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Review

Mechanisms governing the therapeutic effect of mesenchymal stromal cell-derived extracellular vesicles: A scoping review of preclinical evidence

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ABSTRACT

Compelling evidence supports the therapeutic benefit of extracellular vesicles (EVs). EVs are nanostructures with a lipid bilayer membrane that are secreted by multiple cells, including mesenchymal stromal cells (MSCs), as means of cellular communication. MSC-EVs, resembling their MSC origin, carry protected immunomodulatory and pro-regenerative cargoes to targeted neighboring or distant cells and tissues. Though treatments focused on MSC-EVs have emerged as greatly versatile approaches to modulate multiple inflammatory-related conditions, crucial concerns, including the possibility of increasing therapeutic outcomes by pre-conditioning parental MSCs or engineering derived EVs and clarification of the most relevant mechanisms of action, remain. Here, we summarize the large amount of preclinical research surrounding the modulation of beneficial effects by MSC-EVs.

1. Introduction

The potential of mesenchymal stromal cells (MSCs) in regenerative medicine has been explored extensively over the past few decades. Multipotent MSCs were originally identified from mouse bone marrow according to their differentiation plasticity and hematopoietic-supportive function 50 years ago [1,2]. The initial interest in tissue replacement and regeneration by MSCs was mainly based on their self-renewing capabilities and differentiation plasticity but has dwindled with low cell engraftment and short lifespan of cells after infusion [3]. Instead, their tissue supportive functions and immune modulation capabilities prevail as the currently accepted mechanism of action, which has been characterized for a variety of human adult and perinatal tissue sources [4]. Specifically, MSCs are recognized as hypoimmunogenic or 'immune privileged' cells with the possibility of being transplanted across major histocompatibility barriers as advanced therapeutic medicinal products in the context of multiple human indications [5–7]. However, MSC-based therapies remain a challenge due to relatively poor/limited success in clinical trials [3,8], and the size of cells is associated with a risk of pulmonary thromboembolism after in vivo infusion [9,10].

Remarkably, the tissue-supportive functions of MSCs are thought to be conferred by a number of secreted factors and double-layer phospholipid membrane vesicles termed extracellular vesicles (EVs), rather than a direct contribution of their differentiation ability [11–14]. Thus, MSC-EVs are envisioned to be a valuable alternative to parental MSCs because they are neither replicative nor respond to the host microenvironment (e.g., the inflammatory milieu) once delivered in vivo. They also exhibit low alloreactivity, easy usage and storage, and better biodistribution [14]. Specifically, despite MSC-EVs infusions can be partially entrapped in filter organs, they can be engineered and would have a better chance to reach targeted tissues because of their small size, reducing the possibility of capillary obstruction when infused and, thus, more likely to transfer their molecular cargo associated functions [15,

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16].

In light of the large amount of existing literature, what evidence supports the potential exhibited by MSC-EVs? Is their benefit based on well-established or biologically relevant properties, or on speculative assumptions or unproven expectations? As a rule, determining the strength or level of evidence for the potential risks and efficacy of novel therapeutic interventions or agents is important in making the decision to advance to human clinical trials. Thus, we provide a summary to map the pre-clinical research surrounding the therapeutic effect of MSC-EVs. In particular, we examine the progress using in vitro and in vivo models studying whether MSC-EVs are powerful effectors of pivotal MSC functions and therapeutic benefit. We also present relevant issues in the development of more effective therapies based on MSC-EVs, including specific bioengineering and pre-conditioning of parental MSCs or derived EVs.

2. Major remarks on EVs

EVs include a diverse array of double-layer phospholipid membrane vesicles used by most cells to encapsulate and transfer biologically active molecules, such as lipids, proteins, and nucleic acids, as a means of communication and regulating multiple patho/physiological processes [14,17,18]. In this regard, EVs are increasingly being identified as fingerprints of originating cells because EV cargo is closely dependent on the lineage of the origin cell and specifically represents the state of the parental cell; thus, they are also considered a potential source of circulating biomarkers [19]. For example, the content of EVs depend on different conditions, including cell viability, stage of activation, infection, stress, and neoplastic transformation [20].

2.1. EV classification

The classification of EVs remains controversial, and categorization based on unique parameters should proceed with caution [21,22]. EVs are currently distributed into three major categories according to their biogenesis, though other EV subpopulations are possible when classifying for specific cell types or pathological conditions (e.g., *microparticles* from platelets or *oncosomes* from tumor cells). The first major category is *exosomes*, which are intraluminal vesicles with a diameter between 30 and 150 nm that are formed by the internal budding of endosomal membrane during maturation, referred to as intracellular multivesicular bodies (MVBs). Specifically, the use of 'small EVs' to designate exosomes is becoming more common. The second major category are *microvesicles*, which sprout directly from the plasma membrane and are released into the extracellular space. They have a wider size range than exosomes, with a diameter of 50 nm up to 1 μm. They can also be referred to as *ectosomes*. The third major category, *apoptotic bodies,* have a diameter of 1–5 μm and are externally secreted following the apoptotic cell disassembly process [23–26]. Recent discoveries have also unveiled *exomeres*, non-membranous nanovesicles $<$ 50 nm in size, as a novel type of EV [19,27]. Due to the existence of overlapping physicochemical characteristics, the majority of EV preparations include a mix of the different EV subtypes. In this regard, though many efforts are directed towards demonstrating sufficient EV subtype enrichment [19], researchers must keep in mind the various strengths and weaknesses of each extraction procedure and the unmistakable fact that there is a lack of pan-specific and subpopulation-specific EV markers for the results to be sufficiently comparable and robust. For this reason, the International Society of Extracellular Vesicles has made numerous efforts in agreeing on a common minimal consensus on EV characterization and reporting [26,28].

2.2. EV composition and biological relevance

In terms of biological functions, EVs actively participate in regulating many biological processes, including cell motility and adhesion, membrane fusion, intracellular signal transduction, antigen presentation, protein trafficking, and MVB biogenesis, as well as patho/physiological conditions, such as coagulation, pregnancy, male reproduction, bone calcification, liver homeostasis, tumor progression, immunomodulation and acquired immunity, and tissue repair and regeneration [14,15,29–32]. Many of these findings have been collected in vitro or in vivo with EVs that were generated in the laboratories and administered to animals.

Theoretically, EVs can exert similar functions as parental cells due to the cells having the required machinery to shuttle part of the content that is bound to the external membrane or inside of EVs, where it is protected from the highly modifiable external microenvironment. The protection that EVs confer to their effector molecules is the key to maintaining the integrity of their cargo and associated functions over time. Thus, EVs are capable of transferring information between neighboring or distant cells and can influence the function of target cells [33,34]. In general, EVs transport several types of bioactive compounds, including metabolites; lipids and RNA species, mostly small non-coding microRNAs (miRNAs) that may post-transcriptionally regulate the expression of hundreds of target genes; or cytosolic and transmembrane proteins in appropriate functional orientations, resembling the molecules present in the parental cells [14,32,33,35–37]. In this sense, critical aspects related to EV research include well-planned approaches to understand the functional diversity of EVs. For example, in a given mixture of EVs, each EV subset may carry different molecular cargoes and lead to specific outcomes that could lead to either positive or negative, synergistic, or even neutral effects due to compensatory phenomena. These considerations are especially relevant when deciding the degree of purity and corresponding isolation method to be employed to avoid cross-contamination with co-precipitated molecules or between EV categories to diminish the risk of biased results and enhance further use of the resulting EV preparations.

2.3. EV enrichment methods

The isolation of EVs raises several technical challenges, such as to successfully separate EVs or even EV subtypes from non-vesicular particles or protein aggregates of similar size, to maintain EV's physical properties, and to allow downstream analysis of the preparation [38]. Currently, EVs are isolated following distinct laboratory methods that range in different degrees of specificity and recovery [28]. Specific isolation methods include differential ultracentrifugation (dUC), flotation separation, precipitation-based procedures, immunoaffinity isolation using antibodies coupled with magnetic beads, flow cytometry, size-exclusion chromatography (SEC), microfluidics technology, ultrafiltration, and field-flow fractionation [38,39]. The most common technique for EV isolation has been dUC, which is based on the application of high centrifugation force to induce the sedimentation of co-solutes [40,41]. An important disadvantage of this methodology is the high disruption of EV integrity, the formation of aggregates, and co-precipitation of unwanted heterogeneous mixtures of overabundant soluble proteins and EVs due to high pressure [42–44], in addition to the isolation of all EVs regardless of their subtype or parental cell. Alternative isolation strategies based on density gradient flotation can be applied following dUC in order to enhance EV purity. However, these approaches are expensive, time-consuming, and have low performance. On the other hand, precipitation methods afford high yields and are far more user-friendly and less time-consuming, but result in lower purity EV samples and can affect the cell viability of target cells due to precipitating agent carryover [45–47]. Immunoaffinity-based methods, which are based on the capture of EVs expressing a specific marker on their surface, can be more stringent for isolating distinct EV subcategories. Nevertheless, downstream functional assays performed with these EVs must consider the influence of the capturing antibodies and would be biased towards the EVs bearing the chosen marker. Thus, given the lack of a pan-EV marker, a combination of capturing markers is

recommended, most commonly the combination of CD9, CD63, and CD81 [48,49].

SEC is an increasingly common EV isolation method that minimally alters EV integrity and subsequent applications. In particular, SEC is based on the distinct elution velocities of differently sized particles carried by a mobile phase through a porous polymer, constituting the stationary phase (gel filtration matrix or resin) of the SEC column. Small particles, including soluble proteins, are slowed by entering the pores of the polymer and elute later than EVs. Conversely, because EVs are bigger than the polymer's pores, they travel more quickly along the column and are collected first. In terms of advantages, SEC removes most of the contaminating soluble proteins or particles from in vitro cultured cell-conditioned medium and biological fluids, leading to purer EV-based preparations, as demonstrated by low protein content and electron microscope evaluation [38]. Notably, when EVs are isolated from serum or plasma samples, the resulting preparation may also contain lipoproteins, and SEC can then be complemented by other techniques, such as anion exchange, if they need to be removed [50–52].

Furthermore, single EV analysis by flow cytometry, which allows the separation of single particles based on their protein surface expression profile, still needs to be optimized and standardized because of the small size and low refractive index of EVs [53], whereas microfluidic technology for high throughput analysis and low sample input, and field-flow fractionation of EVs for high sample input and size resolution, require expensive, specialized equipment [54]. Specifically, tangential field-flow fractionation (TFF) can be used either as a pre/post-processing method to concentrate, or for buffer exchange instead of ultrafiltration. Ultrafiltration is an easy, non-time-consuming separation technique for sample concentration based on dead-end filtration through semi-permeable membranes with a defined pore size or molecular weight limit [55,56], whereas during TFF, the fluid is pumped tangentially along the surface of the membrane. As the applied pressure forces a portion of the fluid and small molecules through the membrane, larger particles are retained and swept along by the tangential flow; thus, they do not build up at the membrane surface, making TFF an ideal technology for large sample processing. In addition, as particles within the flow are separated according to their hydrodynamic radius, TFF allows for highly precise purification of distinct EV populations according to size [57,58].

3. MSC-EVs: new biologicals for immune regulation

MSC-EVs are thought to be central to novel therapies that modulate the host immune response. In general, adding EVs derived from different MSC sources (i.e., bone marrow, adipose tissue, and umbilical cord) to stimulated T lymphocyte culture prevents T-cell stimulation and reduces the levels of pro-inflammatory cytokines, including IL-2, IL-6, IL-12p70, and IFNγ [59–63]. Thus, MSC-EVs have the potential to act through a variety of pathways to induce an anti-inflammatory response.

3.1. Mechanisms of action governing immunomodulation by MSC-EVs

A number of in vitro and in vivo studies have contributed to our understanding of the mechanisms of action by which MSCs and derived EVs regulate inflammation in a variety of inflammatory diseases. Though studies do not agree on a universal enzyme or regulatory mechanism, probably due to the great heterogeneity in MSC sources and lack of standardized EV isolation methodology, as well as the diverse disease-specific pathways, they have shown a common potential to modulate inflammation and have beneficial effects. Here, we summarize the most representative pre-clinical evidence of the mechanisms through which MSC-EV-mediated immunomodulation occurs, generally promoting a shift in the pro-inflammatory milieu and functional changes in recipient immune cells (i.e., monocytes/macrophages, dendritic cells [DCs], T cells, B cells, and natural killer [NK] cells; Table 1). One of the specific EV cargoes observed to exert an important role in modulating the inflammatory response corresponds to miRNAs (Fig. 1). However, EVs isolated using standard preparations do not enclose a great number of miRNA copies (between 0.02 and 1 molecule per EV) [64–66], and MSC-EVs probably owe their functionality to proteins rather than the RNA [67]. Proteomic characterization studies support the importance of protein cargo as the main driver of angiogenesis induction [68] or inflammation regulation [69], but little few pre-clinical evidence exists [70]. Moreover, others have reported that intracellular pathways may be activated by direct surface contact of EV-associated proteins [71,72]. This is underpinned by the observation that a small amount of EVs is enough to affect target cell function. One explanation for this controversy is that a large proportion of studies (76.2%) have used dUC as a strategy for MSC-EV isolation with promising beneficial results (Fig. 2). An important criticism of this technique is that the active substance may not be contained in the EVs, but swept during the dUC process and administered in pre-clinical studies, making it difficult to determine the effective molecule and perform standardized therapy in patients.

One of the main targets of miRNA present in MSC-EVs is the pro-inflammatory transcriptional regulator nuclear transcription factor-κB (NF-κB), which is responsible for the synthesis and release of inflammatory factors [73]· Thus, its inhibition reduces the pro-inflammatory response. For example, Zhang et al. demonstrated that human MSC-EVs reduce inflammation in a mouse model of cecal ligation and puncture (CLP) through the up-regulation of miR-146b levels. The authors transfected human renal tubular epithelial cells (HK-2) with miR-146b mimics and inhibitors to study the regulatory role of miR-146b. When human MSC-EVs were added, they observed increased levels of miR-146b in kidney tissue, which targeted and reduced interleukin (IL)-1 receptor-associated kinase (IRAK1) expression, inhibiting the activation of NF-κB [74]. Similarly, MSC-EV-derived miR-27b inhibited the recruitment of NF-κB and JMJD3 at gene promoter regions, decreasing the expression of pro-inflammatory genes. In accordance, the production of the corresponding cytokines was reduced in LPS-treated bone marrow-derived macrophages and a septic mouse model [75]. miR-22-3p was also involved in the attenuation of acute lung injury by MSC-EVs; MSC-EV administration enhanced miR-22-3p expression and decreased FZD6 (target gene of miR-22-3p) expression. Furthermore, in vitro transfection of LPS-treated lung cells for either the overexpression of miR-22-3p or depletion of FZD6 reduced the inflammatory reaction, oxidative stress response, and apoptosis rate, and increased cell proliferation. In an acute lung injury rat model, EVs reduced apoptosis and FZD6 and NF-κB expression, suppressing lung damage [76]. In the context of spinal cord injury, MSC-EVs are able to reduce apoptosis and pro-inflammatory cytokines (TNF-α and IL-1β). The suggested mechanism of action is the inhibition of PTEN and NF-κB signaling via miR-181, which is enriched in MSC-EVs, as demonstrated by the overexpression of PTEN in vitro and in vivo, which reverts the miR-181c protective effect [77]. Moreover, in vitro experiments demonstrated that MSC-EVs can attenuate LPS-induced acute lung injury via the Nrf-2/ARE and NF-κB signaling pathways [78] and alleviate inflammation and apoptosis by inhibiting the high mobility group box 1 (HMGB1)/NF-κB pathway in smoke inhalation lung injury [79] and spinal cord injury [80]. NF-κB activity can be inhibited by sirtuin 1 (SIRT1) protein, with increased levels after administration of EVs in a sepsis-induced acute kidney injury mouse model [81].

The Toll-like receptor 4 (TLR4)/CD14 signaling pathway has been suggested to be an inflammatory-driving mechanism modulated by MSC-EVs. The TLR4/CD14 signaling pathway is upstream of NFκB activation, signaling through ERK1/2, p38, and JNK, members of the mitogen-activated protein kinase (MAPK) family and regulators of activation protein 1 (AP-1). MSC-EVs prevent the degradation of NFκB inhibitor IκBα and suppress the phosphorylation of MAPK family members to inhibit NFκB- and AP-1-dependent transcription of pro-inflammatory genes [82]. However, MSC-EVs have been found to downregulate TLR4-mediated inflammation by enhancing IL-10 levels and reducing TNF-α and IL-1β levels [83]. Moreover, MSC-EVs can

Table 1
Summary of pre-clinical studies using extracellular vesicles derived from MSC for immune cell regulation and their mechanism of action.

	Disease model (specie)	MSC source (specie)	EV term	EV isolation method	Controls	EV characterization (method)	EV dose, cell equivalent	Injections, Interval, Route	EV tracking	Disease outcome	Effect	MoA	Ref.
GvHD	GvHD (mouse)	BM (human)	EV	UC 10 kg	EV from native NHDFs	CD63+, CD81+ (WB), tunable resistive pulse sensing (qNano), albumin (Bradford), 110 nm (TEM)	200 μL EV derived from 2×10e6 cells/kg	1, IV	ND	Increased survival rate, reduced pathological damage in multiple organs	Increase in CD62L+ CD44- naive T cells. CD4+ CD25+ Foxp31+ Treg population preservation. Suppression of CD3+ T cell activation. Upregulation of miRNA-125a-3p	Suppression of T cells proliferation via miRNA-125a-3p	[119]
	GvHD (mouse)	BM (human)	Exosome	UC 10 kg + 110 kg	EV from native NHDFs	CD9+ CD63+ CD81+ (WB), 100 nm (TEM)	100 μL (1 μg/μL)/week	6, 1w, IV	PKH26 EV labeling (in vitro)	Prolonged survival and less clinical and pathological scores. Less fibrosis in the skin, lung and liver	Reduced pro-inflammatory cytokines (IL-17A, IL-21, IL-2) and increased anti-inflammatory cytokine IL-10. Less CD4+T cells infiltration. Reduction of RORγt (Th17 cells) and STAT3 (RORγt expression and IL-21 production). Increased Foxp3 (Treg differentiation)	Immunomodulatory effects via the inhibition of Th17 and induction of Treg. Inhibition of STAT3 activation	[89]
Sepsis	Sepsis CLP (mouse)	BM (mouse)	Exosome	UC 100 kg + gradient	EV from native MSCs	CD29+, Sca-1+ and CD34- (IF), dynamic light scattering, albumin (BCA), CD63+, CD81+ (WB),	2 μg/g body weight in 150 μL of incomplete culture medium	1, IV	PKH26 EV labeling	Cardiac dysfunction attenuation and animal survival improvement	Inhibition of TNF-α, IL-1β, and IL-6 secretion from macrophages. Cardioprotection.	Cardioprotection by higher levels of miR-223 in exosomes, which could be delivered to cardiomyocytes, resulting in down-regulation of Sema3A and Stat3	[88]
	Sepsis CLP (mouse)	BM (mouse)	Exosome	UC 110 kg	EV from mouse fibroblasts	< 100 nm (TEM), 80 ± 15 nm (NTA), CD63+ CD81+ TSG101+ calnexin- (WB)	30 μg/mouse MSC-EXO-miR-27b-mimic,	1, IV	PKH67 EV labeling	Inhibition of sepsis occurrence. Decreased liver, kidney, and lung injury scores. Higher survival.	TNF-α, IL-1β, and IL-6 decrease and IL-10 increase. Low levels of ALT, AST, and SCr.	Via inhibition of JMJD3/NF-κB/p65 axis	[75]
Hepatic damage	Hepatic IRI (mouse)	BM (human)	EV	UC 100 kg	EV from human fibroblasts, MSC or fibroblasts	160 ± 57 nm (NTA), CD44+, CD90+, CD9+, CD63+, TSG101+(WB)	10^9 EV particles (MSC or FB) or 10^6 MSC or FB in 200 μL PBS,	1, IV	ND	Liver necrosis	Reduction of AST and ALT (serum transaminases), Hmgb1 and Ho1 (inflammatory mediators). IL-6, phosphorylated STAT3 and ICAM1 increase. Early hepatocyte proliferation.	Hepatocyte proliferation via phosphorylated STAT3 (downstream target of IL-6). Prevention of neutrophil extravasation by ICAM-1. Reduced inflammation by HMGB1 downregulation	[97]

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Table 1 (*continued*)

	Disease model (specie)	MSC source (specie)	EV term	EV isolation method	Controls	EV characterization (method)	EV dose, cell equivalent	Injections, Interval, Route	EV tracking	Disease outcome	Effect	MoA	Ref.
	Hepatic IRI (mouse)	BM (mouse)	EV	UC	PBS	115 ± 48 nm (NTA)	2×10^{10} particles/ body in 200 μL	1, IV	DiR EV labeling	Reduction of tissue necrosis extent. Increased cell viability	Apoptotic cells decrease. Reduction of AST, ALP and urea nitrogen levels in serum and expression of IL6. Increased expression of NACHT, LRR, and PYD domains-containing protein 12, and the CXCL1. Attenuation of oxidative stress and NF-κB activity in hepatocytes.	CXCL1 increase hepatocyte proliferation and could contribute to recruitment of NLRP12-releasing cells	[110]
	Hepatic IRI (rat)	UC (human)	EV	UC 100 kg	EV from native MSC, PBS	178.6 ± 64.2 nm (NTA, SEM), shed from MSC (TEM), CD63+ CD9+(WB)	10 mg/kg (EVs and siMnSOD-Evs) and 3×10^6/ rat (MSC)	1, IV	ND	Hepatic IRI protection	Reduction of neutrophil infiltration in hepatic tissue. Lower levels of ALT, AST, and ALP. Caspase-3–positive cells significantly reduced. Reduced levels of IL-1β, IL-6, TNF-α, CCL12, IFN-γ, and TLR4. Reduced ROS generation and 8–isoprostane. Significantly increased the levels of copper/zinc SOD, MnSOD and EcSOD.	IRI reduction by alleviation of oxidative stress and apoptosis suppression via MnSOD	[102]
Lung damage	ARDS (mouse)	BM (human)	EV	UC 100 kg	PBS	< 4 μm, CD44 + (FC)	2.5×10^5 alveolar macrophages cultured with EVs	1, IN	MitoTracker MSC labeling	Lung injury amelioration	Suppression of proinflammatory cytokine secretion, enhancement of phagocytic capacity, and promotion of M2 macrophage marker expression (CD206).	Promotion of anti-inflammatory and highly phagocytic macrophage phenotype through EV-mediated mitochondrial transfer and enhancement of macrophage oxidative phosphorylation.	[94]
	ALI (mouse)	BM (human)	Microvesicle	UC 100 kg	MV from native NHDFs	Ang1 and KGF (RT-PCR), 200 nm SEM, albumin (BCA)	30 μL MV released by 1.5×10^6 serum starved MSCs	1, IV; 1, IT; 2, IT (+ KGF Ab)	ND	Survival increase. Reduced pulmonary edema and protein permeability in the lungs. Reduction in the weight loss of endotoxin-injured mice by 29%	Decrease in inflammatory protein-2 levels of neutrophils and macrophages. Reduction in the influx of white blood cells and neutrophils. MIP-2, KGF and	Via transfer of KGF mRNA to the injured alveolar epithelium and protein expression	[103]

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Table 1 (*continued*)

	Disease model (specie)	MSC source (specie)	EV term	EV isolation method	Controls	EV characterization (method)	EV dose, cell equivalent	Injections, Interval, Route	EV tracking	Disease outcome	Effect	MoA	Ref.
											protein levels increase.		
	Escherichia coli pneumonia (mouse)	BM (human)	Microvesicle	UC 100 kg	EV from native NHDFs	200 nm (SEM), 90 ± 48 μg protein, 97 ± 90 ng mRNA, CD44+(WB)	90 μL MVs released by 9 × 10^6 MSCs	1, IV; 1, IT; IV (+KGF and CD44 Abs)	Fluorescent MV labeling	Survival increase. Reduced total bacterial load, inflammation, and lung protein permeability in the injured alveolus. Decreased histological severity score.	Increased KGF protein levels in the injured alveolus. Reduced influx of white blood cells, neutrophils, total protein concentration and MIP-2 levels in the BAL fluid. Decreased TNF-a secretion associated with PGE2 levels increase by monocytes. lower levels of M1 marker (nitric oxide synthase), and higher levels of M2 marker (transglutaminase 2).	Antimicrobial effect mediated in part through an increase in monocytes phagocytosis. Immunomodulatory effect on monocytes and alveolar macrophages mediated by CD44 and KGF	[104]
	ARDS (mouse)	BM (mouse)	Exosome	UC 100 kg	PBS	cup, round or oval shape (TEM), 80–150 nm (NTA), CD63+ CD81+ (WB), albumin (BCA)	50 μg or 100 μg in 10 μL PBS	1, IT	ND	Reduced lung injury score. Alleviation of alveolar wall impairment, interstitial edema, and inflammatory cell infiltration. Improved survival rate and decreased PaO2/FiO2 ratio.	Decreased number of inflammatory cells and macrophages. Inhibition of HIF-1a, HK2, PKM2, GLUT1, and LDHA upregulation in the lung tissue. Decreased mRNA expression of IL-1b, IL-6, TNF-a. Upregulation of HK2, PKM2, GLUT1, and LDHA (proteins essential for glycolysis) and Ym-1, CD206, and Arg1.	Alveolar macrophages polarization by glycolysis downregulation via inhibition of HIF-1a.	[85]
	Hyperoxic lung injury (rat)	UC (human)	EV	100,000 r.p.m	MSC, fibroblasts, MSC-EVs, fibroblasts-EVs and saline	Spheroidal (TEM), 50–70 nm (NTA), CD63+ CD9+(WB), albumin (BCA)	20 μg of MSC-EVs, scramble siRNA-transfected MSC-EVs, VEGF siRNA-transfected MSCs-EVs or fibroblasts-EVs in 50 μL of saline, 5 × 10^6 MSCs in 50 μL of saline	1, IT	PKH26 EV labeling	Attenuation of hyperoxic lung injuries such as impaired alveolarization, angiogenesis, increased cell death, and activated macrophages and proinflammatory cytokines.	Attenuation of IL-1α, IL-1ß, IL-6, TNF-α, and ED-1-positive alveolar macrophages.	Engulfment of EVs into pericytes and pro-survival cross-talk between pericytes and endothelial cells. Improvement of angiogenesis via VEGF.	[98]
	Hypoxic pulmonary	UC (human)	Exosome	UC 100 kg + PEG + SEC	Fibroblasts-exosomes	30–100 nm (TEM), Dicer +	10 mg protein in 50 mL PBS	1, IV	ND		Prevention of pulmonary influx of	Suppression of the upregulation of the	[90]

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Table 1 (*continued*)

	Disease model (specie)	MSC source (specie)	EV term	EV isolation method	Controls	EV characterization (method)	EV dose, cell equivalent	Injections, Interval, Route	EV tracking	Disease outcome	Effect	MoA	Ref.
	hypertension (mouse)				and MSC-exosomes from depleted medium, serum-free cultured media, PBS	CD63+ Alix + flotillin-1 + CD81+(WB)				Inhibition of vascular remodeling and hypoxic pulmonary hypertension	macrophages. Abrogation of FIZZ-1/HIMF elevation and STAT3 activation in the lung. Reduction of MCP-1, IL-6, Galectin-3, and HIMF, in cell-free BALF. MiR-17 superfamily downregulation.	miR-17 superfamily increasing miR-204. Suppression of hyperproliferative pathways, including STAT-3 mediated signaling induced by hypoxia.	
	LPS ALI (mouse)	BM (human)	Microvesicle	UC 100 kg	PBS, MSC, Negative SiRNA MSC MVs	Spheroids 200 nm (TEM), Ang-1 (mRNA)	30 μL EVs from 3×10^6 cells	1, IT	ND	Improved survival. Lung inflammation amelioration. Pulmonary capillary permeability restoration.	Amelioration of the influx of white blood cells and neutrophils, and MIP-2 secretion. Higher expression of Ang-1 protein. Suppressing of TNF-α, and inflammatory cytokines secretion, and promotion of IL-10, an anti-inflammatory cytokine secretion.	Partly mediated by *Ang-1* mRNA, probably toward the injured endothelial cells. Ang-1 protein participation in the immune-modulation of macrophages.	[99]
	LPS ALI (rat)	UCB (human)	Exosome	UC 100 kg	EV from native MSC	Round or oval 40–100 nm (TEM), CD81+ CD63+ CD166+ CD90+ CD73+ CD105+ CD29+ (FC)	100 μg diluted in 200 μL PBS	1, IV	PKH26 EV labeling	Suppression of pathological changes	Suppression of apoptosis and NF-κB expression. Attenuation of inflammatory factors levels and oxidative stress response. Raised NR8383 cell proliferation activity.	Exosomes enhanced miR-22-3p expression and decreased its target gene (FZD6) expression	[76]
	Smoke inhalation lung injury (mouse)	BM (rat)	Exosome	Precipitation kit	ND	Cup-shaped (TEM), 30–200 nm (NTA), CD9+ CD63+ CD81+ (WB), albumin (BCA)	250 μg	1, IV	CM-Dil EV labeling	Reduced lung injury scoring. Inhibition of inflammatory cell infiltration increases, partial alveolar septum thickening and diffuse hemorrhage induced by smoke inhalation. Suppression of lung wet-to-dry weight ratio increase	Reduced apoptosis (cleaved caspase-3, Bax and c-Jun) and inflammation (TNF-α, IL-1β, and IL-6). Suppression of LDH levels increase. p-NF-κB and HMGB1 levels reduction	Via inhibition of the HMGB1 and NF-κB pathways	[79]
Cardiac damage	Myocardial IRI (mouse)	BM (mouse)	Exosome	UC 100 kg	PBS	Cup-shaped morphology (TEM), CD9+ CD63+ TSG101+ Alix+(WB), albumin (BCA), 50–150 nm (NTA)	50 μg of exosomes in 25 μL PBS	3, IM	DiI exosome labeling	Infarct size and inflammation reduction. Cardiac function improvement, fibrosis attenuation and, reduced hypertrophy of cardiomyocytes.	Neutrophil (Ly6g+) infiltration reduction. IL6 reduction and IL10 increase in heart tissue and serum.	miR-182 modulates macrophage phenotype to M2 polarization through targeting TLR4.	[84]
			Exosome	TFF + SEC	Saline	ND		1, IV					[93]

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Table 1 (*continued*)

	Disease model (specie)	MSC source (specie)	EV term	EV isolation method	Controls	EV characterization (method)	EV dose, cell equivalent	Injections, Interval, Route	EV tracking	Disease outcome	Effect	MoA	Ref.
	Myocardial IRI (mouse)	ESC (human)					1, 4, or 16 ug/kg		GFP or 111In-oxinate MSC labeling	Infarct size reduction by 45%. Enhanced viability of the ischemic/reperfused myocardium. Preservation of left ventricular geometry and contractile performance. Decreased thinning of the infarct area during scar maturation.	Increased levels of ATP and NADH, phosphorylated-Akt and phosphorylated-GSK-3β. Decreased oxidative stress and phosphorylated-c-JNK. Local and systemic inflammation reduction. Phoshorylation of Akt/GSK3 pathway and inhibition of c-JNK. Reduced neutrophil and macrophage infiltration. Reduced white blood cell counts.	Replenishment of depleted glycolytic enzymes to increase ATP production and reduce oxidative stress in the reperfused cardiomyocytes activating adenosine-mediated RISK pathway to reduce cell death.	
	Myocardial IRI (rat)	BM (rat)	Exosome	Precipitation kit + UF 100 kDa	No injection	(TEM), 50–150 nm (NTA), albumin (BCA)	5 μg in 10 μL PBS	2, IM	PKH26 EV labeling	Improved cardiac function. Infarct size reduction	Apoptosis reduction. Up-regulation of myocardial LC3B-II and Beclin-1 expression and decreased p-mTOR/mTOR expression. Enhancement of the p-AMPK/AMPK ratio and reduction of the p-Akt/Akt and p-mTOR/mTor ratios in H9C2 cells after exposure to H2O2.	In vitro enhanced autophagy via the AMPK/mTOR and Akt/mTOR pathways.	[92]
Brain damage	SCI (rat)	BM (rat)	Exosome	UC 100 kg	Native MSC or PBS	Cup-shaped (TEM), 99.02 nm (NTA), CD9+ CD63+(WB)	200 μg	30 min, 7d, 14 d, IV	ND	Increased Basso–Beattie–Bresnahan score	Pro-inflammatory cytokines (TNF-α and IL-1β) decrease. Elevated microRNA-181c levels in tissue. Suppression of NF-κB signal phosphorilation. Elevated microRNA-181c expression in microglia. Cleaved-caspase 3 and Bax decrease and Bcl-2 partially increase.	Via microRNA-181c and inhibition of PTEN and suppression of NF-κB signal.	[77]
	SCI (rat)	BM (rat)	Exosome	UC 110 kg	Native MSC or PBS	saucer-shaped (TEM), 30–150 nm (TRPS), CD9+, CD63+	200 μL exosomes (200 μg/mL from ~ 1×10^6 MSCs)	0d 1d 200 μL/min 30 min, IV	PKH26 cell labeling	Lesion size attenuation and functional recovery improvement.	Reduction of the A1 astrocytes proportion (in ventral horn spinal cord. Attenuation of	Reduce A1 astrocytes via inhibiting the nuclear translocation of NFκB p65.	[80]

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Table 1 (*continued*)

	Disease model (specie)	MSC source (specie)	EV term	EV isolation method	Controls	EV characterization (method)	EV dose, cell equivalent	Injections, Interval, Route	EV tracking	Disease outcome	Effect	MoA	Ref.
						CD81+(WB), albumin (BCA)					proinflammatory cytokine expression in ventral spinal cord. Decrease of TNF-α, IL-1α and IL-1β levels. Neuroprotective and anti-apoptotic effects.		
	ACI (rat)	BM (rat)	Exosome	UC 100 kg	Saline	Round or cup-like shapes 30–150 nm (TEM), CD63+ CD81+(FC), albumin (Bradford)	200 μL exosomes	1, 2 h, IV	ND	Improvement in fear memory, learning and locomotor ability. Attenuated brain injury.	Pro-inflammatory factors (NO, IL-1β, IL-12, and TNF-α) decreased and anti-inflammatory cytokines and neurotrophic factors (TGF-β, IL-10, and BDNF) increased in ischemic tissue. CysLT2R expression and ERK1/2 phosphorylation downregulation. M1 microglia polarization inhibition and M2 microglia cells increase.	Via reversing CysLT2R-ERK1/2 mediated microglia M1 polarization.	[87]
	Perinatal brain injury (rat)	UC (human)	Exosome	UC 100 kg	Saline	Circular shape 42.93 nm (negative-staining EM), CD63+ CD81+ Flotilin 1+ EpCam+ ICAM+ TSG101+ ANXA5+ ALIX+ (Exo-Check Exosome Antibody Array)	50 mg/ kg in PBS	1, 2 h, IN	PKH26 EV labeling	Reduce neuroinflammation in part through peripheral immunomodulation	Reduced microglia-mediated neuroinflammation. Decrease of the injury-related TNFα and IL-1β expression. Reduced area occupied by CD68 (microglia) cells in injured area.	Modulate the microglial response by interfering with the TLR4/CD14 signaling pathway and preventing the degradation of the NFκB inhibitor IκBα and the phosphorylation of MAPK family to prevent NFκB- and AP-1-dependent transcription of pro-inflammatory cytokines	[82]
	TBI (rat)	AT (human)	Exosome	UF 100 kDa + UC 100 kg	Native MSC or PBS	Cup-like morphology (TEM), 101.4 nm (NTA), Hsp90+ Hsp70+ Tsg101+ CD63+ (WB)	20 μL exosomes from 5×10^5 cells	1, CV	DiR EV labeling	Functional recovery promotion.	Neuroinflammation suppression, neuronal apoptosis reduction, and neurogenesis increase. Reduced levels of proinflammatory factors (MCP-1, TNF-	Suppression of microglia/ macrophage activation by NFκB and P38 MAPK signaling inhibition.	[86]

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Table 1 (*continued*)

	Disease model (specie)	MSC source (specie)	EV term	EV isolation method	Controls	EV characterization (method)	EV dose, cell equivalent	Injections, Interval, Route	EV tracking	Disease outcome	Effect	MoA	Ref.
											α, IL-1β and IL-6). Lower proportion of CD68+(activated microglia/ macrophage). Greater number of newly generated neurons in the hippocampal dentate gyrus. Inhibition of the morphological transformation from the M0 to the M1 state and promotion of M2-associated cytokines (arginase 1, CD206, insulin-like growth factor 1 and IL-10).		
Kidney damage Joint damage	AKI (mouse)	BM (mouse)	EV	UC 100 kg	PBS	double-layered membrane vesicles (TEM), ~160 nm (NTA), Alix+ TSG101 + HSP70 + GM130- (WB),	~6.96 × 10^10 particles in 100 μL of PBS	1, IV	PKH26 EV labeling	Attenuation of renal lesion, mitochondrial damage, and inflammation.	Reduced levels of renal cytokine mRNAs (IL-6, IL-1β, and ICAM-1), serum cytokine levels (TNF-α and TWEAK) and CD68+ macrophage infiltration.	Partially dependent on the mitochondrial transcription factor A (TFAM) pathway.	[100]
	Allogenic kidney transplantation (mouse)	BM (mouse)	Microvesicles	Precipitation kit	Native MSC, medium	NA	NA	1, IV	ND	Improvement in allogenic kidney transplantation survival.	Enhanced miR-146a expression in mature and immature dendritic cells in vitro. Enhanced miR-146a expression and reduced IL-12 production of mature dendritic cells and serum creatinine.	Via inhibition of dendritic cells maturity by miR-146a.	[120]
	AKI CLP (mouse)	AT (human)	Exosome	UC 100 kg	Saline	Goblet or spherical morphology (TEM), 30–150 nm (NTA), CD9+ CD63+ CD81+ (WB)	100 μg (200 μL) exosomes	1, IV	ND	Improved survival rat, better mobility, no pilo erection and purulent stool, and less purulent secretions in the corners of the eyes. Improved kidney function and tissue morphology, and kidney inflammation inhibition.	Reduced degree of blood nitrogen urea (BUN), serum creatinine (SCr), TNF-α, IL-6, MCP-1, HIF-1α, and NF-κB p65 protein. Increased SIRT1 and VEGF proteins. Lower caspase-3, caspase-9, and Bax proteins and higher Bcl-2.	Via SIRT1 signaling pathway. The substrate of SIRT1, NF-κB can reduce the inflammatory response through the deacetylation of SIRT1.	[81]
Joint damage	RA (rat)	BM (human)	EV	UC 10 kg	GW4869 or EV from native MSC	Uniform distribution and different sizes	75 μg/mL EVs	1, IV	PKH26 EV labeling	Reduced rheumatoid arthritis inflammation and abnormal rheumatoid	Inhibition of TNF-α, IL-6 and IL-8 expression. Increased	miR-34a could target cyclin I to activate the ATM/ATR/p53	[91]

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Table 1 (*continued*)

	Disease model (specie)	MSC source (specie)	EV term	EV isolation method	Controls	EV characterization (method)	EV dose, cell equivalent	Injections, Interval, Route	EV tracking	Disease outcome	Effect	MoA	Ref.
						(TEM), 93 nm (NTA), CD63+ CD81+ calnexin- (WB), albumin (BCA)				arthritis fibroblast-like synoviocytes growth.	miR-34a expression in the fibroblast-like synoviocytes. Enhanced cyclin I expression.	signaling pathway, thus inhibiting cell proliferation and cell apoptosis resistance.	
Skin damage	Wound healing (rat))	UC (human)	Exosome	UF 100 kDa + UC 100 kg	PBS or native MSC or native HFL1	Spherical (TEM), 100 nm (NanoSight), CD9+ CD63+ CD81+(WB), albumin (BCA)	200 μg in 200 μL PBS	3 sites, SC	ND	Enhanced re-epithelialization.	Increased collagen I/ collagen III ratio. Higher expression of CK19 (epithelial) and PCNA (proliferation). Heat stress-induced apoptosis inhibition and proliferation enhancement in vitro. Increased expression of b-catenin, cyclin-D1, cyclin-D3, and N-cadherin.	Parallel activation of Wnt4/b-catenin and AKT/GSK3b signaling to promote wound healing.	[70]

ACI, acute cerebral ischemia; AKI, acute kidney injury; ALI, acute lung injury; ARDS, Acute respiratory distress syndrome; AT, adipose tissue; BDNF, brain-derived neurotrophic factor; BM, bone marrow; CLP, cecal ligation and puncture; CV, cerebroventricular; ESC, embryonic stem cells; EV, extracellular vesicles; GvHD, guest versus host disease; IM, intramyocardial; IN, intranasal; IR, intrarenal; IT, intratracheal; IV, intravenous, IRI, ischemia reperfusion injury; LPS, lipopolysaccharide; MoA, mechanism of action; MSC, mesenchymal stem cells; NTA, nanoparticle tracking analysis; RA, rheumatoid arthritis; SEC, Size-Exclusion Chromatography; SCI, spinal cord injury; TBI, traumatic brain injury; TEM, transmission electron microscopy; TFF, tangential flow filtration; UC, umbilical cord; UCB, umbilical cord blood; UC, ultracentrifugation; UF, ultrafiltration; WB, western blot.

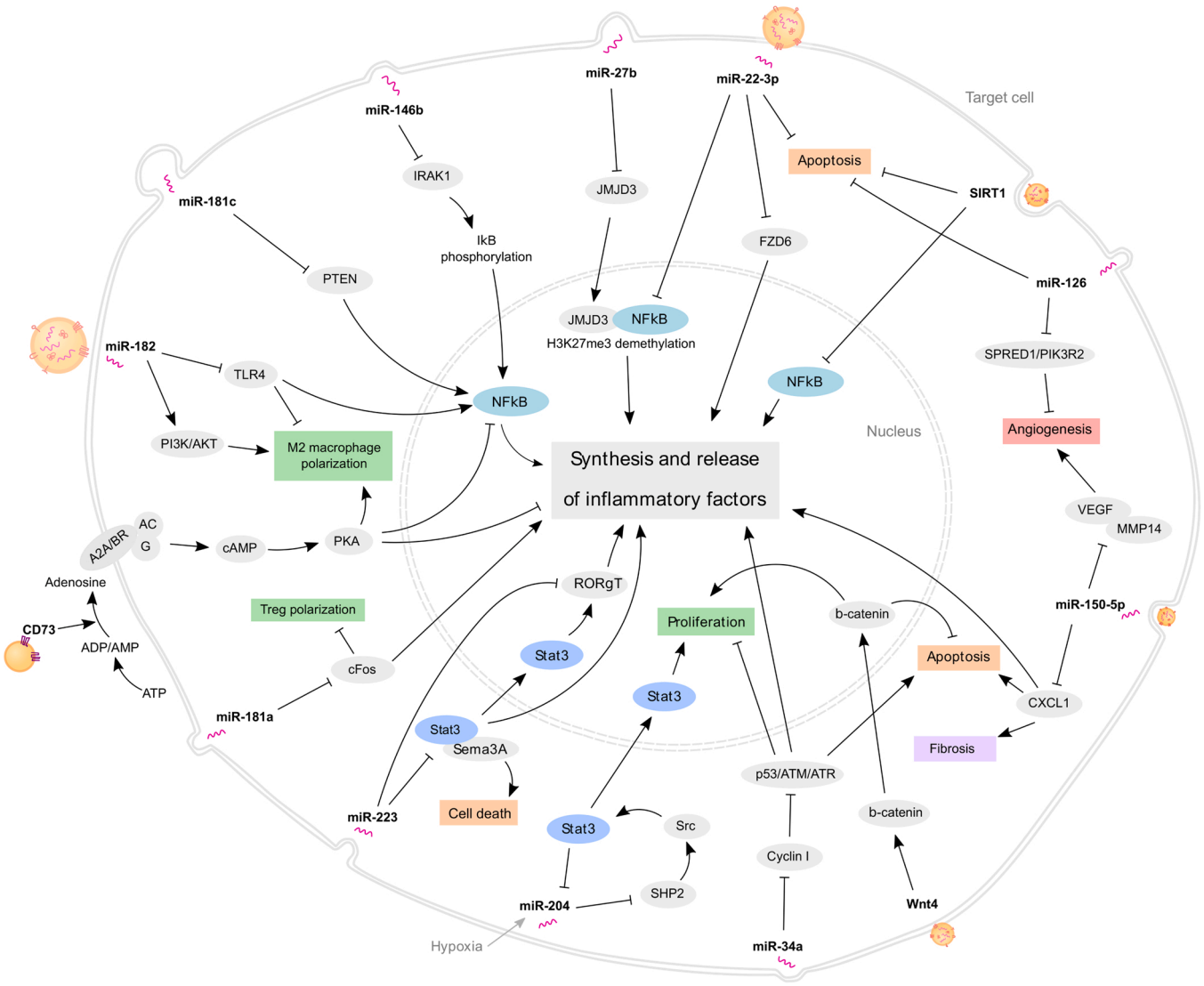

Fig. 1. Schematic representation of cell pathways activated or inhibited by MSC-EV-associated miRNA and proteins.

concomitantly reduce TLR4 activation and promote macrophage polarization into an anti-inflammatory phenotype (M2 polarization) via the action of miR-182 [84]. EVs can also promote M1 to M2 macrophage polarization by inhibiting cellular glycolysis, in which HIF-1α suppression plays a key role [85]. In a rat model of traumatic brain injury, MSC-EVs taken up by macrophages and microglia have been shown to promote M2 microglial polarization by suppressing classical NF-κB and MAPK signaling and the potent inflammatory mediator cysteinyl leukotriene receptor 1 (CysLTR1) [86,87].

Another important target altered by MSC-EVs is the STAT3 pathway, which plays an important role in hypoxia-derived inflammation. Wang X et al. demonstrated that miR-223 contained in MSC-EVs improves animal survival and attenuates cardiac dysfunction when administered to a mouse model of sepsis by CLP. To investigate the mechanisms of action, they used a loss-of-function approach and administered EVs from miR-223 knock-out MSCs, observing higher expression of Sema3A and Stat3, which are targets of miR-223 (5p & 3p). Both proteins were transferred from the EVs to cardiomyocytes, inducing inflammation and cell death. In native EVs, miR-223 downregulated Sema3A and Stat3, leading to reduced inflammation and greater cell survival [88]. Similarly, MSCs-EV treatment in a mouse model of graft versus host disease (GvHD) reduced STAT3, which is required for RORγt expression, as well as production of the pro-inflammatory IL-21 cytokine necessary for Th17 polarization [89]. EVs also inhibited hypoxic activation of STAT3 and halted upregulation of the miR-17 superfamily, but increased the levels of miR-204 in the lungs of a murine model of hypoxic pulmonary hypertension. Specifically, MSC-EVs reduced pulmonary hypertension by suppressing the induction of pro-inflammatory and hyperproliferative pathways, such as STAT3 signaling induced by hypoxia and

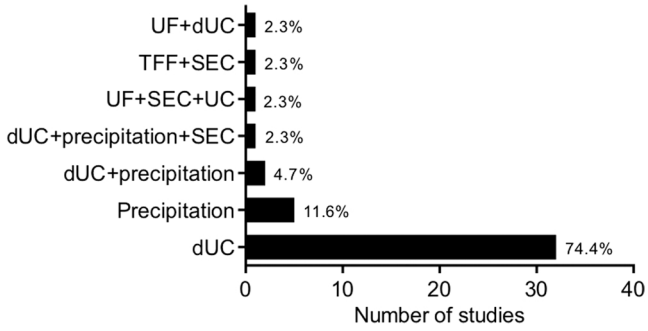

Fig. 2. Isolation methods used in the included studies. The most common were differential ultracentrifugation (dUC; 74.4%) and precipitation methods (11.6%). TFF: tangential flow filtration; SEC: size exclusion chromatography; UF: ultrafiltration.

the influx of macrophages to the damaged lung [90]. Another mechanism described to inhibit proliferation and resistance to apoptosis is via miR-34a, which is highly expressed in MSC-EVs. MiR-34a inhibits cyclin I, which would activate ATM/ATR/p53 signaling [91].

MSC-EVs have also been suggested to be able to reduce rat heart ischemia-reperfusion (I/R) injury by inducting cardiomyocyte autophagy via the AMPK/mTOR and Akt/mTOR pathways. Specifically, in vitro experiments with H9C2 cells under H_2O_2 exposure and MSC-EV treatment have demonstrated increased expression of LC3B-II and Beclin-1, an increased p-AMPK/AMPK ratio, and reduced p-mTOR/mTOR and p-Akt/Akt ratios. These results suggest that MSC-EVs enhance cardiomyocyte autophagy via the AMPK pathway, resulting in certain protection from hypoxia and/or ischemic stress [92]. In the same disease model, Arslan et al. found that the administration of MSC-EVs prior to reperfusion increased ATP and NADH levels, decreased oxidative stress, increased the phosphorylated Akt/GSK3 pathway, and inhibited pro-apoptotic activator c-JNK. Notably, both systemic and myocardial inflammation were significantly reduced, as well as cell death and infarct, and bioenergetics were significantly restored. Moreover, ex vivo Langendorff heart perfusion experiments demonstrated that EVs were able to diminish the fibrotic area to a similar extent as in vivo, suggesting that MSC-EVs enhance the viability of cardiac tissue not requiring blood circulation. The authors proposed that EVs may replenish the myocardium with glycolytic enzymes that increase ATP and NADH levels, supplement cardiomyocytes with new components of the cellular antioxidant system, and activate the adenosine-mediated RISK pathway to ultimately reduce apoptosis [93]. In this context, other authors defended that MSCs modulate human macrophage function by transferring functional mitochondria via EVs and enhancing macrophage oxidative phosphorylation. Pre-treatment of macrophages with MSC-EVs increased basal mitochondrial respiration and ATP turnover, switching macrophage metabolism towards oxidative phosphorylation and decreasing the production of pro-inflammatory cytokines, increasing the expression of M2 polarization marker CD206, and enhancing their phagocytic capacity [94].

Additional in vitro and in vivo experiments have described alternative molecules involved in the immunomodulatory properties of MSC-derived EVs. For example, key players in liver regeneration induced by EV treatment in the hepatic IRI mouse model are increased expression of IL6 and downregulation of intercellular adhesion molecule 1 (ICAM-1) and HMGB1. Succinctly, IL6 triggered hepatocyte proliferation during liver regeneration after IRI through its binding to gp130 and consequent STAT3 activation by phosphorylation [95]. The inhibition of adhesion molecule ICAM-1 prevented neutrophil extravasation and decreased further liver injury. Moreover, HMGB1 acts as an essential damage-associated molecular pattern molecule (DAMP) activating pro-inflammatory signaling and cytokine release through TLRs [96]. Thus, its downregulation could protect the liver from IRI damage [97]. Other molecular effectors modulated by MSC-EVs include VEGF, which reduces pro-inflammatory cytokines through improvements in angiogenesis [98]; angiopoietin-1, which has been found to exert benefits in a mouse LPS lung injury model [99]; mitochondrial transcription factor A (TFAM), which is fundamental for the attenuation of mitochondrial DNA damage and inflammation in the kidney [100]; IL-10, which is key to M2 polarization and recovery from chronic renal inflammation [101]; manganese superoxide dismutase (MnSOD), which is implicated in oxidative stress and neutrophil infiltration regulation [102]; and keratinocyte growth factor (KGF), which increases bacterial phagocytosis by monocytes [103,104].

3.2. Potential of MSC-EVs: engineered versus native

Research on the molecular machinery triggered by MSC-EVs in targeted cells and tissues has increasingly focused on the use of engineered MSC-EVs. The strategies to produce bioengineered MSC-EVs vary from increasing the levels of key functional molecules in their specific cargoes by direct transfection to the pre-stimulation of parental MSCs with different soluble factors or environmental/physical conditions (Table 2). As a result, the upregulation of miRNAs or other molecules substantially improves the beneficial effect of the resulting MSC-EVs compared to native secreted MSC-EVs. This approach also serves to unravel potential MSC-EV-associated mechanisms, such as those implicated in the modulation of undesired inflammatory responses after in vivo infusion. To this end, Wei and co-workers used computational techniques to recognize miR-181 as crucial to improving cardiac function in a myocardial IRI model. The authors concluded that its effect on inflammatory modulation and Treg polarization was via c-Fos targeting, exerting stronger therapeutic function than native MSC-EVs [105]. Moreover, MSCs were transfected to increase the amount of miR-126 in secreted EVs and tested them in the recovery of spinal cord injury. Remarkably, miR-126-loaded EVs attenuated inflammation, stimulated neurogenesis and angiogenesis, and reduced cell apoptosis via inhibition of Sprouty-related EVH1 domain-containing protein 1 (SPRED1) and phosphoinositide-3-kinase regulatory subunit 2 (PIK3R2) expression [106]. Engineered MSC-EVs overexpressing CD73 have also been used to ameliorate inflammation in a mouse model of spinal cord injury. In particular, CD73 hydrolyzes ADP/AMP into adenosine, which activates adenosine A2A/B receptors and promotes the cAMP/PKA signaling pathway, inhibiting NF-kB-driven gene expression. As a result, phenotype polarization from M1 to M2 and a reduction in neuroinflammation is promoted [107]. Another interesting miRNA that increases the therapeutic effect of MSC-EVs is miR-150-5p. In vitro, its overexpression markedly reduced tube formation in HUVECs through both MMP14 and VEGF downregulation and, when miR-150-5p-EV was administered in a rheumatoid arthritis model, a reduction in joint damage by synoviocyte hyperplasia and angiogenesis inhibition was observed [108]. MiR-150-5p has also been overexpressed in MSC-EVs and transferred to hepatic stellate cells (HSCs), targeting C-X-C motif ligand 1 (CXCL1) and subsequently suppressing hepatic fibrosis development both in vitro and in vivo. MiR-150-5p inhibited CXCL1 expression, which prevented the activation of HSCs induced by TGF-β in hepatic fibrosis and reduced collagen deposition, levels of pro-inflammatory cytokines (TNF-α, IL-6, and IL-17), and apoptosis and hepatic injury markers (ALT, AST, and TB), all indicating hepatic recovery [109]. Conversely, experiments by Haga et al. showed a beneficial effect of native MSC-EVs through enhanced CXCL1 expression during in vivo hepatic IRI. The dual role of CXCL1 as a secreted chemokine to recruit and activate leukocytes as well as increase hepatocyte proliferation may explain this phenomenon. The high hepatic expression of CXCL1 may also contribute to recruitment of PYD domain-containing protein 12 (NLRP12)-releasing cells that, in this case, negatively regulates the inflammatory activity by attenuating non-canonical NF-κB signaling. Thus, MSC-EVs were able to suppress oxidative stress, NF-κB activity in hepatocytes, and expression of pro-inflammatory cytokines transcriptionally regulated by NF-κB [110].

Pre-treatment of in vitro primary MSC cultures with certain molecules is useful to enhancing MSC immunomodulatory functions [111] by enriching the cargo of their secreted EVs with specific miRNAs or proteins. In this sense, the use of pro-inflammatory cytokines to pre-condition MSCs is one of the most efficient strategies. In particular, pre-treatment of MSCs with TNF-α enhanced the content of miR-299-3p in derived EVs, fostering their well-described anti-inflammatory functions. Administration of MSC-EVs expressing miR-299-3p in a mouse model of acute liver failure was shown to attenuate liver damage by inactivating the NLRP3 inflammasome pathway in macrophages. This effect drastically inhibited caspase-1 activation, which in turn blocked the maturation of pro-inflammatory cytokines IL-1β and IL-18 [112]. Pre-treatment of MSCs with IL-6 is another strategy showing promising results. Briefly, IL-6 stimulates an inflammatory environment and enriches EVs derived from MSCs with miR-455-3p, which are able to suppress monocyte/macrophage activation and improve liver damage in an animal model of liver fibrosis. MiR-455-3p has a binding inhibitory site in the phosphoinositide-3-kinase regulatory subunit 1 (PIK3r1) gene

Table 2
Summary of pre-clinical studies using engineered extracellular vesicles derived from MSC for immune cell regulation and their mechanism of action.

	Disease model (specie)	MSC source (specie)	EV term	EVs Modification	EV isolation method	Controls	EV characterization (method)	EV dose, cell equivalent	Injections, Interval, Route	EV tracking	Disease outcome	Effect	MoA Ref	Ref.
MiRNA or protein overexpression	AKI CLP (mouse)	UC (Human)	Exosome	**miR-146b overexpression**	UC 100 kg	PBS	110.6 ± 41.9 nm (TEM), 110.6 ± 41.9 nm (NTA), CD9+ CD81+ HSP70+ (WB), albumin (BCA)	120 μg protein in 100 μL PBS	1, IV	ND	Improved survival and sepsis-associated AKI alleviation.	Decreased serum creatinine, blood urea nitrogen, and IL-1 receptor-associated kinase (IRAK1) levels. Increased miR-146b expression in kidney. Decreased IL-1b, TNF-a, and cleaved-caspases3 expression expressions in kidney.	miR-146b regulates NF-kB activity by targeting IRAK1.	[74]
	Myocardial IRI	UC (human)	Exosome	**miR-181 overexpression**	UC 50 kg	PBS or EV from native MSC	Cup-shaped (TEM), 87.6 nm (DLS), CD9+ CD63+ TGS101+ ALIX+(WB), albumin (BCA)	200 μg, miRNA-181a-EXO-treated and EXO	1, IM	ND	Improved ejection fraction and fractional shortening, especially in miRNA-181a-EXO-treated. Reduced inflammatory cell infiltration.	TNF-α and IL-6 downregulation and IL-10 increase. c-Fos protein by miRNA-181a mimic and miRNA-181a-EXO. Higher CD25+ Foxp3 Treg cells/total CD4+ cells ratio.	Via miRNA-181a targeting to c-Fos.	[105]
	SCI (rat)	BM (rat)	Exosome	**miR-126 overexpression**	UC 100 kg	PBS or miR-control EV	Cup-shaped (TEM), 75 nm (NTA), albumin (BCA), CD9+ CD63+ TSG1+(WB),	100 μg exosome protein in 0.5 mL PBS, approximately 1×10^{10} particles	1, IV	PKH67 EV labeling	Reduced lesion volume and improved functional recovery	miR-126-loaded exosomes promoted angiogenesis, neurogenesis and reduced cell apoptosis. Increased VEGF expression. miR126 exos inhibit SPRED1 and PIK3R2 expression in the injured spinal cord tissue, but not control exos. Higher number of NeuN-, Sox2- and Nestin-positive cells in the spinal cord. Reduced number of TUNEL-positive cells, lower levels of Bax and	miR-126 promotes angiogenesis by inhibiting SPRED1 and PIK3R2.	[106]

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Table 2 (*continued*)

	Disease model (specie)	MSC source (specie)	EV term	EVs Modification	EV isolation method	Controls	EV characterization (method)	EV dose, cell equivalent	Injections, Interval, Route	EV tracking	Disease outcome	Effect	MoA Ref	Ref.
												cleaved caspase-3 expression, and higher levels of Bcl-2 expression.		
	RA (mouse)	BM (human)	Exosome	**miR-150 overexpression**	Precipitation kit	PBS or cell-miR-67–transfected exosomes	(TEM) (DLS), CD9+ CD63+ (WB)	50 μg exosomes in 100 μL PBS	2, week, IP	DiO EV labeling	Arthritis alleviation. Reduced clinical arthritic scores, hind paw thickness, and joint destruction.	Exo-150 inhibited migration and invasion and downregulated tube formation. MMP14 and VEGF downregulation in fibroblast-like synoviocytes.	Exo-150 inhibited migration and invasion and downregulated tube formation by targeting MMP14 and VEGF by directly binding to their 3′-UTRs. MMP14 and VEGF downregulation.	[108]
	Hepatic fibrosis (mouse)	AT (mouse)	EV	**miR-150-5p overexpression**	UC 110 kg	PBS or EVs containing miR-NC (negative control) or EVs containing overexpression-NC	Round or oval-shaped (TEM), 30–200 nm (NTA), TSG101+ CD63+ CD81+ GRP94- (WB) albumin (BCA)	0.4 μg/μL, 100 μL	16, 3–4d, IV	PKH26 EV labeling	Hepatic fibrosis alleviation.	Decreased expression levels of ALT, AST and TB. Reduced levels of Bcl-2, collagen I collagen III, vimentin, fibronectin CXCL1, TNF-α, IL-6 and IL-17. miR-150-5p delivered by ADMSCs-EVs alleviated TGFβ-induced hepatic satellite cells activation.	MiR-150-5p transport to HSCs by EVs and prevention of hepatic satellite cells (HSC) activation by CXCL1 inhibition.	[109]
	SCI (mouse)	UC (human)	EV	**CD73 overexpression**	UC 100 kg + UF 100 kDa+SEC	PBS or EV from native MSCs	Spherical (TEM) 103.41 ± 42.03 nm native EVs and 106.51 ± 53.99 nm CD73 Evs (NTA), albumin (BCA), CD9+ CD63+ CD81+ TSG101 + ALIX+ calnexin- (WB)	20 μg EVs were diluted to 50 μg/mL	1daily, 10d, spinal cord wound	DiR EV labeling	Improved the functional recovery	Reduced extracellular ATP, increased adenosine levels and A2bR activation. Improved M1 to M2 phenotype polarization. TNF-α, IL-1β, and IL-6 downregulation	A2bR and cAMP/PKA signaling pathway promotion	[107]

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Table 2 (*continued*)

	Disease model (specie)	MSC source (specie)	EV term	EVs Modification	EV isolation method	Controls	EV characterization (method)	EV dose, cell equivalent	Injections, Interval, Route	EV tracking	Disease outcome	Effect	MoA Ref	Ref.
												and IL-10 and IL-4 upregulation.		
MSC pre-treatment	TBI (rat)	BM (rat)	Exosome	**BDNF pre-treatment**	UC 100 k + 120 kg	PBS or EV from native MSC	Flat vesicular structure 30–150 nm (TEM), MSC Exos 8.31 × 10^8 particles/frame, 110 nm; BDNF-induced MSCs Exo was 9.0 × 10^8 particles/frame 18 nm (NTA), CD63+ CD9 + (WB)	100 μg exosomes	1, IV	ND	Improvements in promoting the recovery of sensorimotor function and spatial learning ability. Inflammation inhibition and neuronal regeneration promotion.	Higher miR-216a-5p levels in BDNF-induced MSCs-Exo than in MSCs-Exo. Improved cell migration, increase immature neurons and inhibition of apoptosis and oxidative stress injury.	Via miR-216a-5p acts by targeting HMGB1/NF-kB pathways and NEUROG2.	[116]
	ALF (mouse)	UC (human)	Exosome	**TNFα pre-treatment**	UC 100 kg	EV from native MSC	Exosome morphology (TEM), 100 nm (NTA), CD9+ CD63+ CD81+ Tsg 101+(WB), > 90% CD63-positive cells (FC),	100 mg	1, IV	PKH26 EV labeling	Acute liver failure alleviation and tissue repair promotion.	Reduced serum levels of ALT, AST and proinflammatory cytokines. Up-regulation of anti-inflammatory-related miRNA-299-3p. No increase of NLRP3, caspase-1 and ASC expression. Decreased levels of IL-6, IL-1β and IL-18.	MicroRNA-299-3p inhibit activation of NLRP3 inflammation-associated pathway proteins.	[112]
	MI (rat)	BM (rat)	Exosome	**Atorvastatin pre-treatment**	UC 120 kg	EV from native MSC	Cup-shaped 100 nm (TEM), normal size distribution (NTA), Alix+ TSG101+ CD81+ CD63+(WB)	10 μg, in 100 μL PBS	3 sites, intramyocardial	PKH26 EV labeling	Improved recovery in cardiac function, further reduction in infarct size and fibrosis.	Reduced cardiomyocyte apoptosis. Angiogenesis promotion and inhibition of IL-6 and TNF-α elevation in the peri-infarct region. Migration acceleration, tube-like structure formation, and increased survival of endothelial cells but not cardiomyocytes in vitro. Col1a1 and Col3a1 expression levels downregulation	lncRNA H19 as a mediator of miR-675 expression regulation and activation of proangiogenic factor VEGF and ICAM-1. ATV pretreatment increased MSCs lncRNA H19 expression and its release via exosomes.	[117]

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Table 2 (*continued*)

	Disease model (specie)	MSC source (specie)	EV term	EVs Modification	EV isolation method	Controls	EV characterization (method)	EV dose, cell equivalent	Injections, Interval, Route	EV tracking	Disease outcome	Effect	MoA Ref	Ref.
												in cardiac fibroblasts. Atorvastatin pretreatment increased MSCs lncRNA H19 expression and its release via exosomes.		
	ALF (mouse)	UC (human)	Exosome	**IL6 pre-treatment**	Precipitation kit	EV from native MSC	50–150 nm (TEM, NTA), TSG101+ CD63+ CD81+ β-actin- (WB)	miR-455-3p agomir or agomir negative control	0 min or 6 h, IV	PKH26 EV labeling in vitro	Improved systemic disorder and liver histology. Acute liver injury alleviation.	miR-455-3p reduced the levels of inflammatory factors to varying degrees, especially IL-6. Monocyte/ macrophage suppression.	miR-455-3p inhibits macrophage activation by inhibiting IL-6-related signaling pathways and regulating PIK3r1.	[113]
	Sepsis CLP (mouse)	UC (Human)	Exosome	**IL-1β pre-treatment**	UC 10 kg+ 110 kg	Native MSC	Cup-shaped (TEM), MSCs 92 ± 34.1 nm, βMSCs 106 ± 45 nm (NTA), CD63+ Alix+(WB)	30 μg in 150 μL PBS	1, IV	PKH67 or CM-Dil EV labeling	Symptoms amelioration and survival rate increase.	M2 macrophage polarization. Strong upregulation of miR-146a by IL-1β stimulation.	IL-1β mediates the up-regulation of miR- 146a, which is transferred to macrophages, where it induces the down-regulation of M1 markers and up-regulation of M2 markers.	[114]
	Sepsis CLP (mouse)	BM (mouse)	Exosome	**IL-1β pre-treatment**	UC 100 kg	EV from native MSC, miR-21 mimic-transfected MSCs or miR-21 inhibitor-transfected MSCs	100 nm (TEM), naive MSC 92 ± 34.1 nm, βMSCs 106 ± 45.2 nm (NPTA), CD63+ and Alix+(WB)	40 μg/mouse, from MSCs IL-1β pre-treated	1, IV	PKH67 or CCM Dil cell labeling	Symptoms attenuation, improved survival rate.	Elevated levels of TNF-α and IL-10. Lower levels of ALT and AST. M2 polarization.	Via exosomal miR-21 transfer to macrophages and inhibition of the target gene PDCD4, leading to M2 polarization.	[115]
	Metabolic syndrome and renal artery stenosis (swine)	AT (swine)	EV	**pre-silenced IL-10 pre-treatment**	UC 100 kg	EV from native MSCs	Abundant (TEM, SEM), 1×10^{10} EVs/mL, ~ 150 nm (NTA), CD9+ CD29+ CD63+(WB)	200 μg from 10×10^6 MSCs	1, IR	PKH26 EV labeling	Attenuated renal inflammation, improved medullary oxygenation and fibrosis. Restored renal blood flow and glomerular filtration rate.	Decreased number of infiltrating inflammatory M1 macrophages and normalized number of reparative M2 macrophages. M1/M2 ratio decreased. Reduced MCP-1,	Polarization of macrophages from M1 to M2 likely mediated by IL-10 and IL-4, enriched in EVs. EVs internalization by macrophages and by tubular cells, which produce	[101]

(*continued on next page*)

Table 2 (*continued*)

	Disease model (specie)	MSC source (specie)	EV term	EVs Modification	EV isolation method	Controls	EV characterization (method)	EV dose, cell equivalent	Injections, Interval, Route	EV tracking	Disease outcome	Effect	MoA Ref	Ref.
												(TNF)-α, IL-6, and IL-1β. Normalized tissue IL-10 and IL-4.	inflammatory cytokines that contribute to macrophage polarization.	
Hypoxia	SCI (mouse)	AT (mouse)	Exosome	**Hypoxia**	UC + sucrose/ D2O cushion	PBS or EV from native MSC	Rounded 50 –150 nm (TEM), 100 nm (NTA), CD81+ CD63+ TSG101 + (WB)	200 μg exosome protein in 200 μL PBS	1, IV	Dil EV labeling	BBB and BMS scores partially restored. Exosomes from hypoxia pretreated cells promote functional recovery more effectively than native exosomes.	Exosomes from hypoxia pretreated cells are more effective than native exosomes downregulating TNF-α, IL-6, and IL-1β, and shifting microglia from M1 to M2 polarization. Increased number of NeuN-positive neurons, decreased rate of iNOS positive microglia(M1) and promoting Arg1 positive microglia (M2). Higher expression of lncGm37494.	LncGm37494 upexpression in HExos promotes microglial M1/ M2 polarization by inhibiting miR-130b-3p and promoting PPARγ expression.	[118]

ACI, acute cerebral ischemia; AKI, acute kidney injury; ALI, acute lung injury; ARDS, Acute respiratory distress syndrome; AT, adipose tissue; BDNF, brain-derived neurotrophic factor; BM, bone marrow; CLP, cecal ligation and puncture; CV, cerebroventricular; ESC, embryonic stem cells; EV, extracellular vesicles; IM, intramyocardial; IN, intranasal; IR, intrarenal; IT, intratracheal; IV, intravenous, IRI, ischemia reperfusion injury; LPS, lipopolysaccharide; MoA, mechanism of action; MSC, mesenchymal stem cells; NTA, nanoparticle tracking analysis; RA, rheumatoid arthritis; SEC, Size-Exclusion Chromatography; SCI, spinal cord injury; TBI, traumatic brain injury; TEM, transmission electron microscopy; TFF, tangential flow filtration; UC, umbilical cord; UCB, umbilical cord blood; UC, ultracentrifugation; UF, ultrafiltration; WB, western blot.

that is crucial in the subsequent inhibition of exacerbated IL-6-related signaling activation [113]. When MSCs are pre-treated with IL-1β, the miR-21 and miR-146a content in EVs is significantly elevated. Notably, administration to septic mice attenuated the symptoms and increased survival. In vitro, miR-21 or miR146a inhibition showed that previous stimulation of MSCs with IL-1β generated EVs that promoted M2 macrophage polarization. Specifically, miR-21 suppressed the effects of its target gene, programmed cell death 4 (PDCD4) [114,115]. Moreover, pre-treatment of MSCs with brain-derived neurotrophic factor (BDNF) increased the capacity of derived EVs to inhibit inflammation and induce neuronal regeneration in a traumatic brain injury rat model. In addition, miR-216a-5p was significantly overexpressed in BDNF-induced MSC-EVs and could protect neurons from oxidative damage by targeting the HMGB1/NF-κB pathways [116]. Furthermore, EVs collected from MSCs pre-treated with atorvastatin promoted cardioprotective effects in a mouse model of MI. MSC-EV-treated animals had reduced levels of cell apoptosis and IL6 and TNFα in peri-infarct areas, smaller infarct area, increased angiogenesis, and improved cardiac function parameters compared to controls. As a potential mechanism of action, the authors suggested that atorvastatin increases lncRNA H19 in MSC-EVs, which directly participates in regulating miR-675 expression and in the activation of VEGF and ICAM-1. In contrast, no cardioprotection was observed when lncRNA H19 was specifically depleted, whereas its overexpression restored beneficial effects [117].

An alternative strategy to improve the effectiveness of secreted EVs is to expose MSCs to hypoxic conditions. In this way, EVs isolated from MSCs under hypoxia were demonstrated to be more effective than naturally secreted EVs in repairing spinal cord injury by suppressing pro-inflammatory factors (TNF-α, IL-6, and IL-1β), inducing polarization of M1 macrophages to M2 macrophages, and increasing the number of NeuN-positive neurons. Furthermore, hypoxic MSC-EVs greatly decreased the iNOS-positive microglia rate (M1-associated) and promoted Arg1-positive microglia (M2-associated). High-throughput sequencing revealed increased levels of lncGm37494 in hypoxic MSC-EVs that were able to inhibit miR-130b-3p and promote PPARγ expression, leading to M2 macrophage polarization [118]. Similarly, serum starvation induced MSCs to secrete EVs with marked angiogenic potential through NF-κB signaling [68].

Collectively, overrepresented proteins and miRNAs in MSC-EVs exert benefits via multiple mechanisms of action, mainly converging in NF-κB or STAT3/SEMA3A signaling (Fig. 1). All of these molecular pathways can be enhanced by promoting the presence of certain molecules in EV cargo or by conferring to the specific preconditioning of parental MSCs, such as those resulting from hypoxia, serum starvation, or pre-treatment modifications.

4. Conclusions and perspectives

MSC-based therapies are a promising strategy for treating a variety of human conditions, including exacerbated inflammation and severe immune-related diseases. However, limited cell engraftment and lifespan rates suggest indirect effects of administered MSCs through paracrine signaling. Among the known paracrine factors secreted by MSCs, EVs are a diverse range of naturally released membranous nanovesicles traveling long distances from the producing cells to specific target tissues and organs. At the targeted sites, EVs have the ability to release their inner content, modulating multiple processes, such as immune responses, senescence, angiogenesis, proliferation, and differentiation. Despite the discrimination of an assortment of EVs into subtypes of distinct sizes, compositions, and biogenetic origins, we highlighted the potential of MSC-EVs in the context of a large number of pre-clinical studies, with a plethora of associated mechanisms of action. In this context, several issues remain unclear for improving the potential of MSC-EVs and achieving clinical application, including increasing our knowledge of how they manage to target damaged tissues and how their internal contents can be modified and interact with the molecular machinery of targeted cells or tissues to induce their benefit. For this purpose, our effort here has focused on describing the mechanism of action and pre-conditioning approaches reported in the existing pre-clinical literature. In particular, some of the presented data advocate for the presence of a variety of miRNAs into MSC-EVs that are capable of inducing marked effects in recipient animal models of disease. Others assume that it is improbable that such low quantities of miRNAs present in MSC-EVs accounts for most of the biological effects observed, as EV-associated proteins are the main drivers of their functionality. Indeed, EVs constitute discrete mixtures of molecular information that is packaged and sent a distance by MSCs with enormous therapeutic potential.

Competing Interests Statement

The authors declare that there are no conflicts of interest.

Data availability

Data will be made available on request.

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