (97, 98). It forms the "red complex" along with *T. forsythia* and *T. denticola*, which are accessory pathogens with complementary or supplementary functions (99, 100).

Gram-negative bacteria can selectively export toxins and other virulence factors to host cells through vesicles named OMVs. In light of our current knowledge, OMVs are double-layered spherical membrane-like structures with a diameter ranging from 20 to 250 nm. OMVs contain bacterial parts and products such as fimbriae, lipopolysaccharides (LPS), toxins, outer membrane proteins, peptidoglycans, and bacteria's DNA and RNA (101–107). Yet it is unclear how these elements are packed into OMVs, and how the cargos are selected (101). OMVs can directedly fuse with target cells or be internalized by lipid rafts, micropinocytosis, and clathrin-dependent endocytosis (108–110).

After entering host cells, OMVs can exhibit a variety of virulences (111), and host-derived proteases have little effect on them (112). While requiring much energy, OMVs are crucial for maintaining bacterial virulence, colony formation, material transfer inside bacteria, immune escape, and host cell immune regulation (101, 103, 113–116).

The gingival epithelium is a physical barrier against invasion by biofilms and other nonautologous substances and is the first line of defense in the oral cavity (117, 118). There have been reports of *P. gingivalis* OMVs invading oral epithelial cells (119). By the endocytic pathway, *P. gingivalis* OMVs can efficiently infiltrate human epithelial cells and interfere with their function by destroying signaling molecules necessary for cell migration, such as transferrin receptor, paxillin, and focal adhesion kinase (120, 121). *T. denticola* can disrupt the function of the epithelial barrier and penetrate the epithelial layers by degrading tight junctional proteins like ZO-1 (122). According to Bartruff (123) et al., OMVs derived from *P. gingivalis* significantly inhibited the proliferation of cultured gingival fibroblasts and human umbilical vein endothelial

cells (hUVECs), as well as hUVECs' ability to form capillaries, which restrained periodontal tissue healing.

OMVs could be oral microbial communication between *P. gingivalis* and other oral bacteria (119). Kamaguchi (124) et al. demonstrated that *P. gingivalis* OMVs significantly promoted oral bacteria coaggregation. Grenier (125) noticed that *P. gingivalis* OMVs could mediate the coaggregation between *T. denticola* and *L. saburreum*. *P. gingivalis* and *T. denticola* co-inoculation synergistically triggered host immune responses and alveolar bone loss in a murine experimental periodontitis model (126). According to Inagaki (127) et al., *P. gingivalis* OMVs play an important role in virulence by enhancing *T. forsythia's* adherence and penetration of epithelial cells. *P. gingivalis* OMVs have explicitly been enriched for the heme-binding lipoproteins HmuY and IhtB, which can provide micronutrients to several other subgingival biofilms, resulting in community benefits that encourage biofilm proliferation (128). In addition, *P. gingivalis* OMVs can suppress and disperse rival biofilms in a gingipains-dependent manner to create a favorable environment for *P. gingivalis* (119).

In brief, these findings indicate that OMVs, which can mediate the interaction between biofilms and host cells and hasten the destruction of periodontal tissue, are substantially responsible for the pathogenicity of biofilms (Figure 1). Nevertheless, the precise mechanisms by which OMVs alter the nature of biofilms remain unclear. Further research is required to determine the specific function and associated mechanisms of OMVs in periodontitis.

3.2 Pathogenic role of OMVs in periodontitis by affecting immunity and inflammation

Pattern recognition receptors (PRRs), expressed by host immune cells, are essential molecules that trigger local immune responses. OMVs from periodontal pathogens can induce PRRs reactions (112). OMVs can activate PRRs in gingival epithelial cells, causing the secretion of pro-inflammatory and anti-inflammatory cytokines and activating neutrophils, T and B lymphocytes, and osteoclasts. These reactions promote connective tissue destruction and alveolar bone resorption (129). Choi (130) et al. derived that OMVs secreted by major periodontal pathogens transferred microRNA (miRNA) to immune cells to suppress target genes related to immune response, thereby evading the host adaptive immune responses. The potent but flexible immunostimulatory effects of *P. gingivalis* OMVs may help manipulate and dysregulate host immune responses to initiate disease, and the pro-inflammatory effects of other bacteria may contribute to the disease progression (100). *P. gingivalis* OMVs can selectively promote tumor necrosis factor (TNF) tolerance in a Toll-Like Receptor 4 and mTOR-Dependent manner, leading to local immune evasion (131). *P. gingivalis* OMVs can selectively entrap and activate human neutrophils to initiate degranulation without being destroyed by neutrophils, and they can also breakdown components of secretory granules with antibacterial activity,

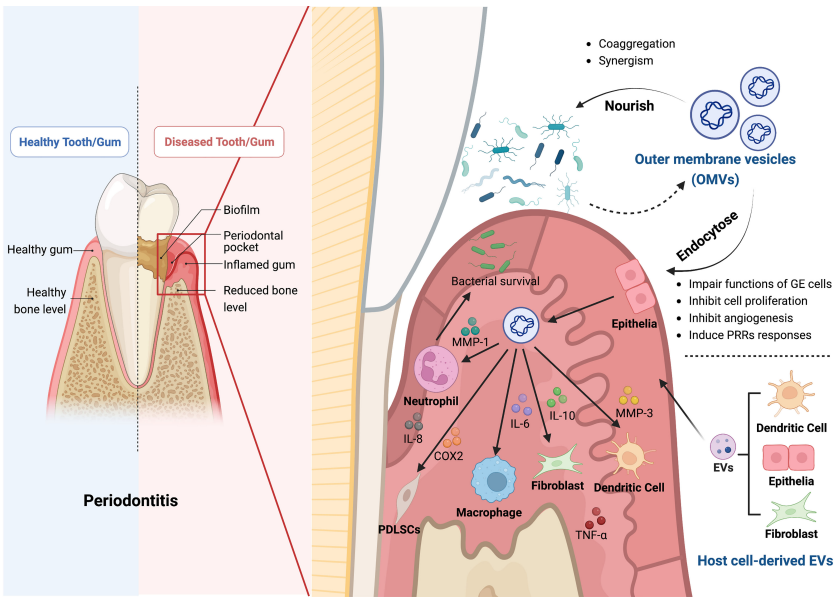

FIGURE 1
The roles of bacterial outer membrane vesicles (OMVs) and host cell-derived EVs in the pathogenesis of periodontitis. OMVs produced by Gram-negative bacteria contain bacterial components and bacterial products, such as outer membrane proteins, peptidoglycan, and lipopolysaccharide, which play a crucial role in the pathogenesis of periodontitis. Firstly, OMVs can enter the gingival epithelial cells through endocytosis and thus impair the function of gingival epithelial cells, inhibiting epithelial cell proliferation, slowing down angiogenesis, and inducing the response to PRRs. Secondly, OMVs can also inhibit bacterial clearance by immune cells by affecting a variety of cellular functions through different inflammatory mediators, including neutrophils, macrophages, fibroblasts, periodontal stem cells, and dendritic cells, which impede the host immune response. OMVs can cause aggregation of bacteria and act synergistically with them to induce the onset and progression of periodontitis. In addition, the EVs secreted by the host cells, such as dendritic cells, fibroblasts, and epithelial cells, can also cause alveolar bone loss, periodontitis and tissue damage. The Graph was created with BioRender.com. PRRs, pattern recognition receptors; MMP, matrix metallopeptidase; IL, interleukin; COX2, cyclooxygenase 2; TNF-α, tumor necrosis factor-α.

especially LL-37 and myeloperoxidase (MPO), to ensure bacterial survival (132).

The levels of inflammatory mediators, particularly interleukin (IL)-1, TNF, prostaglandin E2, and cyclooxygenase-2 (COX-2), are correlated with the severity of inflammatory response and periodontal disease (133, 134). Kou (135) et al. discovered that co-culturing immortalized human gingival epithelial cells with *P. gingivalis* OMVs elevated the production of inflammatory factors as COX-2, IL-6, IL-8, matrix metalloproteinase (MMP)-1, and MMP-3. Another study demonstrated that *P. gingivalis* OMVs enhanced the expression of IL-6 and IL-8 in human gingival epithelial cells *via* activating the signaling pathways Erk1/2, JNK, MAPK, STING, and NF-κB (136). Fleetwood (137) et al. confirmed that *P. gingivalis* OMVs could penetrate the gingival tissue and stimulate macrophages to produce large amounts of TNF-α, IL-12, IL-6, IL-10, IFN-β, and NO, resulting in tissue inflammation and damage. OMVs from *P. gingivalis* and *T. forsythia* induced the expression of pro-inflammatory cytokines like IL-1β, IL-6, IL-23, and IL-12p70 in bone marrow-derived dendritic cells (DCs) (138). Human monocyte cell line U937 and periodontal ligament fibroblasts were activated by *T. forsythia* OMVs in a concentration-dependent manner to produce pro-inflammatory mediators, and the inflammatory response was noticeably greater than that induced by whole *T. forsythia* cells (139).

OMVs should be considered as part of a larger picture because they not only contribute to the local problem of periodontitis (40). As EVs communication is not confined to species, OMVs from periodontal pathogens are also involved in human systemic diseases (104), for instance, Alzheimer's disease (140), neuroinflammation and neurodegeneration (141), cardiovascular disease (142), and diabetes mellitus (98). Investigating interkingdom communication of EVs from different origins may help discover new pathologic mechanisms and innovative therapies.

OMVs derived from probiotic strains contain immunomodulatory molecules that decrease pro-inflammatory cytokines and strengthen epithelial barriers (143, 144). According to reports, OMVs are protease resistant, can withstand long-term storage, and their structural stability makes it easier for them to deliver contents into the host immune system. Moreover, OMVs are attractive vaccines against pathogenic bacteria due to their immunogenicity (145, 146). Specific antibodies against *P. gingivalis* can be produced in mice's blood and saliva by intranasal inoculating OMVs (147, 148). Whereas, because they are still in the very early stages of development, periodontal vaccines face obstacles such as limited yield, unfavorable toxicity, and insufficient immunogenicity (149).

3.3 Host cell-derived EVs in the pathogenesis of periodontitis

Immune senescence plays a pivotal role in the pathophysiology of experimental periodontitis. *P. gingivalis* directly invades DCs to cause premature senescence and dramatically accelerates the senescence of normal bystander DCs by secreting inflammatory exosomes (150). Exosomes of the *P. gingivalis*-invaded DCs transmit senescence to normal bystander DCs and T cells, resulting in the loss of alveolar bone, according to recently published research (151). Another study demonstrated that biofilms could contribute to inflammation and periodontal destruction by promoting gingival fibroblasts to exhibit a tissue-destructive phenotype *via* increased secretion of epithelial EVs (152). Otherwise, LPS-treated periodontal ligament fibroblasts induce inflammation and inhibit the osteogenic activity of osteoblasts by releasing exosomes (153). Presumably, EVs secreted by host cells significantly impact periodontal disease.

In conclusion, OMVs from periodontal pathogens and host cell-derived EVs are critical in the pathogenesis of periodontitis. Nevertheless, there is still much to learn about the precise molecules and mechanisms by which EVs mediate innate and acquired immune response in periodontitis (6).

4 EVs and diagnosis of periodontitis

4.1 Diagnostic role of EVs in saliva and GCF

Saliva, a hypotonic solution composed of GCF, serum, salivary glands secretion, oral mucosal secretion, and microorganisms, is responsible for oral cleaning, antibacterial effect, and host's resistance to oral infections (154, 155). Another important oral biofluid is GCF, a serum exudate of periodontal tissue, presented in the healthy gingival sulcus or the periodontal pocket. Saliva and GCF are rich in biomolecules from the host and microorganisms, such as inflammatory mediators, cytokines, tissue breakdown products, DNA, RNA, EVs, etc. (23, 154, 156–158). Therefore, saliva and GCF can be applied as promising non-invasive indicators for periodontitis (159).

Genetic analysis of saliva sEVs showed that innate immune response proteins were considerably enriched in patients with severe periodontitis, particularly the complement component 6 (C6) (160). One study proposed that the DNA methylation pattern of 5-methylcytosine (5mC) in saliva EVs was comparable in the groups with healthy gums, gingivitis, and periodontitis (161). According to another study, patients with periodontitis had considerably fewer CD9+ and CD81+ saliva exosomes than healthy controls (162). CD63+ exosomes in GCF were increased in patients with periodontitis compared with those without periodontitis (157). In addition to EVs surface markers, miRNAs were abundant in EVs, and the level of hsa-miR-199a-3p decreased with the development and progression of periodontitis (163). There was a decrease in miR-223-3p concentration in periodontitis (164). In contrast, periodontitis dramatically enhanced the expression of hsa-miR-140-5p, hsa-miR-146a-5p, hsa-miR-628-5p, and PD-L1 mRNA (165, 166). A *P. gingivalis* saliva diagnostic kit for the detection of *P. gingivalis* and *P. gingivalis* OMVs has been developed by researchers using monoclonal antibodies that identify the conserved *P. gingivalis* virulence factor RgpA-Kgp complex (167).

These findings demonstrate that EVs released by saliva and GCF can reveal alterations in the local microenvironment and may indicate periodontitis (157, 168, 169). Owing to that, saliva and

GCF EVs may one day become simple and fast chair-side methods for diagnosing and evaluating periodontitis activity.

4.2 Collection of saliva and GCF and isolation of EVs

Patients should refrain from drinking and eating for at least one hour before sampling to avoid contamination of saliva and GCF with food and drink (23, 161, 163). Saliva can be collected either stimulated or unstimulated. Stimulated saliva is collected by chewing or gustatory stimulation (such as chewing paraffin or placing citric acid). In contrast, unstimulated saliva is collected by spitting or drooling without chewing or gustatory stimulation (170). Techniques used to collect saliva can affect its composition and the ultimate determination of particular biomarkers (171). As a result, saliva collection should closely resemble actual clinical conditions, and sample collection and processing should be consistent throughout.

Subgingival biofilms should be removed, and teeth should be blown dry to exclude saliva interference before GCF collection. After gently sampling with filter paper strips to prevent contamination from bleeding, GCF is eluted with PBS (172). Researchers should make it clear whether the GCF is from the healthy or the diseased site and correctly record the clinical parameters of each site, which is of great value in parsing the biochemical information (159, 168).

Although there is no optimal method for isolating saliva and GCF EVs, several researchers have compared different isolation protocols. In a comparison of saliva sEVs obtained by ultracentrifugation (UC) and ExoQuick-TC (TM) (EQ) precipitation, Zlotogorski - Hurvitz (173) et al. found that EQ generated a larger shape/aggregation pattern and a higher CD63/CD9/CD81+ sEVs subset than UC. Other investigators compared the particle production and particle/protein ratio of UC-sEVs and SEC-sEVs in saliva, showing that SEC-sEVs were superior in both categories (23).

In summary, mounting evidence points to the possibility that EVs generated from GCF and saliva may serve as vital diagnostic biomarkers for periodontitis owing to their cargo of proteins, RNA, and DNA (Table 1). But there is still a long way to go before EVs can be used for clinical diagnosis because the techniques for collecting EVs are currently only in-vitro or pre-clinical. The primary challenge is standardized techniques for isolating and characterizing EVs. More specific, sensitive, and practical biomarkers should be developed (174). An analysis with a large

TABLE 1 Application of exosomes in the diagnosis of periodontitis.

Author (Year)	Research type	Origin of EVs	Contents/ markers	Groups	Sample size	Conclusions	Reference
Chaparro Padilla A, et al. (157)	cross-sectional case-control study	saliva, GCF	CD9, TSG101, Alix	Periodontitis(stages II, III, and IV)	41	hypersecretion, pro-inflammatory	(141)
				Gingival health or Gingivitis	45		
Huang X, et al. (160)	cross-sectional case-control study	saliva	CD9, CD81	Severe Periodontitis (SP)	11	protein expression difference, pro-inflammatory	(144)
				Periodontal health	11		
Han P, et al. (161)	cross-sectional case-control study	saliva	CD9, TSG101	Periodontitis	8	hypersecretion, pro-inflammatory	(145)
				Gingivitis	7		
				Periodontal health	7		
Tobon-Arroyave SI, et al. (162)	cross-sectional case-control study	saliva	CD9, CD81	Periodontitis	104	hyposecretion	(146)
				Periodontal health	45		
Nik Mohamed Kamal NNS, et al. (163)	cross-sectional case-control study	saliva, plasma	unspecified	Chronic Periodontitis(CP)	8	miRNA expression difference, expression downregulated	(147)
				Periodontal health	8		
Xia Y, et al. (164)	cross-sectional case-control study	saliva	miR-223-3p	Periodontitis(stages III and IV)	none	expression downregulated, anti-inflammatory	(148)
				Periodontal health	none		
Yu J, et al. (165)	prospective observational investigation	saliva	PD-L1	Periodontitis	61	expression upregulated, pro-inflammatory	(149)
				Periodontal health	30		
Han P, et al. (166)	cross-sectional case-control study	saliva	unspecified	Periodontitis(stages III and IV)	10	expression upregulated	(150)
				Gingivitis	9		
				Periodontal health	10		

sample size is required to establish proper EVs-periodontitis diagnostic criteria matched with different ages, genders, etc.

5 MSCs-derived EVs and therapy of periodontitis

5.1 Role of MSCs-EVs in periodontitis treatment *via* anti-inflammatory and immune regulation

As an essential component of the innate immune system, macrophages mediate the onset and progression of periodontitis (175). Macrophages can differentiate into either a pro-inflammatory (M1) or an anti-inflammatory (M2) phenotype in reaction to local microenvironments, with each playing a unique role in a variety of physiological or pathological conditions (176, 177). Cytokines like TNF- and IL-6, which are produced by M1 macrophages, increase inflammation, activate osteoclasts, and result in the resorption of alveolar bone. In contrast, factors such as IL-10 and transforming growth factor (TGF) -β, produced by M2 macrophages, have anti-inflammatory and angiogenic effects and can activate osteoblasts (175, 176, 178, 179). Consequently, modulating the macrophage M1/M2 polarization ratio is an effective strategy for intervening in diseases.

In a rat calcaneus defect model, TNF-α-pretreated MSCs-EVs possess stronger immunomodulatory properties that can suppress M1 macrophages markers like IL-1β and iNOS and increase M2 macrophages markers like Arg1 and CD206, thereby promoting bone formation (180). MSCs-derived exosomes can improve the treatment of periodontitis by reestablishing the equilibrium of T helper 17/regulatory T cells (Th17/Treg) in inflamed periodontal tissues (181).

Bone mesenchymal stromal cells (BMSCs)-derived EVs regulate the inflammatory immune response and promote periodontal regeneration by inhibiting osteoclast activity, influencing macrophage polarization to M2, and regulating the production of TGF-β1 (68). Xu (182) et al. found that upon LPS stimulation, BMSCs-EVs converted macrophages from M1 to M2 phenotype *in vitro*, which decreased inflammation. In addition, ApoEVs derived from BMSCs can inhibit the polarization of macrophages into the M1 phenotype, reduce the COX-2 expression, down-regulate the TNF-α secretion, and inhibit adjacent osteoclasts, which serve as the foundation for the treatment of periodontitis (183). BMSCs-EVs have become promising therapeutic strategies for managing periodontitis (184).

The TNF-α-pretreated exosomes derived from gingiva mesenchymal stem cells (GMSCs) are notable for their ability to induce M2 polarization and prevent osteoclast formation (185). Wang (186) et al. observed that macrophages co-cultured with GMSCs exosomes in the inflammatory microenvironment showed significantly lower levels of M1 markers but somewhat raised levels of M2 features. In other words, GMSCs exosomes could trigger the transformation of M1 macrophages into M2 macrophages and lessen the pro-inflammatory substances that M1 macrophages release (186). Another study showed that GMSCs-sEVs significantly improved periodontal regeneration by inhibiting the release of pro-inflammatory cytokines from T cells and monocytes/macrophages, blocking T cells activation, and inducing the creation of Tregs (187).

Zheng (188) et al. discovered that periodontal ligament stem cells (PDLSCs)-derived exosomes alleviated the inflammatory microenvironment in chronic periodontitis *via* the Th17/Treg/miR-155-5p/SIRT1 regulatory network. EVs derived from LPS-pretreated PDLSCs induced M1 polarization of macrophages, whereas DNase I-treated EVs abolished M1 polarization. EVs derived from PDLSCs may be a potential therapeutic target for periodontal inflammation (189).

LPS-pretreated dental follicle stem cells (DFSCs)-sEVs polarized macrophages to an M2 phenotype through the ROS/ERK signaling pathway, inhibiting the alveolar bone loss and promoting periodontal regeneration in dogs with experimental periodontitis (69).

DCs-derived exosomes are relevant to immune therapy of periodontitis (190, 191). It has been demonstrated that engineered EVs derived from DCs can modulate the immune response in periodontitis and prevent inflammatory bone loss (192).

Consequently, studies have presented that MSCs-EVs from various sources can promote M2 macrophage polarization, restrict osteoclast activity, and reduce alveolar bone resorption, which paves the way for the development of periodontitis therapy (Figure 2; Table 2).

5.2 Role of MSCs-EVs in periodontal regeneration

To achieve functional periodontal regeneration, periodontal ligament fibers need to be inserted between the newly produced cementum and alveolar bone (193). Recent studies are concerned mainly with the osteogenic and angiogenic properties of MSCs-EVs, which are critical elements of periodontal regeneration.

Zhu B (194) et al. co-cultured MSCs-Exo with PDLSCs and noticed increased proliferation and osteogenic differentiation of PDLSCs. Furthermore, *in vitro* experiments demonstrated that MSCs-Exo promoted the PDLSCs proliferation and migration by activating AKT and ERK signaling pathways (70). Hypoxic preconditioning of MSCs-sEVs significantly enhanced the proliferation, migration, and angiogenesis of human umbilical vein endothelial cells (UVECs) and promoted the formation of vascularized bone (32). In a rat model of the alveolar bone defect, Chew (70) et al. transplanted a collagen sponge loaded with MSCs-Exo and observed the regeneration of alveolar bone and functional periodontal ligament fibers.

Wei (195) et al. proposed that BMSCs-Exo derived from different stages of osteogenic induction could exert a sustained anti-inflammatory effect during osteogenesis, up-regulate genes associated with osteogenesis at the early stage, and promote MSCs migration at the later stage. EVs derived from neural

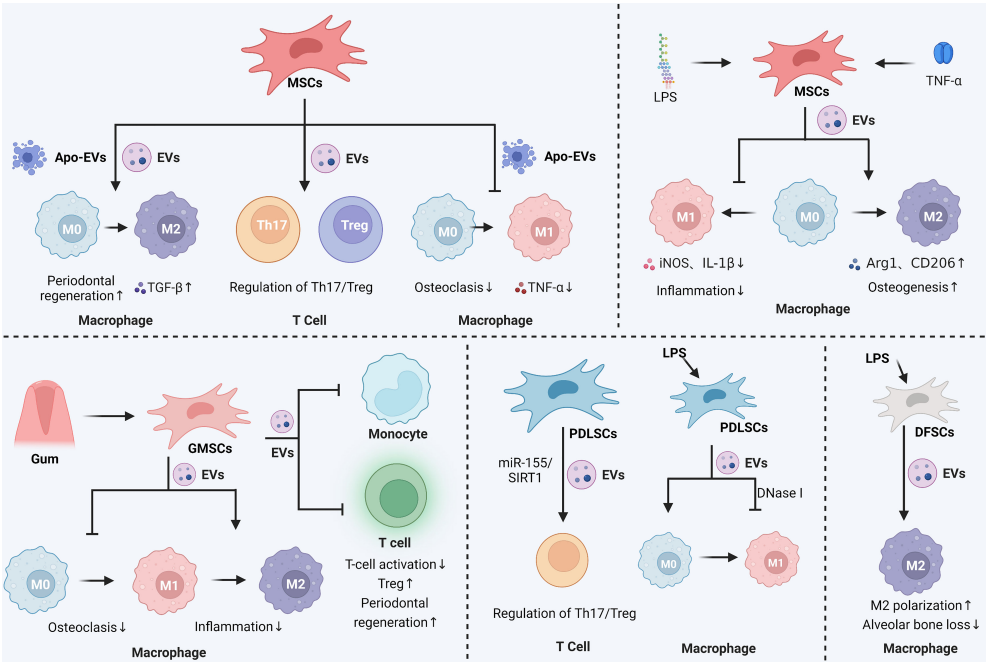

FIGURE 2
Immunomodulatory and anti-inflammatory effects of stem cell-derived EVs in periodontitis. In periodontitis, MSCs secreted EVs were able to promote the M2 polarization of macrophages and maintain the balance of Th17 and Treg cell ratio. MSCs secreted apoptotic vesicles were also able to inhibit the M1 polarization of macrophages, reduce the level of inflammatory mediators such as TNF-α, and promote the M2 polarization of macrophages. Pretreatment of MSCs with LPS or TNF-α enhanced the effect of MSCs-EVs, mainly by affecting macrophage polarization to control the inflammatory response and promote osteogenesis. Gingival mesenchymal stem cells (GMSCs) secrete EVs that affect macrophage polarization and inhibit osteoclastic and inflammatory reactions in periodontitis. GMSCs-EVs also inhibit monocyte and T-cell activation and promote periodontal tissue regeneration. Periodontal membrane stem cells (PDLSCs)-derived EVs could regulate the Th17/Treg ratio *via* the miR-155/SIRT1 axis and inhibit macrophage M1 polarization. LPS-stimulated dental follicular stem cells (DFSCs)-derived EVs also promoted macrophage M2 polarization and reduced alveolar bone loss. The Graph was created with BioRender.com. MSCs: mesenchymal stem cells; EVs, extracellular vesicles; Th, helper T cells; Treg, regulatory T cell; LPS, lipopolysaccharide.

TABLE 2 Anti-inflammatory and immunomodulatory effects of stem cell exosomes in periodontitis.

Author (Year)	MSCs source	Pretreatment of MSCs or EVs	Recipient of EVs	Experimental model	EVs administration	Functional outcome	Reference
Chew JRJ, et al., (70)	hMSC	/	rPDLSCs	①cells co-culture; ②Experimental periodontal defect rat;	①cells co-culture; ②Experimental periodontal defect rat: transplant/ implant with exosome-loaded collagen sponge (CS/Exosome) or control collagen sponge (CS/ Control);	repair periodontal defects, increase PDLSCs migration and proliferation	(67)
Cebatariuniene A, et al., (71)	hPDLSC	/	hPDLSCs	cells co-culture	cells co-culture	suppress basal and LPS-induced activity of NFκB	(68)
Liu L, et al., (68)	rBMSC	/	hPDLSCs/ RAW264.7 cells	①cells co-culture; ②Experimental Porphyromonas-induced periodontitis rats;	①cells co-culture; ②Experimental Porphyromonas-induced periodontitis rats: inject in periodontal pocket;	promote PDLSCs migration, proliferation and osteogenic differentiation	(65)
Huang Y, et al., (69)	hDFMSC	LPS pretreatment	hPDLSCs	①cells co-culture; ②Experimental Porphyromonas-induced periodontitis dogs;	①cells co-culture; ②Experimental Porphyromonas-induced periodontitis dogs: inject into the periodontal pocket;	promote PDLSCs proliferation and migration and macrophage proliferation	(66)

(Continued)

TABLE 2 Continued

Author (Year)	MSCs source	Pretreatment of MSCs or EVs	Recipient of EVs	Experimental model	EVs administration	Functional outcome	Reference
Kang M, et al., (180)	hBMSC	TNF-α pretreatment	mBMMs	①cells co-culture; ②Experimental calvaria defect rat;	①cells co-culture; ②Experimental calvaria defect rat: place on the wound by a clinical grade collagen scaffold (OraPLUG, Salvin);	immunoregulation, anti-inflammatory	(164)
Zhang Y, et al., (181)	hDPSC	3D culture	mouse naive CD4+ T cells	①cells co-culture; ②Experimental Ligature-induced periodontitis mice;	①cells co-culture; ②Experimental Ligature-induced periodontitis mice: inject into the palatal gingiva;	miR-1246 expression upregulated, reactive Th17 cell/ Treg balance, anti-inflammatory	(165)
Xu R, et al., (182)	rBMSC	LPS pretreatment	Raw264.7 cells	①cells co-culture; ②Experimental myocardial infarction mice;	①cells co-culture; ②Experimental myocardial infarction mice: intramyocardial injection at four sites around the infarct border zone;	promote M2 macrophage polarization, attenuat the post-infarction inflammation and cardiomyocyte apoptosis	(166)
Ye Q, et al., (183)	mBMSC	/	mBMDMs	Porphyromonas gingivalis derived LPS (Pg-LPS) induced inflammation of mouse bone marrow-derived macrophages (mBMDMs)	cells co-culture	inhibit M1 macrophage polarization and TNF-α secretion	(167)
Yue C, et al., (184)	hBMSC	/	RAW264.7 cells	cells co-culture	cells co-culture	regulate macrophage metabolism, differentiation, and inflammation resolution	(168)
Nakao Y, et al., (185)	hGMSC	TNF-α pretreatment	hPBMCs	①cells co-culture; ②Experimental wound healing mice; ③Experimental Ligature-induced periodontitis mice;	①cells co-culture; ②Experimental wound healing mice: subcutaneously inject into the cutaneous wounds; ③Experimental Ligature-induced periodontitis mice: inject into the palatal gingiva of the ligated second maxillary molar;	promote M2 macrophage polarization, immunoregulation	(169)
Wang R, et al., (186)	hGMSC	/	THP-1 cells	cells co-culture	cells co-culture	promote M2 macrophage polarization, anti-inflammatory	(170)
Zarubova J, et al., (187)	hGMSC	/	macrophages	cells co-culture	cells co-culture	reactive Th17 cell/ Treg balance, anti-inflammatory	(171)
Zheng Y, et al., (188)	hPDLSC	LPS pretreatment	CD4+ T cells	cells co-culture	cells co-culture	reactive Th17 cell/ Treg balance, anti-inflammatory	(172)
Kang H, et al., (189)	hPDLSC	LPS pretreatment	THP-1 cells	cells co-culture	cells co-culture	inhibit M1 macrophage polarization and TNF-α secretion	(173)

EGFL-like 1 modified BMSCs were more capable of stimulating BMSCs osteogenesis due to the downregulation of miR-25-5p (196). Huang (197) et al. demonstrated that EVs derived from BMP2-overexpressing BMSCs preserved the essential physical and biochemical characteristics of BMSCs-EVs but showed greater bone regeneration capability in a rat calvarial defect model. The ApoEVs from dying BMSCs can effectively promote the viability of endogenous BMSCs and repair bone defects (198). In critical-size calvarial bone defects, BMSCs-EVs positively regulate osteogenic genes and osteoblast differentiation *in vitro* (199). It has also been reported that BMSCs-derived exosomes overexpressing hypoxia-inducible factor (HIF)-1α can increase the packaging of Jagged1 and angiogenesis of endothelial cells (ECs) via the Notch signaling pathway (200). BMSCs-derived Nidogen1-enriched EVs enhanced the migration and angiogenic capacity of rat arterial endothelial cells (AECs) and promoted bone regeneration in rat femoral defect models (201). Hui (202) et al. coated BMSCs-EVs on a demineralized bone matrix to create a functional scaffold with enhanced pro-angiogenic and pro-bone regeneration activities.

Through a rat periodontitis model, Mohammed (203) et al. found that the injection of adipose-derived mesenchymal stem cells (ADSCs) exosomes suspension can be used as an auxiliary tool to promote periodontal regeneration, specifically periodontal fibers, blood vessels, and alveolar bone. The polydopamine-coated poly (lactic-co-glycolic acid) (PLGA/pDA) scaffold combined with ADSCs-EVs significantly induced the alveolar bone defect repair in the rat model (204). The ADSCs exosomes immobilized on the PLGA/pDA scaffolds promote the repair of critical-size skull defects in rats by stimulating osteogenesis and promoting BMSCs migration and homing (205).

In the inflammatory microenvironment, dental pulp stem cells (DPSCs)-EVs may shutter LMBR1-targeting miR-758-5p via the BMP signaling pathway to promote osteogenic and odontogenic differentiation of PDLSCs and provide a potential strategy for bone regeneration (206). DPSCs exosomes can effectively reduce periodontal bone loss by stimulating the migration of human DPSCs and mouse osteoblasts (207). It is addressed that DPSCs-EVs can induce the regeneration of experimental bone defects by enhancing the phosphorylation of ERK 1/2 and JNK and promoting the osteogenic differentiation of ADSCs (208). Xian (209) et al. found that DPSCs exosomes could stimulate endothelial cell proliferation and pro-angiogenic factors production, such as FGF-2, VEGF-A, KDR, and MMP-9. It has been demonstrated that DPSCs-EVs isolated from periodontally healthy and unhealthy teeth can enhance endothelial cell angiogenesis activity, accelerate wound healing, and encourage angiogenesis in mouse skin lesions (210). The co-injection of DPSCs-EVs with collagen, β-tricalcium phosphate, or hydroxyapatite can stimulate new bone formation in rat skull defects (211).

Invitro studies performed by Wang (212) et al. demonstrated that stem cells of human exfoliated deciduous teeth (SHED)-Exo under osteogenic induction conditions could up-regulate the expressions of osteogenic markers like osteopontin (OPN), osteocalcin (OCN), and Runx2 during the osteogenic differentiation of PDLSCs and enhance the osteogenic differentiation of PDLSCs *via* Wnt and BMP signaling pathways. Wei (213) et al. observed that exosomes repaired the defect to the same extent as original stem cells, increased the osteogenic impact of BMSCs, and inhibited adipogenesis after injecting SHED-derived exosomes into the bone defect area of a mouse periodontitis model.

Purified PDLSCs-EVs were discovered to reduce LPS-induced NF-B activity in PDLSCs and enhance osteogenic mineralization in PDLSCs, which may be helpful for the targeted treatment of chronic inflammation in periodontitis (71). Engineered EVs from PDLSCs promoted bone regeneration and angiogenesis of skull defects in rats (214). Collagen membrane enriched with PDLSCs-EVs or polyethylenimine (PEI)-engineered EVs (PEI-EVs) can activate osteogenesis and promote bone regeneration (215). PDLSCs-EVs immobilized in matrigel accelerated bone tissue repair by inducing BMSCs proliferation and migration through increasing the phosphorylation of AKT and ERK1/2 (216).

GMSCs-exosomes were shown to contain a variety of growth factors, such as transforming growth factor-β (TGF-β) and vascular endothelial growth factor (VEGF), which were shown to promote pre-osteoblast migration and osteogenic differentiation (217). 3D-engineered scaffolds complexed with GMSCs-EVs exhibited strong osteogenic induction ability (218).

According to Ma L (219) et al., DFSCs-sEVs boosted PDLSC migration, proliferation, and osteogenic differentiation, which offers a novel approach to periodontal regeneration in the future.

In conjunction with the current investigations, it is proposed that MSCs-EVs possess the potential to treat periodontitis and promote periodontal regeneration (Figure 3; Table 3). While the effectiveness of MSCs-EVs on periodontal ligament fibers and cementum regeneration needs to be further studied. Moreover, effectively alleviating the homeostasis imbalance is the key to periodontal regeneration. The application of MSCs-EVs in dentistry is restricted to fundamental research, and its clinical use requires more rigorous evidence. There is still a long way to go before MSCs-EVs can be used as an effective and safe dental clinical treatment method (220).

6 Summary and prospect

Several findings imply that OMVs can interfere with host gingival epithelium functions, affect angiogenesis, and induce PRRs reactions. OMVs also play a role in the pathogenicity of biofilms, such as promoting bacteria coaggregation, providing micronutrients to subgingival biofilms, and dispersing rival biofilms. These indicate that OMVs can promote connective tissue destruction and alveolar bone resorption. More research is required on the precise function and related mechanisms of OMVs in periodontitis. For example, the molecules and pathways by which OMVs affect innate and acquired immune defense (6).

Growing evidence suggests that saliva and GCF-derived EVs may serve as periodontitis biomarkers. Although the potential of saliva and GCF-derived EVs is promising, many obstacles must be solved before EVs can be translated into chair-side or off-the-shelf diagnostic tools. The major challenges are: (1) The need for standardized

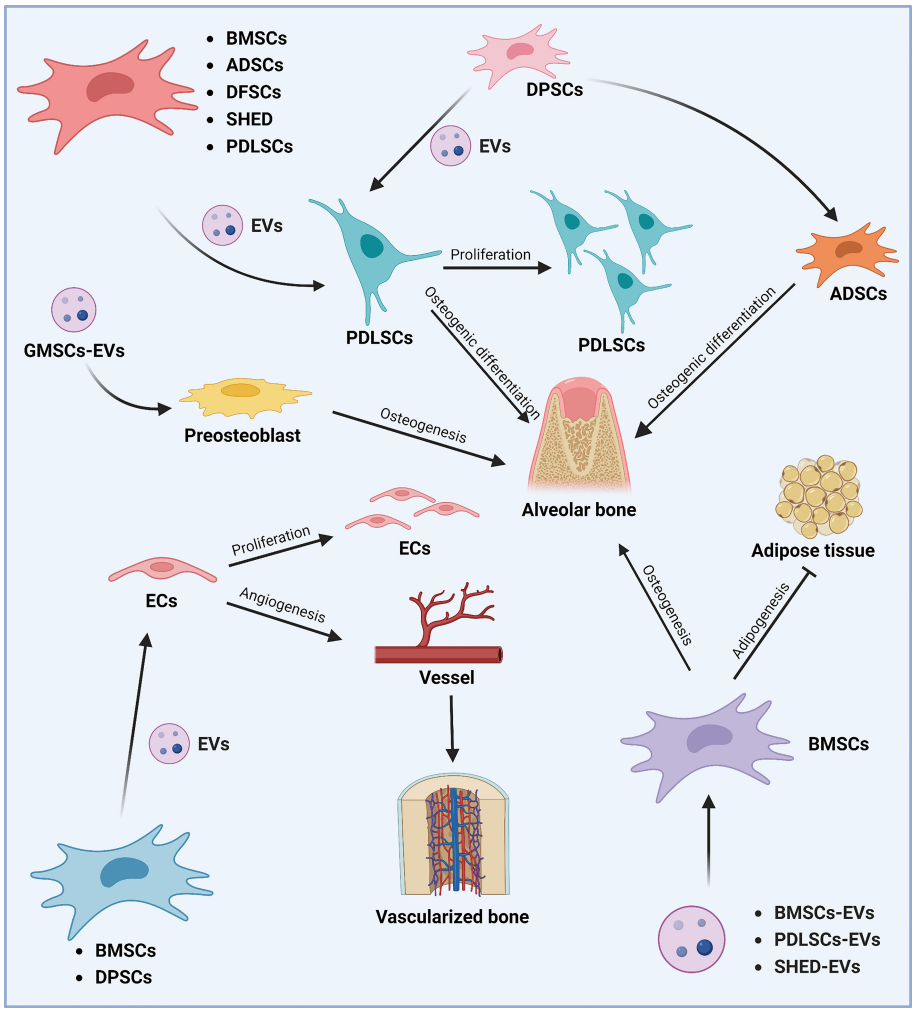

FIGURE 3
Role of stem cell-derived EVs in periodontal tissue regeneration. Different sources of MSCs can promote periodontal tissue regeneration and thus play a role in the treatment of periodontitis. Various sources of MSCs-EVs (including BMSCs, ADSCs, DFSCs, SHED, DPSCs, and PDLSCs) can promote the proliferation of periodontal membrane stem cells and alveolar bone osteogenesis by regulating osteogenic differentiation. DPSCs-derived EVs can promote alveolar bone regeneration by affecting the osteogenic differentiation of ADSCs. In addition, GMSCs-derived EVs can promote alveolar bone regeneration by regulating osteoblast precursor cells. In addition, BMSCs, PDLSCs, and SHED-derived EVs can promote osteogenic differentiation and inhibit lipogenic differentiation of BMSCs, thus promoting alveolar bone regeneration. The Graph was created with BioRender.com. BMSCs, bone marrow-derived mesenchymal stem cells; ADSCs, adipose tissue-derived mesenchymal stem cells; DFSCs, dental follicle stem cells; SHED, stem cells of human exfoliated deciduous teeth; PDLSCs, periodontal ligament stem cells; EVs, extracellular vesicles; GMSCs, gingiva-derived mesenchymal stem cells.

TABLE 3 Tissue regeneration of stem cell exosomes in periodontitis.

Author (Year)	MSCs source	Pretreatment of MSCs or EVs	Recipient of EVs	Experimental model	EVs administration	Functional outcome	Reference
Zhu B, et al. (194)	hPDLSC, hIBMMSC, JBMMSC	/	hPDLSCs	①cells co-culture; ②nude mice;	①cells co-culture; ②nude mice: transplant/implant into;	pro-osteogenic, osteoimmunomodulatory	(178)
Zhuang Y, et al. (32)	hox-rBMSC	hypoxia pretreatment	HUVECs	①cells co-culture; ②Experimental calvarial defect rat;	①cells co-culture; ②Experimental calvarial defect rat: transplant/ implant into;	promote HUVECs proliferation, migration and angiogenesis, pro-osteogenic	(179)
Wei F, et al. (195)	hBMSC	/	hBMDMs and RAW264.7	cells co-culture;	cells co-culture;	pro-osteogenic, osteoimmunomodulatory	(180)

(Continued)

TABLE 3 Continued

Author (Year)	MSCs source	Pretreatment of MSCs or EVs	Recipient of EVs	Experimental model	EVs administration	Functional outcome	Reference
			cells (macrophages)				
Lan Y, et al. (196)	rBMSC	neural EGFL-like 1 (Nell1) pretreatment	rBMSCs	①cells co-culture; ②Experimental calvarial defect rat;	①cells co-culture; ②Experimental calvarial defect rat: transplant/ implant into;	pro-osteogenic	(181)
Huang CC, et al. (197)	hBMSC	genetically modified by constitutively expressing BMP2 (bone morphogenetic protein 2)	hBMSCs	①cells co-culture; ②Experimental calvarial defect rat;	①cells co-culture; ②Experimental calvarial defect rat: place on the wound;	pro-osteogenic	(182)
Li M, et al. (198)	mBMSC	/	rBMSCs	①cells co-culture; ②Experimental calvarial defect rat;	①cells co-culture; ②Experimental calvarial defect rat: transplant/ implant into;	promote rBMSCs proliferation, migration, and osteogenic differentiation	(183)
Qin Y, et al. (199)	hBMSC	/	human osteoblasts (hFOBs)	①cells co-culture; ②Experimental calvarial defect rat;	①cells co-culture; ②Experimental calvarial defect rat: transplant/ implant into;	promote hFOBs proliferation, migration, and osteogenic differentiation	(184)
Gonzalez-King H, et al. (200)	hDPSC	hypoxia pretreatment	HUVECs	①cells co-culture; ②nude mice;	①cells co-culture; ②nude mice: inject subcutaneously into the flanks;	pro-angiogenic	(185)
Cheng P, et al. (201)	rBMSC	/	RAECs	①cells co-culture; ②nude mice;	①cells co-culture; ②nude mice: inject subcutaneously into the bac;	enhance RAECs migration, pro-angiogenic	(186)
Xie H, et al. (202)	rBMSC	/	HUVECs	①cells co-culture; ②nude mice;	①cells co-culture; ②nude mice: nude mice: implant into subcutaneously;	promote grafts vascularization, pro-angiogenic, pro-osteogenic	(187)
Mohammed E, et al. (203)	hADSC	/	/	Experimental periodontal defect rat;	Experimental periodontal defect rat: inject in periodontal pocket;	immunomodulatory, anti-inflammatory, pro-osteogenic	(188)
Yang Y, et al. (204)	hADSC	/	hPDLSCs	①cells co-culture; ②Experimental alveolar Bone Defects rat;	①cells co-culture; ②Experimental alveolar Bone Defects rat: transplant/implant into;	pro-angiogenic	(189)
Li W, et al. (205)	hADSC	/	hBMSCs	①cells co-culture; ②Experimental calvarial defect mice;	①cells co-culture; ②Experimental calvarial defect mice: transplant/ implant into;	promote hBMSCs osteogenic, proliferation and migration, pro-angiogenic	(190)
Yan C, et al. (206)	ihDPSC	/	hPDLSCs	cells co-culture;	cells co-culture;	promote hPDLSCs osteogenic and osteogenic differentiation	(191)
Shimizu Y, et al. (207)	hDPSC	/	mouse osteoblastic MC3T3- E1 cells	①cells co-culture; ②Experimental periodontal defect mice;	①cells co-culture; ②Experimental periodontal defect mice: directly appliy onto the silk ligature;	promote MC3T3- E1 cells migration	(192)
Jin Q, et al. (208)	hDPSC	/	hADSCs	①cells co-culture; ②Experimental mandible defect rat;	①cells co-culture; ②Experimental mandible defect rat: injecte into the hydrogel scaffold material and sutur;	promoted hADSCs migration, mineralization and osteogenic differentiation	(193)
Xian X, et al. (209)	hDPSC	/	HUVECs	cells co-culture;	cells co-culture;	promote HUVECs proliferation and tube	(194)

(Continued)

TABLE 3 Continued

Author (Year)	MSCs source	Pretreatment of MSCs or EVs	Recipient of EVs	Experimental model	EVs administration	Functional outcome	Reference
						formation, pro-angiogenic	
Zhou H, et al. (210)	hDPSC	/	ECs	①cells co-culture; ②Experimental skin wound healing mouse;	①cells co-culture; ②Experimental skin wound healing mouse: subcutaneously inject into the full-thickness excisional skin wound;	pro-angiogenic	(195)
Imanishi Y, et al. (211)	rDPSC	/	/	Experimental calvarial defect rat;	Experimental calvarial defect rat: transplant/ implant into;	pro-angiogenic, pro-osteogenic	(196)
Wang M, et al. (212)	hSHED	/	hPDLSCs	cells co-culture;	cells co-culture;	promote hPDLSCs osteogenic differentiation	(197)
Wei J, et al. (213)	hSHED	/	mBMSCs	①cells co-culture; ②Experimental periodontal defect rat;	①cells co-culture; ②Experimental periodontal defect rat: inject into the buccal and lingual sides of the first molar;	promote mBMSCs osteogenesis, differentiation, and bone formation	(198)
Pizzicannella J, et al. (214)	hPDLSC	/	hPDLSCs	①cells co-culture; ②Experimental calvarial defect rat;	①cells co-culture; ②Experimental calvarial defect rat: transplant/ implant into;	pro-angiogenic, pro-osteogenic	(199)
Diomede F, et al. (215)	hPDLSC	/	hPDLSCs	①cells co-culture; ②Experimental calvaria defect rat;	①cells co-culture; ②Experimental calvaria defect rat: transplant/ implant into;	pro-angiogenic, pro-osteogenic	(200)
Zhao B, et al. (216)	hPDLSC	/	hBMSCs	①cells co-culture; ②Experimental calvaria defect rat;	①cells co-culture: none; ②Experimental calvaria defect rat: transplant/ implant into;	promote hBMSCs proliferation, migration, and osteogenic differentiation	(201)
Jiang S, et al. (217)	hPDLSC	/	mouse osteoblastic MC3T3- E1 cells	cells co-culture;	cells co-culture;	promote pre-osteoblasts migration and MC3T3-E1 cells osteogenic differentiation	(202)
Diomede F, et al. (218)	hGMSC	/	hGMSCs	①cells co-culture; ②Experimental calvaria defect rat;	①cells co-culture; ②Experimental calvaria defect rat: transplant/ implant into;	pro-osteogenic	(203)
Ma L, et al. (219)	hDFSC	/	hPDLSCs	①cells co-culture; ②Experimental periodontal defect rats;	①cells co-culture; ②Experimental periodontal defect rats: transplant/ implant into;	promote hPDLSCs proliferation, migration, osteogenic differentiation	(204)

methods for EVs isolation and characterization. (2) More useful, sensitive, and specific biomarkers must be developed. (3) A large sample size analysis is required to establish the diagnostic standards for EVs-periodontitis that are appropriate for individuals of different ages and genders (23, 174). Despite the current challenges, the diagnostic potential of saliva and GCF-derived EVs is compelling, and future clinical applications can be expected.

MSCs-derived EVs have massive advantages, particularly convenient access, a wide range of sources, immunomodulatory ability, and tissue repair and regeneration capacities. EVs derived from MSCs have emerged over the past decades as an alternative therapy for stem cells in the field of regenerative medicine (221), and they are expected to be a novel therapeutic tool for periodontal regeneration (222). However, the oral cavity is a highly complex environment constantly changing, and it is unclear how several elements, including temperature, pH, oxygen, inflammation, and microbiota species, affect EVs. The majority of current MSCs-EVs studies use animal models. Yet the clinical application of MSCs-EVs in periodontal regeneration has not been reported. There are no standardized methods for the clinically graded manufacture and quality control of EVs medicines, which are crucial in following EVs clinical trials (223). In addition, there are still no low-cost technologies to swiftly produce an abundance of highly homogenous MSCs-EVs (224).

It is anticipated that more comprehensive research on EVs affecting periodontitis will be produced, demonstrating tremendous promise for clinical treatment, and opening up new doors for the advancement of stomatology.

Author contributions

CM and JP were responsible for constructing the concept of the paper. JP and WZ contributed to the literature search and analysis. CM, RC, and LW were responsible for writing the manuscript draft. BL and YW participated in editing and finalizing the manuscript. All authors have agreed with the final version of the manuscript before submission. All authors read and approved the final manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 26 April 2023
DOI 10.3389/fimmu.2023.1136964

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Cytokines and Soluble Mediators in Immunity, a section of the journal Frontiers in Immunology

RECEIVED 03 January 2023
ACCEPTED 31 March 2023
PUBLISHED 26 April 2023

CITATION
Homma K, Bazhanov N, Hashimoto K, Shimizu M, Heathman T, Hao Q, Nawgiri R, Muthukumarana V, Lee JW, Prough DS and Enkhbaatar P (2023) Mesenchymal stem cell-derived exosomes for treatment of sepsis.
Front. Immunol. 14:1136964.
doi: 10.3389/fimmu.2023.1136964

Mesenchymal stem cell-derived exosomes for treatment of sepsis

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Introduction: The pathogenesis of sepsis is an imbalance between pro-inflammatory and anti-inflammatory responses. At the onset of sepsis, the lungs are severely affected, and the injury progresses to acute respiratory distress syndrome (ARDS), with a mortality rate of up to 40%. Currently, there is no effective treatment for sepsis. Cellular therapies using mesenchymal stem cells (MSCs) have been initiated in clinical trials for both ARDS and sepsis based on a wealth of pre-clinical data. However, there remains concern that MSCs may pose a tumor risk when administered to patients. Recent pre-clinical studies have demonstrated the beneficial effects of MSC-derived extracellular vesicles (EVs) for the treatment of acute lung injury (ALI) and sepsis.

Methods: After recovery of initial surgical preparation, pneumonia/sepsis was induced in 14 adult female sheep by the instillation of *Pseudomonas aeruginosa* (~1.0×10^{11} CFU) into the lungs by bronchoscope under anesthesia and analgesia. After the injury, sheep were mechanically ventilated and continuously monitored for 24 h in a conscious state in an ICU setting. After the injury, sheep were randomly allocated into two groups: Control, septic sheep treated with vehicle, n=7; and Treatment, septic sheep treated with MSC-EVs, n=7. MSC-EVs infusions (4ml) were given intravenously one hour after the injury.

Results: The infusion of MSCs-EVs was well tolerated without adverse events. PaO_2/FiO_2 ratio in the treatment group tended to be higher than the control from 6 to 21 h after the lung injury, with no significant differences between the groups. No significant differences were found between the two groups in other pulmonary functions. Although vasopressor requirement in the treatment group tended to be lower than in the control, the net fluid balance was similarly increased in both groups as the severity of sepsis progressed. The variables reflecting microvascular hyperpermeability were comparable in both groups.

Conclusion: We have previously demonstrated the beneficial effects of bone marrow-derived MSCs (10×10^6 cells/kg) in the same model of sepsis.

However, despite some improvement in pulmonary gas exchange, the present study demonstrated that EVs isolated from the same amount of bone marrow-derived MSCs failed to attenuate the severity of multiorgan dysfunctions.

KEYWORDS

mesenchymal stem cell-derived exosomes, mesenchymal stem cell, exosome, sepsis, ovine model

1 Introduction

The pathogenesis of sepsis involves an imbalance between the pro-inflammatory and anti-inflammatory components of the immune system. It leads to an overproduction of pro-inflammatory cytokines and an overactivation of the immune system (1), a compromised anti-inflammatory state leading to immunosuppression and hemodynamic and coagulation changes, with cell injury, leading to the development of multiple organ failure (MOD) (2–5). The lung is among the most frequently injured organ in the development of sepsis. As lung injury gets worse clinically, patients will develop acute respiratory distress syndrome (ARDS), with a mortality rate of up to 40% (6, 7). Moreover, the mortality rate for sepsis in the intensive care unit is 40 to 60% (8–11). Currently, there are no effective treatments for sepsis. Because the pathogenesis of sepsis is extremely complex, the ideal therapy for sepsis would need to combine multiple targets, including early immunomodulation, cell protection, and prevention of end-organ damage. Based on extensive pre-clinical data, cell-based therapy with mesenchymal stem cells (MSC) has entered clinical trials for both ARDS and sepsis (12, 13). However, there remain long-term concerns about tumor risk in patients with administration of up to 10 million MSC/kg of body weight per treatment. Recent studies demonstrating the efficacy of MSC-EVs in pre-clinical studies in acute lung injury (ALI) and sepsis suggest a superior therapeutic than MSCs. Although less potent, MSC-EVs have a similar phenotype to their parent cells in suppressing inflammation and increasing bacterial clearance (14–20). Based on their small size (<200 nm), MSC-EVs cause fewer hemodynamic changes with administration than MSCs (with sizes up to 10μm), such as a rise in pulmonary artery pressure. In addition, MSC-EVs do not require a bone marrow transplant facility for storage or a preservative such as DMSO, which may affect the potency of the therapeutic. And most importantly, due to the anuclear properties of the EVs, MSC-EVs pose minimal long-term tumor risk (21, 22). However, many of these pre-clinical studies were performed in rodent models and had limited relevance to human sepsis. Hence, large animal models are required to evaluate respiratory and circulatory dynamics in similar clinical situations. Therefore, we investigated the effect of MSC-EVs in a clinically relevant ovine model of sepsis.

2 Materials and methods

2.1 Characterization of mesenchymal stem cell

2.1.1 Isolation of mesenchymal stem cell extracellular vesicles

Human bone marrow-derived MSCs were purchased from a National Institutes of Health repository from Texas A&M Health Science Center (Temple, TX) (23).

MSC-EVs were isolated from the conditioned medium of human bone marrow-derived MSCs using ultracentrifugation as we previously described (24, 25). Briefly, MSCs were grown in a T175 flask until 90% confluent and then serum starved in α-MEM supplemented with 0.5% Bovine Albumin Fraction (MP BioMedicals, LLC, Santa Ana, CA, http://www.mpbio.com). After 48 hours, the conditioned medium was collected and centrifuged at 3500 rpm 4℃ for 30 min to remove whole cells, cellular debris, and larger particles and then at 100,000 × g (Beckman Coulter Optima L-100XP Ultracentrifuge) to isolate the MSC-EVs at 4°C for 1 hour. The pellet was resuspended and washed in phosphate-buffered saline (PBS) and then ultracentrifuged again under the same conditions. After the second ultracentrifugation, they were collected at the bottom of tubes, resuspended with PBS (10μl per MSC-EVs released by 1×10^6 cells), and stored at −80℃.

2.1.2 Characterization and the dose of mesenchymal stem cell extracellular vesicles

In our prior publications, MSC-EVs were well characterized by morphology, size, protein, RNA content, and surface receptors (17). For consistency of preparation, we measured vesicle concentration from different MSC-EV isolations. By Nanosight, different MSC-EV preparations gave vesicle concentration of 10^{11} particles per ml. We chose the dose (4000 ul of MSC-EVs) for the sheep model based on an extrapolation of the dose of MSCs from our previous ex vivo perfused human model to the sheep. Ex vivo perfused human ALI model required a dose of 5-10 million MSCs (26). Also the ex vivo perfused human ALI model required a dose of 100- 200 ul of MSC-EVs for an equivalent effect (27). We have also reported that the sheep model of ALI required a dose of 5-10 million cells/kg (200-400 million MSCs) (28).

2.1.3 Animal model

Fourteen adult female Merino sheep were studied. The care and use of sheep followed the guidelines for using laboratory animals from the National Institutes of Health and the American Physiological Society (29). Approximately three-year-old females weighing 35 to 40 kg were purchased from Talley Ranch, Bastrop, TX. The Institutional Animal Care and Use Committee at the University of Texas Medical Branch approved the protocol for the study. All sheep were screened by a veterinarian and group housed at the Animal Research Center with free access to food and water until the day before the surgical procedures.

2.1.4 Surgical procedures

After at least 14 days of quarantine, sheep were housed in individual cages and transferred to the Translational Intensive Care Unit for the surgical procedures as described previously (30). Briefly, sheep were sedated with intramuscular ketamine (500mg) injection followed by its intravenous bolus injection (300mg; KetaVed; Vedco, St. Joseph, MO). Then, the endotracheal tube was placed with isoflurane inhalation *via* a mask (2–5%; Piramal Healthcare, Digwal, India). Afterward, the anesthesia was maintained with inhaled isoflurane *via* the endotracheal tube to effect (2-5%). For pain control, a subcutaneous injection of long-acting (72 h) buprenorphine (0.1mg/kg; Buprenorphine SR; ZooPharm, Laramie, WY) was given. After shaving the fur and weighing, the sheep were transferred to the operating room. A polyvinyl chloride catheter (Park-Davis, Sandy, UT) was implanted through the right femoral artery to monitor heart rate (HR) and mean arterial pressure (MAP) and draw blood samples. A 7 Fr Swan-Ganz thermodilution catheter (Edwards Lifesciences, Irvine, CA) was inserted into the common pulmonary artery through the right external jugular vein to monitor pulmonary arterial pressure (PAP), pulmonary capillary wedge pressure (PCWP), central venous pressure (CVP), intermittent cardiac output, and core body temperature. A Silastic catheter (Dow Corning, Midland, MI) was inserted into the left atrium of the heart through a left thoracotomy at the fifth intercostal space. After the surgical procedure, anesthesia was discontinued, and sheep were extubated when they could maintain adequate spontaneous breathings and transferred to the ICU. Pre- and post-surgical analgesia were provided with intravenous administration of long-acting (72 h) buprenorphine (0.1mg/kg). During the recovery period of about a week, sheep received intravenous fluid resuscitation (lactated Ringer's solution; 2 mL·kg body wt-1·h-1) and free access to food and water. All implanted catheters were continuously flushed with heparinized saline using a transducer (Truwave PX4X4; Edwards Lifesciences) and pressure infusor (Clear Cuff MX4710; Smiths Medical) to prevent a clot from forming in the lines.

2.1.5 Induction of pneumonia

After surgical recovery, fasted sheep (24 h) were again sedated with an intravenous bolus injection of ketamine (500mg) followed by a subcutaneous injection of buprenorphine (0.1mg/kg) as pre- and post-surgical analgesia. Then, anesthesia was maintained by isoflurane inhalation (2-5%) through a face-fitting mask. The tracheostomy was performed, a 10 mm tube (Shiley; COVIDIEN) was placed in the trachea, and isoflurane inhalation was switched from the face mask to the tracheostomy tube. A Foley catheter was placed in the urinary bladder of female sheep. Then, a total of 30 mL of live *P. aeruginosa* (PA,~1.0×10^{11} CFU) mixed with saline was instilled into the lungs (10 mL in the right middle, 10 mL in the right lower, and 10 mL in the left lower lobes) by a bronchoscope (model BF-P40; Olympus, Tokyo, Japan). The number of PA bacteria was determined based on our previous studies (31, 32).

2.1.6 Post-injury care

Immediately after the PA instillation, mechanical ventilation (Avea ventilator system; CareFusion, Yorba Linda, CA) started with pressure-regulated volume-controlled mode. The tidal volume (TV), positive end-expiratory pressure (PEEP), and respiratory rate were 12 mL/kg, 5 cmH_2O, and 20 breaths/min, respectively. FiO_2 was initially (for three h) set at 100% and further adjusted to keep PaO_2 around 100 mmHg. The respiratory rate was first set at 20 breaths/min and further adjusted to control $PaCO_2$ between 30-40 mmHg. The cardiopulmonary variables were monitored for 24 h in a conscious state. During the study, sheep had free access to food but not water to calculate the fluid balance accurately. To enhance translational aspects of the study, sheep were treated with an antibiotic (Cefazoline, 2g), titrated norepinephrine to keep MAP close to baseline (10 mmHg below the baseline) but not to exceed it, and fluid resuscitated. For the fluid resuscitation, lactated Ringer's solution was initiated with an initial rate of 2 mL/kg/h, which was further adjusted every 3 h to keep hematocrit close to baseline (± 3%).

2.1.7 Mesenchymal stem cell extracellular vesicle treatment

Sheep were studied in pairs to provide side-by-side assessment and were randomized to treatment with MSC-EVs (treatment: n=7) or saline (control: n=7). The MSC-EVs were stored frozen, and on the study day, the vial with MSC-EVs was thawed gently. The total volume (4mL) of a solution containing MSC-EVs was transferred into a sterile infusion bag containing 100 mL of USP-grade 0.9% NaCl using a 14-gauge needle and a syringe. At 1 h after the injury, MSC-EVs were administered by intravenous infusion through a central vein catheter within 30 minutes. Control sheep received 0.9% NaCl infusion at a matching rate. Physiologic measurements were performed at baseline, 0.5, 1, 5, 10, 15, 20, 30, 40, 50, and 60 min after initiation of MSC-EVs infusion in awake sheep.

2.1.8 Measured variables

Cardiopulmonary hemodynamics, mechanical ventilation readouts, fluid input, urine output, and arterial and mixed venous blood gas analysis were recorded at baseline and every three hours after that. In each graph, 0 h was the baseline, and the baseline measurement was performed immediately before bacterial inoculation. Hemodynamic variables included PAP, PCWP, CVP,

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systolic blood pressure (SBP), MAP, LAP, and HR. (hemodynamic monitor, IntelliVue MP50; Philips Medizin Systeme Boeblingen, Boeblingen, Germany). Cardiac output (CO) was determined three times by standard methods, and two relative values were used to calculate the mean cardiac index (CI). CI and systemic vascular resistance index (SVRI) were calculated according to the standard formula (33). The mechanical ventilation readouts included FiO_2, TV, respiratory rate (RR), peak and plateau pressure, and dynamic compliance. The mean airway pressure, PaO_2/FiO_2 (P/F) ratio, static compliance, and pulmonary shunt fraction (Qs/Qt) were calculated according to the standard formula. The urine output in female sheep was measured *via* a Foley catheter. The blood gas analysis was performed using a blood gas analyzer (RAPID Point 500; Siemens Healthcare, Erlangen, Germany). Blood samples were taken from a femoral artery to determine the white blood cells count (WBC) and C-reactive protein (CRP) and centrifuged under 4,000 rpm at 4℃ for plasma and serum separation to measure creatinine, and total bilirubin. Postmortem, bloodless lung wet-to-dry weight (W/D) ratio was determined by the method described by Pearce et al. (34). Microvascular hyperpermeability was indirectly evaluated by measuring lung extravascular water content and fluid balance.

2.1.9 Euthanasia criteria

The sheep were euthanized at the end of 24 h monitoring or at the time of reaching the euthanasia criteria by using intravenous infusion of ketamine (40mg/kg), xylazine (3.0mg/kg), and buprenorphine (0.01 mg/kg) (35). The euthanasia criteria included reduced mean arterial pressure (MAP) <40 mmHg, reduced heart rate (HR) <40 beats/min, increased $PaCO_2$ > 90 mmHg, or decreased PaO_2 <50 mmHg at 100% FiO_2 for at least an hour.

2.1.10 Bacterial clearance assay

As previously described (36), 1g of the lung tissue was taken from the dorsal edge of the right middle lobe, and 1g of the spleen was taken during the necropsy and homogenized in 3 mL of 1×PBS. Then, a ten-fold serial dilution was performed on each of the tissue homogenates. 10μL of each tissue homogenate dilution was pipetted and streaked onto Tryptic soy agar (TSA) plates. Bronchial alveolar lavage fluid was also taken from the left lower lobe during the necropsy and pipetted with ten-fold serial dilution onto the TSA plates. The plates were incubated for 24 h at 37°C for bacterial CFU counts (32).

2.1.11 Statistical analysis

All statistical analyses were performed using GraphPad Prism version 9.4.1 (Graph-Pad Software, Inc., La Jolla, CA). Results were compared between the groups at each time point by a two-way ANOVA with a mixed-effects model with *post hoc* Bonferroni or Sidak multiple comparison tests. The values measured at a single time point were compared by unpaired t-test or Mann–Whitney U test, based on the normality of the data distribution (Shapiro–Wilk test). All values are expressed as mean ± standard error of the mean (mean ± SEM). Statistical significance was considered for p-value < 0.05.

3 Results

3.1 Mortality rate

The mortality rate was calculated by dividing the number of non-survival (euthanized upon reaching euthanasia criteria) sheep within 24 h by the total number of sheep in each group. Six sheep out of seven survived in both groups during the study period. One control sheep were euthanized at 18 h, and one treatment sheep was euthanized at 16 h upon reaching the euthanasia criteria. The mortality rate was similar between control and treatment (14% vs. 14%) (Figure 1).

3.2 The severity of pulmonary dysfunction

No significant differences between the groups were found in the variables evaluating pulmonary functions. There was a tendency for the treatment group to have a higher PaO_2/FiO_2 ratio and static compliance from 6 h to 21 h throughout the study period. Mean airway pressure and plateau airway pressure tended to be higher in the control group sheep from 15 h to 24 h. Both groups were comparable in respiratory rate, actual tidal volume, pulmonary shunt fraction, and dynamic compliance (Figure 2).

3.3 Hemodynamic changes

Hemodynamic variables such as HR PAP, LAP, CVP, PWCP, lactate, and SVRI are comparable in both groups. The treatment group tended to have a slightly higher MAP from 12 h to 18 h and Cardiac Index from 6 h to 18 h than the control; however, no significant differences were found between the variables (Figure 3).

3.4 Microvascular hyperpermeability

The hematocrit was shown in actual numbers and numbers adjusted to the baseline as a percentage. Although treatment sheep tended to have higher hematocrit (one sheep in control group had an

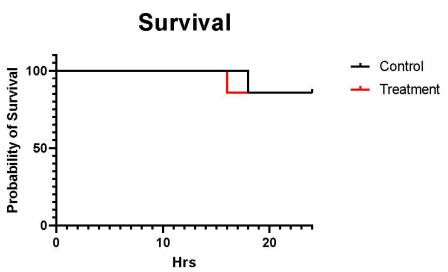

FIGURE 1
The Kaplan-Meier curve of the mortality rate. The black line shows control (n=7 at 0-18 h and 6 at 19-24 h), and the red line shows treatment (n=7 at 0-16 h and 6 at 17-24 h). The mortality rate was calculated by dividing the number of sheep that not survived within 24 h by the total number of sheep in each group. The mortality rate was similar between males and females (14% vs. 14%).

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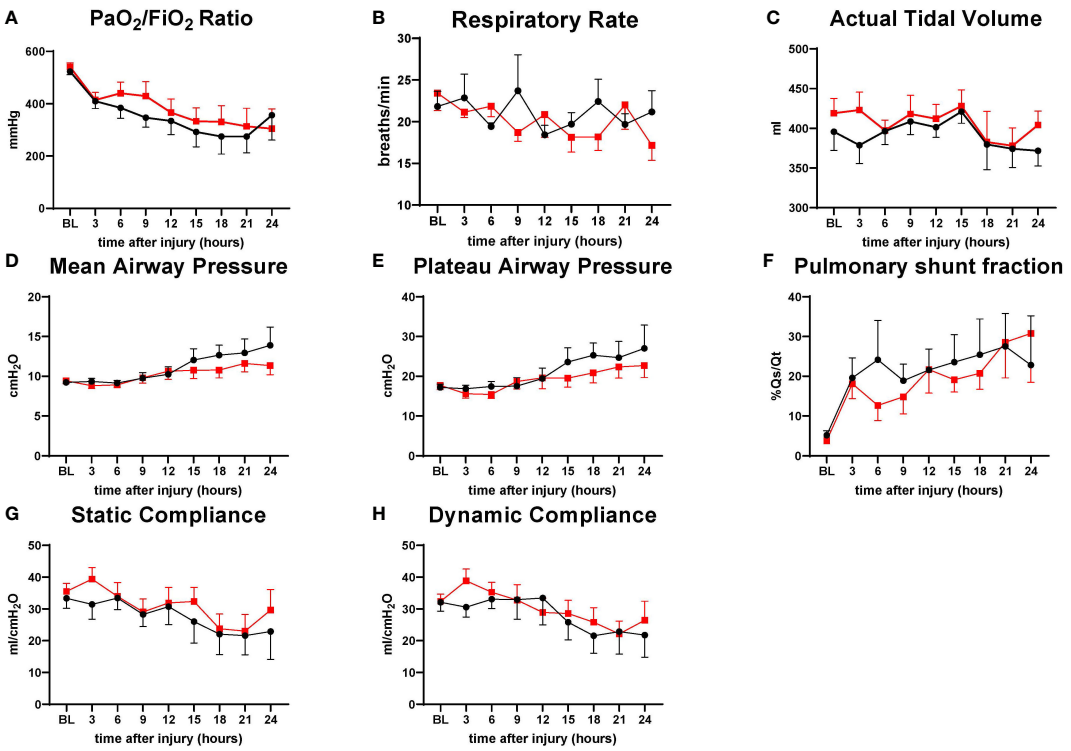

FIGURE 2
The severity of lung injury. ● Control (n=7 at 0-18 h and 6 at 19-24 h), ■ Treatment (n=7 at 0-16 h and 6 at 17-24 h. There were no differences in pulmonary function. PaO_2/FiO_2 ratio (P/F ratio) **(A)**, respiratory rate (RR) **(B)**, actual tidal volume **(C)**, mean airway pressure **(D)**, plateau airway pressure **(E)**, pulmonary shunt fraction (Qs/Qt) **(F)**, static compliance **(G)**, dynamic compliance **(H)**. Data are expressed as mean ± SEM.

unusual pattern of low hematocrit, resulting in lower mean hematocrit value) at baseline and post-injury time points, overall changes in hematocrit were comparable in both groups. The net fluid balance, a measure of accumulated fluid over time, was similarly increased in both groups as the severity of sepsis progressed. The lung bloodless wet-to-dry weight ratio, wet lung weight per body weight, and thoracic fluid volume were comparable in both groups, and no significant difference was found between the groups. Accumulation of exudate in the thoracic cavity tended to be lower in the treated group. Control sheep required more vasopressor to maintain MAP than treatment sheep after 10 h post-injury (Figure 4).

3.5 Altered mental status

The neurological status of the animal was assessed by the Simplified Sheep Neurological/Alertness Assessment score (SSNAA). The SSNAA is scored by summing the scores for response to approach (1,2,4), response to sound (1,2,4), and response to mechanical stimulations (0,1,2,3) (total score 2-11) (Supplement Table 1). The SSNAA score tended to be slightly higher in treatment than in control from 15 h to 24 h (Figure 5).

3.6 Bacterial clearance assay

The number of bacteria in lung tissue at 24 h after PA instillation in control was $1.68\times10^9 \pm 1.58\times10^9$ CFUs/g, and in the treatment group, it was $5.48\times10^8 \pm 5.19\times10^8$ CFUs/g. The number of bacteria in the spleen in control was $1.52\times10^6 \pm 7.23\times10^5$ CFUs/g, and in treatment, it was $1.08\times10^7 \pm 4.95\times10^6$ CFUs/g. The number of bacteria in BALF in control was $5.13\times10^4 \pm 2.90\times10^4$ CFUs/g, and in treatment, it was $2.03\times10^5 \pm 1.66\times10^5$ CFUs/g. Although treatment sheep tended to have lower bacterial numbers in lung tissue than control sheep, treatment sheep tended to have higher bacteria numbers in the spleen and BALF, and no significant difference was found between the groups in each tissue (Figure 6).

3.7 Inflammatory marker

WBC in the treatment tended to be lower than the control after 18 h (n=4, each group). However, no statistical significance was noted between the groups. CRP changes were comparable in both groups (Figure 7)

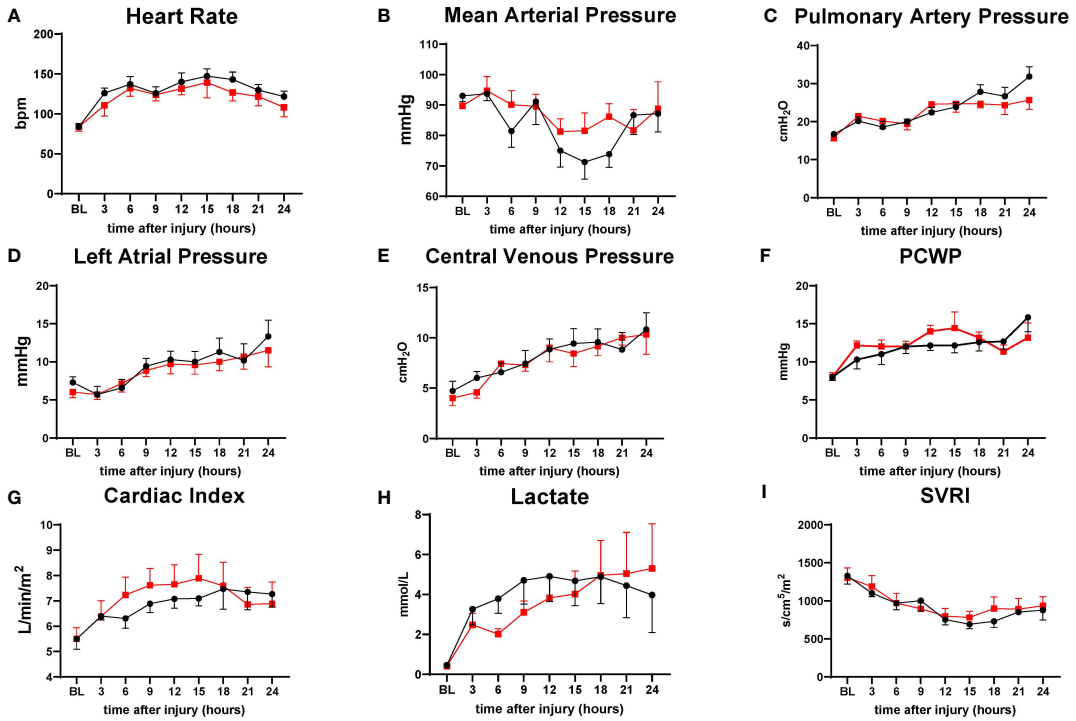

FIGURE 3
The severity of lung injury. ● Control (n=7 at 0-18 h and 6 at 19-24 h), ■ Treatment (n=7 at 0-16 h and 6 at 17-24 h). There were no differences in hemodynamic changes. Heart rate (HR) **(A)**, mean arterial pressure (MAP) **(B)**, pulmonary artery pressure (PAP) **(C)**, left atrial pressure (LAP) **(D)**, central venous pressure (CVP) **(E)**, pulmonary capillary wedge pressure (PCWP) **(F)**, cardiac index (CI) **(G)**, lactate **(H)**, systemic vascular resistance index (SVRI) **(I)**. Data are expressed as mean ± SEM.

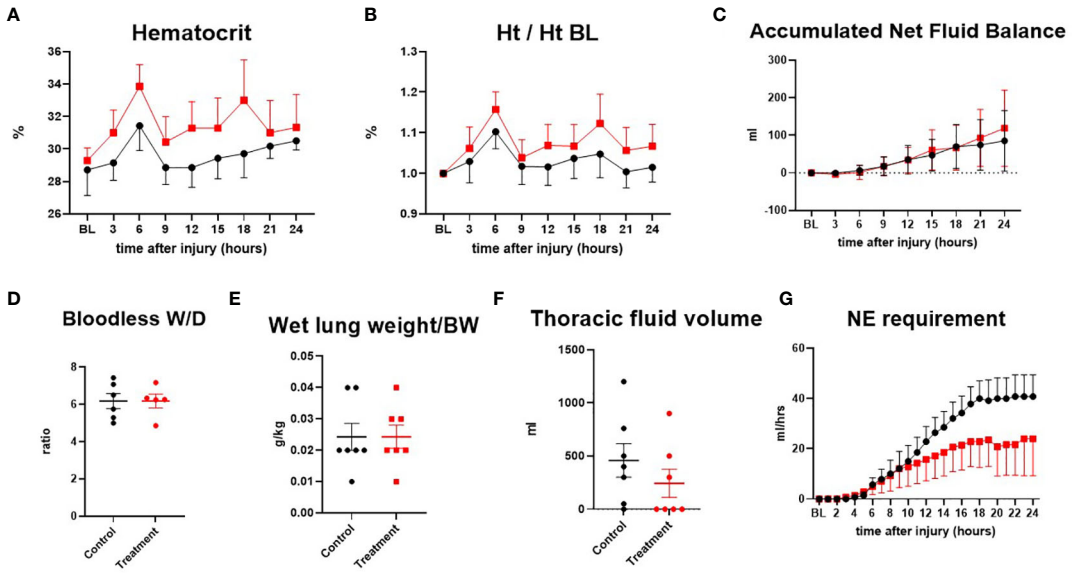

FIGURE 4
The severity of lung injury. ● Control (n=7 at 0-18 h and 6 at 19-24 h), ■ Treatment (n=7 at 0-16 h and 6 at 17-24 h). Hematocrit (Ht) **(A)**, hematocrit/hematocrit at baseline (Ht/Ht BL) **(B)**, accumulated net fluid balance **(C)**, bloodless wet-to-dry weight ratio (W/D ratio) **(D)**, wet lung weight/body weight **(E)**, thoracic fluid volume **(F)**, Norepinephrine requirement **(G)**. Data are expressed as mean ± SEM.

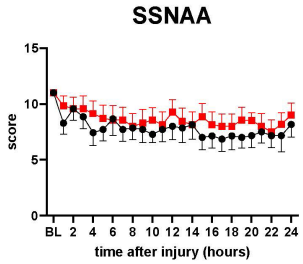

FIGURE 5
SSNAA: A simplified sheep neurological/alertness assessment scale. The severity of lung injury. ● Control (n=7 at 0-18 h and 6 at 19-24 h), ■ Treatment (n=7 at 0-16 h and 6 at 17-24 h). The SSNAA score tended to be slightly higher in treatment than in control from 15 h to 24 h, but there was no significant difference between the groups. Data are expressed as mean ± SEM.

4 Discussion

Cell-based therapy with stem cells may be important for physiologic maintenance and organ repair in the event of injury. This has been extensively studied for the lung. Stem cell therapy using mesenchymal stem cells (MSCs), endothelial progenitor cells (EPCs), embryonic stem cells (ESCs), and induced pluripotent stem cells (iPSCs) are novel treatment options for ALI. MSCs possess multipotency and repair functions and can be harvested from almost every postnatal tissue. Their easier accessibility, improved safety profile, and nonexistent ethical concerns make them a superior candidate for the cell-based therapy compared to ESCs and iPSCs (37). Special attractiveness arises from the immune-privileged status of MSCs. They do not trigger a host response or cell rejection because they are less sensitive to the effects of HLA-II expression by inflammatory IFN-γ (38). In various pre-clinical ALI models, MSCs have been shown to secrete multiple paracrine factors that reduce lung endothelial and epithelial permeability, decrease inflammation, promote tissue repair, inhibit bacterial growth, and ultimately reduce mortality. However, concerns about using stem cells, specifically the risk of iatrogenic tumor formation, remain unresolved. Currently, the accumulating evidence suggests that new cell-free therapies involving MSC-derived conditioning medium and extracellular vesicles released from MSCs might be alternative therapies.

In comparison with MSCs, MSC-EVs possess hypoimmunogenic properties, low tumorigenesis, and higher stability (39). Functions similar to those of their parental cells, such as antimicrobial effects, immunomodulatory properties, and the ability to repair damaged tissues, have been observed in MSC-EVs (40).The paracrine effect mediated by secreted growth factors, cytokines, and extracellular vesicles is mainly responsible for the efficacy of MSCs (41). MSC-EVs have been identified as the main parts responsible for the paracrine effect. They transfer functional molecules such as messenger RNA (mRNA), microRNA (miRNA), lipids, mitochondria, and proteins to tissue-specific cells in need of repair. These molecules in MSC-EVs play a critical role in modulating immune responses and repairing lung injury in ALI/ARDS. MiRNAs in MSC-EVs have been considered critical to exert efficacy in sepsis (14).

Some researchers showed that intratracheal administration of MSC-EVs had therapeutic effects in hyperoxia-induced lung injury, demonstrating that MSC-EVs could ameliorate impaired alveolarization in both short and long-term bronchopulmonary dysplasia models and activate M2 macrophages (42, 43). The effects of MSC-EVs on COVID-19, a pandemic disease for which no specific antiviral medication, is being investigated in two clinical trials. MSC-EVs are administered intravenously (NCT04798716) or by inhalation (NCT04276987). Allogeneic bone marrow MSC-derived exosomes (ExoFloTM) were shown to be safe and effective in restoring oxygenation, downregulating cytokine storm, and reconstituting immunity in severe COVID-19 patients in a prospective, non-randomized, open-label cohort study (44).

The results of our present study demonstrated that the use of MSCs-EVs failed to attenuate pneumonia/sepsis-induced multiorgan dysfunctions in a clinically relevant ovine model despite some tendency in improving pulmonary gas exchange and reducing the vasopressor requirement to maintain the blood pressure. Currently, the reason for the inefficiency of these EVs is not clear. As mentioned, we have treated septic sheep with EVs harvested from 10×10^6/kg body weight one time, 1 h after the injury. It is possible that the dose of the EVs was too small, or repeated treatment was needed. It is also possible that IV-administered MSCs continuously secrete EVs while circulating within the body, thus producing more EVs than ones harvested from 10^6/kg cells at a given time. This notion is supported by findings by Silva et al., who reported that EVs were less effective

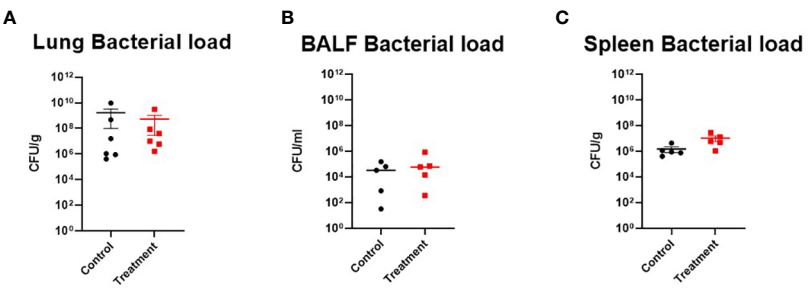

FIGURE 6
Bacterial clearance assay. **(A)** Lung bacterial load ● Control (n=6), ■ Treatment (n=6), **(B)** BALF bacterial load ● Control (n=5), ■ Treatment (n=5), **(C)** Spleen bacterial load ● Control (n=5), ■ Treatment (n=5). There was no significant difference between the groups in the bacterial clearance assay. Data are expressed as mean ± SEM.

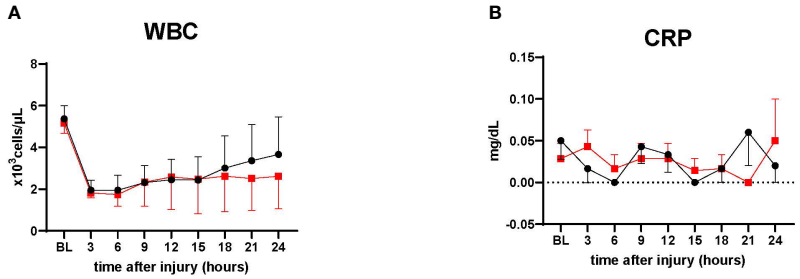

FIGURE 7
Inflammatory marker. **(A)** White blood cells count (WBC), ● Control (n=4 at 0-24 h), ■ Treatment (n=4 at 0-24 h). **(B)** C-reactive protein measurement. ● Control (n=7 at 0-18 h and 6 at 19-24 h), ■ Treatment (n=7 at 0-16 h and 6 at 17-24 h). WBC in the treatment tended to be lower than the control after 18 h. CRP showed relatively low in both groups, with no significant differences. Data are expressed as mean ± SEM.

than parental MSCs at reducing lung injury (45). Monsel et al. reported that higher EVs concentration was needed to obtain similar therapeutic effects as MSCs (46). Zhu and collaborators reported a modest effect of EVs when they were given based on the final MSC cell count. The authors achieved enhanced therapeutic effect only with the increased doses of EVs (16). In general, we estimate that MSC-EVs are 5-10× less potent than MSCs in pre-clinical ALI/sepsis models.

The homing of the EVs to the site injury can also be of concern. The limitation of the study is also related to the lack of information on the kinetics of the IV-injected EVs within the body, especially in the septic environment. Nevertheless, we report that EVs harvested from 10^6/kg cells do not produce benefits against PA-induced sepsis. Future dose-dependent studies should be carried out to eliminate the limitations mentioned above. Another limitation of this study is the duration of our studies was relatively short (24 h), which precluded comparison of the extent of recovery over time in both groups and the more prolonged efficacy of MSC-EVs in the septic sheep.

Despite the equivocal results, there is still the potential for using MSC-EVs clinically for ARDS or sepsis. For example, Sengupta et al. (44) found improved oxygenation with an average pressure of arterial oxygen to fraction of inspired oxygen ratio (PaO_2/FiO_2) increase of 192% ($P < 0.001$) in 24 patients with COVID-associated ARDS who were administered 15 mL of ExoFlo (Direct Biologics, LLC). Their study was a multicenter, double-blinded, placebo-controlled, randomized control trial where patients received normal saline 90 mL or ExoFlo 10-15 mL, which contains approximately 800–1,200 billion EVs released by MSCs (44). These studies raise the question of whether MSC-EVs may be better suited as a potential therapeutic for patients with sterile ARDS induced by various etiology factors, i.e., ventilator-induced lung injury, trauma, transfusion-associated ALI, etc. Regardless, the issue of potency and pharmacokinetics of administered MSC-EVs remains the main barrier to bringing this very promising therapeutic to clinical use.

5 Conclusion

We have previously demonstrated the beneficial effects of bone marrow-derived MSCs (10×10^6 cells/kg) in the same model of sepsis as well as the smoke inhalation-induced ARDS model. The results of our present study indicate that EVs harvested from the same amount of MSCs do not produce equivalent efficacy, suggesting that higher doses of EVs are required to be isolated from a greater number of MSCs to achieve the same therapeutic effects obtained by parent MSCs that exerted benefits.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by The Institutional Animal Care and Use Committee at the University of Texas Medical Branch.

Author contributions

KeH and PE conceived the experiment. KaH, NB, and KeH conducted the experiment and analyzed the data. MS, QH, and JL contributed to the preparation of MSC-EVs. KeH and MS drafted the manuscript and figures and carried out the literature search. JL and PE carried out the manuscript modification. DP helped perform the manuscript with constructive discussions. TH helped with bacterial clearance assay and manuscript editing. All authors contributed to the article and approved the submitted version.

Funding

PE received grant from NIH R01GM097480. R01HL148781. JL received grant form R01HL113022-06. R01HL148781.

Acknowledgments

We want to thank the translational intensive care unit personnel for their professionalism and excellent work done to complete this study.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1136964/full#supplementary-material

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frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 16 May 2023
DOI 10.3389/fimmu.2023.1166536

OPEN ACCESS

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RECEIVED 15 February 2023
ACCEPTED 04 May 2023
PUBLISHED 16 May 2023

CITATION
Zhao Z, Zhang L, Ocansey DKW, Wang B and Mao F (2023) The role of mesenchymal stem cell-derived exosome in epigenetic modifications in inflammatory diseases.
Front. Immunol. 14:1166536.
doi: 10.3389/fimmu.2023.1166536

The role of mesenchymal stem cell-derived exosome in epigenetic modifications in inflammatory diseases

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Epigenetic modification is a complex process of reversible and heritable alterations in gene function, and the combination of epigenetic and metabolic alterations is recognized as an important causative factor in diseases such as inflammatory bowel disease (IBD), osteoarthritis (OA), systemic lupus erythematosus (SLE), and even tumors. Mesenchymal stem cell (MSC) and MSC-derived exosome (MSC-EXO) are widely studied in the treatment of inflammatory diseases, where they appear to be promising therapeutic agents, partly through the potent regulation of epigenetic modifications such as DNA methylation, acetylation, phosphorylation, and expression of regulatory non-coding RNAs, which affects the occurrence and development of inflammatory diseases. In this review, we summarize the current research on the role of MSC-EXO in inflammatory diseases through their modulation of epigenetic modifications and discuss its potential application in the treatment of inflammatory diseases.

KEYWORDS

epigenetic modification, inflammatory bowel disease, osteoarthritis, systemic lupus erythematosus, mesenchymal stem cell-derived exosome

1 Introduction

MSCs are widely used in the field of regenerative medicine and have the potential to differentiate into adipose, bone, cartilage, and other tissues under specific *in vitro* conditions. Classical MSCs express CD105, CD73, CD90, CD34, and CD44 and these surface markers are commonly used in research to identify the purity of MSCs by flow cytometry (1). There are many sources of MSCs, mainly bone marrow, adipose, and umbilical cord blood (2). Currently, MSCs are widely being explored in the treatment of autoimmune diseases, inflammatory diseases, and many other diseases (3). MSCs secrete exosomes, a type of extracellular vesicle (EV) with a diameter of 40-200 nm, and are the

best-defined secretory vesicle of all EVs. Exosomes are produced when the endosomal membrane invaginates to form multi-vesicular bodies (MVBs) and are released when the MVBs fuse. The exosome membrane is structurally similar to the cell membrane, rich in signaling molecules and surface antigens, and it also contains a typical lipid raft structure. The main mechanisms by which exosomes bind to target cells include ligand-receptor binding, endocytosis, and direct binding (4). MSC-EXO has similar functions to MSCs as recent evidence suggests that MSCs act primarily through paracrine effects and the use of MSC-EXO as a vehicle for cell-free therapy reduces the concern of injecting live cells (5, 6). Therefore, it has been suggested that MSC-EXO is superior to MSCs in clinical treatment, and how to apply MSC-EXO in the clinical setting is a trending topic of research today (7).

Epigenetics has been a headline of research in various fields worldwide, and the study of epigenetic modifications has contributed to the development of medicine, biology, and other disciplines. It has also led to breakthroughs in the study of disease pathogenesis, diagnosis, and treatment. The study of epigenetics includes two main aspects; the regulation of selective transcriptional expression of genes and the post-transcriptional regulation of genes. From a broader perspective, epigenetics is the study of the mechanisms by which gene expression is regulated and, the mechanisms by which genes interact with each other. The core of epigenetic research and the key question to be addressed is the central law of regulation of the transmission of genetic information from the genome to the transcriptome (8). Epigenetic modifications include histone-related modifications such as histone methylation, acetylation phosphorylation, DNA methylation, and expression of regulatory non-coding RNAs (9, 10). Epigenetic modifications are dynamic and can be caused by genetic factors or induced by environmental factors. Studies have shown that epigenetic modifications are closely associated with the development and progression of many diseases. The interplay and intermodulation between epigenetic remodeling and metabolic reorganization are thought to be one of the hallmarks of inflammatory diseases, Various metabolic and epigenetic changes, to a certain extent, promote the development of inflammation, leading to the onset of chronic inflammatory diseases (11). For example, fibrosis, a consequence of chronic inflammatory disease, is caused by a variety of factors including epigenesis. The expression of DNA methyltransferase (DNMT) in patients with idiopathic pulmonary fibrosis (ITP) is significantly higher than that in normal people, indicating that the abnormal expression of DNMT is one of the important factors leading to the generation of ITP (12). In addition, the degree of DNA methylation of peroxisome proliferator-activated receptor γ in plasma is one of the important indicators used to assess the severity of pulmonary fibrosis clinically (13). A number of drugs that use modulation of epigenetic modifications to treat diseases are in development or already in clinical use, and drugs that modulate DNA methylation and histone modifications are already widely used in the treatment of diseases such as cancer (Table 1).

Inflammation is a biological response that occurs when the body is stimulated by pathogens, microorganisms, damaged cells, and other internal and external environmental factors. Under normal physiological conditions, inflammation activates the body's immune system to eliminate the corresponding stimulus, initiates the healing process, minimizes tissue damage and infection caused by the stimulus, helps restore homeostasis, and is an important defense mechanism in maintaining host health. However, prolonged failure of inflammation to subside can result in chronic inflammation and tissue damage, which contributes to the pathogenesis of many inflammatory diseases such as atherosclerosis, IBD, and OA (10). Inflammatory diseases are characterized by a dramatic increase in the expression of pro-inflammatory cytokines (20, 21) such as Interleukin-6 (IL-6) and Interleukin-β (IL-β) and activation of the NOD-like receptor thermal protein domain associated protein 3 (NLRP3) inflammasome. Inflammatory diseases are often difficult to treat clinically, difficult to cure, and prone to recurrence (22). Therefore, there is an urgent need to explore new and effective therapeutic approaches for inflammatory diseases. This review summarizes previous studies on epigenetic abnormalities in inflammatory diseases and how MSC-EXO affects the occurrence and development of inflammatory diseases by influencing epigenetic modification.

2 Epigenetic modifications

Epigenetic modification is a complex physiological mechanism that is important for maintaining homeostasis. It regulates gene

TABLE 1 Drugs used in clinical practice for cancer and inflammatory disease.

Modifying objects	Mechanism	Drugs	Disease	Reference
DNA	DNA methylation	Azacytidine decitabine EGCG Clofarabine	Leukemia	(14, 15, 113)
Histones	Acetylation, methylation, ubiquitination, benzoylation, deimination, and poly(ADP) ribosylation	Panobinostat tazemetostat Pracinostat Vorinostat	MM refractory B cell lymphoma AML CTCL	(16, 17)
RNA	RNA interference	Alicafforsen AZD8601	IBD Ischemic heart disease	(18, 19)

expression without changing the DNA sequence. Epigenetic changes are dynamic, and influenced by age, environment, and many other factors (23). Epigenetics includes DNA methylation, histone modification, and RNA-based mechanism. DNA methylation is the transfer of a methyl group from the methyl donor to the fifth carbon of DNA cytosine residue to form a specific methylation structure(5-MC), occurring mostly on CPG islands in the gene promoter region, and methylation in gene promoter region can lead to transcriptional silence. This process is catalyzed by the DNA methyltransferase family (DNMT). Different DNMTs play different roles in DNA methylation (24). Abnormal DNA methylation is closely related to a variety of diseases. For example, abnormal hypermethylation is the most important epigenetic modification mechanism for atherosclerosis (25). Overexpression of DNMT1 and DNMT3A can lead to abnormal methylation of DNA of tumor suppressor genes (TSGs), leading to pituitary adenoma invasion. Therefore, DNA methylation is closely related to the occurrence and development of invasive pituitary adenoma (26). DNA methylation-based biomarkers and epigenetic therapy play an important role in the early diagnosis and prognosis of a variety of diseases.

A nucleosome consists of DNA and a histone octamer and the N-terminus and C-terminus of histones can be modified by posttranslational modification such as the addition of methyl groups to lysine residues of histones H3 and H4 to induce methylation of histones or phosphorylation of histones at serine and tyrosine residues. In addition to this, there are acetylation and ubiquitination as types of histone modifications (27). Histone modification is catalyzed by a variety of enzymes called histone acetyltransferases (HATs) and histone deacetylases (HDACs), regulating histone acetylation. Histone methyltransferase (HMT) catalyzes the methylation of histones by transferring methyl groups from S-adenosyl methionine (SAM) to lysine residues of histones (28). Histone modification has been shown to play a role in a variety of diseases such as non-small lung cell carcinoma (NSCLC), periodontitis, and breast cancer (29, 30). Di Zhang et al. reports a novel post-translational modification of proteins, histone lactate modification. Their study showed that the accumulated lactic acid in the body can catalyze the lactating modification of histone lysine to further regulate gene expression (31), and found that histone lactating modification plays an important role in the regulation of inflammation and tumor metabolism. Histone lactate modification in the tumor microenvironment can generate immunosuppression to promote the immune escape of tumors (32). Histone lactate modification also improves cardiac function after myocardial infarction by promoting repair and gene transcription to regulate the dual activities of anti-inflammation and angiogenesis by monocyte-macrophage (33).

RNA modification is also an important part of epigenetic modification (34). Similar to DNA modification, cellular RNA also has a variety of chemical modifications, such as N6-methylladenosine (m6A) of mRNA. m6A modification is the most abundant epigenetic modification of RNA and is mainly catalyzed by the methyltransferase complex, which is composed of METTL3, METTL4, and other protein subunits. Abnormal modification of m6A leads to abnormal transcription, resulting in an abnormal translation program, and promotes the occurrence and development of tumors. Studies have shown that m6A modification can also affect the occurrence and development of IBD by regulating immune cells and RNA. YTH domain family 2 (YTHDF2) is an m6A-binding protein and its knockout increases inflammation. The expression of YTHDF2 is closely related to the development of IBD (35, 36). The chemical modification of these RNAs plays a very important role in RNA metabolism. The types of epigenetic modification are briefly shown in Figure 1.

3 The effect of MSCs and MSC-EXO on epigenetic modifications in inflammatory diseases

The relationship between epigenetic modifications and MSCs is one of mutual influence and regulation. Epigenetic modifications play an important role in the differentiation of MSCs as DNA methylation, histone modifications, and miRNAs are all important mechanisms that regulate MSC differentiation (37). MSC-EXO can also mitigate the onset and progression of inflammatory diseases by regulating the epigenetic modifications of related molecules. Changes in epigenetic modification are common in inflammatory diseases (Table 2). MSC-EXO can influence the expression of epigenetic modifications related molecules by regulating DNA methylation levels to suppress inflammation *in vivo*, leading to the amelioration of inflammatory diseases (55). It can also be used to activate relevant signaling pathways through RNA modifications such as mRNA m6A modifications to suppress inflammation and restore homeostasis in the body's internal environment (56). Moreover, MSCs play an immunomodulatory and homeostatic role in inflammation, and MSCs-EXO can contribute to the resolution of inflammatory diseases by regulating the polarization of macrophages from pro-inflammatory M1 to anti-inflammatory M2 (57) and inducing cytokine secretion to participate in the immunomodulatory of T cells (58) (Figure 2).

3.1 Inflammatory bowel disease

Inflammatory bowel disease (IBD) is a chronic, idiopathic, non-specific inflammatory disease of the gastrointestinal tract, mainly divided into two categories: UC and CD. The specific pathogenesis of IBD is not yet clear but a combination of genetic, immunological, and microbial factors certainly contribute to its onset and progression (59, 60). The main clinical manifestations of IBD are gastrointestinal symptoms such as abdominal pain, diarrhea, and bloody stools, with some patients presenting with extra-intestinal symptoms (EIM). EIM most often affects the joints, skin, or eyes, causing peripheral arthritis and erythema nodosum, but can also affect other organs such as the liver and pancreas (61). There are various clinical strategies for the treatment of IBD such as the use of traditional methods like immunomodulators and the ongoing exploration of fecal microbiota transplantation (15). The incidence of IBD in Western countries is entering the

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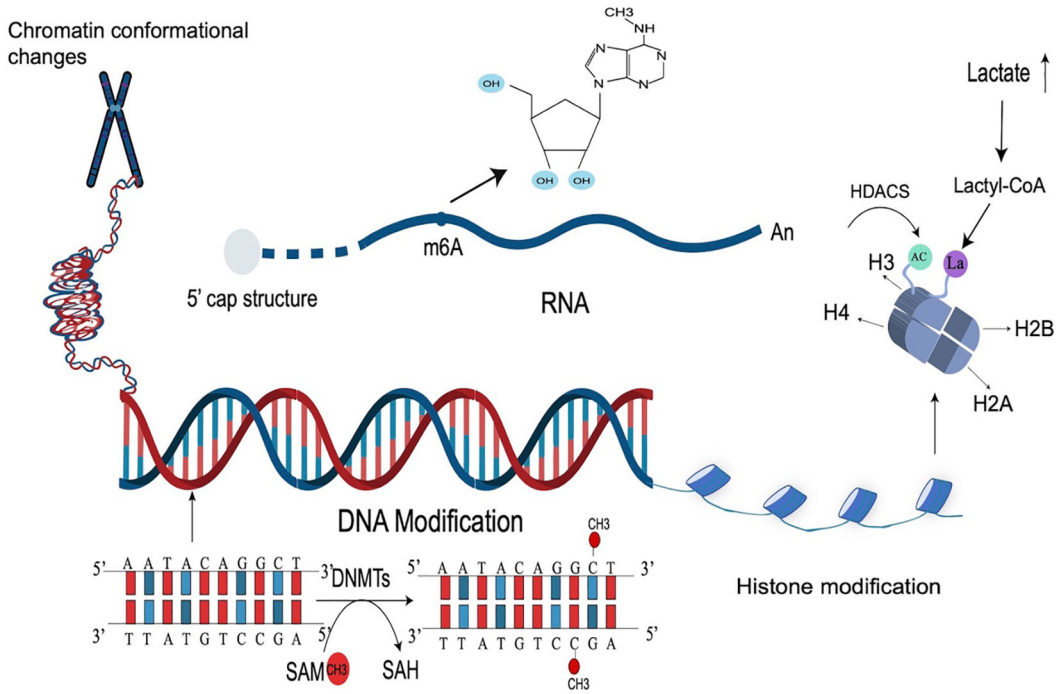

FIGURE 1
Types of epigenetic modifications. Epigenetic modification is a very complex mechanism that regulates gene expression, mainly including DNA modification, histone, and RNA modification, as well as chromosome changes. The most common epigenetic modification is DNA methylation (catalyzed by DNMTs), RNA m6A modification, and histone acetylation modification (catalyzed by HDACs). Lactate-induced histone modification is a newly discovered histone modification.

Compounding Prevalence stage, heading towards an eventual Prevalence Equilibrium stage, whereas, in some Asian countries, the incidence is steadily increasing as technology and living standards increase. Thus, there is an urgent need to explore the pathogenesis of IBD and new effective treatments (62, 63).

Epigenetic alterations are closely related to the development and progression of IBD. Gloria et al. found that the rate of 3H-methyl group admixture in the DNA of UC patients was 10 times higher than normal and that patients with the histologically active disease also had significantly higher rates, demonstrating that DNA methylation is associated with the pathogenesis of UC. Histone deacetylase (HDAC) inhibitor can promote acetylation of intestinal epithelial cells and the deficiency of HDAC1 exacerbates DSS-induced colitis in a mouse model of IBD (64, 65). Thus, repairing abnormal epigenetic expression in patients with IBD may also be one of the possible therapies for IBD, and controlling or reversing abnormal epigenetic modifications may provide some relief from the symptoms of IBD.

Studies have shown that MSCs can alleviate IBD by modulating epigenetic modifications of related molecules, Yi-Jun et al. found that enterocytes that were co-cultured with Methyltransferase-like 3 (METTL3) and Insulin-like growth factor 2 mRNA binding protein 3 (IGF2BP3) overexpressed or decreased MSC-EXO. Methylated RNA immunoprecipitation-quantitative polymerase chain reaction (MeRIP-qPCR) showed that the level of m6A modification of pre-miR-34A was significantly increased in the METTL3 overexpression group and promoted a marked increase in the expression of miR-34a-5p, which upregulated the tight junction proteins ZO-1, Occludin, Zonulin, and Claudin. Tight junction proteins play a critical role in maintaining intestinal function and the integrity of the intestinal barrier (66, 67), and miR-34a-5p is closely associated with anti-apoptosis (68). TUNEL and TER results showed that the apoptosis of intestinal epithelial cells in IBD mice was decreased. In contrast, the deletion of METTL3 and IGF2BP3 resulted in a significant decrease in the level of m6A modification of pre-miR-34A, which in turn led to reduced levels of miR-34a-5p and increased apoptosis. The same results were shown in animal experiments, where mice that received tail vein injections of MSCs that overexpressed METTL3 and IGF2BP3 showed reduced damage to the colorectal mucosa and increased levels of miR-34a-5p. In contrast, mice that received tail vein injection of METTL3 or IGF2BP3 knockout MSCs showed no alleviation or even increase in colorectal damage, and the level of miR-34a-5p was reduced. This suggests that MSCs can modulate the level of pre-miR-34a m6A modification through METTL3/IGF2BP3 to improve the stability of pre-miR-34a, promote miR-34a-5p secretion, maintain intestinal function, and protect the integrity of the intestinal barrier Maintaining the integrity of the intestinal barrier and intestinal function is also very important in the treatment of IBD, and MSCs play a therapeutic role in IBD by regulating the epigenetic modification of pre-miR-34A through METTL3 and IGF2BP3 (69).

MSC-EXO has also been widely used in the study of IBD, as it regulates the immune response by inducing Treg cells and tumor necrosis factor-α stimulated gene 6 (TSG-6) to repair the intestinal

TABLE 2 Abnormal epigenetic modification common in inflammatory diseases.

Disease	Epigenetic modification	Therapeutic target	Key outcome	Reference
Atherosclerosis	Abnormal DNA methylation mediated by DNMT1 and DNMT3a	DNA methylation inhibitors (5-azil and its nucleoside analogues)	In mice treated with DNA methylation transferase inhibitors, macrophage activation was inhibited and macrophage invasion of atherosclerotic plaques was reduced	(38, 39)
Atherosclerosis	Loss of HDAC3 results in abnormal acetylation of histones	HDAC3 inhibitors	HDAC3 inhibitor suppresses EndMT *via* modulating inflammatory response in ApoE-/- mice and HUVECs.	(40, 41)
Atherosclerosis	Increased trimethylation of histone H3K27	Histone methylation inhibitors (e.g., EZH2 inhibitors)	Mice treated with EZH2 inhibitor GSK126 showed significant reductions in atherosclerotic plaque	(42, 43)
Inflammatory bowel disease	Abnormal modification of mRNA m6A	m6A eraser	FTO protects IBD patients from adverse effects after treatment with thiazolate	(44, 45)
Inflammatory bowel disease	Abnormal DNA methylation	DNA methylation inhibitors	Several immuno-active genes show significant correlations between methylation and gene expression in IBD, and multi-omic integration of the methylome, genome, and transcriptome also implicate specific pathways in immune activation, response, and regulation at disease inception.	(46)
Inflammatory bowel disease	Abnormal histone acetylation levels	HDAC inhibitor	In mice treated with VPA, acetylation of histone 3 at the inflammatory site increased, alleviating DSS-induced colitis	(47, 48)
Osteoarthritis	Abnormal DNA methylation	DNA methylation inhibitors	Compared with normal cartilage, OA has a reduced methylation signature, which is associated with increased gene expression and leads to ECM degradation	(49)
Osteoarthritis	promoter methylation of BPM-7	methylation inhibitors	Altered expression levels of BMP-7 may lead to abnormal matrix synthesis and cartilage degeneration	(50, 51)
Systemic lupus erythematosus	DNA hypomethylation in T cells	DNA methylation inhibitors	CD4+ T cells convert to autoreactivity when treated with 5-azacytidine, procainamide or hydralazine, all of which inhibit DNA methylation and can lead to a lupus-like syndrome	(52, 53)
Systemic lupus erythematosus	Low acetylation of histones H3 and H4 in CD4+ T cells	Immunomodulator	Mycophenolic acid (MPA) can up-regulate the global acetylation status of histone H3/H4 by regulating HAT and HDAC in CD4(+)T cells in lupus.	(54)

barrier, relieving the occurrence and progression of IBD (70, 71). In addition to the above mechanisms, MSC-EXO, like MSC, can also mitigate IBD by regulating epigenetic modifications. Huashan Liu et al. found that MSCs-EXO can reduce the level of phosphorylated IκBα in macrophages, reduce the nuclear translocation of NF-κB p65 subunit, increase the level of total IκBα protein and mRNA, thus inhibiting inflammation and alleviating IBD. MSC-EXO-transmitted metallothionein-2 to inhibit NF-κB activation by promoting IκBα transcription and inhibiting IκBα phosphorylation, thereby further abrogating inflammation (70). The study of Gaoying Wang and colleagues showed that human umbilical cord MSC-EXO (hUCMSC-EXO) effectively alleviates the symptoms of IBD induced by DSS in mice, where the number of neutrophils in the gut of hUCMSC-EXO treated mice was reduced along with inhibited ERK phosphorylation and increased N2-phenotype. Clinical studies show that patients with IBD have significantly more neutrophilic markers of N1 and fewer N2 markers than healthy individuals. Therefore, the ability of MSC-EXO to impede ERK signal transmission and ERK phosphorylation of neutrophils and promote neutrophil polarization towards the N2 phenotype greatly contributes to alleviating IBD (72). The high expression of miR-378a-5p in HucMSC-EXO results in decreased expression of NLRP3 inflammatory bodies, increased cell survival rate, and abrogates cell pyroptosis, alleviating DSS-induced IBD (73). A study found that Periodontitis and IBD, two types of inflammation, interact with each other. Periodontitis aggravates IBD and MSC-EXO produced by 3D culture promotes the expression of miR-1246, inhibits Nfat5, restores Th17 cell/Treg balance, and relieves the periodontitis of IBD patients (1). In addition, a study by Ting Zhang et al. found that M6A mRNA modification could maintain colonic epithelial cell homeostasis through NF-β-mediated anti-apoptotic pathways and that disruption of intestinal homeostasis is one of the possible triggers of IBD. Thus, m6A modification of mRNA could alleviate IBD by maintaining intestinal homeostasis and reducing apoptosis in intestinal epithelial cells (74). Although this study does not discuss whether MSCs and MSC-EXO contribute to the regulation of m6A modification of mRNA, it has been shown that MSCs and MSC-EXO promote m6A mRNA modification (56, 75). Regardless, more studies are needed to further explore whether

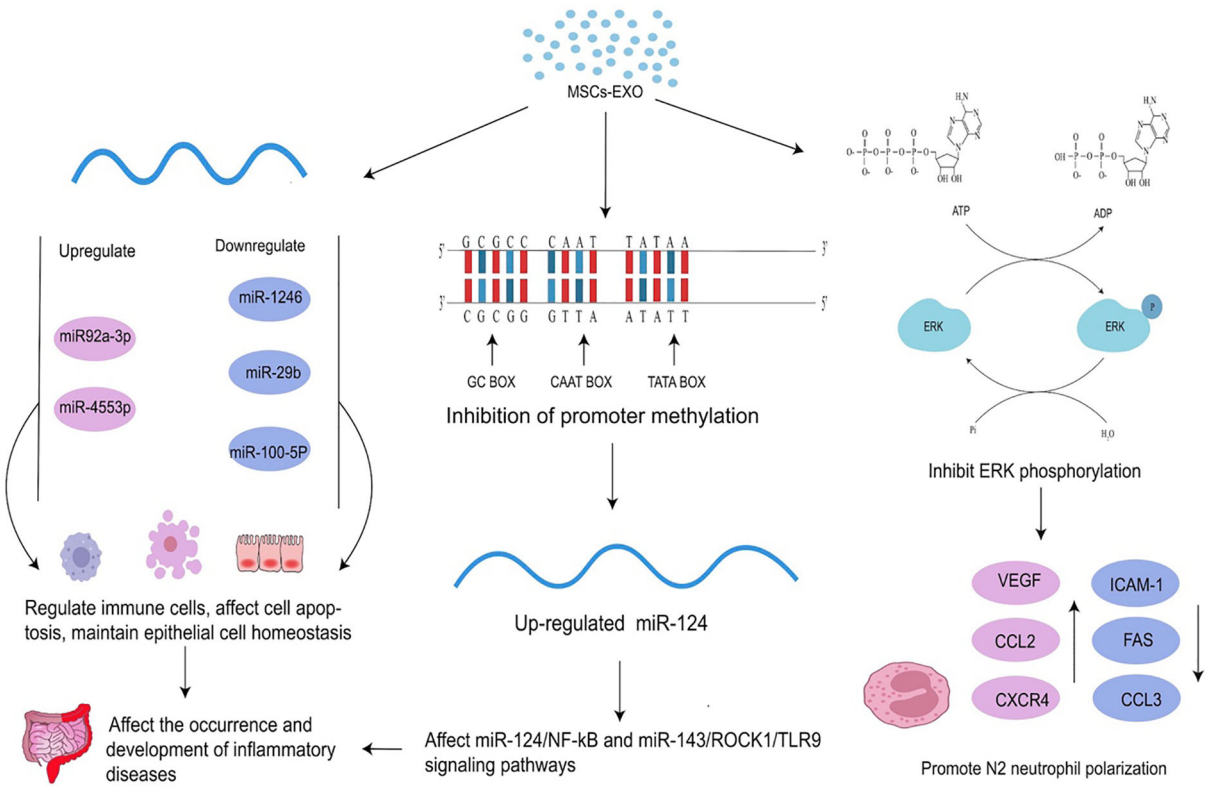

FIGURE 2
Effects of MSC-EXO on epigenetics. MSC-EXO can affect mRNA expression by inhibiting promoter methylation and regulating the expression of related molecules to affect the occurrence and development of diseases. It can also directly regulate the expression of mRNA and other RNA. MSC-EXO can ameliorate the occurrence and development of inflammatory diseases by influencing enzyme activity to up-regulate or down-regulate ERK phosphorylation and promote N2 neutrophil polarization.

MSCs and MSC-EXO can alleviate IBD by regulating m6A mRNA modifications. In summary, although there is evidence that MSCs and MSC-EXO can influence the development of IBD by regulating epigenetic modifications, their relationship, specific mechanisms of action, and pathways still need to be further explored. The regulation of immune cells by MSC in the inflamed gut is summarized in Figure 3.

3.2 Osteoarthritis

Osteoarthritis (OA) is considered to be a chronic inflammatory disease characterized by joint deformation and progressive degeneration of cartilage caused by an imbalance between osteogenesis and bone resorption activities, affecting approximately 15% of the world's population, and accounting for approximately 10% of men and 18% of women over the age of 60. The incidence of OA will further increase with factors such as population aging and obesity rates, placing enormous economic pressure on society and people (76). OA not only damages the knee joint but also the spine, hand, and jaw joints, and is a major cause of disability in the elderly today (77). The development of OA is the result of a combination of environmental factors, genetic factors, and non-genetic factors such as being overweight. The main aim of treating OA is to reduce pain and improve movement. There are various strategies for the treatment of OA, which include options such as exercise, weight control, and other lifestyle treatments, as well as the use of drugs such as Duloxetine, Chondroitin, or Glucosamine. However, all of these treatments have their drawbacks, and when applied clinically the results vary considerably between individuals, do not cure OA, and are prone to relapse (78, 79). Therefore, there is a need to explore new treatment options.

MSCs have been used in recent years for the treatment of OA because of their potential to differentiate into chondrocytes and their ability to produce extracellular matrix. Tofiño-Vian M et al. found that adipose MSC-EXOreduces the expression of inflammatory mediators, particularly IL-6 and prostaglandin E2, to exert anti-inflammatory and protective effects on chondrocytes and suppress inflammation in OA. It has been suggested that chondrocyte destruction and loss of regenerative capacity are important factors in OA, and therefore promoting chondrocyte regeneration plays a very important role in the treatment of OA (80). Yubao Liu et al. found that MSC exosomal lncRNA KLF3-AS1 increases the expression of G-protein-coupled receptor kinase interacting protein-1 (GIT1) by sponging miR-206, promoting the regeneration of chondrocytes in OA through the lncRNA-KLF3-AS1/miR-206/GIT1 axis and inhibiting apoptosis to prevent and

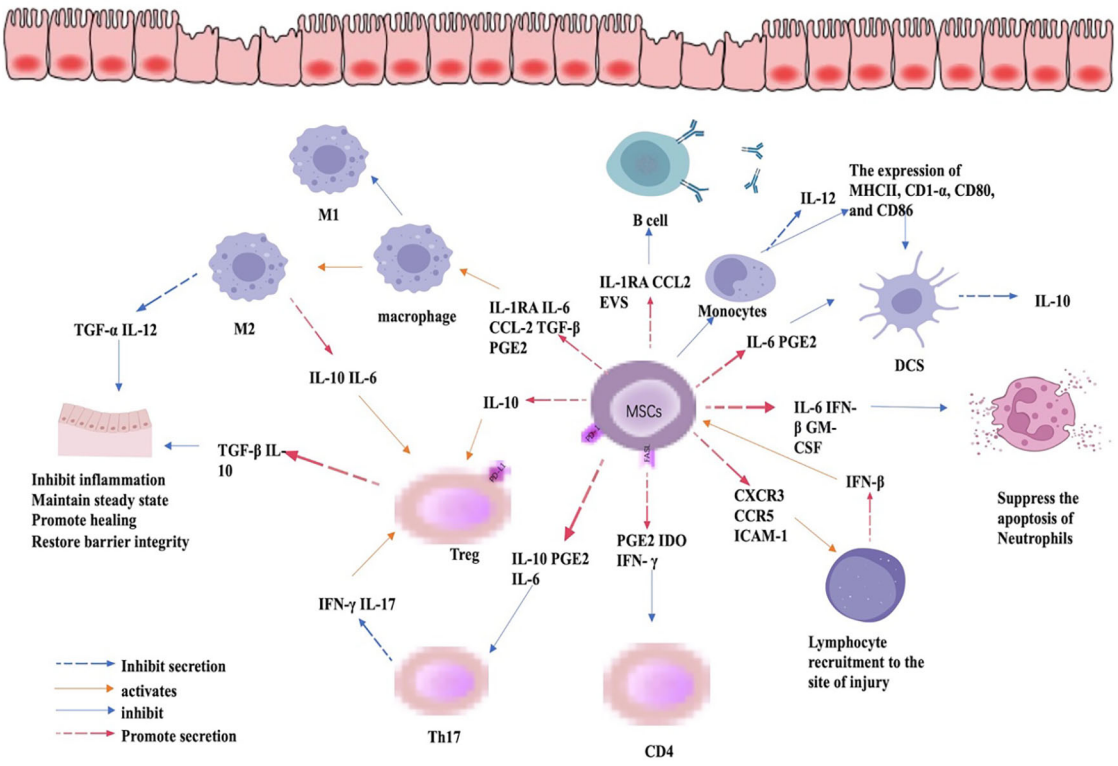

FIGURE 3
Immunomodulatory role of MSC in IBD. By regulating the expression of cytokines, MSCs can inhibit the expression of CD4 and Th17cells, promote the differentiation of Treg cells, the differentiation of macrophages to M2 phenotype, and inhibit the apoptosis of neutrophils and the expression of pro-inflammatory cytokines, thus mitigating inflammation, promoting mucosal healing, and restoring barrier function.

treat OA (81, 82). MSC-EXO is therefore considered a potential new cell-free therapeutic approach for the treatment of OA.

Epigenetic alterations are closely associated with aging and are contributing factors to aging-related diseases such as OA. Thus, age-dependent epigenetic regulation of gene expression plays a very important role in the development and progression of OA. The epigenetics of the growth factor BPM-7 (bone morphogenetic proteins 7), which is important for human articular chondrocyte activity, has been found to change with age, and promoter methylation of BPM-7 increases with age. The hypermethylation of the promoter of BPM-7 leads to abnormal bone formation and degradation eventually leading to OA (9, 83).

It was found that MSC-EXO could treat OA by modulating the DNA methylation of target molecules. The expression levels of NF-κB and associated coiled-coil-containing protein kinase (ROCK) are direct targets of miR-124 and miR-143 and were reduced in the MSC-EXO treatment groups, where the clinical manifestations associated with OA in mice were alleviated. NF-κB and ROCK are both implicated in the development of OA, as the activation of ROCK causes degeneration of cartilage, reduces bone production, and inhibits osteoblast degeneration (84) while NF-κB affects the expression of certain apoptosis regulators such as C-caspase3, Cyto-c, and Bax induces apoptosis and promotes the production of ADAMTs-5 and MMP-13, all of which contribute to the pathogenesis of OA (85). The expression of miR-124 is significantly reduced in IL-1β-induced arthritic mice, whereas the expression of NF-κB and ROCK is increased. MSC-EXO affects miR-124/NF-kB and miR-143/ROCK1/TLR9 signaling pathways by regulating the DNA methylation of the miR-124 promoter, which inhibits the expression of NF-kB and ROCK1, thereby affecting the onset and development of OA (86). It was found that MSC-EXO up-regulates the expression of miR-92a-3p, enhances chondrocyte formation, and inhibits chondrocyte degradation by targeting WNT5A. miR-92a-3p plays an important role in cartilage formation and degradation, and the expression of miR-92a-3p in OA cartilage is significantly lower than that in normal cartilage (87, 88). In addition, miR-92a-3p is an important regulator of Aggrecanase-1 (ADAMTS-4) and aggrecanase-2 (ADAMTS-5), and ADAMTS-4/5 plays a very important role in the development of OA (89). MSC-EXO affects the development of OA by up-regulating the expression of miR-92a-3p (87).In addition, the expression of circRNA_0001236 in OA cartilage is significantly lower than that in normal cartilage and is associated with the incidence of OA. The expression of circRNA_0001236 in chondrogenic MSC-EXO is significantly higher than that in undifferentiated MSC-EXO. MSC-EXO alleviates OA by up-regulating circRNA_0001236 expression in chondrocytes (90). Infrapatellar fat pad (IPFP) MSC-derived exosomes (MSCIPFP-Exos) inhibits the expression level of miR-100-5p and the binding of mTOR to miR-100-5p. MSCIPFP-Exos reduces the autophagy level of chondrocytes and improves the severity of OA *in vivo* by inhibiting the mTOR autophagy pathway (91). It is demonstrated

that TGF-β1-stimulated BMSC-derived exosomes (BMSCs-ExoTGF-β1) are highly expressed with miR-135b, which promotes M2 polarization of macrophages, inhibits M1-polarization, and repairs cartilage damage (92). All the above studies suggest that MSC-EXO can alleviate the occurrence and development of OA by regulating epigenetic modification

Interestingly, it has been shown that certain molecules can influence the differentiation of MSCs and the secretion of MSC-EXO through their own epigenetic modifications to affect OA. For example, microRNAs are involved in the subtle control of gene expression in osteoblasts/chondrocytes, promoting MSCs toward osteogenesis. A study by Feng Liu et al. found that the knockdown of the lncRNA CIR gene results in significantly higher expression of the chondrogenic markers SOX9, Aggrecan, and COL2A1 than controls, demonstrating that the presence of lncRNA CIR inhibits the differentiation of hUC-MSCs into chondrocytes and that there is a relationship between lncRNA CIR and the development of OA. Further studies revealed that lncRNA CIR promotes EZH2-mediated methylation of the ATOH8 promoter, which inhibits the expression of ATOH8, thereby suppressing the differentiation of hUC-MSCs towards chondrocytes (93). In summary, although the specific mechanisms and molecular pathways between epigenetic modifications and MSCs still need further exploration and validation, it is undeniable that there is a link between them, and this link may be a breakthrough point in the treatment of inflammatory diseases such as OA.

3.3 Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a chronic inflammatory disease involving multiple systems and organs, mediated by the autoimmune system, with varying degrees of presentation, course, and prognosis, ranging from mild skin and mucosal tissue damage to severe damage to multiple organs and the central nervous system. Environmental factors play a role in the pathogenesis of SLE (94). Defects in the early complement components C1q, C1r, C1s, C4, C2, and TREX1 are high-risk factors associated with the development and progression of SLE. Although the exact gene has not been identified, the risk of SLE is 10 times higher in women than in men, and the risk in women with Klinefelter syndrome (47, XXY) is 14 times higher than in men, which is sufficient evidence to link the development of SLE to genes located on chromosome X (95). Severe and persistent inflammation in SLE causes damage to a variety of organs, which is one of the causes of SLE complications. Complications associated with SLE include atherosclerosis, neurological defects, and kidney disease. About half of all SLE patients develop nephritis, which is a major cause of death in SLE patients (96). Studies have found that autoantibodies often appear before the clinical manifestations of SLE when they cause damage to the immune system and normal physiological functions, so early diagnosis and treatment can help improve the remission rate and prognosis of SLE patients. Immunomodulators and immunosuppressants are used to improve the immune system and treat SLE to maintain minimum activity (97). Since steroids can place a significant burden on the body and lead to sequelae of events such as cardiovascular disease, B-cell depletion (BCD) without any or minimal use of steroids is also a possible treatment for SLE therapy (98, 99).

MSC-EXO has also been used in studies of SLE treatment due to their multidirectional differentiation and less immunogenic properties, MSC-EXO can maintain homeostasis of M2-type macrophages by promoting polarization of macrophages towards an anti-inflammatory phenotype and activation of Treg cells. Inhibiting the activation of effector cells involved in the innate and adaptive immune response suppresses the immune response and alleviates the autoimmune response induced by autoantibodies in SLE (100). Reduced expression of CD4, CD25, and FOXP3 is a major genetic defect in SLE and MSCs can promote CD4+, CD25+, and FOXP3+ inducible Treg cells in SLE peripheral blood mononuclear cells (PBMCs) by releasing Transforming Growth Factor Beta 1 (TGFβ1) (101). Jang E et al. showed that human bone marrow (hBM)-MSCs administration in a mouse model of lupus nephritis relieves the condition by inhibiting the development of T follicular helper cells (Tfh)cells and the subsequent activation of humoral immune components, decreasing autoantibody levels and the incidence of proteinuria in mice, and improving survival (102). Clinical trials have also shown the role of MSCs in the treatment of SLE, with results from randomized controlled trials showing some relief of symptoms in the MSCs-treated group, a significant reduction in disease activity index and 24h proteinuria, and an increase in complement C3 (103). Although the results of the study show that MSCs are effective in the treatment of SLE, their efficacy is still low compared to other diseases, and therefore further research is still needed to verify whether MSCs and MSC-EXO can be used in the clinical treatment of SLE patients.

Epigenetic modifications such as abnormal DNA methylation and histone modifications play a key role in the pathogenesis of SLE, and the main epigenetic alteration in SLE patients is the overall hypomethylation of CD4+ T cells. Patients with active SLE show an overall downregulated expression level of H3 and H4 acetylation in CD4+ T cells *in vivo*, which leads to an increased expression of autoimmune-related genes and an elevated risk of developing autoimmune diseases. At the miRNA level, upregulation of miR-148a and miR-21 in the MRL/LPR mouse model results in reduced DNA methyltransferase 1 (DNMT1) expression and decreased DNA methylation levels, exacerbating lupus (104).In SLE patients, the expression level of mRNA methyltransferase NSUN2 in CD4+T cells is decreased, and the Methylation level of mRNA m5 C Methylation in CD4+T cells is abnormally increased. Epigenetic defects, especially the abnormal expression of cytokines and co-receptors caused by abnormal DNA methylation and histone modification, are also considered to be one of the important causes of non-SLE immune activation and tissue damage (105, 106). Therefore, correcting the aberrant epigenetic modifications in SLE patients is a potential therapeutic modality for SLE.

MSC-EXO can relieve SLE by modulating epigenetic modifications. For example, MSC-EXO was found to improve the reduction of bone mass in lupus and alleviate the symptoms of SLE. Fas-deficient-MRL/LPR mice lack FAS, resulting in the failure of miR-29b release, leading to a high level of miR-29b in the cell.

Moreover, the deficiency of FAS results in the decreased expression of DNMT1 in BM-MSCs of MRL/LPR mice, resulting in hypomethylation of the notch1 promoter and activation of NOTCH signaling, which in turn impairs osteogenic differentiation. However, MSC-EXO reduces the levels of miR-29b and upregulates DNMT1 in recipient mouse cells, thereby restoring DNMT1-mediated methylation of the NOTCH1 promoter and downregulating the expression of NOTCH1 and NICD, rescuing the function of BM-MSCs in MRL/LPR mice to some extent. This implies that MSC-EXO could rescue the BM-MSC of MRL/LPR mice by promoting the release of miR-29b and thus regulating the miR-29b/DNMT1/Notch epigenetic cascade to treat SLE to a certain extent (107). In addition, the aging of MSCs is closely related to the incidence of SLE. MiR-146-a can be targeted TRAF6/NF-KB signaling pathway to participate in the aging of MSCs, as a study found that exosomes derived from SLE serum have significantly decreased expression of miR-146-a, along with increased SA- KB-gal positive cells. This is likely linked to MSCS aging mechanisms in SLE patients, where epigenetic modification affects the occurrence and progression of the disease by affecting MSCs (108). However, studies on the treatment of SLE by MSCs and MSC-EXO through the regulation of epigenetic modification are largely lacking. More studies are still needed to verify the idea that MSCs can play a therapeutic role in SLE through epigenetic modification.

3.4 Other diseases

In addition to the above-mentioned inflammatory diseases, MSCs, and MSC-EXO can also act on other inflammatory and non-inflammatory diseases through the regulation of epigenetic modifications. For instance, in acute liver injury, MSC-EXO induced expression of SLC7A11 protein causes an increase in CD44 and OTUB1, which mediates deubiquitination and removes aberrant ubiquitination of SLC7A11, thereby increasing the stability of SLC7A11, activating system XC-, and preventing CCL4-induced hepatic cytophosis. This provides a new idea for the prevention and treatment of acute liver injury caused by cytophosis (109). Another study found that hUC-MSC-EXO promotes miR-4553p expression when subjected to IL-6 stimulation. Western-blot and QRT-PCR analyses showed that PIK3r1 expression is significantly reduced in the presence of miR-4553p at both the macrophage mRNA and protein levels. PI3K is a key factor in inhibiting the activation of IL-6-related signaling pathways, suggesting that miR-4553p inhibits macrophage activation by suppressing the expression of the target gene PIK3r1. In effect, hUC-MSC-EXO inhibits the release of IL6, as well as other inflammatory factors from macrophages by promoting the expression of miR-4553p.Targeting PIK3r1. suppresses the over-activation of immune cells such as macrophages/monocytes, reducing inflammation, ameliorating liver damage, and maintaining systemic homeostasis (110).

Studies have found that lncGm36569 is increased in ASCI mouse models and anoxic cell models treated with MSC-EXO. Bioinformatics analysis and luciferase analysis showed that lncGm36569, as a competitive RNA of miR-5627-5p, induced upregulation of FSP1. The overexpression of miR-5627-5p inhibits the therapeutic effect of lncGm36569 in the treatment of neuronal iron osteoporosis. Therefore, MSC-EXO can inhibit siderosis in neurons by regulating the expression of lncGm36569 and acting on miR-5627-5p/FSP1 axis (111). Nan Zhang et al. found that MSC-EXO could deliver non-coding developmental regulatory RNA (FENDRR) to tissues and cells, FENDRR can also regulate the expression of TEA domain transcription factor 1(TEAD1) by binding with miR-28. Moreover, it was found that when miR-28 was inhibited, atherosclerotic plaques were reduced. The deletion of TEAD1 reduces the inhibition of miR-28 and aggravates AS. Therefore, it indicates that MSC-EXO competitively binds to miR-28 with TEAD1 by secreting FENDRR, and alleviates the occurrence and development of AS (112). Yuli Zhang et al. found that imiquimod (IMQ) induces epidermal proliferation in mice. hucMSC-EXO inhibits IMQ-induced phosphorylation of signal sensors and transcriptional 3 (STAT3) activators in mIce skin and human keratinocyte (HaCaT) cells in addition to reducing the expression of IL-17. Thus alleviating psoriasis-like skin inflammation in mice. Therefore hucMSC-EXO may be an effective treatment for psoriasis (113). Ge Gao et al. found that exosomal circular RNA (circ_0006790), which was carried by MSC-EXO, regulated DNA methylation of S100A11 and inhibited transcription of S100A11 by binding with CBX7 and recruiting methyltransferase to the promoter region. Blocking the immune escape of adenocarcinoma cells is a new prospect for pancreatic ductal adenocarcinoma (PDAC) treatment (114). The role of MSC-EXO in epigenetics is summarized in Table 3.

4 Conclusion and prospects

Epigenetic modification is a very complex mechanism for regulating gene expression without altering DNA sequence, and the interrelationship between metabolic alterations and epigenetic remodeling is one of the hallmarks of cancer and a causal factor in the pathogenesis of many diseases (115). The study of epigenetic modification may provide new ideas for the treatment of certain refractory diseases through the regulation of DNA methylation, m6A modifications, and other epigenetic modifications by MSCs and MSC-EXO. It also provides new insights into the study of the mechanisms of MSCs and MSC-EXO for the treatment of inflammatory diseases. Although epigenetic modifications include DNA, non-coding RNA, and histone-related modifications, few studies have examined the role of MSCs and MSC-EXO through modulation of histone modifications, and most studies have focused on DNA methylation modifications and non-coding RNA modifications. However, studies have shown that histone modifications can also affect inflammatory diseases through certain pathways. For example, the TLR signaling adapter BCAP can regulate the conversion of inflammatory reparative macrophages by promoting histone lactation modifications. Moreover, lactate and histone modifications lead to the expression of tissue repair genes, restoring the tissue repair properties of macrophages, as mice recover from DSS-induced enteritis (11). Impaired histone modification of T cells and CpG

TABLE 3 Effects of MSC-EXO on epigenetics.

Disease	Study model	Function	Reference
Inflammatory bowel disease	*In vivo* model/mice	Inhibits the phosphorylation of IκBα	(70)
Inflammatory bowel disease	*In vivo* model/BABL/C mice *In vitro* model/FHC	Inhibits ERK phosphorylation	(72)
Inflammatory bowel disease	*In vivo* model/C57BL/6J mice	Decreases the expression of miR-1246	(10, 73)
Osteoarthritis	*In vivo* model/mice *In vitro* model/Primary chondrocytes	Regulates DNA methylation of miR-124 promoter	(86)
Osteoarthritis	A sample of articular cartilage from a patient with OA knee joint. Normal cartilage samples were taken from patients with no history of OA or rheumatoid arthritis	Upregulates the expression of miR-92a-3p	(87–89)
Osteoarthritis	Human clinical samples	Upregulates the expression of circRNA-0001236	(91)
Osteoarthritis	*In vivo* model/SD rats at 12 weeks old *In vitro* model/BMSC	Inhibits the expression of miR-100-5P	(92)
Systemic lupus erythematosus	*In vivo* model/C3MRL-Faslpr/J(MRL/lpr) *In vitro* model/BMSC	Reduces the levels of miR-29b	(107)
Acute liver injury	*In vivo* model/C57BL/6 J mice	Mediates SAC7A11 deubiquitination	(109)
Liver damage	*In vivo* model/C57BL/6 mice *In vitro* model/THP-1	Promotes the expression of miR-4553p	(110)
Acute spinal cord injury	*In vivo* model/C57BL/6 mice *In vitro* model/HT-22 and HEK-293 T	Promotes the expression of lncGm36569	(111)
Atherosclerosis	Clinical samples *In vivo* model/ApoE−/− mice *In vitro* model/HUV-EC-C	Triggers the release of lncRNA and regulates TEAD1 expression	(112)
Psoriasis	*In vivo* model/C57BL/6 mice *In vivo* model/HaCaTcells	Inhibits the phosphorylation of STAT3	(113)
Pancreatic ductal adenocarcinoma	*In vivo* model/BALB/c mice *In vivo* model/PANC-1(CL-0184)and CFPAC-1(CL-0059)	Regulates the DNA methylation of S100A11	(114)

DNA methylation in SLE increases the expression of IL-17a, breaks the balance between cytokines, and aggravates the pathological damage of SLE, exacerbating the pathological damage in SLE (116). In addition, the influence of histone lactate modification on diseases is bidirectional. Studies have found that under the condition of LPS-induced inflammation, glycolysis activity is enhanced, lactic acid production is increased, and histone lactate modification is significantly up-regulated, promoting the occurrence and development of inflammation. However, in the late stage of M1 macrophage polarization, the increase of histone lactate modification promotes the expression of homeostasis genes involved in the damage repair process (32). Whether and how MSC-EXO promotes histone modification towards alleviating inflammation by regulating the bidirectional effect of histone modification remains to be further investigated. Although many studies have shown that MSC-EXO can alleviate inflammatory diseases by regulating epigenetic modifications, there are still fewer papers on the specific signaling pathway through which MSC-EXO affects epigenetic modifications. The discovery of specific upstream or downstream signaling pathways through which MSC-EXO affects epigenetic modification to relieve IBD will likely provide a more promising foundation for the development of relevant therapeutics for IBD and other inflammatory diseases. Further research exploring the relationship between epigenetic modification and MSC-EXO is necessary, and disease pathogenesis and therapeutic research will bring great advances.

At present, there are studies on MSCs and epigenetics, most of which are about the effect of epigenetic modification on MSCs differentiation, while there are few related contents about how MSCs regulate epigenetic modification. There are also studies on MSC-EXO alleviating inflammatory diseases by regulating epigenetic modification, but most of such studies focused on the MSC-EXO effect through modulating certain molecules and mRNA expression and not directly regulating epigenetic modification. Although studies have shown that MSC-EXO can affect the DNA methylation of promoters, more studies are needed to prove the reliability of this conclusion. As a cell-free therapy, MSC-EXO is superior to MSC in treatment because it is more stable and could reduce the inherent safety risks associated with the administration of cell-based therapy,

including the risk of occlusion in the microvasculature, as well as possible immune recognition by the host system. Moreover, MSC-EXO possesses enhanced delivery of exogenous biological particles to the target site and directly into the cytosol, circumventing the lysosomal-endosomal pathway, and consequently elevating transfection efficiency (117). As a result of their small sizes and other camouflage strategies, exosomes are capable of evading the mononuclear phagocytic system's clearance, leading to extended circulatory time for passive targeting of inflammatory and cancerous cells (15). These special properties among others give MSC-EXO enormous potential over the parental cell therapy in regenerative medicine and cancer therapy. Considering the crucial role of epigenetic modification in the occurrence and development of inflammatory diseases and the efficient anti-inflammatory effect of MSC-EXO, the application of MSC-EXO to regulate epigenetic modification may be a potential therapy for inflammatory diseases.

Author contributions

Conceptualization, ZZ, LZ, and BW; funding acquisition, DO; project administration, FM; software, DO; visualization, FM; writing—original draft, ZZ, LZ, and BW; writing—review & editing, DO. All authors contributed to the article and approved the submitted version.

Funding

National Natural Science Fund of China (Grant no.82250410378), the 2022 Jiangsu Excellent postdoctoral program (Grant no.2022ZB634), the open project of clinical medical research center of Gynecology and Traditional Chinese Medicine of Zhenjiang (Grant no. SS202204-KFC01), and Zhenjiang key research and development plan (social development) (Grant no.SH2022062; Grant no.SH2022091).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 15 June 2023
DOI 10.3389/fimmu.2023.1188830

OPEN ACCESS

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RECEIVED 17 March 2023
ACCEPTED 23 May 2023
PUBLISHED 15 June 2023

CITATION
Seim RF, Herring LE, Mordant AL, Willis ML, Wallet SM, Coleman LG Jr and Maile R (2023) Involvement of extracellular vesicles in the progression, diagnosis, treatment, and prevention of whole-body ionizing radiation-induced immune dysfunction. *Front. Immunol.* 14:1188830. doi: 10.3389/fimmu.2023.1188830

Involvement of extracellular vesicles in the progression, diagnosis, treatment, and prevention of whole-body ionizing radiation-induced immune dysfunction

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Acute radiation syndrome (ARS) develops after exposure to high doses of ionizing radiation and features immune suppression and organ failure. Currently, there are no diagnostics to identify the occurrence or severity of exposure and there are limited treatments and preventative strategies to mitigate ARS. Extracellular vesicles (EVs) are mediators of intercellular communication that contribute to immune dysfunction across many diseases. We investigated if EV cargo can identify whole body irradiation (WBIR) exposure and if EVs promote ARS immune dysfunction. We hypothesized that beneficial EVs derived from mesenchymal stem cells (MSC-EVs) would blunt ARS immune dysfunction and might serve as prophylactic radioprotectants. Mice received WBIR (2 or 9 Gy) with assessment of EVs at 3 and 7 days after exposure. LC-MS/MS proteomic analysis of WBIR-EVs found dose-related changes as well as candidate proteins that were increased with both doses and timepoints (34 total) such as Thromboxane-A Synthase and lymphocyte cytosolic protein 2. Suprabasin and Sarcalumenin were increased only after 9 Gy suggesting these proteins may indicate high dose/lethal exposure. Analysis of EV miRNAs identified miR-376 and miR-136, which were increased up to 200- and 60-fold respectively by both doses of WBIR and select miRNAs such as miR-1839 and miR-664 were increased only with 9 Gy. WBIR-EVs (9 Gy) were biologically active and blunted immune responses to LPS in RAW264.7 macrophages, inhibiting canonical signaling pathways associated with wound healing and phagosome formation. When given 3 days after exposure, MSC-EVs slightly modified immune gene expression changes in the spleens of mice in response to WBIR and in a combined radiation plus burn injury exposure (RCI). MSC-EVs normalized the expression of certain key immune genes such as *NFκBia* and *Cxcr4* (WBIR), *Map4k1*, *Ccr9* and *Cxcl12* (RCI) and lowered plasma TNFα cytokine levels after RCI. When given prophylactically (24 and 3 hours

before exposure), MSC-EVs prolonged survival to the 9 Gy lethal exposure. Thus, EVs are important participants in ARS. EV cargo might be used to diagnose WBIR exposure, and MSC-EVs might serve as radioprotectants to blunt the impact of toxic radiation exposure.

KEYWORDS

ionizing radiation, extracellular vesicles, mesenchymal stem cells, burn injury, radiation syndrome, immune dysfunction

Introduction

The likelihood of a radiological incident occurring in the general population is growing due to the increased reliance on nuclear power, the risk of sophisticated terrorist attacks, and the current threats of nuclear warfare (1, 2). Such exposures can result in the development of Acute Radiation Syndrome (ARS) in affected individuals that are exposed to high doses of radiation over most or the entire body within a short period of time (3). Exposure to whole-body ionizing radiation (WBIR) impacts all organ systems, with rapid induction of a systemic inflammatory response, mediated by many mechanisms such as induction of the Acute Phase Response (APR) and peripheral and central (bone marrow) cell death; however, rapidly dividing cells are the most radiosensitive (4). Thus, the three main ARS syndromes exist: hematopoietic, gastrointestinal, and neurovascular (3). After exposure to 2 grays (Gy), bone marrow cells are depleted, resulting in death after about 30-60 days (3). Thus, peripheral immune function is greatly impaired (5, 6). Similar ARS syndromes occur in rodents, though higher doses are required than in humans for mortality (~9 Gy) (7, 8). Since symptoms can take days or weeks to develop after exposure, it can be difficult to determine if individuals have been exposed and their level of exposure. There are also delayed effects of acute radiation exposure (DEARE) resulting in multiple chronic conditions affecting multiple organ systems and bacterial susceptibility (9), and it is thought that reducing the amplitude of ARS can also reduce DEARE. There is also a pressing need for field-deployable approaches to detect and determine radiation dose after a large-scale radiological event using an assay that is as minimally invasive as possible. Therefore, given the increasing risks of WBIR exposure and its profound biological consequences, research gaps include a lack of biomarkers to identify radiation exposure, and a corresponding paucity of radiation medical countermeasures (MCM) that can act as mitigators of acute (and chronic) radiation-induced immune dysfunction. In addition, as we have described, radiation and polytrauma models share a common theme, with increased tissue damage and resultant signals driving the central immune dysfunction leading to increased infection susceptibility and aberrant wound healing (10–12). Polytrauma patients have a greater amplitude of dysfunctional responses. Indeed, radiation combined with burn injury (RCI) causes a more severe ARS and occurs when patients are exposed to high doses of radiation in addition to burn injury. Historically, 65-70% of the survivors of a nuclear incident will have significant burn injuries in addition to exposure to high doses of radiation (13). RCI results in a higher level of lethality and the exacerbation of physiological complications associated with either burn or radiation alone (14, 15). Due to the severity of RCI, there is an extremely intense inflammatory response early after injury that is one of the major contributors to the high mortality rate observed in people (16). We have previously described a pre-clinical mouse model of RCI that exhibits these phenotypes and utilized this model to understand the cellular and molecular elements that exist to control immune recovery after polytrauma such as RCI (10, 12).

A key aspect of radiation injury is the “Bystander Effect”, wherein irradiated cells transmit signals that can cause damage to non-irradiated cells (17, 18). Nagasawa found that irradiating 1% of cells would cause DNA damage in more than 30% of other nearby cells. Further, serum isolated from Chernobyl survivors was found to cause chromosomal damage in cultured cells (9), with effects that can last for up to 30 years after the initial exposure (19). The bystander effect can result in genomic instability, cellular stress responses, oxidative damage, apoptosis, and immune activation (18, 20). The secretion of clastogenic factors, cytokines, damage-associated molecular patterns, miRNAs, and lipid rafts may contribute (20). Extracellular vesicles (EVs) are multimodal signaling mediators that carry these factors, and are implicated in immune dysfunction in various settings (18, 21). We hypothesized that EVs contribute to post-WBIR immune dysfunction.

EVs are phospholipid enclosed structures secreted by almost all cell types. EVs transport a diverse array of cargo that includes miRNA, DNA, histones, long non-coding RNAs, proteins, lipids, and both damage associated molecular patterns (DAMPs) and pathogen associated molecular patterns (PAMPs). Due to their ability to harbor a diverse array of cargo, EVs can induce powerful and complex biological effects on downstream or recipient cells. They have also been identified as critical mediators across a wide range of pathologies, including traumatic injuries, central nervous system disorders, cancer, autoimmune diseases, and cardiovascular disease (21–25). In the context of radiation, recent attention has been placed on EVs as one of the contributors of the Bystander Effect (18, 26). EVs released from irradiated cells caused

chromosomal damage in naïve cells (27), and transfer of EVs isolated from mice exposed to WBIR to naïve mice induced immune signaling changes that were comparable to the effects of direct radiation (18). However, the influence of WBIR on plasma EV cargo is unknown, as well as the ability of EVs to be used as biomarkers of WBIR. We hypothesized that EV protein and miRNA cargo would be impacted by WBIR and could be used to inform the level of radiation in exposed individuals.

Given the likely detrimental role of EVs in ARS, it is reasonable to posit that administration of protective EVs might improve symptoms. Mesenchymal stem cell-derived EVs (MSC-EVs) carry trophic and immunomodulatory signals that have shown therapeutic benefit across a range of diseases (21). Administration of MSC-EVs after WBIR have been found to slow the progression of ARS (28–30). However, the impact of MSC-EV treatment on immune dysfunction is unknown. Further, it is unknown whether MSC-EVs could be given prophylactically as a radioprotectant to prevent ARS in those at increased risk for exposure. This would be a valuable tool that could be administered to soldiers, emergency personnel, and nuclear power-plant workers during radiological attacks or incidents (31). In addition, we further utilized our murine model of RCI (10, 12) to evaluate if treating mice with mesenchymal-stem cell derived extracellular vesicles (MSC-EVs) could improve immune outcomes due to their inherent regenerative and anti-inflammatory properties (32). We hypothesized that MSC-EVs would improve immune dysfunction caused by WBIR and RCI, and that MSC-EV administration prior to WBIR would reduce ARS.

Materials and methods

Mouse irradiation injury model

The protocols described here were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institute of Health. This protocol was approved by the University of North Carolina Institutional Animal Care and Use Committee with ethically approved experimental design. All animals were housed in an American Association for Accreditation of Laboratory Animal Care accredited facility with full time veterinary staff. All animals were monitored closely throughout the duration of the experiments.

Female C57BL/6J mice between 6-8 weeks of age with a weight of 15-20g were used for all experiments. Mice were placed in a mouse pie cage before receiving either 2 Gy or 9 Gy of radiation (dose rate of about 0.8 Gy/min) from a Cs^{137} based irradiator developed by Best Theratronics Ltd. (Kanata, Ontario). After the irradiation procedure, mice were returned to their cages and monitored closely following the procedure. They were provided with food and water ad lib throughout the procedure and if the mice showed any overt symptoms of radiation sickness (hunched, dehydrated, difficulty breathing, loss of > 20% body weight, inactivity, or lesions), then they were euthanized immediately with inhaled isoflurane (drop method), followed by cervical dislocation.

Mouse model of combined irradiation and burn injury model

The model of murine burn injury combined with irradiation has been previously described (10, 12). Female C57BL/6J mice between 6-8 weeks of age with a weight of 15-20g were used for all experiments. Briefly, for the burn injury procedure, mice were anesthetized with tribromoethanol (avertin; 475 mg/kg) and the dorsum and flank of the mouse was shaved, and morphine sulfate (3mg/kg) was injected subcutaneously into the dorsum of the mouse. Following injection and anesthesia, a copper rod, heated to 100°C by a boiling water bath, was applied to the dorsum and flank of the mouse for ten seconds. This was repeated four times with a 65g copper rod (1.9 cm in diameter) to achieve a full-thickness contact burn of 20% total body surface area (TBSA). After the burn procedure, mice were resuscitated with an intraperitoneal injection of Lactated Ringer's solution (0.1 mL/g of body weight). Within one hour of the burn procedure, the mice were exposed to 9 Gy of WBI from a Cs^{137} (dose rate of about 0.8 Gy/min) based irradiator developed by Best Theratronics Ltd (Kanata, Ontario). Sham groups went through an identical procedure but were not burned or irradiated. The mice were returned to individual cages and given food and morphinated water *ad lib*. The mice were monitored twice daily throughout the experiments and if mice developed overt symptoms of injury that could not be easily treated (dehydration, hunched posture, difficulty breathing, >20% body weight loss, or inactivity), then the mice were immediately euthanized utilizing inhaled isoflurane (drop method) followed by cervical dislocation. Following exposure to WBIR or radiation combined with burn injury (RCI), the plasma and spleen was harvested from these mice and stored at -80°C before further processing.

Extracellular vesicle isolation, quantification, and sizing

EVs were isolated from the plasma of mice 3 days and 7 days following exposure to either 2 Gy or 9 Gy of WBIR *via* differential centrifugation as previously described (33–35). Mice were euthanized by inhaled isoflurane and blood was collected *via* cardiac puncture and collected in tubes containing 40% trisodium citrate. The plasma was centrifuged for 2000xg for 20 min to remove cells. Following this, the supernatant was collected and spun at 10,000xg for 30 min to remove cellular debris. Finally, the supernatant from this spin was removed and spun at 21,000xg for 1 hr to pellet EVs. The EV containing-pellet was resuspended in 30 µl of phosphate buffered saline (PBS) that was filtered with a Whatman™ 0.02 µm syringe filter and frozen at -80°C before analysis. To assess the quality and concentration of our isolations, Nanoparticle Tracking Analysis (NTA) was performed on the final EV products using the ZetaView QUATT instrument (Particle Metrix, Mebane, NC) and ZetaView (version 8.05) software. The EV pellets were diluted 1:1000 in 0.02 µm syringe filtered PBS. The mean concentrations

(EV/ml) and size were determined by taking 10 videos with a 488 nm laser, pump speed 30, camera shutter of 100. Each measurement from the videos was screened for quality control and all videos that failed were excluded.

Unbiased proteomic assessment of EVs isolated from mice following WBIR

EVs were isolated from mice exposed to either 2 Gy and 9 Gy of WBIR 3 days and 7 days following exposure as well as sham (uninjured) mice and prepared for unbiased proteomic assessment using LC-MS/MS (36). Following the last spin of EVs, the EV-containing pellet was resuspended in 20 mM Tris buffer (pH 7.5). Next, 8 M Urea was added to the protein samples (about 10-20 µg per sample), then reduced with 5mM dithiothreitol (DTT) for 30 min. After reduction, the samples were alkylated with 15 mM iodoacetamide for 45 minutes. The samples were then diluted with 1 M urea before digestion with mass-spec grade Trypsin (Promega, Madison, WI) at 37°C overnight. Following the overnight incubation, the peptide samples were acidified with 1% trifluoroacetic acid (TFA) before desalting with Pierce™ C18 spin columns (ThermoFisher Scientific, Waltham, MA). Peptide quantification was then performed utilizing a Pierce™ bicinchoninic acid assay (BCA) fluorometric peptide quantitation assay. The samples were dried *via* vacuum centrifugation and resuspended in 0.1% formic acid. Samples were normalized to 0.1 µg/µl. Pooled samples were used to assess technical reproducibility and were prepared by combining a small portion of each sample. 0.5 µg of sample was analyzed by LC-MS/MS using a ThermoFisher Easy nLC 1200 coupled to a QExactive HF (ThermoFisher Scientific, Waltham, MA) with an Easy Spray PepMap C18 column (75 µm id × 25 cm, 2 µm particle size) (ThermoFisher Scientific, Waltham, MA). The samples were separated over a 90-minute period where the gradient of separation consisted of 5-32% mobile phase B kept at a flow rate of 250nL/min and a mobile phase A consisting of 0.1% formic acid in acetonitrile. The QExactive HF identified the 15 most intense precursors and selected them for subsequent Higher-energy C-trap dissociation (HCD). For the precursor scan, the resolution was set to 60,000 with a target value of 3×10^6 ions, 100 ms inject time; for MS2 scan, the resolution was set to 15,000 with a target value of 1×10^5 ions, 75 ms inject time. The collision energy set to 27% for the HCD, with an isolation window of 1.6 *m/z*. Peptide match was set to preferred and the precursors with an unknown charge or a charge state of 1 and ≥7 were excluded. The proteins were identified and quantified with Proteome Discoverer 2.5 utilizing a Uniport Mouse database (~ 17,000 sequences). The peptide false discovery rate (FDR) was set to 1% and only proteins with >1 peptide were used for downstream analyses. Proteins were media-normalized within Proteome Discoverer. The level of lcp2 protein was also measured by ELISA (MyBioSource) according to the manufacturer's instructions as we have done previously (36).

Transmission electron microscopy of EVs

To visualize EVs, isolated EVs were prepared for negative-stain transmission electron microscopy. A glow-discharged formvar/carbon-coated 400 mesh copper grids (Ted Pella, Inc., Redding, CA) was floated on a droplet of the sample suspension for 12 minutes, transferred quickly to 2 drops of deionized water followed by a droplet of 2% aqueous uranyl acetate stain for 1 minute. The grid was blotted with filter paper and air-dried. Samples were observed using a JEOL JEM-1230 transmission electron microscope operating at 80kV (JEOL USA INC., Peabody, MA) and images were taken using a Gatan Orius SC1000 CCD camera with Gatan Microscopy Suite version 3.10.1002.0 software (Gatan, Inc., Pleasanton, CA).

Cytokine and chemokine detection

Bio-Plex Immunoassays (Hercules, CA, USA) were utilized to analyze the cytokine/chemokine levels of TNF-α, IL-2, and MCP-1 according to the manufacturers protocols in the mouse plasma following RCI. The data was acquired on a Bio-plex 200 system with Bio-Plex Manager and Bio-Plex Pro Software and analyzed using a five-parameter logistic spline-curve fitting method. The data are presented as picograms/ml.

RAW 264.7 cell culture and EV exposure

RAW 264.7 (ATCC, Manassas, VA, USA) mouse macrophage cells were grown in culture according to the manufacturer's recommendations. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin at 37°C and 5% $C0_2$. For the EV exposure, a total of $1x10^6$ cells were plated in a 24-well plate and allowed to adhere overnight. The following day, $1x10^7$ EVs were added to the cells in the presence of 1 µg/ml lipopolysaccharide (LPS) from *Escherichia coli* 0111: B4 for 24 hr. Cellular mRNA was harvested for analysis.

C57BL/6J mouse mesenchymal stem cells culture, EV isolation, and *in vivo* transfers

C57BL/6J mouse bone marrow mesenchymal stem cells (MSCs) (Cyagen, Santa Clara, CA, USA) were grown in OriCell™ MSC Growth Medium (Cyagen, Santa Clara, CA, USA) containing 10% FBS and 1% penicillin/streptomycin at 37°C and 5% CO_2 according to the manufacturer's recommendations. MSCs were allowed to grow for three days before the media was removed and centrifuged at 2000xg for 20 min to remove cells. The supernatant was collected, and the media was spun at 10,000xg for 30 min to remove debris. Lastly, the supernatant was centrifuged at 21,000xg for 1 hr to pellet EVs and the supernatant was removed. The pellet was resuspended

in 30 µl of phosphate buffered saline (PBS) that was filtered with a 0.02 µm syringe filter and frozen at -80°C before analysis. To assess the quality and concentration of our isolations, Nanoparticle Tracking Analysis (NTA) was performed on the final EV products using the ZetaView QUATT instrument (Particle Metrix, Mebane, NC) and ZetaView (version 8.05). Samples were concentrated to $1x10^{10}$ EVs and intravenously injected into the mice prior to WBIR or after RCI and WBIR.

RNA isolation and immune gene quantification

RNA was isolated using Qiagen's (Hilden, Germany) RNeasy kit. RAW 264.7 cells were lysed with RLT lysis buffer and processed using the RNeasy kit. Cell suspensions were prepared from the spleens of mice. Red blood cells were lysed with ACK Lysis Buffer (0.15M NH_4Cl_4, 1mM $KHCO_3$, and 0.1 Mm Na_2 EDTA in water) before lysing with RLT lysis buffer and processed using the RNeasy kit. Total RNA was quantified using a nanodrop 2000 spectrophotometer (Waltham, MA). NanoString Technology and the nCounter Mouse Immunology Panel (Nanostring, Seattle, WA) was used to assess 561 mRNAs with relevance to immune function (37). All samples were run in triplicate. 100ng of mRNA was hybridized to report-capture probe pairs (CodeSets) at 65°C for 16hrs. After hybridization, the nCounter Prep Station was used to process the samples. During this stage, excess probe was removed, aligned with the probe/target complexes, and these complexes were immobilized in the nCounter cartridge. The nCounter cartridge was placed in a digital analyzer for image acquisition and data processing. The expression levels of each gene was analyzed by quantifying the number of times the color-coded barcode was detected for each gene. For data analysis, nSolver V4.0 was utilized to normalize the data and calculate fold changes, the resulting ratios, and differential expression. The resulting data was analyzed using Ingenuity Pathway Analysis (IPA) software to identify pathway-specific responses.

miRNA analysis

To assess changes in the miRNA content of EVs following exposure to WBIR, nanoString technology and the nCounter mouse miRNA panel v2 that allows for the evaluation of 577 miRNAs was used (38). Following differential centrifugation, the EV pellets were disrupted using Qiazol Lysis Reagent (Qiagen, Hilden, Germany) and the miRNA easy kit was used for the isolation of mRNA and miRNA. Nanodrop ND1000 (NanoDrop Technologies, Waltham, MA) was used to assess the quantity and quality (A260/280 and A260/A230) of mRNA. A total of 100 ng of mRNA was used for the mouse nanoString nCounter miRNA microarray assay according to the manufacturer's instructions. Briefly, the miRNAs were hybridized to probes at 65°C for 30 hrs. Afterwards, the hybridized probes were extended and quantified using the nCounter Prep Station and Digital Analyzer. The data was analyzed using the nSolver 4.0 software based on the manufacturer's instructions for analyzing miRNA data.

Phagocytosis assay

For the phagocytosis assay, $2.5x10^5$ RAW cells were plated in a 96-well flat bottom plate and were allowed to adhere for one hour. Utilizing the Vybrant™ (Thermo Fisher Scientific) phagocytosis assay kit, the cells were subsequently exposed to killed *E.coli* (K-12 strain), which were labelled with fluorescein in the presence of LPS from *Escherichia coli* 0111:B4 (10 µg/ml). Phagocytosis occurred for 2 hrs before aspirating the extracellular fluorescent *E. coli* and quenching the reaction in trypan blue. The intracellular fluorescence was quantified at an excitation of 480 nm and 520 nm emissions using a BioMek plate reader. In accordance with the manufacturer's instructions, we subtracted the average fluorescence units of no-cell negative-control wells from all wells. We then defined phagocytosis response to the experimental effector (% Effect) as: % Effect = Net experimental phagocytosis × 100% x Net positive control phagocytosis.

Statistical analysis

For the proteomics, the UNC Mass Spectrometry Core handled the date processing and statistical analyses. Briefly, the raw data files were processed using Proteome Discoverer 2.5 and searched against the Mouse Uniprot database (containing 16,940 sequences) (39). Trypsin was specified as the enzyme and only up to two missed cleavage sites were allowed. The carbamidomethylation of Cys was set as the fixed modification and oxidation of Met was used for variable modification. A 1% false discovery rate (FDR) was used to filter data and label-free quantification (LFQ) of unique peptides was used. At minimum, there had to be 2 unique peptides per protein and >50% non-zero values across all data sets were essential for all quantification. Further data analysis was conducted in Perseus (Gene Ontology Cellular Component was used for the annotation and imputation) and Argonaut was used for Log2 transformation and statistical tests (40). For all of the nanoString analyses, nSolver v4.0 was used to normalize the miRNA and mRNA fold changes to reveal ratios and differential expression data. Ingenuity Pathway Analysis was used to identify canonical pathways that were impacted and Z-scores greater than 2.0 and p-values <0.05 were considered to be significant (37). For the rest of the analyses, One-way ANOVAs with Dunnett's *post-hoc* test were performed in GraphPad Prism version 9.0 for Windows. Data is displayed as mean ± standard error of the mean (*P<0.05, P**<0.01, and ***P<0.001).

Results

Whole body irradiation alters EV numbers in a dose dependent manner

Multiple studies have found changes in the number of circulating EVs after traumatic injuries (21, 41, 42). In order to determine the impact of radiation dose and time after injury on circulating EV numbers and size, mice received either 2 or 9 Gy of WBIR with

sacrifice at 3 or 7 days after exposure (Figure 1A), which are moderate/survivable and lethal doses for mice respectively (8, 18). The total number and average diameter of the plasma EVs was measured by NTA. 2 Gy of WBIR had no impact on total EV number or size distribution up to 7 days after exposure compared to sham mice (Figures 1B, C). The total EV concentration was significantly reduced, however, 3 days after exposure to 9 Gy (Figures 1B, D). At 7 days after WBIR, there were no significant differences in total EV concentrations with either dose (Figure 1B). EVs were then isolated by sequential centrifugation which results in reliable isolation of ~0.05-500nm EVs (exosomes and microvesicles), with characteristic EV size and markers that we have measured by transmission electron microscopy (TEM), NTA, western blot, and flow cytometry (34, 36, 38, 43–45). Here, we confirmed the isolation of characteristically shaped EVs using TEM (Figures 1E, F). Next, we assessed EV protein and miRNA content using LC-MS/MS and nanoString analyses (Figure 2A). These data suggests that in our mouse model of WBIR, circulating EV concentrations are dependent on WBIR dose and time after exposure.

Whole body irradiation alters protein and miRNA cargo in a dose and time-dependent manner

In order to determine the impact of radiation dose and time after injury on EV cargo, and therefore test the usefulness of EV as a source of "radiosensitive" biomarkers as outlined in Figure 2A, we assessed EV protein (Figures 2B–J) and miRNA (Figures 2K, L) content using LC-MS/MS and nanoString analyses, respectively. For the proteomic analysis, we found that across both doses and timepoints, exposure to WBIR significantly altered EV protein cargo compared to sham mice (Figures 2B–F). Two Gy of WBIR caused robust changes in protein expression at both 3 and 7 days after WBIR (Figures 2B, C) compared to sham mice. Three days after 2 Gy exposure, 508 proteins were significantly increased, and 80 proteins were reduced (Figure 2B) compared to sham mice. Similarly, 7 days after 2 Gy of WBIR, there were 408 upregulated proteins, and 80 downregulated proteins in EVs (Figure 2C). In order to identify canonical pathways inferred from the proteomic analysis, we employed Ingenuity Pathway Analysis (IPA) with resulting Z-scores reflecting increased or decreased numbers of gene members of each canonical pathway compared to sham mice. IPA predicted several protein categories were altered by 2 Gy, with similar changes at days 3 and 7 (Supplemental Figures 1A, B). Interestingly, the most impacted categories are often observed as being disrupted in tissues after WBIR, such as intracellular and second messenger signaling, cell proliferation and growth, cellular stress and injury, apoptosis, and cellular immune responses (Supplemental Figures 1A, B) (5). Proteins involved in integrin signaling, actin cytoskeletal, and RHOGDI signaling proteins were notably altered, as were proteins associated with phagosome formation. Next, we performed analysis of a higher WBIR dose to test the hypothesis that EV-bound protein changes will reflect the

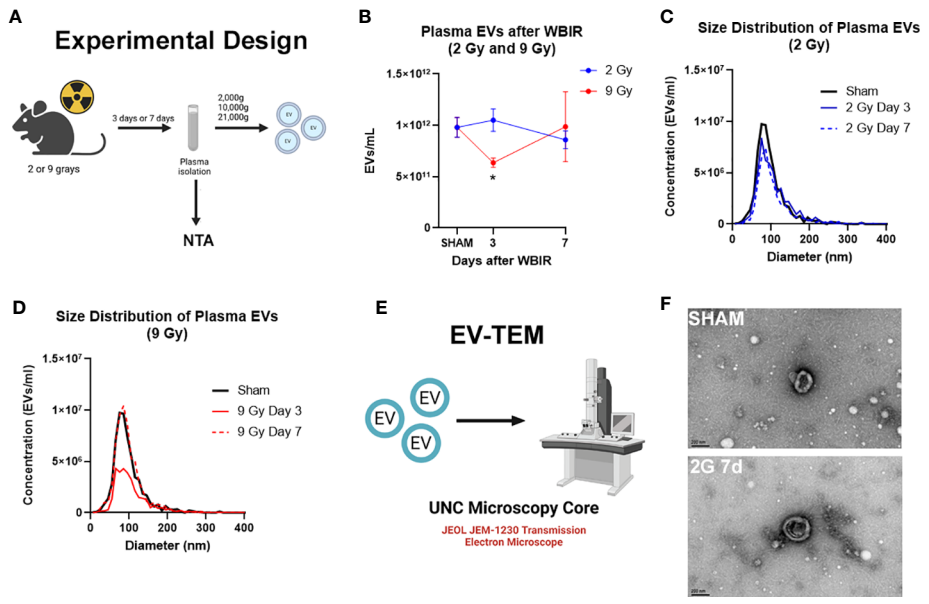

FIGURE 1
Total concentration of plasma extracellular vesicles are altered following WBIR. Assessing the plasma concentrations of extracellular vesicles (EVs) and confirmation that EVs were isolated following exposure to either 2 Gy or 9 Gy of whole-body ionizing radiation (WBIR). **(A)** Experimental design. C57BL/6 mice were exposed to either 2 Gy or 9 Gy of whole-body ionizing radiation from a Cesium-137 irradiator. EVs were isolated from the plasma of these mice 3 and 7 days after exposure (n=6 for each group). **(B-D)** Nanoparticle Tracking analysis (NTA) was used to measure the frequency and size distribution of EVs isolated following exposure to WBIR. **(B)** NTA found a reduction in plasma EVs at 3 days after WBIR. *$p<0.05$. One-way ANOVA with Dunnett's *post-hoc* test. **(C)** Size Distribution of Plasma EVs at 3 and 7 days after 2 Gy exposure showed no shifts in the size of the EV pools. **(D)** Size Distribution of Plasma EVs at 3 and 7 days after 9 Gy exposure display a decrease in total EVs 3 Days after exposure to 9 Gy but there were not any shifts in the size distribution of these EVs **(E)** Approach for assessment of EVs by Transmission Electron Microscopy (TEM). **(F)** Representative TEM micrographs of plasma EVs isolated following sham or exposure to 2 Gy WBIR. Scale bar = 200nm. Created with BioRender.com.

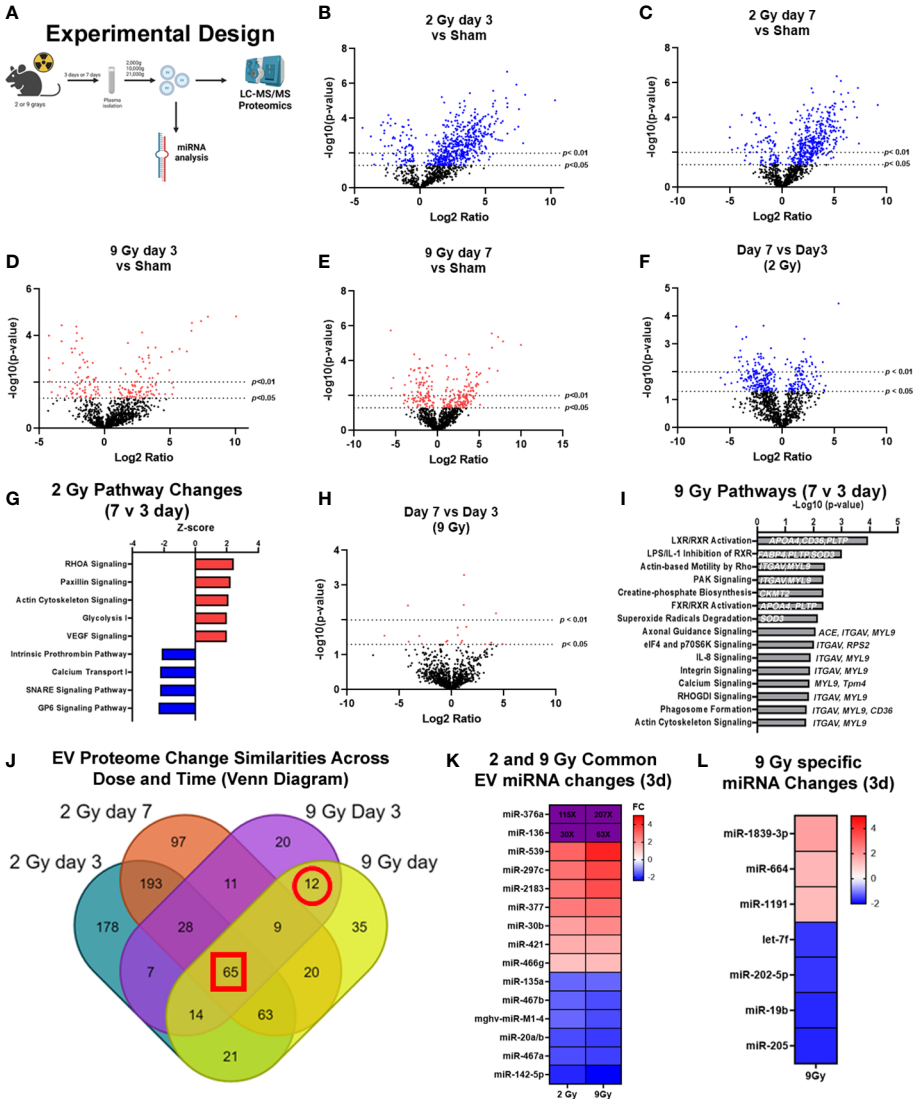

FIGURE 2
Exposure to WBIR induces alteration in the proteomic cargo of extracellular vesicles in a dose and time-dependent manner. **(A)** Experimental overview for the proteomic analysis of EVs isolated following WBIR. EVs were isolated from the plasma of mice 3 days and 7 days after exposure to 2 and 9 grays of WBIR. Protein content was measured by LC-MS/MS and all doses and treatment groups were compared to sham (uninjured) mice (n=3 for each experimental group). **(B)** Differential protein expression changes between EVs isolated 3 days after 2 Gy of WBIR compared to sham mice. **(C)** Differential protein expression changes between EVs isolated 7 days after 2 Gy of WBIR compared to sham mice. **(D)** Differential protein expression changes between EVs isolated 7 days after 9 Gy of WBIR compared to sham mice. **(E)** Differential protein expression changes between EVs isolated 7 days after 9 Gy of WBIR compared to sham mice. **(F)** Temporal changes in the proteomic cargo of these EVs following exposure to 2 Gy of WBIR. **(G)** Ingenuity Pathway Analysis (IPA) displaying the most predicted pathways to be activated or inhibited and associated Z-scores from day 7 to day 3 following exposure to 2 Gy of WBIR. **(H)** Temporal change in the protein cargo from day 7 to day 3 following exposure to 9 Gy of WBIR. **(I)** IPA analyses displaying the -Log10(p-values) of pathways that are predicted to be impacted from day 7 to day 3 following exposure to 9 Gy of WBIR. **(J)** Venn diagram displaying the overlap in significantly altered proteins between groups. **(K)** Heat map displaying significant fold changes in the miRNA cargo of EVs isolated 3 days after exposure to 2 and 9 Gy of WBIR. **(L)** Heat map displaying the significant miRNA alterations that were specific to EVs isolated 3 days after 9 Gy of WBIR. Created with BioRender.com.

WBIR dose received. Indeed, EVs isolated after 9 Gy exposure had significantly different changes in protein cargo compared to EVs isolated at 2 Gy. This was the case at both timepoints after exposure (Figures 2B–E). After the 9 Gy exposure, EV protein cargo changed quite differently when compared to 2 Gy, with fewer changed proteins compared to the 2 Gy treatment group. Three days after 9 Gy of WBIR, only 94 proteins were significantly increased, and 72 proteins significantly decreased (Figure 2D). Seven days after 9 Gy WBIR, 148 proteins were significantly up-regulated and 98 proteins were down-regulated (Figure 2E) compared to sham mice. IPA analysis found significant increases in proteins related to estrogen receptor signaling and the complement system.

In order to refine the potential use of EV-bound proteins as biomarkers, we performed several further analyses. First, we assessed specific EV-bound proteins that were altered over time at the same WBIR dose. We found that 7 days after 2 Gy exposure, there were 100 proteins increased and 173 proteins decreased compared to day 3 (Figure 2F). IPA identified that these protein

changes were associated with RHOA signaling, actin cytoskeleton signaling, VEGF, and glycolysis, while there were fewer proteins involved in the intrinsic prothrombin pathway, calcium transport, SNARE signaling, and glycoprotein 6 signaling (Figure 2G). After 9 Gy exposure, only 12 proteins significantly changed between days 7 and 3 (Figure 2H). Although there were not enough significantly altered proteins for IPA to assign a Z-score, we found that these proteins were involved in several pathways, notably LXR/RXR signaling, cytoskeleton-related pathways, and phagosome formation (Figure 2I). In an effort to identify proteins that might serve as biomarkers of exposure, we assessed the similarity in changes across both doses and timepoints (Figure 2J). Across all the timepoints and doses, 65 shared protein changes were found with 35 increased and 30 were reduced (Figure 2J red square, Tables 1, 2). Among these, thromboxane-A synthase (~10-fold), fibrinogen alpha and gamma chains (~7-8-fold), and lymphocyte cytosolic protein 2 (lcp2, ~6-fold) were the most increased (Table 1). The increase in lcp2 was also seen by ELISA (Supplemental Figure 1E). Among the shared reduced proteins, vasorin and alpha-1-antitrpysin 1-5 showed the greatest reduction in EVs after WBIR (Table 2). To identify potential biomarkers of high doses of radiation exposure that could be used at different times after exposure, we compared the protein changes at 3 and 7 days following 9 Gy WBIR. Twelve proteins overlapped (Figure 2J, red circle). Notably, suprabasin and sarcalumenin were increased, whilst cathelicidin antimicrobial peptide (Camp) and copine-1 showed the greatest decrease (Table 3).

Beyond protein changes, we and other groups have also demonstrated that EV-bound miRNA can also serve as potential biomarkers (38, 46). Therefore, we assessed alterations in the miRNA content of EVs using nanoString's nCounter mouse miRNA panel. Three days after injury there were 15 miRNAs that changed similarly at both doses (Figure 2K). Notably, miR-376a and miR-136 were increased over 100 to 200-fold and 30 to 60-fold at 2 Gy and 9 Gy respectively. Further there were 7 miRNAs specific to the high dose 9 Gy, exposure (Figure 2L). Taken together, these unbiased assessments of EV cargo have identified proteins and miRNAs that could serve as biomarkers for radiation exposure, and they may also act as possible drivers of the immune and physiologic dysfunction observed during ARS.

WBIR-induced EVs induce immune dysfunction consistent with exposure to radiation

EVs have emerged as potential contributors to the bystander effect that is observed following exposure to radiation (27). Therefore, to determine the effect of EVs on immune dysfunction/immune reprogramming after WBIR, EVs were isolated after exposure (3 or 7 days) to WBIR (2 or 9 Gy). WBIR-EVs were then administered to RAW 264.7 macrophages treated with LPS using our *ex-vivo* protocols previously used in the setting of burn injury (35, 36). Immune gene expression was measured 24 hours after exposure using nanoString technology (Figure 3A) which allows for the simultaneous quantification of 561 immune genes. WBIR-EVs blunted immune responses to LPS, with the most severe impact seen with 9 Gy exposure. EVs isolated 3 days after 2 Gy exposure resulted in a significant down-regulation in seven immunoregulatory genes: *NFκBia*, *NFκBiz CD274*, *Ifnar1, Itgb2, Tollip, and Irak2* (Figure 3B), compared to sham EVs. Although there were not enough significantly altered genes for IPA to assign Z-scores, we found that 3 day WBIR-induced EVs, compared to sham-EV, induced significant changes in key regulatory genes with an overall predicted increase in pro-inflammatory TLR, IL-1 and iNOS signaling with a corresponding increase in NFκB signaling (Figure 3C). These changes are consistent with the hyper-inflammatory response associated with ARS). EVs isolated 7 days after 2 Gy, however, showed almost no significant changes with only a slight reduction in *CD274* (Figure 3D) compared to sham EVs. This return to baseline by day 7 is possibly consistent with the survivability of this level of exposure. In contrast, EVs isolated 3 (Figure 3E) and 7 days (Figure 3F) after 9 Gy exposure showed profound suppression of macrophage responses to LPS, with IPA predicting inhibition of wound healing, inflammatory signaling, leukocyte extravasation, nitric oxide production, aryl hydrocarbon receptor signaling, and phagosome formation (Figures 3G, H). PPAR signaling, however was predicted to be activated after exposure to EVs from all time points compared to sham EVs.

Since IPA predicted an inhibition of phagosome formation, and we have previously shown that EVs released after burn injury also influence the phagocytic capability of macrophages (35), we assessed the effect of WBIR-EVs on phagocytosis. RAW macrophages were co-cultured with LPS, fluorescein-labelled killed *E. coli* (K-12 Stain), and WBIR-Induced EVs or Sham EVs. EVs isolated 3 days after 2 Gy exposure significantly inhibited phagocytosis compared to sham EVs; however 2 Gy EVs isolated at 7 days had no impact on phagocytosis compared to sham EVs (Figure 3I). EVs induced by 9 Gy exposure did not result in a significant reduction in phagocytosis when isolated both 3 and 7 days after injury (Figure 3J). Since these data indicates that EVs may contribute to ARS-associated immune dysfunction, we next investigated if MSC-EVs, shown in many systems to restore overt immune reprogramming to homeostasis, could be used to reduce the immune dysfunction associated with ARS.

MSC-EVs restore immune function to homeostasis when given after either WBIR or radiation combined with burn injury

To determine if MSC-EVs can act as a putative MCM to restore ARS-induced immune function to one closer to health, we utilized the WBIR model plus a model of polytrauma, RCI, in which we have observed a greater amplitude of immune dysfunction than WBIR alone (Figure 4A). Mouse MSC-EVs were purified from MSCs and isolated by differential centrifugation. $1x10^{10}$ MSC-EV/mouse resuspended in sterile saline, or sterile saline alone, were injected *via* tail vein into mice 72 hours after WBIR, RCI or sham injury. Forty-eight hours later, mice were sacrificed, and we harvested the spleen to assess the transcription of immune genes using nanoString, and plasma for quantification of peripheral

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TABLE 1 Significantly up-regulated proteins across all doses and timepoints.

Gene Name	Protein Description	Log2 Fold Change 2 Gy Day 3	Log2 Fold Change 2 Gy Day 7	Log2 Fold Change 9 Gy Day 3	Log2 Fold Change 9 Gy Day 7
Tbxas1	Thromboxane-A synthase	10.34	9.14	10.07	9.96
Fga	Fibrinogen alpha chain	8.05	6.80	7.91	7.82
Fgg	Fibrinogen gamma chain	7.38	6.30	7.39	7.23
Lcp2	Lymphocyte cytosolic protein 2	6.59	5.82	6.65	6.50
Fgb	Fibrinogen beta chain	6.67	5.61	6.68	6.48
Hsp90aa1	Heat shock protein HSP 90-alpha	6.20	5.54	6.29	6.18
P4hb	Protein disulfide-isomerase	6.29	5.18	4.63	3.59
Cyb5a	Cytochrome b5	6.69	3.98	4.08	3.05
Cavin1	Caveolae-associated protein 1	5.27	3.87	4.17	3.92
Esyt1	Extended synaptotagmin-1	5.71	4.01	3.43	3.43
Ubash3b	Ubiquitin-associated and SH3 domain-containing protein B	3.10	5.63	3.71	3.67
Actn1	Alpha-actinin-1	3.59	6.02	1.74	4.25
Tmed10	Transmembrane emp24 domain-containing protein 10	5.94	3.44	3.65	2.24
Cd81	CD81 antigen	5.00	3.91	3.67	2.51
Pon3	Serum paraoxonase/lactonase 3	5.48	2.57	3.30	3.61
Cyb5r3	NADH-cytochrome b5 reductase 3	5.30	3.68	3.30	2.63
Tapbp	Tapasin	5.59	3.78	2.96	2.22
Aadacl4	Arylacetamide deacetylase-like 4	4.94	2.44	3.32	3.75
Hsp90b1	Endoplasmin	4.15	4.28	3.33	2.10
Pdia3	Protein disulfide-isomerase A3	4.61	3.55	3.27	2.16
Clic1	Chloride intracellular channel protein 1	3.33	4.31	2.25	2.79
Mvp	Major vault protein	4.87	2.91	3.13	1.75
Atp2a1	Sarcoplasmic/endoplasmic reticulum calcium ATPase 1	3.94	2.62	2.50	3.48
Gp1bb	Platelet glycoprotein Ib beta chain	4.08	3.71	1.82	2.73
Atp1a2	Sodium/potassium-transporting ATPase subunit alpha-2	3.10	2.57	2.76	3.33
Mmp2	72 kDa type IV collagenase	2.21	2.65	3.38	3.25
Thsd4	Thrombospondin type-1 domain-containing protein 4	5.60	2.23	2.69	0.69
F13a1	Coagulation factor XIII A chain	3.06	1.96	2.89	1.79
Rps6	40S ribosomal protein S6	2.85	1.76	2.70	1.92
Ca1	Carbonic anhydrase 1	2.00	2.42	2.33	1.76
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	1.81	1.44	1.94	1.53
Vamp8	Vesicle-associated membrane protein 8	1.98	1.26	1.29	1.00
F13b	Coagulation factor XIII B chain	1.66	0.91	1.78	1.10
Pros1	Vitamin K-dependent protein S	1.84	0.88	1.24	1.36

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TABLE 2 Significantly down-regulated proteins across all doses and timepoints.

Gene Name	Protein Description	Log2 Fold Change 2 Gy Day 3	Log2 Fold Change 2 Gy Day 7	Log2 Fold Change 9 Gy Day 3	Log2 Fold Change 9 Gy Day 7
Vasn	Vasorin	-3.78	-5.05	-4.28	-5.53
Serpina1e	Alpha-1-antitrypsin 1-5	-2.87	-4.83	-4.21	-3.63
Gda	Guanine deaminase	-2.75	-3.54	-3.29	-3.97
Prg4	Proteoglycan 4	-2.99	-3.88	-3.30	-3.21
C1sb	Complement C1s-B subcomponent	-3.02	-3.61	-3.20	-3.20
Ttr	Transthyretin	-4.41	-2.08	-2.54	-2.14
Capn1	Calpain-1 catalytic subunit	-1.53	-2.82	-2.46	-2.81
Serpina1d	Alpha-1-antitrypsin 1-4	-2.27	-2.25	-2.49	-2.29
Serpina3k	Serine protease inhibitor A3K	-2.10	-2.52	-2.22	-2.07
C1sa	Complement C1s-A subcomponent	-1.94	-2.64	-2.16	-2.11
Hspa9	Stress-70 protein, mitochondrial	-2.42	-1.12	-2.91	-2.08
Serpina1a	Alpha-1-antitrypsin 1-1	-1.90	-2.16	-2.28	-2.16
C5	Complement C5	-2.25	-2.24	-1.64	-1.80
C1ra	Complement C1r-A subcomponent	-1.56	-2.75	-1.91	-1.69
A2m	Alpha-2-macroglobulin-P	-2.48	-1.13	-1.90	-2.23
Park7	Parkinson disease protein 7 homolog	-2.84	-0.84	-1.88	-2.17
Serpina3n	Serine protease inhibitor A3N	-2.74	-2.47	-1.10	-1.27
Serpina3m	Serine protease inhibitor A3M	-2.04	-2.02	-1.72	-1.73
Bdh1	D-beta-hydroxybutyrate dehydrogenase	-1.40	-1.65	-1.49	-1.43
Spp2	Secreted phosphoprotein 24	-1.42	-1.44	-1.72	-1.34
Masp2	Mannan-binding lectin serine protease 2	-1.28	-1.42	-1.26	-1.56
Psma1	Proteasome subunit alpha type-1	-1.16	-0.71	-1.29	-2.05
Serpina1b	Alpha-1-antitrypsin 1-2	-1.02	-1.22	-1.34	-1.18
Gpx3	Glutathione peroxidase 3	-1.32	-0.85	-0.86	-1.30
Ica	Inhibitor of carbonic anhydrase	-1.35	-0.77	-0.78	-0.72
Cfp	Properdin	-0.74	-0.92	-0.75	-1.09
Ifnar2	Interferon alpha/beta receptor 2	-0.71	-1.10	-0.84	-0.73
Prdx2	Peroxiredoxin-2	-0.56	-0.71	-0.92	-1.12
Atp5f1e	ATP synthase subunit epsilon	-0.91	-0.76	-0.77	-0.66
Azgp1	Zinc-alpha-2-glycoprotein	-1.03	-0.66	-0.51	-0.60

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TABLE 3 Significantly altered EV proteins after 9 Gy WBIR.

Gene Name	Protein Description	log2 Fold Change day 3	log2 Fold Change day 7
Sbsn	Suprabasin	2.16	3.65
Srl	Sarcalumenin	1.95	2.53
Plxna4	Plexin-A4	-1.41	-1.72
Pf4	Platelet factor 4	-1.51	-2.31
Pdcd6	Programmed cell death protein 6	-2.44	-1.76
Vcam1	Vascular cell adhesion protein 1	-1.93	-1.72
Cat	Catalase	-1.91	-2.36
Aadac	Arylacetamide deacetylase	-2.13	-2.35
Steap3	Metalloreductase STEAP3	-4.23	-2.19
Dnpep	Aspartyl aminopeptidase	-2.45	-3.54
Cpne1	Copine-1	-3.59	-4.05
Camp	Cathelicidin antimicrobial peptide	-4.27	-5.62

immune cytokines and chemokines by Bio-plex multiplex assay (Figure 4A). Firstly, to demonstrate the specific immune gene reprogramming associated with WBIR versus sham injury, in the absence of MSC-EV treatment, nanoString analysis revealed that WBIR caused profound alterations in peripheral immune gene expression with 264 genes significantly altered (Figure 4B, $\log_2$FC range -10 to 10) compared to sham mice. Of note, *Camp* was significantly reduced ($\log_2$FC= -9.8) by WBIR and was also the most significantly reduced protein in EVs after WBIR (Table 3). IPA revealed a downregulation of several key immune pathways related to ARS (Figure 4C), *versus* sham injured mice, with many similarities to the IPA of RAW cells exposed to 9 Gy WBIR-induced EV (Figure 3C). Particularly, an "immunosuppressed phenotype" was observed with reduced T cell signaling, TNF and NFκB, as well as wound healing and significantly increased PPAR signaling (Figures 3G–H). The immune checkpoint inhibitor CTLA4 pathway was also increased by WBIR, compared to sham mice, congruent with the reductions in T cell signaling.

We then investigated the effect of MSC-EV treatment on the peripheral immune responses after sham or WBIR injury. Firstly, we compared MSC-EV-treated WBIR mice to untreated sham-injured mice. Utilizing splenic mRNA, we found that MSC-EV treatment normalized certain key WBIR-induced immune gene changes with 245 total genes changing (Figure 4D), compared to untreated sham injured mice (contrast these data with the 264 genes altered in untreated WBIR mice versus sham mice, Figure 4B). Therefore, to complete this analysis, we also examined the effect of MSC-EV treatment of WBIR injured mice compared to untreated WBIR injured mice (Figure 4E). This comparison directly identified the key genes which were altered specifically by MSC-EV treatment in injured mice (e.g. *NFκBia, Cxcr4, Socs1* were significantly upregulated, and *Stat5a* was significantly downregulated) *versus* untreated mice. In addition, MSC-EV treatment increased aryl hydrocarbon signaling which was shown to be inhibited in RAW macrophages exposed to EVs isolated following exposure to 9 Gy of WBIR (Figures 3G, H, 4E). Taken together, these data suggest the majority of genes were not significantly altered by MSC-EVs, however, there were specific genes returned to homeostasis. These findings were reflected in the IPA of the MSC-EV treated *versus* untreated WBIR-injured mice (Figure 4F), with immune signaling pathways mainly remaining unchanged. Comparing this analysis with the specific immune gene reprogramming associated with WBIR versus sham injury (Figure 4F *versus* Figure 4C), it is clear that most WBIR-dependent signaling changes, such as downregulation of T cell Receptor, NFκB and upregulated PPAR signaling were not altered by MSC-EV treatment. However, the wound healing pathway, thrombin signaling were not reduced when MSC-EVs were given, and a downregulation of the P70s6K and "FCγRIIB signaling in B lymphocytes" pathways occurred with MSC-EV treatment of WBIR injury compared to untreated WBIR mice.

Turning next to our RCI model, we observed that RCI *versus* sham injury caused similar widespread immune gene changes with 233 genes changing (Figure 4G) and similar pathway disturbances (Figure 4H), both comparable to the gene and pathway changes induced with WBIR (Figures 4B, C). Also similar to WBIR, we found that MSC-EV treatment normalized certain key RCI-induced immune gene changes with 240 total genes changing (Figure 4I) and minimal changes to immune signaling pathways (not shown) compared to untreated sham injured mice (compare Figure 4I with Figure 4G). When we treated RCI mice with MSC-EV and compared the splenic immune gene expression with untreated RCI-mice, we found a slight yet significant reprogramming of the immune response (Figure 4J), with more genes impacted than in the WBIR model (26 genes compared to 10 in the WBIR model). In comparison to the untreated RCI response (Figure 4G) certain key immune regulatory genes reduced by RCI were improved by MSC-EVs such as *Map4k1*, *Ccr9*, *s100a8/9*, and *Cxcl12*. IPA of these data revealed a significant re-programming of the immune signaling pathways in MSC-EV treated mice compared to untreated mice (Figure 4K), with a shift towards a regenerative anti-inflammatory Th2 responses and a reduction in the highly inflammatory Acute

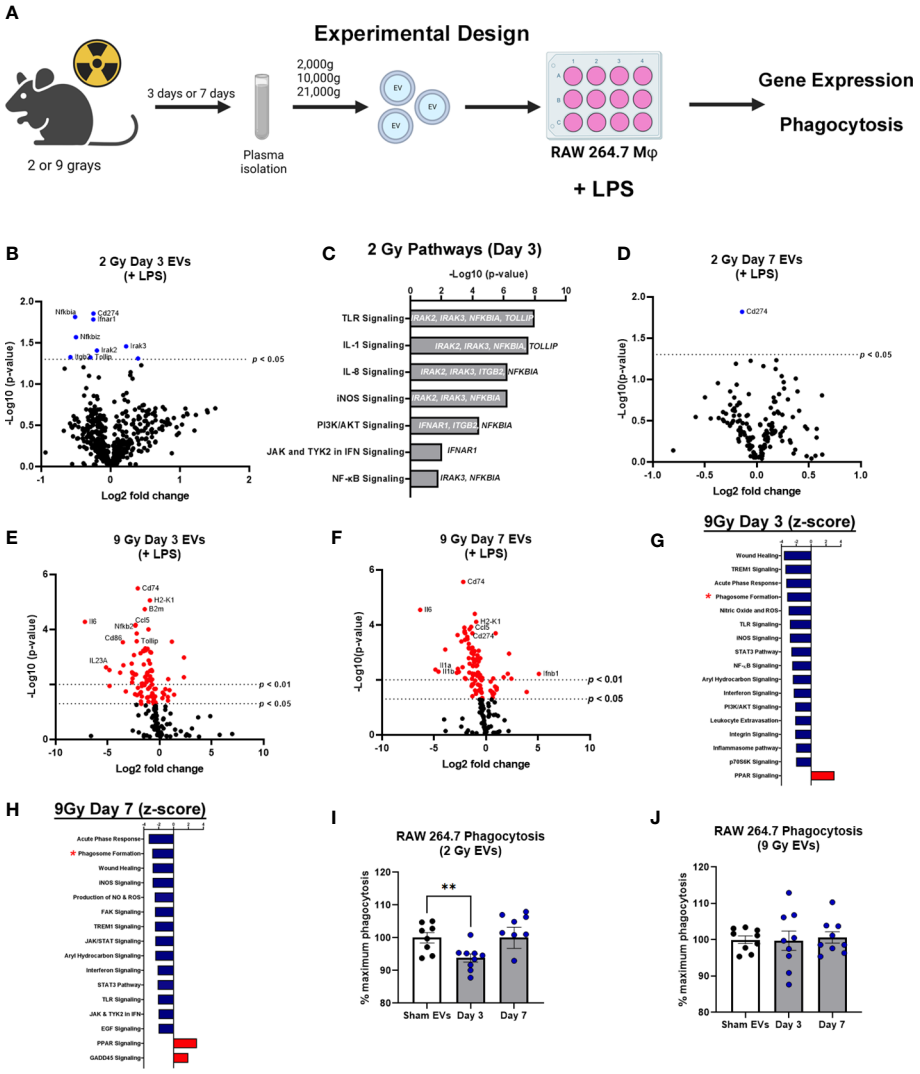

FIGURE 3
EVs released following WBIR induce immune gene expression changes that reflect exposure to radiation. **(A)** Experimental overview for the *in vitro* exposures and analyses involving WBIR-induced EVs. **(B)** Volcano plots displaying the immune gene changes in RAW macrophages exposed to EVs isolated 3 days after 2 Gy of WBIR compared to Sham EVs in the presence of LPS (n=3 for each experimental group). **(C)** IPA analysis of the pathways that were determined to be most impacted. **(D)** Volcano plots displaying the immune gene changes in RAW macrophages exposed to EVs isolated 7 days after 2 Gy of WBIR compared to Sham EVs in the presence of LPS (n=3 for each experimental group). **(E, F)** Volcano plots displaying the immune gene changes in RAW macrophages exposed to EVs isolated **(E)** 3 days or **(F)** 7 days after 9 Gy of WBIR compared to Sham EVs in the presence of LPS (n=3 for each experimental group). Canonical immune pathways identified to be most impacted by IPA with their associated Z-scores after **(G)** 3 days or **(H)** 7 days. **(I, J)** Quantification of the phagocytic capability of macrophages during co-culture of EVs released after 2 Gy **(I)** and 9 Gy **(J)** of WBIR (n=8-9 for each experimental group); average fluorescence units of no-cell negative-control wells from all wells. We then defined phagocytosis response to the experimental effector (% Effect) as: % Effect = Net experimental phagocytosis x 100% x Net positive control phagocytosis. *p<0.05, One-way ANOVA with Dunnett's *post-hoc* test. Created with BioRender.com.

Phase Response pathways, so intrinsically involved with the induction of the hyper-inflammation associated with both ARS and burn injury.

We then tested whether these gene changes translated into differences in functional protein expression. Multiplex cytokine and chemokine analysis of the plasma harvested from RCI-mice after MSC-EV treatment or treatment revealed a significant reduction in TNFα protein levels after RCI with MSC-EVs (Figure 4L), a trend toward a reduction in IL-2 (Figure 4M, N). MCP-1 did not approach statistical significance (p=0.36; Figure 4N). These findings are consistent with partial normalization of immune changes by MSC-EVs given 3 days after RCI injury. Taken together, these data demonstrate in two different pre-clinical models of radiation injury that MSC-EV may act to normalize the profound immune dysfunction associated with ARS.

MSC-EVs can significantly improve survival if given before WBIR exposure

The *in vivo* stability of EVs combined with the homeostatic effects of EVs as described above suggest that they may act as potent

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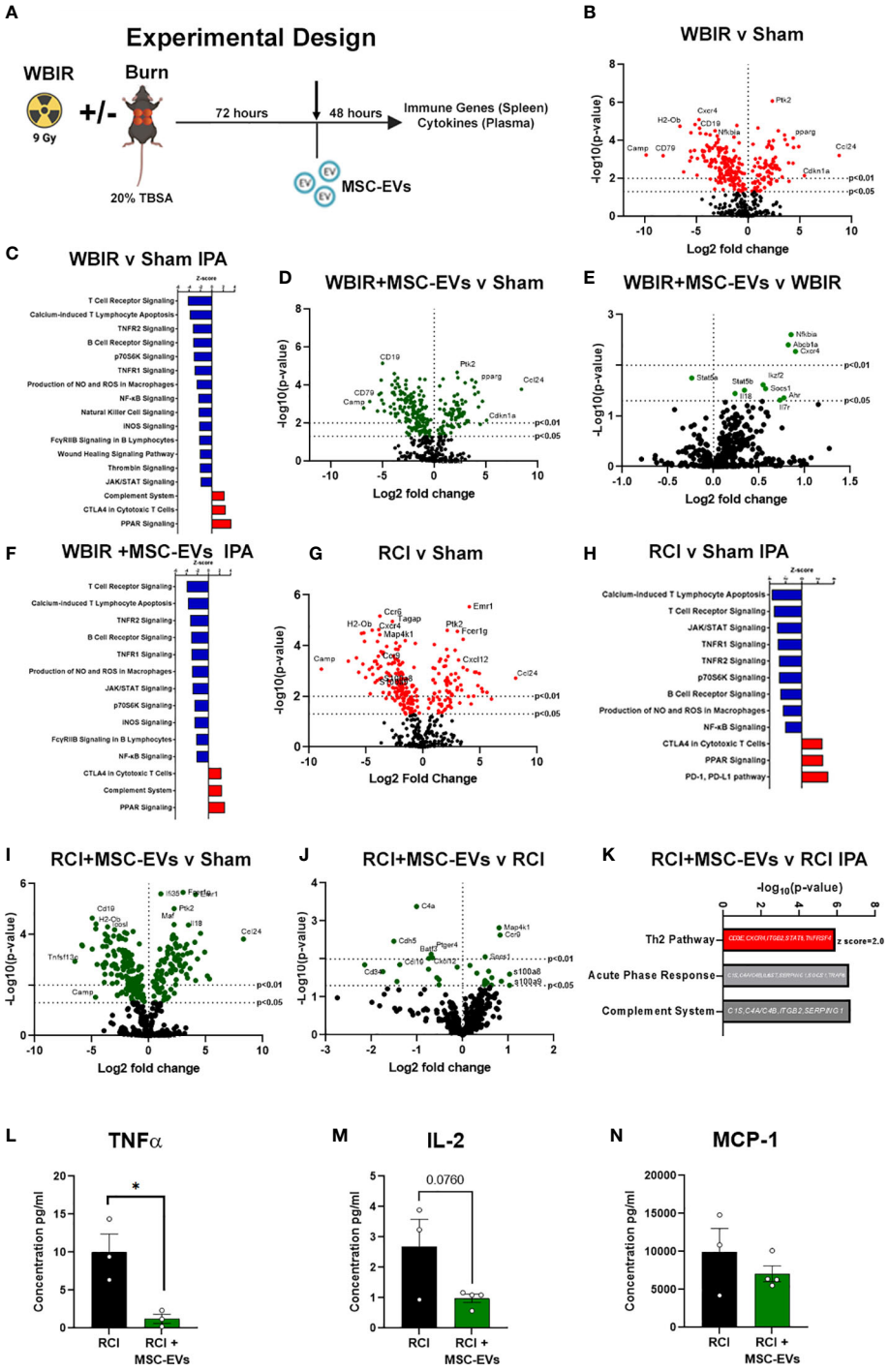

FIGURE 4
MSC-EVs restore immune function to homeostasis when given after either WBIR or radiation combined with burn injury (RCI). **(A)** Experimental design displaying the models of both WBIR and RCI and the treatment with MSC-EVs 3 days after injury before the harvesting of spleens and cytokines. **(B)** Volcano plot displaying the immune gene changes and **(C)** IPA pathways that were most impacted following exposure to WBIR (n=3 for each experimental group). **(D, E)** Volcano plots displaying gene expression changes in mice treated with MSC-EVs following exposure to WBIR compared to **(D)** sham and **(E)** untreated WBIR mice (n=3 for each experimental group). **(F)** IPA analysis displaying the most significantly impacted pathways with MSC-EV treatment compared to sham mice. **(G)** Volcano plots illustrating immune gene alterations in mice exposed RCI compared to sham mice and the **(H)** IPA analysis of these immune pathways. **(I, J)** Volcano plots demonstrating the impact of MSC-EVs on immune gene changes following **(I)** RCI compared to sham mice **(J)** vs RCI alone mice. **(K)** IPA analysis of RCI+MSC-EVs vs RCI alone. The Th2 pathway was increased by MSC-EVs. Acute phase response and complement showed gene changes depicted. **(L–N)** Plasma cytokine levels of **(L)** TNFα, **(M)** IL-2, and **(N)** MCP-1 mice treated with MSC-EVs following RCI (n=3-4 for each experimental group). *p<0.05, One-way ANOVA with Dunnett's *post-hoc* test. Created with BioRender.com.

radioprotectants with long clearance time. To the best of our knowledge, there have not been any studies evaluating if MSC-EVs can act as a radioprotectant if given prophylactically before lethal doses of WBIR. Therefore, we treated mice with MSC-EVs (i.v, 10^{10}/mouse in saline) twice prior to exposure (24 and 3 hrs) to 9 Gy WBIR (Figure 5A). The 9 Gy exposure was fatal with all mice dying within 3 weeks. Pre-treatment with MSC-EVs significantly prolonged survival (Figure 5C, 1.8 *versus* 2.6 weeks, 9 Gy *versus* 9G +MSC-EVs). MSC-EV pretreatment accordingly slowed the progression of weight loss after exposure (Figure 5B). Thus, MSC-EV pretreatment can slow the progression of mortality after WBIR injury.

Discussion

Due to the heightened threats of nuclear warfare, terrorist attacks, or nuclear power plant accidents, there is an increasing likelihood of a radiological incident occurring within the general population. Because of the severity of these scenarios, it is likely that clinicians may have to treat thousands of patients that were exposed to high doses of radiation (47). Currently, there are not any effective bio-dosimetry markers for predicting the radiation dose received and very few therapeutics for treating ARS. In this study, we demonstrate that EVs released after exposure to WBIR have altered proteomic and miRNA cargo that is related to the dose of radiation received and time after exposure. This data could be utilized to assist in triaging and the clinical treatment of patients. Furthermore, we demonstrate that these EVs induce immune gene changes in cultured monocytes which dampened pro-inflammatory signaling and inhibited phagocytosis. We also assessed if treatment with MSC-EVs could be used as an effective therapeutic for treating both WBIR and RCI. We found that MSC-EVs slightly improved immune homeostasis following both WBIR and RCI and reduced pro-inflammatory cytokine levels after RCI. Further, we found that MSC-EVs could act as a radioprotectant if given prior to WBIR. Taken together, this data demonstrates that EVs contain cargo that promotes radiation-induced immune effects and that they could be utilized as a potential bio-dosimetry marker. Prophylactic or therapeutic treatment with MSC-EVs limits the harmful effects of exposure to WBIR (Figure 6).

Following exposure to WBIR, we found there was a significant reduction in circulating plasma EV levels 3 days after exposure to 9 Gys (Figure 1A). However, there were not any significant differences in circulating EV levels across the other timepoints and doses. Other conditions associated with tissue injury such as trauma and burn injury have been typically associated with an increase in the number of plasma EVs, we were surprised to observe only a transient reduction of plasma EVs after 9 Gy of WBIR (36, 42). This could be due to the massive cell death seen after 9 Gy of WBIR or a transient cellular shock caused by the injury resulting in the shedding of fewer EVs. This has been similarly observed in cancer cells exposed to 9 Gy of gamma irradiation which resulted in dramatic decreases in EV secretion (48). We also found that there were alterations in EV proteomic and miRNA cargo that reflect the dose of WBIR received and time after exposure. Interestingly, the more dramatic alterations in the EV protein cargo occurred following exposure to 2 Gy while the protein changes for the 9 Gy dose were much less pronounced (Figures 2B–E). However, there was a significant reduction in the total number of EVs 3 days after exposure to 9 Gy of WBIR and no significant changes in the total number of EVs 3 days after 2 Gy of WBIR. It is important to note that exposure to 2 Gy of WBIR for a mouse is not lethal and mice are able to recover from this exposure while a 9 Gy dose of WBIR is lethal and the mice are unable to recover (8). Thus, the lower number of circulating EVs three days after 9 Gy could be more detrimental than the lack of proteomic changes. Based on the proteomic changes following exposure to 2 Gy of WBIR, IPA identified a plethora of pathways that were impacted (Supplemental Figures 1A, B). These changes may represent a functional adaptation during recovery from 2 Gy that does not occur with the larger 9 Gy dose due to massive cell death and fewer healthy cells secreting EVs into the periphery. When comparing the temporal proteomic changes between day 7 and day 3 following exposure to 2 Gy, some of the notable pathways that were activated were related to Rhoa and actin cytoskeleton signaling (Figure 2G). Consistent with this finding, previous studies has found that exposure to ionizing radiation in melanoma leads to actin rearrangements and the thickening of actin fibers which may be mediated by Rhoa signaling (49, 50). Based on these protein changes in our EV cargo, it is possible that EVs could be mediating these effects of ionizing radiation. Further, there was a down-regulation in pathways associated with prothrombin and glycoprotein-6 signaling, a key platelet pathway, 7 days after exposure to 2 Gy of WBIR (Figure 2G). Bleeding diathesis is one of the most common symptoms associated with radiation exposure (51) and an inhibition of these pathways by EVs could be mediating these symptoms.

Regarding the use of EVs as biomarkers of exposure, there were 65 proteins that were significantly altered across all the doses and timepoints (Tables 1, 2). Among the proteins that had the highest fold-change across all groups were thromboxane A synthase and lymphocyte cytosolic protein 2 which play essential roles in platelet function and T-cell receptor signaling, respectively (52, 53). Among proteins decreased across all groups, the transforming growth factor, vasorin, was significantly decreased (54). For the 9 Gy dose there were only 12 proteins that were significantly altered between timepoints and suprabasin and sarcalumenin were increased, while Camp and copine-1 were decreased (Table 3). Based on these findings, a panel for detecting increases of EV proteins that were increased among all groups (e.g. thromboxane A synthase and lymphocyte cytosolic protein 2) could be used to identify if there was any radiation exposure, while the increases of proteins in the 9 Gy group (suprabasin and sarcalumenin) might be useful as an indicator for a high dose of radiation. Interestingly, Camp was significantly decreased in the EV protein cargo while the *Camp* gene was also found to be down-regulated in spleens of irradiated mice (Table 3, Figure 4B). Beyond proteins, EVs are rich in miRNAs. Therefore, we assessed if there were changes in the miRNA cargo of these EVs following WBIR exposure.

Between both the 2 Gy and 9 Gy dose, there were 15 significantly altered EV-bound miRNAs across both groups

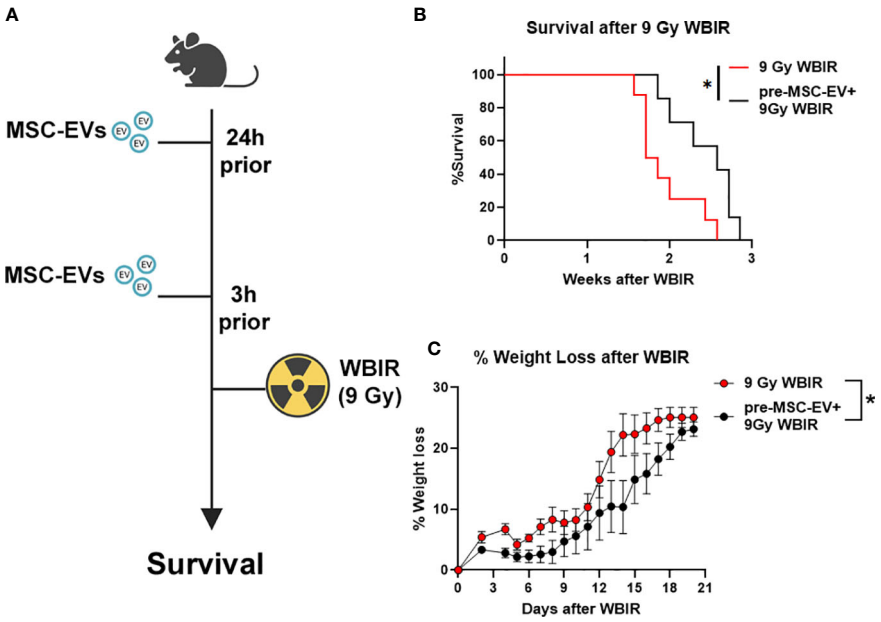

FIGURE 5
MSC-EVs act as a radioprotectant if given before exposure to 9 Gy of WBIR. **(A)** Experimental design for assessing the efficacy of MSC-EV pre-treatment for protecting against WBIR. **(B)** MSC-EV pre-treatment prevents mortality and limits **(C)** weight loss following exposure to a lethal dose of WBIR (n=7-8 for each experimental group). *p<0.05, One-way ANOVA with Dunnett's *post-hoc* test. Significant interaction between time after radiation and treatment was assessed using a Mixed-effects model (REML), $F_{19,234} = 2.105$, $p=0.0055$. Created with BioRender.com.

(Figure 3K). In addition, the directionality of these miRNAs was similar between both the 2 and 9 Gy doses. With both doses, there was an enormous fold-change increase in miR-136 and miR-376a. The fold changes in these miRNAs were slightly more dramatic in the 9 Gy dose as expected. For instance, miR-136 was increased 30-fold in EVs isolated following 2 Gy, while it was increased 63-fold in EVs after 9 Gy. In cancer, miR-136 has been shown to promote apoptosis and actively represses anti-apoptotic genes (55). Direct exposure to ionizing radiation causes the mass apoptosis of numerous different cell types and can be mediated by the bystander effect (27). It is possible that the increase in miR-136 in EVs after WBIR could be involved in these effects. Prior work has found that miR-376a sensitizes cells to DNA damage, making them unable to repair DNA breaks which causes genomic instability (56). Since exposure to ionizing radiation causes DNA damage, an up-regulation in this miRNA may indicate that these EVs are exacerbating some of the effects of exposure to ionizing radiation. Future studies will investigate the role of these miRNAs in post-WBIR cell death and DNA damage. In addition, there were seven miRNAs that were significantly changed in the 9 Gy dose alone (Figure 3L). Based on this data, we propose that these EV-bound miRNA changes could be used as biomarkers for detecting radiation dose. For instance, the detection of miR-136 and miR-376a in EVs could be used to identify if there was any exposure to radiation, while the identification of miRNAs specific to the 9 Gy dose could indicate that there was an exposure to a high-dose of radiation. Others have identified a significant down-regulation in EV bound miRNA 142 after 2 Gy of WBIR which we also observed (18). Experiments are underway to further identify classes of biomarkers and a multiparametric bio-dosimetry algorithm(s) with the potential to detect IR exposure, time since exposure and/or exposure dose. Also, outside of miRNAs, EVs are also rich in IncRNAs and CircRNAs and future experiments will explore if there are alterations in these RNA species (57). The feasibility of use in non-invasive (e.g., use of salivary EV) screening for radiation exposure will then be explored. Since these alterations in EV cargo were shown to impact numerous pathways (Supplemental 1A–D), we next performed *in vitro* exposures to evaluate if WBIR-induced EVs influenced immune gene expression and functional alterations.

Previous work in our lab and others have demonstrated that macrophages play an important role in immune dysfunction following severe burn injuries (35, 36). Macrophages are also important following radiation exposure, where they are responsible for the removal of apoptotic cells and elicit phagocytic functions (58). EVs isolated 3 and 7 days after 9 Gy of WBIR robustly reduced the macrophage immune transcriptome response to LPS, suggesting these EVs may physiologically dampen macrophage responses in the setting of ARS. These immune gene changes and IPA analysis were remarkably similar between macrophages exposed to EVs isolated 3 or 7 days after exposure to 9 Gy (Figures 3E–H). Interestingly, while we observed the most dramatic protein changes in the EVs released after 2 Gy, only seven immune genes were blunted in the macrophage response to LPS. By day 7, the influence of these EVs on gene expression were essentially gone, with *Cd74* being the only gene that was significantly different. A limitation of this study is that we only analyzed transformed macrophage immune transcriptome responses to EV, and therefore these data can only be extrapolated to physiological responses in primary immune cells. We are currently testing if these transcriptomic responses do indeed translate to *in vivo* immune functional alterations.

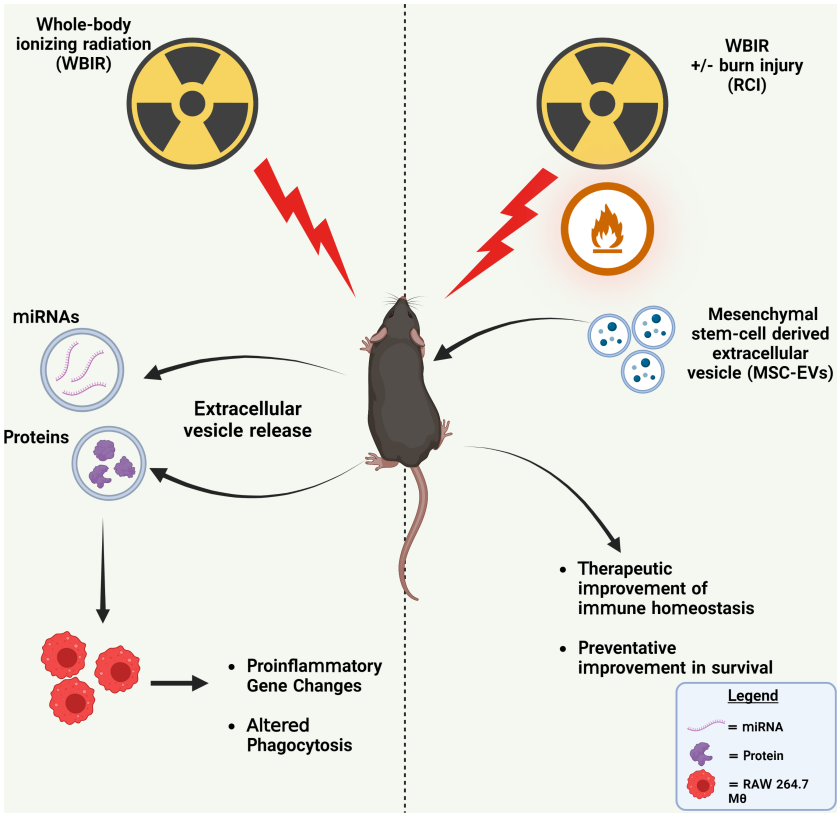

FIGURE 6
Summary of findings. Left: WBIR causes alterations in miRNA and protein cargo of circulating EVs that suppressed proinflammatory immune gene induction and phagocytic responses to LPS in RAW 264.7 macrophages. Right: The preventative and therapeutic administration of MSC-EVs with either WBIR or RCI protected against WBIR exposure and improved immune homeostasis. Created with BioRender.com.

Since the 2 Gy dose of radiation is survivable, this is consistent with a return to homeostasis by day 7. This was also observed with the phagocytosis assay. EVs isolated 3 days after 2 Gy inhibited phagocytosis whereas EVs isolated 7 days after 2 Gy did not (Figure 3I). While we only looked at phagocytosis in this study, future studies should evaluate if the inhibition of these pathways also correlate with other functional assays. For instance, assessing if these EVs released after WBIR limit healing of damaged tissue or promote barrier dysfunction, as the IPA predicted. While WBIR-induced EVs can cause immune dysfunction, we found that MSC-EVs could serve as therapeutic that could reverse or prevent these effects.

MSC-EVs have emerged as potent immunomodulatory molecules that are anti-inflammatory, promote wound healing, and regeneration (59). There are no FDA-approved treatments for the clinical outcomes of ARS after high dose IR-exposure. While stem cell therapy has been used to develop radiation MCM, studies have suggested that the secretome of these stem cells contained the critical growth factors and signaling molecules for the stem cell-driven regeneration *via* EVs. (60). In the context of radiation exposure, MSC-EVs have been shown to mitigate intestinal and hematopoietic damage when given after exposure to WBIR (28, 29). In addition, MSC-EVs are a promising therapeutic for the treatment of these forms of injury because they can be produced on a mass scale, lack histocompatibility complexes (i.e. low risk of donor incompatibility), and can be administered rapidly in response to emergencies (61). We found that MSC-EVs given 3 days after WBIR slightly and selectively restore immune homeostasis after RCI and WBIR. For instance, *Cish1* and *Socs1* (both members of the Suppressors of Cytokine Signaling (SOCS) family, involved with negative regulation of cytokine inhibition) and *Nfκbia* (NFκB Inhibitor Alpha) were upregulated in IR-injured mice following MSC-EV treatment, while *Stat5a* is significantly downregulated. These data are interesting as they are consistent with data from other animal models which also showed that *Cish1*, *Socs* and *Nfκbia* are associated with restoration of immune homeostasis and were slowly upregulated after long periods (weeks) during recovery from IR exposure (62). We also show a treatment-dependent increase in PPARγ gene expression and PPARγ signaling pathways, which reside at the intersection of immune and metabolic pathways (63–65); PPARγ is a negative regulator of mTOR which is activated after TLR/MyD88 engagement. Reduction in mTOR signaling also reduces myeloid-derived suppressor cell production, also thought to play a role post-irradiation immune suppression against infection (66). Downregulation of P70S6K after MSC-EV treatment compared to untreated mice also suggests that mTOR activation has been reduced (67). We have shown that the mTOR/PPARγ axis is partly responsible for the acute and chronic (analogous to

DEARE) immune dysfunction in burn injury (68–70), and experiments are underway to determine if MSC-EV can modulate this response in an mTOR-dependent fashion. It is therefore tempting to speculate that a key component of the reprogramming capacity of MSC-EVs is mTOR dependent. We found that MSC-EVs were also able to re-program immune response in a more severe polytrauma model (RCI), previously published findings demonstrate a greater amplitude of immune dysfunction compared to WBIR or burn alone (10), and were able to partially restore systemic cytokine patterns. We are currently testing the potential therapeutic use of MSC-EV in burn injury monotrauma models. While MSC-EV treatment has shown to be effective for mitigating the harmful effects of WBIR, we wanted to assess if MSC-EVs can simultaneously act as a radioprotectant for exposure to lethal doses of irradiation. We found that MSC-EVs administered prophylactically before exposure to 9 Gy of WBIR prolonged survival and mitigate weight loss (Figure 5). Due to the potential use of nuclear weapons in conflict and nuclear power plant accidents, there is a need to identify therapeutics that protect soldiers, emergency responders, and nuclear power plant workers to allow them to perform their duties in these hazardous environments. In addition, many cancer patients undergoing radiotherapy have to undergo less intense treatment plans, which are less effective for treating cancers, due to radiation toxicity. The use of a radioprotectant in these situations would be extremely beneficial as it would allow cancer patients to undergo more rigorous radiotherapy treatment regimens (31, 71). Future work will investigate the effects of combined pre-treatment and post-treatment of MSC-EVs, as well as their effects on cellular survival.

In November 2020, Klyachko et al. (72) reported that *clinicaltrials.gov* contained ~180 studies involving EVs as interventions or as a study object. Among these clinical trials, multiple timings and routes of administration were being utilized including oral, inhalation, nasal drop, i.v., and topical (72). As i.v. administration does not lend itself to being easily administered in the field, further work is required to assess the optimal dosage, efficacy of oral, intramuscular (i.m.) and intraperitoneal (i.p.) administration of MSC-EV in alleviating ARS and ultimately, DEARE. We present foundational data demonstrating the use of MSC-EV as a prophylactic therapy. Further experiments are in progress to examine the cellular and molecular mechanisms behind the increased survival after MSC-EV therapy before injury; regardless, these findings present an exciting avenue for the use of MSC-EVs in various applications, such as their use by military, firefighters and radiotherapy patients, and possibly in environmental situations to mitigate accrual of the damaging effects of low-level IR occupational exposure.

Conclusions

Here, we demonstrate that proteomic and miRNA cargo of WBIR-induced EVs is altered depending on the dose and time after exposure. These EVs produce functional effects that are consistent with similar immune alterations observed in ARS. Lastly, we demonstrate that MSC-EVs can restore immune homeostasis following radiological injury and for the first time demonstrate that MSC-EVs can act as a radioprotectant.

Data availability statement

The data presented in the study are deposited in the NIH GEO repository, accession numbers GSE234375, GSE233688 and GSE233692. The mass spectrometry proteomics data was deposited in the PRIDE database with the dataset identifier PXD041837.

Ethics statement

The animal study was reviewed and approved by UNC Institutional Animal Care and Use Committee (IACUC).

Author contributions

Conceptualization: RM, RS. Methodology: RM, LC, RS, SW, MW, LH, AM. Investigation: RM, LC, RS, LH, AM. Visualization: RM, LC, RS. Funding acquisitions: RM, LC, SW. Project administration: RM, LC, SW. Supervision: RM, LC. Writing-original draft: RM, LC, RS. Writing – review & editing: RM, LC, RS. All authors contributed to the article and approved the submitted version.

Funding

NIH NIAID U19AI067798 RADCCORE Subaward 203-2315 (to RM); NIH NIAID U19AI067798 RADCCORE Subaward 203-2367 (to RM); NIH NIEHS T32ES007126 (to RS and MW); NIH NIGMS R01GM131124 (to RM and SW); NC Jaycee Burn Trust (to RM, RS, and MW); Bowles Center for Alcohol Studies (to LC).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1188830/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Alterations in EV proteomic cargo following WBIR impact multiple physiological pathway categories. From the proteomic data, IPA was utilized to generate bubble charts displaying the general pathway categories that were most impacted. The size of the bubble denotes the number of proteins that overlap with that pathway and pink colors are associated with the activation of these pathways and blue is indicative of an inhibition of that pathway. **(A)** Bubble chart displaying the canonical pathways impacted based on the proteomic cargo of EVs released 3 days after 2 Gy of WBIR. **(B)** Bubble chart displaying the canonical pathways impacted based on the proteomic cargo of EVs released 7 days after 2 Gy of WBIR. **(C)** Bubble chart displaying the canonical pathways impacted based on the proteomic cargo of EVs released 3 days after 9 Gy of WBIR. Bubble chart displaying the canonical pathways impacted based on the proteomic cargo of EVs released 7 days after 9 Gy of WBIR. (E) Enzyme linked-immunosorbent assay (ELISA) for lymphocyte cytosolic protein-2 (LCP2) content in extracellular vesicles isolated 3 and 7 days after 9 Gy of WBIR.

SUPPLEMENTARY FIGURE 2
EVs released following WBIR induce immune gene expression changes in the absence of LPS. **(A)** Volcano plots displaying the immune gene changes in RAW macrophages exposed to EVs isolated 3 days after 9 Gy of WBIR compared to Sham EVs (n=3 for each experimental group). **(B)** Volcano plots displaying the immune gene changes in RAW macrophages exposed to EVs isolated 7 days after 9 Gy of WBIR compared to Sham EVs (n=3 for each experimental group). Canonical immune pathways identified to be most impacted by IPA with their associated -log10 (p-values).

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frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 14 July 2023
DOI 10.3389/fimmu.2023.1214757

OPEN ACCESS

EDITED BY
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RECEIVED 30 April 2023
ACCEPTED 26 June 2023
PUBLISHED 14 July 2023

CITATION
Jia Q, Zhao H, Wang Y, Cen Y and Zhang Z (2023) Mechanisms and applications of adipose-derived stem cell-extracellular vesicles in the inflammation of wound healing. *Front. Immunol.* 14:1214757. doi: 10.3389/fimmu.2023.1214757

Mechanisms and applications of adipose-derived stem cell-extracellular vesicles in the inflammation of wound healing

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Wound healing is a sophisticated process consisting of serial phases with overlaps, including hemostasis, inflammation, proliferation, and remodeling. The inflammation response is an early response that plays a crucial role in eliminating microbes and clearing damaged cell debris. However, in some pathological circumstances, such as diabetes mellitus, ischemia, trauma, deep burn, etc., abnormal inflammation can cause impaired wound healing. Adipose-derived stem cells (ADSCs) belong to the mesenchymal stem cell (MSC) family and exhibit prospective applications in tissue regeneration and dermatological repairs. ADSC-secreted extracellular vesicles (ADSC-EVs) mimic the functions of ADSCs without the concerns of cell survival, immune response, or ethical issues. Studies have revealed that ADSC-EVs can inhibit abnormal inflammation responses and accelerate wound healing through various mechanisms. Moreover, some studies explored modifications in the cargo components of ADSC-EVs to enhance their therapeutic efficacy. Given the increasing studies focusing on the potential of ADSC-EVs in wound healing, how they interfere with different phases of this process has been investigated in pieces. In this review, we summarized all up-to-date evidence to map a clearer picture of the underlying mechanisms of ADSC-EVs in inflammation response. The applications of ADSC-EVs aiming at inflammation in the healing process were also reviewed to provide therapeutic strategies for future investigators.

KEYWORDS

adipose-derived stem cells, extracellular vesicles, inflammation, wound healing, stem cell therapy

Introduction

The skin is the largest organ and exercises the maintenance function of balancing the external and internal environments of the human body. When the integrity of the skin is compromised, a series of repair mechanisms related to wound healing are initiated (1). Wound healing is a sophisticated and dynamic process of the body consisting of serial

phases with overlaps: hemostasis, inflammation, proliferation, and remodeling (2). During the repairing process, the interplay between different cellular components of the skin is crucial and complex for proper wound healing, including fibroblasts, keratinocytes, endothelial cells, and immune cells (residential and recruited). In a physiological state, these cells and the extracellular matrix work together harmoniously to restore barrier integrity. However, factors such as metabolic diseases (insulin resistance and type 2 diabetes), prolonged mechanical stress, or vascular disorders (ischemia and vascular ulcer) can impair the healing ability, leading to chronic wounds or keloids (3).

Inflammation is one of the earliest and classic responses during wound healing. It usually occurs in the first 24 to 48 h after the injury. A moderate inflammation serves as a protective response, aiming to eliminate microorganisms and clear damaged debris, thus accelerating the healing process (4). Upon the skin injury, the coagulation cascade is activated (5). Accumulated platelets not only result in the formation of blood clots but also release various cytokines and substances, like transforming growth factor-β (TGF-β) and arachidonic acid metabolites, which recruit immune cells to the wounding site, building an immune barrier against further infection (6, 7). However, the extent of inflammation is critical, as excessive inflammation, observed in conditions such as obesity or elevated glucose levels, and deficient immune responses can lead to delayed wound healing (8). Studies also suggested that in adults, the disordered inflammatory response can drive fibrosis rather than regeneration during the healing process, potentially leading to hypertrophic scar formation (9). Since both chronic wounds and keloids are associated with reduced quality of life and increased disability rates, it highlights the significance of regulating the inflammatory phase as a potential therapeutic target in wound healing.

In the field of regeneration medicine, mesenchymal stem cells (MSCs) hold tremendous potential in tissue healing. MSCs are a group of adult cells possessing renewing capability, and they can be obtained from multiple different tissues, such as bone marrow, umbilical cord, and adipose tissue (10, 11). MSCs obtained from different sources display similar functions. Among these sources, adipose-derived stem cells (ADSCs) are often preferred as the first choice due to their accessibility and sufficient content. ADSCs can be found in subcutaneous adipose depots. They have the ability to respond to inflammation and interact with immune cells at injured sites to regulate local immune response through secreting chemokines and cytokines (12). Accumulating evidence indicates that ADSCs can release extracellular vesicles, called ADSC-EVs, through paracrine secretion to mimic ADSC behavior (13). These ADSC-EVs are considered a highly promising therapeutic strategy for restoring the balance of wound inflammation and promoting healing. Under certain pathological circumstances, administration of ADSCs directly may lead to lower survival rates due to the over-activation of inflammation. However, ADSC-EVs have resembling functions of their parental cells, while being more resistant to degradation and carrying fewer transplantation risks or ethical concerns. Therefore, ADSC-EVs present themselves as an ideal therapeutic approach for wound healing.

Adipose-derived stem cell extracellular vesicles

Adipose tissue is the most abundant volume and one of the most functionally diverse organs in the body. In addition to its traditional storage sites functions, such as storing excessive energy into its triglyceride pool during feeding and releasing them as free fatty acids during fasting, adipose tissue is also an endocrine organ secreting adipokines, hormones, enzymes, and other bioactive particles in an endocrine, autocrine, and paracrine manner to maintain energy homeostasis. The main components of adipose tissue are mature adipocytes and the stromal vascular fraction (SVF). ADSCs are members of SVFs with self-renewal and differentiation capabilities (14). Extensive studies have proven that ADSCs not only have therapeutic effects on wounds and injuries, including cutaneous wounds, lung injuries, spinal cord injuries, etc. but have also been applied to cosmetic medicine (15, 16). In the exploration of the underlying mechanism, evidence suggested that ADSCs exert their functions through paracrine secretion of cytokines, lipids, and extracellular vesicles. These factors play crucial roles in mediating the therapeutic effects of ADSCs in different contexts.

Extracellular vesicles (EVs) represent a broad category that encompasses various types of vesicles released by living cells, carrying contents similar to those of their parental cells. EVs can be divided into exosomes, microvesicles, and apoptotic bodies based on their sizes. However, for functional purposes, EVs are commonly divided into two main groups: endosomes and exosomes (17). Exos are formed during the endosomal process, which consists of plasma membrane double invagination and the subsequent formation of multivesicular bodies (MVBs) containing multiple intraluminal vesicles (ILVs) inside (18). If the ILVs are secreted into the extracellular environment via MVB exocytosis, these ILVs are named exosomes. During this process, cytoplastic substances, including proteins, lipids, DNAs, mRNAs, and miRNAs, are encapsulated in the exosomes. Therefore, EVs are capable to conduct cell-to-cell communication without increasing the risks of rejection and malignant cell transformation (19).

Human ADSC-EVs are EVs extracted from the supernatant of ADSCs by ultracentrifugation (20). They can be identified by nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM) according to the phospholipid bilayer structure and size of ADSC-EVs (21). ADSC-EVs can also be verified by Western blot with a series of specific protein markers, such as CD9, CD63, TSG101, ALIX, and HSP90 (22, 23). As research on ADSCs continues to expand, there is a growing interest in studying ADSC-EVs. Compared to ADSC treatment, ADSC-EVs not only exhibit similar tissue repair capacities but also eliminate the concern of cellular viability, immune-mediated rejection, and malignant

transformation. Thus, ADSC-EVs could achieve a novel "cell-free therapy" in regeneration medicine and wound healing.

Inflammation in wound healing process

The roles of immune cells in wound healing

Wound healing is a complex process that usually takes weeks or months, depending on the depth of the wound and the homeostasis of its microenvironment. Several key cell components, including immune cells, fibroblasts, and Schwann cells, play crucial roles in the wound microenvironment during the healing response. The inflammation response is one of the earliest phases in wound healing and plays a vital role in orchestrating the entire healing process. In a healthy state, neutrophils are the earliest immune cell lineages to be recruited to the site of injury. Dovi et al. demonstrated that neutrophils are responsible for killing the microorganisms at the wound site (24). After this procedure, neutrophils undergo apoptosis and are ingested by macrophages to maintain cellular homeostasis. However, in pathological conditions such as diabetic wounds, excessive neutrophil accumulation at the healing site might contribute to delayed and impaired wound healing (25).

Unlike neutrophils, macrophages are essential in wound repair. The wound macrophages originate from both circulating monocytes and tissue-resident macrophage precursors. After the injury, monocytes from the circulation are rapidly recruited to the wound and simultaneously acquire macrophage phenotypic traits (26). Meanwhile, the macrophages' precursors mature and migrate to the injured site in response to CCR2/CCL2 and CX3CR1/CXCL1 chemokines (27, 28). The number of macrophages increases, accompanied by a decrease in the number of neutrophils in the wound. This is the result of the apoptosis and phagocytosis abilities of macrophages. Once neutrophils have executed their functions, they sequentially undergo apoptosis. Macrophages recognize and engulf these neutrophils through membrane-bound tumor necrosis factor-alpha (TNF-α) and CD36 receptors (29), becoming the dominant inflammatory cell type in the wound. Studies have confirmed that the depletion of macrophages led to a lessened rate of skin wound healing (30, 31). Especially, macrophage depletion during the initial phase of injury influences the subsequent healing response, including re-epithelialization and scar formation (32). During the transition from inflammation to proliferation, the number of macrophages gradually decreases. Some of the macrophages die at the wound site, and their debris is cleared out with the wound extracellular fluid. Other macrophages migrate to the near-draining lymph nodes (33) (Figure 1).

Macrophages exhibit distinct phenotypes, broadly classified as proinflammatory M1 and anti-inflammatory M2 phenotypes. The two phenotypes of macrophages are able to transition from one phenotype to another depending on the microenvironment (34, 35). Each subset has a spectrum of phenotypes and their symbolic markers. Even though this dichotomous classification has been found inconclusive and arbitrary (36, 37), the markers representing different subsets can still be able to give us

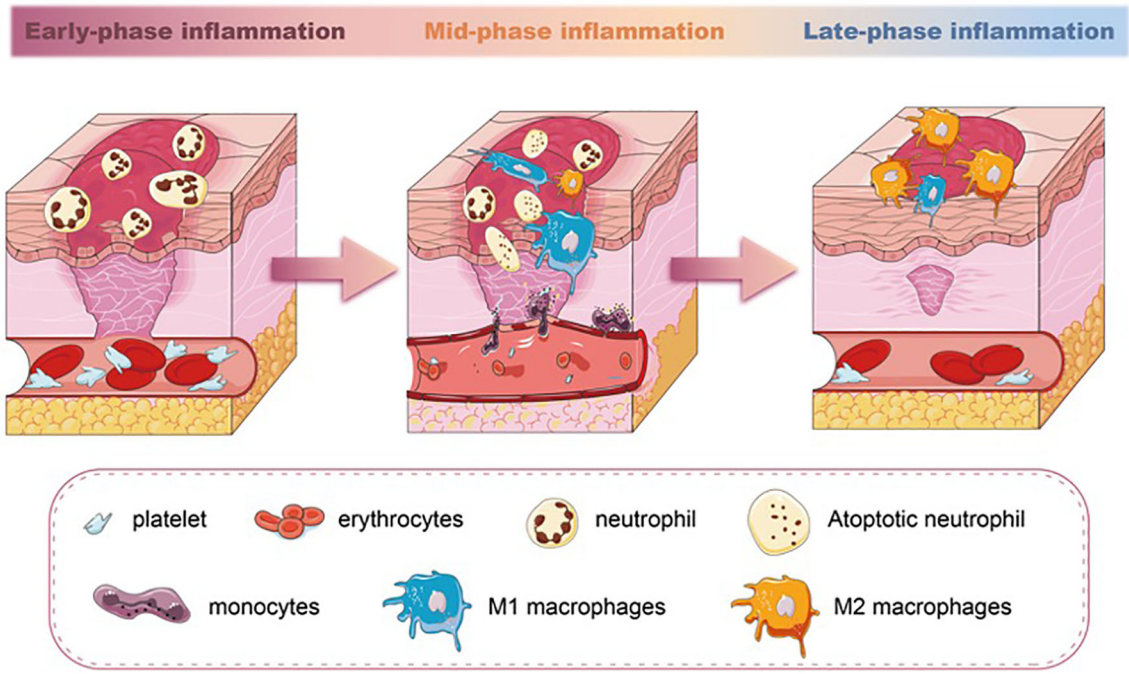

FIGURE 1
The different stages of inflammation and predominated population of immune cells in wound healing. Neutrophils are the earliest immune cell lineages during wound healing. M1 macrophages increase in the mid-phase of inflammation and phagocytose apoptotic neutrophils. In the late-phase inflammation of wound healing, a switch of M1 macrophages towards M2 macrophages is seen.

perspective to evaluate the inflammation severity of the tissue. During the initial stages of wound healing, both M1 macrophages and neutrophils are responsible for pathogen clearance. M1 macrophages also perform functions such as phagocytosis and the production of proinflammatory cytokines like TNF-α and interleukin-1 beta (IL-1β). As the wound progresses toward the resolution phase, M2 macrophages promote tissue remodeling, angiogenesis, and the production of anti-inflammatory cytokines, such as interleukin-10 (IL-10) and transforming growth factor-beta (TGF-β), with the highest levels occurring around day 7 of wound healing (38). M1 macrophages can exacerbate tissue damage if not regulated, and dysregulated or delayed M2 polarization can lead to chronic inflammation and impaired wound healing (39). The balance between M1 and M2 macrophages is crucial for successful wound healing. Studies have documented that the impaired switching of M1 to M2 macrophages in wounds is related to poor wound closure, impaired angiogenesis, and reduced collagen deposition (40–42). Therefore, the macrophages' polarization during the inflammatory phase directly (phagocytosis) or indirectly (cytokines and growth factor secretion) regulates the wound-repairing process (43) (Figure 1).

In addition to macrophages, other immune cell lineages and their roles have also been investigated during wound healing. For example, NK cells have been found to restore the balance of antimicrobial defense in the hypoxic injury site, thereby regulating the process of skin repair (44). However, another study indicates that NK cells play a role in the delay of wound healing due to proinflammatory cytokine production (45). Further research focusing on the functions of immune cell lineages in wound healing remains an interesting and ongoing topic of investigation.

The roles of proinflammatory and anti-inflammatory cytokines in wound healing

Numerous studies suggested that proinflammatory cytokines, including IL-1β, IL-6, and TNF-α, took part in the wound healing process, as their expression showed significantly elevated in the inflammation phase of the wound healing (46, 47). In contrast, there are also anti-inflammatory cytokines, such as IL-10, that respond to the inflammatory response. Proinflammatory cytokines are primarily produced by polymorphonuclear leukocytes and macrophages (48, 49), while anti-inflammatory cytokines are mainly produced by keratinocytes and mononuclear cells of the wound epidermis (50, 51). Despite the names of the two categories, both the appropriate levels of proinflammatory cytokines and the coordinated expression of anti-inflammatory cytokines are crucial for normal wound healing.

TNF-α is a potent proinflammatory cytokine released early in wound healing. It activates endothelial cells, promotes leukocyte recruitment, and induces the production of other cytokines and chemokines, thereby initiating the inflammatory response (52). Knockdown of TNF-α receptor p55-mediated signals has been shown to positively affect wound healing and reduce leukocyte infiltration (53). IL-6, secreted by various cell types, including macrophages and fibroblasts, plays a role in the early stages of wound healing. It stimulates immune cell activation, angiogenesis, and fibroblast proliferation, contributing to tissue repair (54). Gallucci et al. demonstrated that IL-6 deficiency in mice resulted in impaired cutaneous wound healing and a single dose of recombinant IL-6 was able to reverse the situation (55). IL-1β, predominantly produced by macrophages, promotes the recruitment of immune cells, angiogenesis, and the production of matrix metalloproteinases (MMPs) (56). On the other hand, the anti-inflammatory cytokine IL-10 is a potent anti-inflammatory cytokine produced by immune cells such as macrophages and T cells. It suppresses proinflammatory cytokine production, dampens immune cell activation, and promotes tissue repair and regeneration (57). Studies have demonstrated that fetal cells heal the wound scarlessly, while the absence of IL-10 in fetal cells resulted in scar formation (58). Also, overexpression of IL-10 in adult mice led to decreased inflammation response and wound healing acceleration (59). Taken together, proinflammatory and anti-inflammatory cytokines play distinct yet interconnected roles in wound healing. Proinflammatory cytokines initiate the inflammatory response and recruit immune cells, while anti-inflammatory cytokines resolve inflammation and promote tissue repair and remodeling. Achieving a balanced cytokine response is critical for effective wound healing.

Inflammatory effects of ADSC-EVs in wound healing

A moderate and appropriate inflammation in the wounding site is critical and a footstone for the whole healing course, as we have mentioned. Studies using different inflammatory markers all showed ADSC-EVs have the ability to alleviate the intensity of inflammation response at the injury wound site. In normal and healthy individuals, tissue undergoing wound healing showed significantly decreased infiltration of inflammatory cells when treated with ADSC-EVs (60–62). Furthermore, ADSC-EV administration has been found to substantially downregulate the expression of proinflammation cytokines IL-6 and TNF-α while upregulating anti-inflammation cytokine levels of IL-10 (62, 63). Zhou et al. showed that ADSC-EVs treatment either intravenously or by smearing on the wounding site can both remarkably reduce macrophage CD68 and M1-macrophage CD14 in the skin lesion (64). Similarly, Heo et al. observed a significant increase in the expression of the M2-macrophage marker CD206 in cells treated with ADSC-EVs (65). The polarization of macrophages toward an anti-inflammatory M2 phenotype promoted by ADSC-EVs suppresses the release of proinflammatory cytokines such as TNF-α and IL-1β while enhancing the secretion of anti-inflammatory cytokines such as IL-10, leading to a shift in the macrophage phenotype toward tissue repair and regeneration (66).

In pathological conditions, like diabetic individuals, the effectiveness of ADSC-EVs in ameliorating the inflammation response becomes more prominent. ADSC-EVs can significantly decrease IL-6, TNF-α, and IL-1β expression (67–70), suppress CD14 and CD68 expression levels (68), and remarkably increase CD206 and IL-10 expression levels (69, 71) in diabetic wound lesions.

Histological analysis has also indicated reduced immune cell infiltration, thus preventing the formation of diabetic ulcers (72, 73). In addition to the local effects, ADSC-EVs can reverse the systematic inflammatory condition in diabetes models. Jian et al. found ADSC-EV treatment can significantly decrease serum IL-6, IL-1β, and TNF-α levels (74). Other diseases that are not specifically for wound healing but are involved in similar phases of wound repairment, such as ischemic injury, nerve injury, and epithelial recovery, showed that ADSC-EVs can substantially modulate the balance of inflammation response. Studies revealed that ADSC-EVs impact a number of inflammatory pathways and cytokines in brain and nerve injuries (75, 76). Fewer neutrophils and macrophages infiltrated the fistula after ADSC-EV administration (77), and ADSC-EVs have been shown to modulate neutrophil function by reducing neutrophil activation, oxidative stress, and release of proinflammatory mediators. This modulation helps limit excessive inflammation and tissue damage at the wound site (78), increasing the number of CD206 counted to aid urethral defect recovery after using ADSC-EVs (79). Cao et al. also demonstrated that ADSC-EVs can significantly decrease the number of CD11b-positive macrophages and resolve inflammation after microneedle-induced injury (80).

How ADSC-EVs regulate wound inflammation

There is no doubt that ADSC-EVs can alleviate wound inflammation response. However, the mechanisms underneath this phenomenon are heat topics being discussed. EVs consist of three main substances: RNAs, proteins, and lipids (81). RNAs are the most widely investigated, as exosomes contain a spectrum of RNAs that conduct intercellular communication (Table 1).

TABLE 1 Regulation mechanism of ADSC-EVs in wound and injury inflammation.

Mechanism	Subtypes	Models	Phenotype/pathway	Ref.
Micro-RNA	miR-34a-5p, miR-124-3p, and miR-146-5p	Fibroblast scratch model	IL-6↓, TNF-a↓, IL-8↓, IL-10↑, TSG-6↑, TGF-β1↑; M2 macrophage polarization↑	(65)
	miR-132 and miR-146a	THP-1 cells treated with LPS; a full-thickness skin wound of diabetic nude mice	ROCK1/PTEN signaling pathway↓; M2 macrophage polarization↑	(82, 83)
	miR-21-5p	A full-thickness skin wound of a murine model; a full-thickness excision wound of a diabetic rat	M2 macrophage polarization↑, KLF6↓	(63, 84)
	miR-21-3p, miR-126-5p, and miR-31-5p↑; miR-99b, miR-146-a↓	A full-thickness skin wound of diabetic nude mice	PI3K/AKT signaling pathway↑	(70)
	miR-146a	HUVECs; CD14+ monocytes	IL-1β↓; M2 macrophage polarization↑; NF-κB signaling mediators IRAK1	(85, 86)
	miR-29a	A scald skin wound of mice model	Inflammatory cells infiltration↓	(87)
	miR-10a	Peritonitis in C57BL/6 mice	M1 macrophage polarization↓, NF-κB signaling pathway↓	(88, 89)
	miR-30d-5p	Acute ischemic stroke of rat *in vivo* model and oxygen- and glucose-deprived (OGD) primary microglia *in vitro* model	M2 macrophage polarization↑; IL-6↓, TNF-a↓, iNOS↓; IL-10↑, IL-4↑	(75)
	miR-146a-5p, miR-340-5p, miR-223-3p, miR-125b-5p, miR-16-5p, miR-149-3p, miR-105-5p, miR-181c-3p, miR-146b-5p, and miR-181a-5p	LPS-treated THP-1 cells	Selenium; IL-6↓, TNF-a↓, IL-8↓; IL-10↑, TGF-β1↑, CD206↑, CD163↑, Arg1↑	(90)
	miR-511-3p	Rats with spinal cord injury; PC12 cells under LPS damage	TRAF6/S1P/NF-κB signaling pathway↓	(91)
linc-RNA	lncRNA H19	A full-thickness skin wound of a mouse model	miR-19b↑, SOX9↑, Wnt/β-catenin signaling pathway↑	(61)
	lncRNA GAS5	LPS-treated HDF cells	TLR7 signaling pathway↓	(92)
	linc00511	Rat diabetic foot model	IL-6↓, TNF-a↓, IL-1β↓	(74)
	lncGm37494	*In vitro* and *in vivo* spinal cord injury	miR-130b-3p↓, IL-6↓, TNF-a↓, IL-1β↓; PPARγ↑, M2 macrophage polarization↑	(93)

(Continued)

TABLE 1 Continued

Mechanism	Subtypes	Models	Phenotype/pathway	Ref.
circ-RNA	circ-Snhg11	A full-thickness skin wound of diabetic mice	miR-144-3p↓, STAT3 signaling pathway↓; M2 macrophage polarization↑	(71)
	mmu_circ_0000250	A full-thickness skin wound of diabetic mice	miR-128-3p↓; SIRT1↑	(72)
	circ-Fryl	A sepsis-induced lung injury mouse model and an LPS-induced alveolar epithelium cells damage model	miR-490-3p↓; SIRT3/AMPK↑	(94)
Immunomodulatory characteristics	M2 macrophage polarization	Chondrocytes and synoviocytes	IL-1β↓, p65 nuclear translocation↓, NF-κB signaling pathway↓	(95)
	Neutrophil function	Rat diabetic foot model	Interferon-γ↓, M1↓, ROS↓, Nrf2↑; IL-6↓, TNF-a↓, IL-1β↓; inflammatory cell infiltration↓; EGR-1↑	(67, 96, 97)
	Treg-cell activation	A full-thickness skin wound of a mouse model	Interferon-γ↓, M1 macrophage polarization↓; EFGR↑	(98)
	Reduced B cell proliferation	Peripheral blood mononuclear cell	Proliferation↓, differentiation↓	(99)
	Reduced inflammatory cytokines	HUVECs under high glucose; a full-thickness skin wound of db/db mice model	ROS reduced through SIRT3	(100)
	Vimentin	A full-thickness skin wound of a mice model; human dermal fibroblasts	IL-6↓, TNF-a↓; IL-10↑	(62)

ADSC-EV RNAs modulate wound inflammation

RNAs are commonly divided into three types: messenger RNA (mRNA), transfer RNA (tRNA), and ribosomal RNA (rRNA). These three types of RNAs carry out almost all cellular regulatory processes. RNAs can also be divided into coding RNA (cRNA) and noncoding RNA (ncRNA). The ncRNA can be subdivided into long ncRNAs (lncRNAs) and microRNAs (miRNAs) according to their size. Circular RNAs (circRNAs) are unique from other RNAs due to their 5′ and 3′ ends bonding together and creating a loop (101). Studies confirmed that lncRNAs, miRNAs, and circRNAs are all able to regulate cell physiological functions and realize intercellular communication (102). Recent research found ADSC-EVs to be a vital source of ncRNAs to engage in the inflammation response in wound healing (103). Heo et al. reported that miR-34a-5p, miR-124-3p, and miR-146-5p expressed in ADSC-EVs attenuated IL-6 expression and induced M2-phenotype macrophage polarization in the fibroblast scratch model (65). Further investigation suggested that miR-132 and miR-146a improved the anti-inflammatory responses through ROCK1 and PTEN signaling pathways in THP-1 cells (82). Especially, miR-132 from ADSC-EVs is found to induce M2 polarization in diabetic skin flaps (83). Li et al. observed that ADSC-EV treatment of diabetic foot ulcer wounds could elevate miR-21-5p levels in macrophages, induce M2 polarization, and suppress Keuppel-like factor 6 (KLF6), which has been reported to enhance the inflammatory phenotype in macrophages (63, 84). Wang et al. indicated that miR-21-3p, miR-126-5p, and miR-31-5p upregulation and miR-99b and miR-146-a downregulation in hypoxic ADSC-EVs regulated immune response via phosphatidylinositide 3-kinases (PI3K)/protein kinase B (AKT) signaling pathway, thus accelerating wound healing in the diabetic model (70). Water et al. studied the miR-146a in the inflammatory responses exhibited by endothelial cells. miR-146a secreted by ADSC-EVs is capable of inhibiting inflammatory activation by IL-1β (85), and miR-146 might participant in M2 polarization by targeting NF-κB signaling mediators IRAK1 (86). Yuan et al. reported that miR-29a overexpression in ADSC-EVs could reduce inflammatory cell infiltration and subsequentially forbid keloid formation after skin burn (87). Baglio et al. screened the top 5 miRNAs in ADSC-EVs using small RNA sequencing and found miR-486-5p, miR-10a-5p, miR-10b-5p, miR-191-5p, and miR-222-3p accounted for almost half of the miRNAs (88). Later, Njock et al. confirmed that among these five miRNAs, miR-10a was able to suppress M1 activation by targeting the NF-κB signaling pathway in peritonitis (89). Treatment with ADSC-EVs overexpressed with miR-30d-5p showed a positive effect on M2 polarization, decreased expression of TNF-α, IL-6, and iNOS, and increased IL-4 and IL-10 levels after acute stroke (75). In the spinal cord injury model, hypoxia-pretreated ADSC-EVs enriched miR-511-3p and ameliorated the inflammation response via the TRAF6/S1P/NF-κB pathway (91). Despite the fact that overexpressing miRNAs in the ADSC-EVs can directly verify their roles in enhancing ADSC-EV function in inflammatory regulation of wound healing, Heo et al. found that pretreatment of selenium in ADSC during culture could also improve the inflammatory cytokines through miRNAs. Selenium-treated ADSC-EVs significantly suppressed the inflammatory response in THP-1 cells

due to the elevated miRNAs of miR-146a-5p, miR-340-5p, miR-223-3p, miR-125b-5p, miR-16-5p, miR-149-3p, miR-105-5p, miR-181c-3p, miR-146b-5p, and miR-181a-5p (90).

The circRNAs attenuating inflammation reaction in wounding lesions is supported by circ-Snhg11. Hypoxic treatment significantly increased circ-Snhg11 contents in ADSC-EVs and promoted M2 polarization by inhibiting miR-144-3p expression and the STAT3 signaling pathway in skin wounds (71). The mmu_circ_0000250 in ADSC-EVs was verified to promote miR-128-3p absorption, which can induce inflammatory response, and subsequently increase SIRT1 level and improve hyperglycemic-induced inflamed environment in diabetic wound sites (72). Shen et al. studied the overexpression of circ-Fryl in ADSC-EVs, regulating miR-490-3p to attenuate inflammation-related injury via SIRT3/AMPK pathway (94). Qian et al. studied lncRNA H19 in ADSC-EVs targeting miR-19b and SOX9 to activate the Wnt/β-catenin pathway, thus promoting wound healing and alleviating inflammation responses in skin wounds (61). lncRNA GAS5 in ADSC-EVs was found to also decrease inflammatory response in human dermal fibroblasts (HDF) by downregulating the TLR7 signaling pathway (92). Qiu et al. found that linc00511 overexpression in ADSC-EVs decreased serum IL-6, IL-1β, and TNF-α levels in the diabetic foot model (74). ADSC-EVs overexpressed with lncGm37494 downregulated its downstream target miR-130b-3p and subsequently upregulated PPARγ expression, which is a miR-130b-3p target, shifting M2 polarization and downregulating TNF-α, IL-6, and IL-1β (93).

ADSC-EVs modulate immunomodulatory characteristics

The immunomodulatory characteristics of ADSC-EVs are presented by the influence of immune cell activities and non-ncRNA-related macrophage polarization. ADSC-EVs have been shown to influence macrophage polarization, shifting the balance from a proinflammatory M1 phenotype towards an anti-inflammatory M2 phenotype, indicating the anti-inflammation action (104). The underpinning mechanism, besides the changes of miRNA contents in ADSC-EVs, might be due to the changed proinflammatory cytokine levels in the microenvironment. Proinflammatory cytokines, including IL-1β, IL-6, and TNF-α, can classically activate the M1 phenotype, and suppressing them can reverse M1/M2 switching (105). Cavallo et al. confirmed the mechanism by which ADSC-EVs reverse the proinflammatory microenvironment and induce the expression of anti-inflammation cytokines. After pretreated with IL-1β, ADSC-EV administration could prevent p65 nuclear translocation and NF-κB signaling pathway activation. The decreased expression of IL-1β, IL-6, and TNF-α was consistent with the blockage of the proinflammatory pathway. On the other hand, ADSC-EVs significantly increased IL-10 and IL-4 levels, inducing an alternative action of M2 macrophages (95) (Figure 2).

ADSC-EVs have been found to modulate neutrophil function, leading to the suppression of their activation and subsequent reduction in proinflammatory responses. Neutrophils generate reactive oxygen species (ROS) as part of their antimicrobial defense mechanism. However, excessive ROS production can cause tissue damage and impair wound healing. ADSC-EVs have been shown to reduce oxidative stress in neutrophils by regulating the production of ROS and restoring redox balance (96). Li et al. proved that overexpression of Nrf2 in ADSC-EVs decreased the detoxification effect of ROS under hyperglycemic conditions and proinflammatory cytokines IL-1β, IL-6, and TNF-α levels (67). As we mentioned above, excessive or uncontrolled neutrophil infiltration can prolong inflammation and impair wound healing. ADSC-EVs have been shown to modulate neutrophil migration by influencing the expression of adhesion molecules and chemotactic factors. This regulation helps maintain the appropriate balance of neutrophils at the wound site, preventing excessive tissue damage. Sun et al. demonstrated that ADSC-EVs influenced inflammatory cell infiltration through the expression of early growth response factor-1 (EGR-1) (97). A study employed by Parvanian et al. reported that vimentin-knockout ADSC-EVs showed an extended inflammation phase in sounds and failed to recruit inflammatory cells to the wound site. The absence of vimentin in ADSC-EVs upregulated proinflammatory cytokines IL-6 and TNF-α, and downregulated anti-inflammatory cytokine IL-10 (62). In conclusion, ADSC-EVs exhibit a variety of mechanisms by which they suppress neutrophil activation and modulate inflammation during wound healing.

ADSC-EVs also possess other immunomodulatory properties and can suppress the activation and function of various immune cells involved in wound healing. Nosbaum et al. showed that ADSC-EVs promoted T-regulatory cell activation and facilitated wound healing by inhibiting interferon-γ production and M1 macrophage accumulation in an EFGR signal-dependent manner (98). B cells are involved in antibody production and can contribute to inflammation in certain contexts (106). ADSC-EVs have been reported to modulate B-cell responses, leading to reduced B-cell proliferation and antibody production (99). Zhang et al. found that ADSC-Exos significantly decreased inflammatory cytokines IL-6, TNF-α, and MCP-1 levels by reducing ROS production and protecting mitochondrial function through SIRT3 (100). All these immune modulations help maintain an appropriate balance of immune cells and inflammation during wound healing. Taken together, ADSC-EVs have emerged as potent regulators of wound inflammation through various mechanisms. These include the modulation of macrophage polarization, suppression of neutrophil infiltration, and immune cell suppression. Understanding the intricate mechanisms underlying ADSC-EV-mediated regulation of wound inflammation will pave the way for the development of targeted therapies and improved wound healing outcomes. Further research is needed to uncover additional mechanisms and optimize the use of ADSC-EVs as therapeutic interventions in the context of wound healing.

Application of ADSC-EVs

The therapeutic use of ADSC-EVs shows a promising future as a “cell-free-therapy” approach in wound healing. Nevertheless,

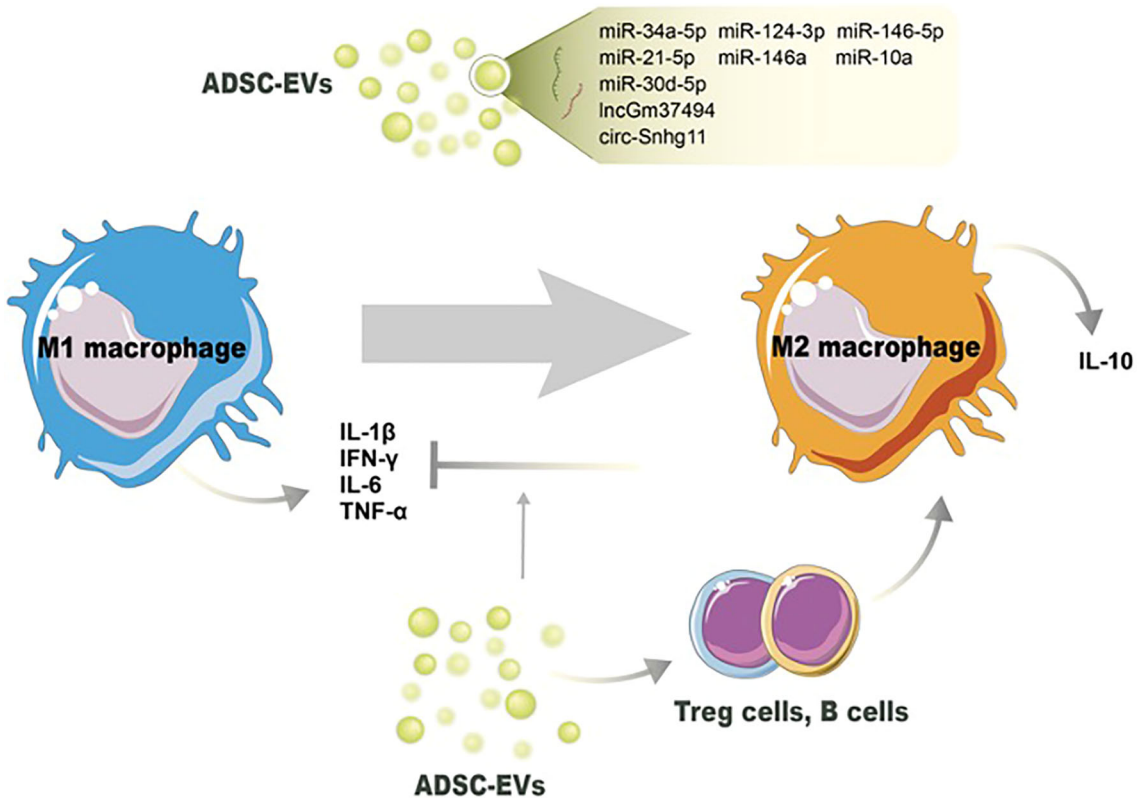

FIGURE 2
Potential mechanisms of ADSC-EVs regulating the switch of M1 macrophages toward M2 macrophages. Noncoding RNAs in ADSC-EVs induce M2 macrophage polarization and decrease the number of M1 macrophages. ADSC-EVs also modulate other immune cells' activation to switch macrophage phenotype and reduce proinflammation cytokines production.

ADSC-EVs usually lead to rapid clearance due to the high metabolic activity of wounds. Therefore, investigators keep making an attempt to combine biotechnology and ADSC-EVs that enable optimized retention and release profiles of ADSC-EVs. Hyaluronic acid (HA) and hydrogels were used as exosome immobilizers as well as ideal wound dressing. By combining HA and ADSC-EVs, a distinct decreased in inflammatory cell infiltration was observed in the wound area (107). Hydrogels are three-dimensional networks that can encapsulate and deliver ADSC-EVs to the wound site. They provide a supportive environment for cell growth, facilitate the sustained release of ADSC-EVs, and enhance their therapeutic effects. ADSC-EV-loaded hydrogels have been shown to alleviate inflammation, promote tissue regeneration, and improve wound healing outcomes. Some hydrogels with modifications are capable of alleviating inflammation responses in wound healing themselves. Silva et al. investigated a thermoresponsive gel-embedded ADSC-EV formulation. The gel itself could significantly reduce neutrophil and macrophage infiltration in the necrotic area, while combining gel and ADSC-EVs could further decrease the immune cell burden (77). Zhou et al. developed a thermosensitive hydrogel, Pluronic F-127, and found this material not only reduced IL-6 level in the cutaneous wound but also significantly reduced inflammation (decreased IL-6, TNF-α, CD68, and increased CD206) when encapsulated ADSC-Exos (108). We also developed a methacryloyl-modified gelatin (GelMA) hydrogel with catechol motifs of dopamine (GelMA-DOPA) and loaded it with ADSC-EVs to treat diabetic wounds. The GelMA-DOPA can relieve IL-6 expression in lesions, and GelMA-DOPA-EVs lower the inflammation burden more efficiently (109).

Another material serving as a structural framework for cell attachment and tissue regeneration is scaffolding. Incorporating ADSC-EVs into scaffolds can enhance their regenerative capacity and immunomodulatory effects. ADSC-EV-loaded scaffolds have been demonstrated to reduce inflammation, enhance angiogenesis, and promote wound closure. A study showed that a 3D scaffold engineered from decellularized cardiac tissue was an ideal support material for ADSC-EVs to employ anti-inflammatory functions in ischemic myocardial infarction (110). A more biocompatible scaffold of the human acellular amniotic membrane (hAAM) revealed effective inflammation regulation functions. Xiao et al. found that hAAM loaded with ADSC-EVs could enhance inflammatory regulation in diabetic wounds (111). Similarly, nanofibers are also considered a desirable material for ADSC-EV bedding due to their high surface area-to-volume ratio, mimicking the extracellular matrix structure. The research found that phosphoethanolamine phospholipid-grafted poly-L-lactic acid micro/nanofibers (DSPE-PLLA) not only could carry ADSC-EVs and release them in a slow manner, but also showed M2 macrophage polarization with increased expression of Arginase1, CD206, and IL-10 (112). These data present strong evidence for the

prospective future of ADSC-EVs in alleviating inflammation responses and promoting wound healing. The association of different biomaterials and ADSC-EVs cannot only preserve their activities and function but also extend their release time, showing promising clinical pharmacotherapy for wound healing. However, blinded, randomized, placebo-controlled, and a larger number of prospective clinical trials need to be carried out to verify the safety and effectiveness of ADSC-EVs in the future.

Conclusion

A considerable number of clinical patients are suffering from prolonged wound healing due to an imbalanced inflammation response, especially under pathological conditions. Inappropriate immune responses in injury lesions affect the whole healing process. Despite the number of studies focusing on the efficacy of ADSC-EV mechanism of alleviating the inflammatory response to promote wound healing, modified applications targeting these mechanistic factors are still vacant. ADSC-EVs obtain the analogous functions of ADSCs and act as a carrier to conduct an action on target cells at the transcription and translation level to transmit proteins, messenger RNA, microRNAs, circ-RNAs, lincRNAs, and cytokines via paracrine manners. Based on these biological characteristics, ADSC-EVs modulate and control the cytokines production, macrophage phenotype polarization, and immune cell lineage infiltration through their contents. Furthermore, with the combination of newly developed bioengineered materials and media, the effect of ADSC-EVs controlling immunomodulation can be persistent and even enhanced in some types of materials. It is undeniable that the significance of ADSC-EVs' effects has presented a new opportunity for wound healing in clinical practice. Nevertheless, further drug development focusing on the targeted mechanism pathway of ADSC-EVs *in vivo* and *in vitro* is needed to expand our clinical options, and clinical trials of ADSC-EVs are needed for wound healing.

Author contributions

QJ and ZZ conceptualized and validated the manuscript. QJ drafted the manuscript. YW, HZ, YC, and ZZ reviewed and edited the manuscript. QJ and HZ drew the figures in the manuscript. QJ and ZZ obtained the funding. ZZ supervised the project. QJ and ZZ are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. All authors have read and agreed to the published version of the manuscript, accepted responsibility for the entire content of this manuscript, and approved its submission.

Funding

This work was supported by grants from Sichuan Natural Science Foundation (2022NSFSC0717, 2022NSFSC1579).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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