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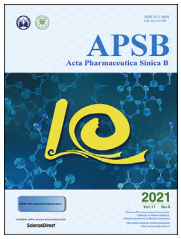

REVIEW

Extracellular vesicle activities regulating macrophage- and tissue-mediated injury and repair responses

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Targeted therapy

Abstract Macrophages are typically identified as classically activated (M1) macrophages and alternatively activated (M2) macrophages, which respectively exhibit pro- and anti-inflammatory phenotypes, and the balance between these two subtypes plays a critical role in the regulation of tissue inflammation, injury, and repair processes. Recent studies indicate that tissue cells and macrophages interact *via* the release of small extracellular vesicles (EVs) in processes where EVs released by stressed tissue cells can promote the activation and polarization of adjacent macrophages which can in turn release EVs and factors that can promote cell stress and tissue inflammation and injury, and *vice versa*. This review discusses the roles of such EVs in regulating such interactions to influence tissue inflammation and injury in a number of acute and chronic inflammatory disease conditions, and the potential applications, advantage and concerns for using EV-based therapeutic approaches to treat such conditions, including their potential role of drug carriers for the treatment of infectious diseases.

Abbreviations: ADSCs, adipose-derived stem cells; AKI, acute kidney injury; ALI, acute lung injury; AMs, alveolar macrophages; BMSCs, bone marrow stromal cells; CLP, cecal ligation and puncture; DSS, dextran sodium sulphate; EVs, extracellular vesicles; HSPA12B, heat shock protein A12B; HUCMSCs, human umbilical cord mesenchymal stem cells; IBD, inflammatory bowel disease; ICAM-1, intercellular adhesion molecule 1; IL-1β, interleukin-1β; iNOS, inducible nitrogen oxide synthase; KCs, Kupffer cells; KLF4, krüppel-like factor 4; LPS, lipopolysaccharides; MHC, major histocompatibility complex; MSCs, mesenchymal stromal cells; MVs, microvesicles; PEG, polyethylene glycol; PMFA, 5,7,30,40,50-pentamethoxyflavanone; PPARγ, peroxisome proliferator-activated receptor γ; SIRPα, signal regulatory protein α; TECs, tubular epithelial cells; TNF, tumor necrosis factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

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1. Introduction

Macrophages are myeloid linage cells involved in innate immune responses that have historically been viewed as terminally-differentiated phagocytic cells that primarily exhibit pro-inflammatory phenotypes[1]. Fate-mapping studies now indicate that macrophages involved in the regulation of tissue homeostasis have distinct origins, which we will herein refer to as tissue-resident and recruited macrophages[2–4]. Most tissue-resident macrophages derive from the yolk sac or fetal liver during embryonic development and migrate to their final site prior to the end of gestation, whereas recruited macrophages derive from circulating monocytes that continue to differentiate from hematopoietic stem cells during the organism's lifetime[3–5]. Monocytes that highly express Ly6c have pro-inflammatory and antimicrobial activity and accumulate at sites of inflammation, while monocytes with low levels of Ly6c expression are considered to be patrolling monocytes that crawl along and survey the vasculature and participate in early inflammatory and tissue repair responses[6]. Tissue-resident macrophages are generally considered to be responsible for maintaining homeostasis, but during tissue injury or infection they can initiate pro-inflammatory responses by secreting chemokines to drive the rapid influx of neutrophils and inflammatory leukocytes[7]. Alveolar macrophages are self-renewing, but decrease in other tissue-resident macrophage populations during infection or tissue injury are replaced by patrolling monocytes that enter the affected tissue and differentiate into macrophages[8]. The primary function of these recruited macrophages is to attenuate inflammation and promoted tissue repair by stimulating angiogenesis, inhibiting neutrophil recruitment, and clearing cell debris. Tissue-resident and recruited macrophages tend to exhibit functionally distinct phenotypes, but can sometimes be functionally interchangeable during certain inflammatory responses[7,9–11]. Both tissue-resident and recruited macrophages play important roles in regulating the development and resolution of pro-inflammatory tissue microenvironments, as described later in this review.

Macrophages, including both tissue-resident and recruited macrophages, are classified as belonging to two distinct functional subsets: classically activated (M1) macrophages, which are primarily associated with pro-inflammatory immune responses; and alternatively activated (M2) macrophages, which are primarily involved in tissue repair (Fig. 1)[12]. Nitric oxide (NO) released by M1 macrophages tends to inhibit cell proliferation and induce tissue damage, while ornithine secreted by M2 macrophages tends to promote cell proliferation and tissue repair responses[12]. M2 polarization appears to be the default option for tissue-resident macrophages, but M2 and M1 macrophages can interconvert in response to specific environmental stimuli[12]. M1 macrophages undergo polarization in response to lipopolysaccharides (LPS) and Toll-like receptor signaling to secrete an array of pro-inflammatory cytokines [*e.g.*, interleukin (IL)-1β, IL-23, IL-12, IL-6, TNF-α] and chemokines (*e.g.*, CXCL2, CCL8, CXCL4 and CCL5). M1 macrophages exhibit increased antigen presenting activity and pro-inflammatory phenotypes, and function to eliminate infections caused by bacterial, viral and fungal factors[13,14]. M2 macrophages are classified into three subpopulations (M2a, M2b and M2c) that arise in response to different stimuli. M2a macrophages are induced by exposure to IL-4 and IL-13, M2b macrophages by immune complex signalizing in conjunction with LPS or IL-1β, and M2c macrophage by anti-inflammatory stimuli, including glucocorticoids, IL-10 or TGF-β. All M2 macrophages express CD14 and secrete high levels of anti-inflammatory factors, including IL-10, arginase-1, CCL24, CCL22, and CCL17[14,15]. These cells play important roles in resolving inflammation at infection and tissue injury sites by suppressing pro-inflammatory immune responses and regulating tissue repair and angiogenesis.

Detecting differences in gene and protein expression is the simplest and most useful method to determine macrophage polarization states. For example, M1 macrophages are characterized by the abundant expression of the class II major histocompatibility

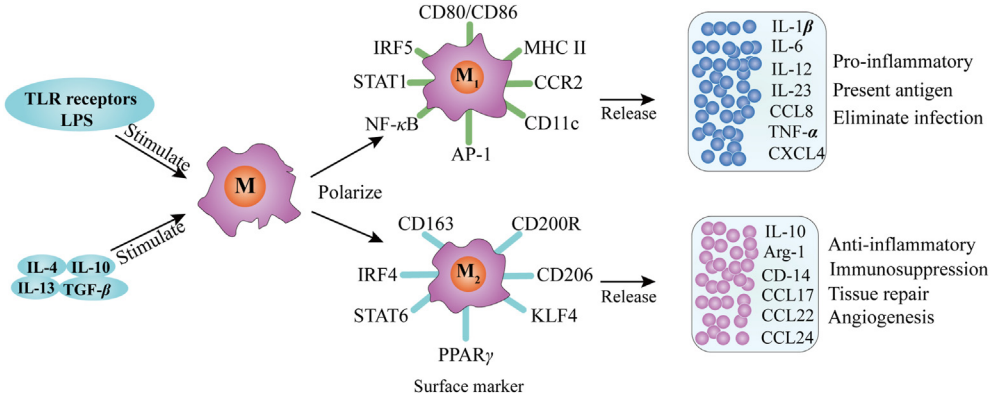

Figure 1 Macrophage polarization and phenotypes. Stimulation by TLR receptor signaling or by exposure to IL-4, IL-10, IL-13 and TGF-β induces macrophages to adopt M1 or M2 phenotypes, respectively, which causes these macrophage subtypes to secrete distinct sets of cytokines and chemokines that exert pro-inflammatory or anti-inflammatory effects.

complex (MHCII), MHCII-associated co-stimulatory molecules CD80/CD86, and CD11c and CCR2 after stimulation with IFN-γ or LPS. Similarly, macrophage expression of CD163, CD200R, and CD206 after treatment with IL-10 or IL-4 is considered a hallmark of M2 polarization[14,16]. Macrophage activation and polarization is mediated by different signaling pathways, and the presence of distinct regulatory molecules involved in the polarization to specific macrophage subtypes can be employed as macrophage phenotype markers. Mediators of TLR-dependent M1 macrophage polarization include the transcription factors activator protein-1 (AP-1), NF-κB, PU.1, STAT1, CCAAT/enhancer-binding protein α (C/EBP-α) and IRF5. Transcription factors promoting M2 activation include C/EBP-β, IRF4, STAT6, Krüppel-like factor 4 (KLF4) and peroxisome proliferator-activated receptor γ (PPARγ)[14,17]. As discussed in the following sections, important interactions between M1 and M2 macrophages and the respective cell types they regulate rely on the activity of EVs as molecular information carriers.

EVs are lipid bilayer-enclosed structures, formed either by outward budding of the plasma membrane or through an intracellular endocytic trafficking pathway[18]. EVs are typically classified into multiple sub-categories, with the most common classification scheme relying on differences of their diameters to segregate them three overlapping groups: exosomes (40−100 nm), microvesicles (MV; 100−1000 nm) and apoptotic bodies (50−5000 nm)[19]. Exosomes derive from the endosomal compartment, while MVs and apoptotic bodies bud from the plasma membrane, and each of these biogenesis processes is subject to different regulatory mechanisms. Given the potential for overlapping these groups during their characterization in the absence of additional information, this review will use "small EV" to refer to EVs populations that could contain exosomes and/or MVs to avoid classification errors in the absence of sufficient detail regarding EV isolation or characterization methods or data. Some small EVs, particularly exosomes, carry miRNAs, mRNAs, and proteins that can influence the inflammatory response[20]. Exosomes are produced by the endosomal compartments of most cells, including multiple cell types that play important roles in immune and pro-inflammatory responses, such as macrophages, dendritic cells, and T and B lymphocytes[21]. Such exosomes can travel throughout the body *via* the circulatory system and traverse the blood−brain barrier and other tissue to boundaries to be taken up by target cells to participate in numerous biological and pathological processes, including tissue damage and repair responses[22]. Small EVs, including exosomes, are also frequently produced in greater abundance stressed *versus* unstressed cells, including cells in tissues experiencing autoimmune responses[21]. Small EVs produced by malignant, infected or otherwise stressed cells and tissues may contain regulatory molecules that are critical for both local and systemic inflammatory responses by promoting the activation and secretion of pro-inflammatory cytokines[23]. Increasing effort is devoted to determining pro-inflammatory factors carried by EVs can regulate inflammatory reactions and could be altered for therapeutic purposes.

Small EVs released by immune cells can stimulate the adaptive immune response by carrying MHCII-antigen peptide complexes[24], but the protective effects of these small EVs can be altered when their source cells are exposed to inflammatory stimuli[25]. For example, under hypertensive conditions exosomes secreted by macrophages stimulate the release inflammatory factors upon their uptake by endothelial cells[26], while endothelial cell EVs can also modulate macrophage phenotypes[24] in one example of an EV-based interaction loops between macrophages and host cells under pro-inflammatory conditions. EVs secreted by macrophages are also able to influence the phenotypes of adjacent macrophages to induce inflammatory tissue responses. For instance, native M1-derived exosomes can effectively shift recipient macrophages from a tumor permissive M2 to a tumor restrictive M1 phenotype[27]. Substantial literature has described interactions between exosomes and macrophages in autoimmune or metabolic diseases[21,24]. Small EVs secreted by macrophages and other cell types can act on their recipient cells to transmit regulation signals and effects through receptor-mediated endocytosis, receptor-mediated membrane fusion, and phagocytosis and by paracrine signaling upon the release of their cargoes to directly interact with specific receptors on the target cells[22]. Less is known about the specific EVs populations that are involved in some of these interactions given that EVs generated from the plasma membrane and endosome compartment may be difficult to distinguish since they are similar in size and may exhibit some degree of compositional overlap[28].

Here, we discuss the roles macrophages play in some inflammation-related diseases, and how EVs secreted by pro-inflammatory M1 macrophages can induce tissue damage, while EVs released by stem cells can alter macrophage phenotypes to regulate anti-inflammatory M2 macrophage responses. Finally, we discuss the potential advantages of employing native or modified EVs as novel therapeutics for the treatment of infectious and inflammatory diseases, as well as additional questions and technical challenges that need to be addressed to allow the clinical translation of such applications.

2. EV-mediated interaction loops between macrophages and tissues in tissue damage

Macrophages can exhibit substantial heterogeneity and plasticity, displaying a continuum of phenotypes, with the extremes represented by the M1 and M2 states[29]. M1 macrophage subtypes predominate at the onset of tissue injury, and can secrete reactive oxygen species and other pro-inflammatory factors that can disrupt normal cell metabolism, induce apoptosis, and exacerbate tissue ischemia to ultimately worsen tissue damage. In the injury-resolution phase, however, M2 macrophages become predominant and produce a variety of effector molecules and growth factors that can regulate myofibroblast activation, stem and tissue progenitor cell differentiation, and angiogenesis[30,31]. Increasing evidence shows that macrophages take part in the regulation of tissue damage by releasing EVs with pro- or anti-inflammatory information to affect inflammation in their microenvironment, and that damaged tissue cells can release EVs to activate local macrophages to initiate these responses (Fig. 2), resulting in an EV interaction loop between macrophages and tissue cells during tissue injury.

2.1. *Cardiac injury*

During the cardiac inflammation, circulating blood mononuclear cells are recruited into cardiac tissue and differentiate into macrophages that secrete exosomes that contribute to cardiac injury by attenuating the induction of angiogenesis and aggravating myocardial damage[32,33]. The contribution of macrophage-derived EVs to this process was identified by findings revealing that while pro-inflammatory miR-155 levels were elevated in macrophages

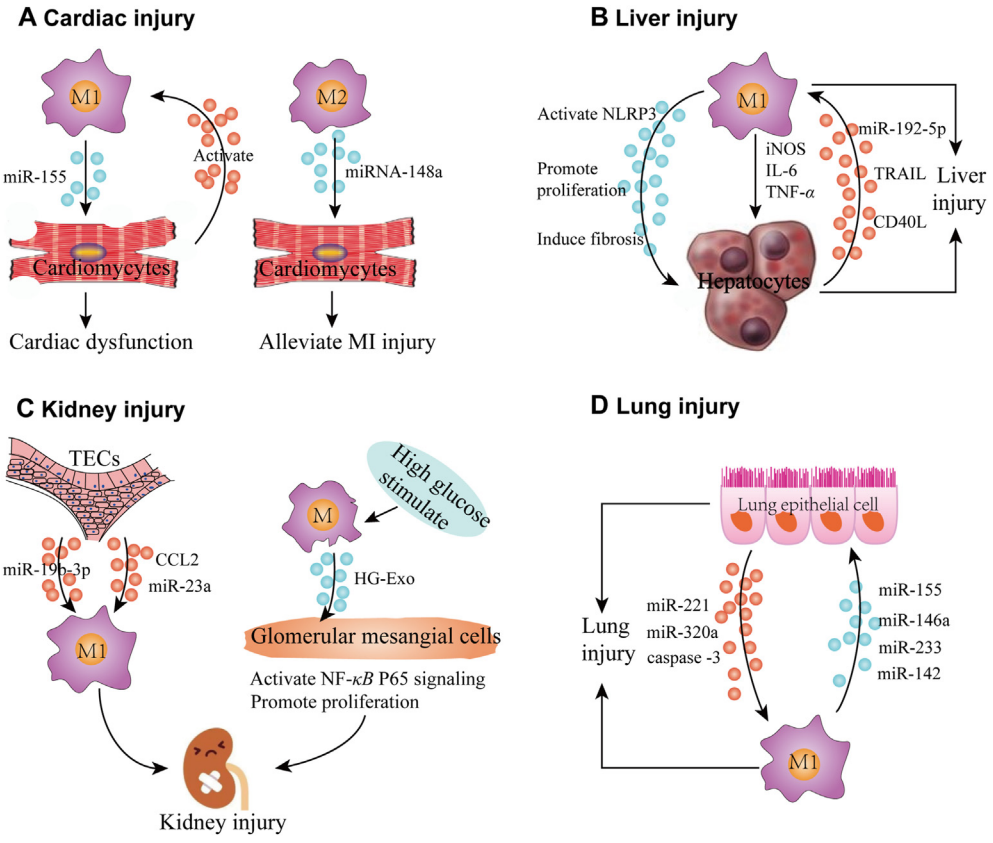

Figure 2 EV-mediated interactions between tissue macrophages and adjacent cells can induce tissue inflammation and injury. (A) Cardiac injury. M1 macrophages secrete extracellular vesicles (EVs) containing miR-155 that induce cardiomyocyte dysfunction, while EVs secreted by injured cardiomyocytes can activate macrophages. Conversely, exosomes secreted by M2 macrophages carry miRNA-148a, which can target cardiomyocytes and reduce myocardial ischemia/reperfusion injury. (B) Liver injury. EVs secreted by M1 macrophages can activate the hepatocyte NLRP3 inflammasome pathway and promote hepatocyte proliferation, liver fibrosis, and the secretion of inflammatory factors, including iNOS, IL-6 and TNF-α, to cause liver injury. Stressed hepatocytes can secrete EV containing miR-192-5p, TRAIL and CD40L to promote M1 macrophage polarization. (C) Kidney injury. Tubular epithelial cells (TECs) secrete EVs enriched with miR-19b-3p, CCL2 and miR-23a that promote M1 macrophage polarization to induce tubulointerstitial inflammation. Conversely, M1 macrophages stimulated by high glucose secrete EVs that can activate NF-κB P65 signaling and promote proliferation of glomerular mesangial cells to cause kidney injury. (D) Lung injury. EVs secreted by lung epithelial cells carry miR-221, miR320a and caspase-3 to promote alveolar macrophages to adopt an M1 phenotype, while M1 macrophage EVs containing miR-155, miR-146a, miR-233 and miR-142 induce lung epithelial cell injury.

and cardiac fibroblasts in injured cardiac tissue, only the cardiac macrophages expressed the miR-155 precursor required to generate miR-155 and that macrophage-derived exosomes contained miR-155 that could be transferred to cardiac fibroblasts[34]. Further, following myocardial infraction cardiac macrophages are reported to express the M1 macrophage markers CD86, CD11c and inducible NO synthase (iNOS) and release pro-inflammatory exosomes containing miR-155, and can transfer miR-155 to endothelial cells, leading to cardiac dysfunction and the inhibition of angiogenesis[26]. Conversely, exosomes derived from IL-4-induced M2 macrophages expressing the M2 macrophage markers CD163 and CD206 carry miRNA-148a, which can alleviate myocardial ischemia/reperfusion injury by inhibiting TXNIP and the TLR4/NF-κB/NLRP3 inflammasome signaling pathway[35]. Finally, EVs secreted by neonatal cardiomyocytes are reported to polarize pro-inflammatory macrophages and exacerbate myocardial ischemia and its associated inflammatory responses[36], suggesting that EV-regulated crosstalk between macrophages and cardiomyocytes may also contribute to cardiac injury. Further studies are required to determine the initiator and relative contribution of EVs from these cells during cardiac ischemia, although EVs derived from neonatal cardiomyocytes under normal and ischemic conditions appear to have similar polarizing affects, whether macrophages stimulate cardiomyocytes or whether cardiomyocytes activate macrophages first in this interaction loop. Additional studies are also necessary to evaluate whether adjusting the ratio of M1 and M2 macrophages in cardiac tissues using small EVs could serve as a therapeutic intervention during myocardial infarction.

2.2. *Liver injury*

Tissue-resident liver macrophages are referred to as Kupffer cells (KC)[37], and several lines of evidence indicate that M1 KCs secrete pro-inflammatory factors that may induce liver damage, including reactive oxygen species, TNF-α, iNOS and IL-6. M2 KCs, conversely, release anti-inflammatory cytokines, which play important roles in liver repair[38]. In addition to KCs, circulating monocytes also have a profound impact on liver injury as they are recruited both during homeostasis and injury-associated

inflammation[37]. Emerging evidence suggests that small EVs, especially exosomes, derived from the recruited macrophages that differentiate from these invasive monocytes, have an important role in mediating liver injury[39]. For example, hepatocytes are reported to take up LPS-induced macrophage-derived exosomes and subsequently activate the NLRP3 inflammasome pathway both *in vitro* and *in vivo*, resulting in liver injury[39]. Exosomes from LPS-treated THP-1 macrophages likewise promote hepatic stellate cell proliferation and induce hepatic fibrosis, a common pathological consequence of repeated liver injury[40]. These results indicate that exosomes secreted by pro-inflammatory KCs and by recruited macrophages alter the phenotypes of hepatic cells to promote hepatic tissue injury.

These interactions appear likely to be bidirectional, since hepatic cells can release small EVs that can activate or polarize macrophages in the hepatic microenvironment. For instance, after lipotoxic injury hepatocytes secrete exosomes that contain miR-192-5p, which can promote macrophages to undergo M1 polarization, while also releasing iNOS, IL-6 and TNF-α into the liver environment to aggravate hepatocyte cellular dysfunction[41]. Small EVs secreted by such hepatocytes contain tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which was found to promote an inflammatory phenotype in recipient macrophages[42]. Hepatocyte dysfunction and pro-inflammatory macrophages induced by EVs-based interactions between hepatocytes and macrophages thus appear to be an important pathological basis for nonalcoholic hepatitis. Other hepatocyte−macrophage interactions have also been demonstrated by exposing hepatocytes to alcohol, which release small EVs carrying CD40 ligand to promote macrophage activation, including the activation of primary murine KC and human monocyte-derived macrophages[43]. Thus, hepatocytes and macrophages can each secrete small EVs that can promote pro-inflammatory activity in the other cell type, with the potential for form a feedback loop to enhance and maintain hepatic inflammation leading to liver injury.

2.3. *Kidney injury*

Acute kidney injury (AKI) is a growing global health concern, and animal models demonstrate that macrophages are major contributors to the inflammatory response associated with AKI[44]. Macrophages from healthy kidneys primarily exhibit an M2-like phenotype, but M1 macrophages predominate shortly after acute kidney injury and act to aggravate renal inflammation, while M2 macrophages play a major role in the eventual attenuation of inflammation and tissue repair response. Both tissue-resident and invasive macrophage generated upon the differentiation of circulating monocytes following their tissue infiltration participate in these responses[29]. Tubular epithelial cells (TECs) are the most populous cell type in the kidney and have key roles in pathological kidney injury[45]. Tubulointerstitial inflammation is a common characteristic of many types of acute and chronic kidney injury, and several studies have demonstrated that TEC-derived exosomes communicate with kidney-resident macrophages to cause kidney damage. For instance, miR-19b-3p in TEC-derived exosomes internalized by primary macrophages and macrophage cell lines are reported to induce M1 polarization and trigger kidney inflammation[45]. A second study indicated that macrophage internalization of exosomes from BSA-treated TECs led to an increased inflammatory response and macrophage migration[46]. This study also found that TEC-derived exosomes enriched in CCL2 mRNA activate macrophages to subsequently induce interstitial inflammation[46]. Similarly, miR-23a-containing exosomes released by TECs exposed to hypoxic conditions were found to induce M1 macrophage polarization and tubulointerstitial inflammation by promoting local inflammatory effects[47]. In combination, these reports indicate that AKI-related inflammation is substantially regulated by communication between TECs and kidney-resident macrophages where TEC-derived exosomes induce M1 macrophage polarization and inflammation to cause or promote tissue injury.

Interactions between kidney-infiltrating macrophages and glomerular mesangial cells are also reported to promote kidney injury. Diabetes-associated chronic kidney inflammation is a consequence of the activation and proliferation of glomerular mesangial cells and the secretion of extracellular matrix and inflammatory cytokines in response to high glucose concentrations[48]. Exosomes from high glucose treated macrophages can transmit pro-inflammatory information between macrophages and glomerular mesangial cells[48], and were found to activate kidney macrophages and accelerate kidney injury *via* the NF-κB P65 signaling pathway[49]. Thus, in tubulointerstitial inflammation-related kidney injury, TECs first release EVs to activate pro-inflammatory macrophages, while in diabetic nephropathy-related kidney injury, EVs secreted by pro-inflammatory macrophages induce pathological changes of glomerular mesangial cells. Both of these processes result in activation cascades which can develop into unchecked interaction loops. Approaches that employ modified small EVs to block the communication between macrophages and renal cells may thus prevent the development of renal tissue injury, and is worth further study.

2.4. *Lung injury*

Alveolar macrophages (AMs), unlike from KCs and other tissue-resident macrophages, are not replenished by *in situ* differentiation of invasive monocytes, so the maintenance of AM abundance requires self-renewal[8]. During the development of acute lung injury (ALI), AMs are pivotal factors in both the initiation and resolution of inflammatory lung responses. AMs are found to constantly communicate with alveolar epithelial cells, with macrophage−epithelial cell crosstalk occurring *via* epithelial cells-derived EVs and their constituent miRNAs[50], where epithelial cells can secrete small EVs to activate pro-inflammatory macrophages. For instance, caspase-3 enriched small MVs derived from lung epithelial cells activate AMs and mediate lung inflammatory responses involved in lung injury, and exposure to caspase-3-deficient MVs can interfere with macrophage activation and reduce lung inflammation[51]. Epithelial cell-derived MV expression of miR-221 and miR-320a is also responsible for triggering macrophage-mediated pro-inflammatory effects[52]. EVs released by lung epithelial cells into the alveolar cavity are primarily MVs, with a lesser amount of exosomes, and few apoptotic bodies[51,52]. Similarly, macrophage-derived exosomes containing miR-155 and miR-146a induce pro-inflammatory cytokine expression in recipient epithelial cells to promote ALI progression[53]. Collectively, epithelial cell-derived MVs can promote macrophage-regulated lung inflammation, causing macrophage-derived EVs to continue to incite epithelial cell activation. Secreted interleukin (IL)-25 from lung epithelial cells can downregulate macrophage Rab27a and Rab27b expression, suppressing macrophage secretion of TNF-α-carrying exosomes and exosome-induced inflammatory effects[54], in which alveolar

Table 1 EV-mediated interactions between macrophages and tissue cells in tissue injury.

MΦ source	Stimulus	MΦ markers	EV origin	EV isolation	EVs characterization	EV marker analyzed	EVs function	Ref.
M1 MΦ	IFN-γ, TNF-α, IL-1β	CD86, CD11c, iNOS	MΦ	UC	TEM (30−150 nm), NTA, WB (Alix, CD9, CD63, CD81 and calnexin)	miR-155	Exacerbate myocardial infarction injury and inhibit cardiac angiogenesis	26
RAW264.7MΦ	ND	iNOS, CD68	MΦ	UC	TEM (80−130 nm), WB (Alix, CD9, CD63)	miR-155	Increase cardiomyocyte pyroptosis	34
M2 MΦ	IL-4	CD68, CD163, CCL22 and PPARγ	MΦ	UC	TEM (mean 100 nm), NTA, WB (CD63, CD81, TSG101)	miR-148a	Reduce myocardial infarction injury	35
MΦ	EVs	p-p38 MAPR, iNOS	Neonatal cardiomyocytes & H9c2 cells	UC	TEM (50−200 nm), NTA, WB (D63, CD8, flotillin andcalnexin)	Cx43	Increase macrophage phagocytic activity and resistance to oxidative damage	36
Raw264.7 MΦ	LPS	ND	MΦ	UC	TEM (112 nm), NTA, WB (CD9, CD63, CD81)	22 NOD-like signaling pathway proteins	Cause hepatocyte damage and NLRPS inflammation activation	39
THP-1 MΦ	LPS	ND	THP-1 MΦ	ND	TEM (30−160 nm), DLS, WB (CD9, CD63)	miR-103-3p	Promote hepatic stellate cells proliferation and liver fibrosis	40
THP-1 MΦ	Exo	CD68, CD11b, CD86, iNOS, IL-6, TNF-α	Lipotoxic hepatocytes	ExoQuick-TC	TEM, NTA, WB (CD63, CD81)	miR-192-5p	Activate the pro-inflammatory macrophages and the progression of NAFLD	41
Mouse BMDMs	EVs	IL-6, IL-1β	Lipotoxic hepatocytes	ND	TEM (40−300 nm), NTA, WB (Alix, TSG101, cd63, ASGPR1, ARF6)	TRAIL	Activate M1 macrophages-associated inflammation in NASH	42
THP-1 MΦ	EVs	TNF-α, IL-6, IL-1β, NOS-2	Hepatocytes	UC	TEM (110 nm), NTA, WB (TSG101, LAmp-1, CD63, RAB5)	CD40 ligand	Promote macrophage activation, contributing to inflammation in ALD	43
MΦ	Exo	MCP-1, IL-1β, IL-6 and TNF-α	Tubular EC	UC	TEM, NTA, WB (Alix, CD9, CD63)	miR-19b-3p	Lead to M1 macrophages	45
RAW264.7 MΦ	Exo	CCL2	Tubular EC	UC	TEM, NTA, WB (Alix, CD9, CD63)	CCL2, TNF-α, IL6	Activate the M1 macrophages and mediate renal inflammation	46
RAW264.7 MΦ	Exo	p-P65	Hypoxic tubular EC	UC	TEM, NTA, WB (Alix, CD9, CD63)	miRNA-223a	Activate the M1 MΦs and tubulointerstitial inflammation	47
RAW264.7 MΦ	High glucose	CD63, TSG101, Calnexin	MΦs	ExoQuick-TC	TEM, WB (CD63, TSG101, calnexin)	TGF-β1/Smad3	Activate glomerular mesangial cells	48
RAW264.7 MΦ	High glucose	p-P65	MΦs	ExoQuick-TC	TEM, WB (CD63, TSG101, calnexin)	IL-1β, iNOS	Activate M1 MΦs and accelerate kidney injury	49
Mouse alveolar MΦ	EVs	MIP-2	Lung EC	UC	TEM, DLS, flow	Caspase-3	Activate the alveolar MΦs	51

Mouse alveolar MΦ & THP-1 MΦ	MVs	TNF-α, IL-1β, IL-10	Lung EC	UC	TEM, DLS, WB	miR-320a and miR-221	*via* ROCK1 pathway	Promote MΦ-regulated lung inflammatory responses	52
Mouse BMDMs	Exo	Rab27a, Rab37b	LPS-induced MΦs	UC	NTA, flow cytometry (CD63)	IL-25		Promote neighboring MΦs to secrete TNF-α.	54
Mouse alveolar MΦ	LPS-induced ALI	TNF-α, IL-1β, IL-6	Alveolar MΦs	UC	ELISA (TNF, IL-1β, IL-6), flow cytometry (CD11c)	ICAM-1		Active epithelial cells and initiate ALI	56
Mouse alveolar MΦ	LPS-induced ALI	NLRP3, Asc	Alveolar MΦs	UC	TEM, NTA, flow cytometry (F4/80, CD11c), WB (CD40L)	miR-223 and miR-142		Activate the inflammatory lung responses	57

ALI, acute lung injury; BMDMs, bone marrow derived macrophages; DLS, dynamic light scattering; EC, epithelial cells; Exo, exosomes; EXO, exosomes; LPS, lipopolysaccharide; MΦ, macrophage; ND, not described; NTA, nanoparticle tracking analysis; TEC, tubular epithelial cells; TEM, transmission electron microscopy; UC, ultracentrifugation; WB, Western blot.

epithelial cells negatively regulate the inflammatory responses of macrophages.

AM-derived MVs may also play a significant role in regulation of ALI associated inflammation, since in LPS-induced animal models of ALI, macrophages were the major source of MVs in bronchoalveolar lavage fluid[55]. These MVs were found to carry biologically active tumor necrosis factor (TNF) and play an important role in initiating ALI[56]. These MVs also contained miR-223 and miR-142 and were found to selectively target pulmonary macrophages[57]. Further study found that MVs released by AMs caused lung injury by activating the NLRP3 inflammatory pathway in adjacent macrophages[57]. Further, bronchoalveolar lavage fluid MVs loaded with miR-223/142 mimics were found to effectively target AMs and inhibit their activation and reduce lung inflammation[57]. Thus, using EVs to target the delivery of regulatory factors to pulmonary macrophages appears to represent a viable approach to prevent pulmonary injury in cases where there is an excessive inflammatory response, although it is possible that the opposite approach could be used in other conditions where a suboptimal inflammatory response was associated with chronic infection or other condition. EV-mediated interactions between macrophages and tissue cells in tissue injury are listed in Table 1[26,34–36,39–43,45–49,51,52,54–57].

3. The role of macrophages and their EVs in inflammatory diseases

As discussed above, M1 macrophages release NO to inhibit cell proliferation during inflammatory responses, while M2 macrophages can secrete factors that induce cell proliferation to promote tissue repair. Similarly, small EVs secreted by M1 macrophages exhibit some of the same pro-inflammatory characteristics of their parental cells, while EVs secreted by M2 macrophages exhibit the ability to promote tissue repair. A major question is whether or not approaches that alter the phenotype of small EVs involved in pro-inflammatory interactions can attenuate the inflammatory responses and tissue injury to serve as potential therapeutic options. This section therefore discusses currently available evidence regarding the nature of EV-mediated regulated macrophage−tissue interactions in several importance inflammatory conditions, and whether these interactions and interventions differ in resolving and non-resolving inflammatory conditions. We discuss examples covering both acute inflammatory responses (AP, AKI, ALI, and sepsis) that can often resolve with supportive care or short-term interventions and non-resolving, chronic inflammatory conditions (IBD, obesity and diabetes) that are more difficult to attenuate and regress.

3.1. Inflammatory bowel diseases (IBDs)

Inflammatory bowel diseases (IBDs), which primarily represent ulcerative colitis and Crohn's disease, are generally considered to be immune homeostasis disorders affecting genetically susceptible individuals, and are characterized by prominent infiltration of the intestines by inflammatory cells such as lymphocytes, T cells, macrophages and neutrophils[58]. At present, the precise etiology of IBD is still unclear. Macrophages, as the most significant components of the intestinal mononuclear phagocyte system, have received much attention in the study of IBD development. Studies indicate that intestinal macrophages, predominantly M1 macrophages, recruit circulating mononuclear cells and other myeloid

cells into the intestinal tract by secreting the chemokines CCL7 and CCL8, and that these recruited monocytes mechanism have pro-inflammatory properties[58]. However, both tissue-resident and recruited macrophages play key roles in intestinal inflammation in IBD, as they serve as a major source of chemokines that trigger further recruitment of pro-inflammatory monocytes, release IL-1β and TNF-α to induce inflammatory response, disrupt the epithelial barrier, and induce epithelial cell apoptosis and drive intestinal inflammation[58,59]. Thus, it has been proposed that attenuating monocyte recruitment or promoting M2 macrophage polarization could limit chronic inflammation in IBD. For example, the natural soya-based product genistein has been shown to promote M1 macrophages to adopt an M2 phenotype, and genistein treatment attenuates the severity of dextran sodium sulphate (DSS)-induced colitis[60]. The induction of M2 macrophages in the intestinal tract was likely IL-10 and TLR4 dependent, given that the effect disappeared in IL-10-, TLR4-and MyD88-deficient mice[61].

Macrophages are thus likely to play a central role in IBD-related inflammation, and studies described below indicate that exosome stimulation is responsible for macrophage activation and polarization in IBD[62]. For example, RAW264.7 macrophages exhibited increases in M1 or M2 macrophage markers[63], respectively, when treated with exosomes purified from the serum of DSS-induced or control C57BL/6 male mice. In-depth study found that serum exosomes of DSS-induced mice caused phosphorylation of p38 and ERK and TNF-α production[62], and released TNF-α promoted M1 macrophage polarization to further aggravate the inflammatory response[63]. Small EVs with M1 polarizing activity are found in the peripheral circulation and the intestinal lumen of IBD patients, where small EVs are absorbed by intestinal macrophages, leading to increased IL-8 expression, the induction of M1 macrophage polarization, and ultimately colitis[64]. Polarization and stimulation of M1 macrophages by small EVs may thus be the major underlying mechanism behind propagation of local inflammation.

Exogenous administration of modified small EVs *via* the peripheral circulation may inhibit the polarization of M1 macrophages or increase the proportion of anti-inflammatory M2 macrophages to alleviate inflammation. For example, *Pediococcus pentosaceus*, a gut-resident bacteria, secrete small membrane vesicles that can promote M2 macrophage polarization and the production of myeloid-derived suppressor cells, and these vesicles have been shown to reduce colon inflammation *in vivo* and *in vitro*[65]. Several reports have also indicated that small EVs secreted by stem cells can also promote the M2 polarization of intestinal macrophages and alleviate colitis[66–68]. Finally, M2 macrophages also secrete small EVs that have therapeutic potential, since analysis of small EVs isolated from different M2 macrophage subtypes, found that M2b macrophage-derived exosomes could reduce the severity of DSS-induced colitis more effectively than those derived from M2a and M2c macrophages[69]. Following treatment with exosomes derived from M2b macrophages, mice with colitis revealed an increase in the number of anti-inflammatory regulatory T (Treg) cells and the level of IL-4 in their spleens, and significant suppression of key cytokines associated with colitis (IL-17A, IL-6, and IL-1β)[69]. In summary, these findings indicate that small EVs have potential to alter local or systematic macrophage M1/M2 macrophage balances and could thus represent an effective treatment strategy to alter and improve inflammation in IBD.

3.2. *Aseptic inflammation: Pancreatitis*

Acute pancreatitis (AP) is an inflammatory disorder of the pancreas characterized by infiltration of a large number of inflammatory cells or inflammatory factors, leading to the amplification of local and systemic inflammation. M1 macrophage infiltration and activation is a critical step during development of AP[70]. Because NLRP3 inflammasome activity and NF-κB activation induce the M1 macrophage phenotype and cytokine production, it is possible that prevention of pro-inflammatory M1 macrophage polarization and inhibition of oxidative stress-induced NF-κB and NLRP3 inflammasome responses in the pancreas could prevent or attenuate AP progression[70].

Conversely, promoting M2 macrophage polarization could prevent AP or alleviate its inflammatory symptoms. After observing that carbon monoxide (CO) can modulate the macrophages to adopt an M2-like phenotype[71], Taguchi et al.[72] used nanotechnology-based CO-bound hemoglobin vesicles (CO-Hbv) to treat a mouse model of AP. They found that CO-Hbv treatment polarized macrophages toward an M2-like phenotype and inhibited neutrophil infiltration, eventually suppressing AP and pancreatitis-associated acute lung injury. Abdominal paracentesis drainage (APD) treatment has a beneficial effect on patients with severe AP before percutaneous catheter drainage, and Liu et al.[73] determined that APD effect to improve the inflammatory environment of the peritoneal cavity in severe AP was mediated by polarizing peritoneal macrophages (PMs) towards the M2 phenotype, resulting in increased levels of anti-inflammatory mediators (IL-4 and IL-10) and decreased levels of pro-inflammatory mediators (IL-1β and L-selectin). In combination, these studies demonstrate that M1 macrophages can promote AP development, whereas M2-polarized macrophages can alleviate AP inflammation. Modifying macrophage polarization may thus represent a promising treatment for AP.

Exosomes appear to play an important role in AP. *In vitro* experiments have demonstrated that culture medium from taurolithocholate-activated AR42J pancreatic acinar cells can enhance macrophage NF-κB activation, but this effect disappears when using a supernatant fraction that lacks exosomes[74]. These results indicate that exosomes from pancreatic acinar cells play a key role in activating pro-inflammatory macrophages in pancreatitis. Further, in an AP rat model, miR-155 in plasma exosomes was found to induce pro-inflammatory activity in macrophages[75]. Thus, both pancreatic acinar cell-derived exosomes and circulating exosomes can activate recipient macrophages. However, few *in vivo* studies have reported that pancreatic acinar cells affect the phenotype of tissue-resident pancreatic macrophages *via* the secretion of small EVs to influence pancreatitis development. However, it has been reported that exosomes derived from pancreatic cancer cells can induce macrophages to adopt the M2 phenotype, which facilitates the migration, invasion, and epithelial-to-mesenchymal transition of pancreatic cancer cells[76]. MiR-501-3p expressed by M2 macrophage-derived exosomes can inhibit tumor suppressor TGFBR3 gene expression and facilitate the development of pancreatic ductal adenocarcinoma by activating the TGF-β signaling pathway[77]. It is therefore possible that, as with pancreatic cancer, there are EVs derived by pancreatic acinar cells that can promote the development of pancreatitis by communicating with adjacent macrophages. However, unlike in

pancreatic cancer where M2 macrophages appear to promote cancer, M1 macrophages appear to promote pancreatitis[73]. Further research may be required and demonstrate the existence of an interaction loop between pancreatic cells and tissue-resident macrophages AP progression.

Severe AP develops rapidly and often causes multiple organ dysfunction. ALI is the most common extra-pancreatic complication associated with severe AP, and it is believed that pulmonary macrophages are involved in pancreatitis-associated ALI by releasing inflammatory mediators and recruiting neutrophils. Sabrina et al.[78] have reported that AMs promote early inflammatory responses, while interstitial macrophages help resolve inflammation, and M1-like AMs are the dominant population in non-resolving lung inflammation[79]. A series of tracking experiments have demonstrated that circulating exosomes effectively reach the alveolar compartment and were internalized by macrophages in an AP rat model to polarize AMs towards a pro-inflammatory M1 phenotype[80]. Thus, circulating exosomes associated with AP appear to communicate with distant macrophages and to promote ALI *via* pro-inflammatory AM polarization.

3.3. *Infection disease: Sepsis*

Sepsis is a systemic inflammatory syndrome caused by an extreme response to pathogen-derived factors that can lead to multiple organ dysfunction and potentially death. Since macrophages play an important role in preventing microbial infection and regulating inflammation, macrophage polarization is thought to be a key process in the pathogenesis of sepsis. In bacterial septicemia progression, M1-polarized macrophages phagocytose bacteria and generate a series of pro-inflammatory cytokines that promote an innate immune response[81], while in patients with severe sepsis, high circulating concentrations of M1-type cytokines correlate with increased mortality.

Approaches designed to attenuate M1 macrophage activity or induce M1 to M2 macrophage polarization have been studied and deemed successful as potential sepsis treatments. For example, treatment of a cecal ligation and puncture (CLP) mouse sepsis model with antibiotics and gold nanoparticles (AuNPs) that exhibit anti-inflammatory activity significantly attenuated CLP-induced bacterial sepsis, at least partially by inducing macrophage polarization toward an anti-inflammatory phenotype[82]. This process was measured by evaluating the decreased numbers of M1 macrophages and increased numbers of M2 macrophages in the spleens of these septic mice, as well as the altered cytokine production of bone-marrow-derived macrophages from these mice, which included reductions in pro-inflammatory factors and increases in anti-inflammatory factors[82]. PMFA (5,7,30,40,50-pentamethoxyflavanone) treatment was also found to promote macrophages to shift from an M1 to an M2 phenotype *via* effects on STAT1/STAT6 signaling. Intraperitoneal administration of PMFA to LPS- or CLP-induced mouse models of sepsis, significantly increased mouse survival rates and dramatically decreased pro-inflammatory cytokine levels[81].

Circulating small EVs have been demonstrated to contribute to systemic signaling and immunomodulation during sepsis. Moreover, these small EVs can induce M1 or M2 macrophage polarization to aggravate or improve sepsis-associated phenotypes. For example, when small EVs derived from the plasma from septic shock patients was injected into animal tissues, there was a marked increase in NF-κB activation and NO synthesis, demonstrating a pro-inflammatory effect[83]. Conversely, another experiment found that human umbilical cord mesenchymal stem cells pretreated with interleukin-1β secreted miR-146a-containing exosomes that improved the phenotype of a mouse sepsis model by promoting M2 macrophage polarization[84]. Some EV-associated miRNAs are reported to regulate EV-induced cytokine production to contribute to pro-inflammatory or anti-inflammatory macrophage phenotypes activated by circulating exosomes in sepsis[85]. *In vitro* experiment have also shown that endothelial cell cultures release small EVs that carry heat shock protein A12B (HSPA12B) and when these EVs are taken up by LPS-stimulated macrophages they increase IL-10 and decrease TNF-α and IL-1β production, while the loss of endothelial HSPA12B expression aggravates inflammatory responses during sepsis[86]. This data suggests that specific modifications to small EV cargoes affecting known functional proteins or miRNAs can generate modified EVs that can employed to promote pro-inflammatory or an anti-inflammatory macrophage phenotypes.

Reducing macrophage secretion of small EVs or attenuating the uptake of these EVs could also have therapeutic potential. For instance, macrophages pre-treated with GW4869—an inhibitor of exosome biogenesis/release—and then challenged with LPS revealed impaired release of several pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) compared to cells treated with LPS alone[87], suggesting that blocking the secretion of macrophage-derived exosomes in sepsis could reduce macrophage-triggered inflammatory responses. Macrophage-derived nanoparticles can also demonstrate anti-inflammatory properties in sepsis to prolong survival, by reducing pro-inflammatory and increasing anti-inflammatory gene expression in macrophages to reduce endothelial cell inflammation[88]. Although there are several known mediators of septic inflammation that could be targeted to downregulate inflammatory responses, macrophages-derived exosomes appear to be the most studied and thus the most likely to serve as the basis for a short-term clinical application.

Similar to previously mentioned examples in AKI and ALI, sepsis can also cause systemic organ damage and like pancreatitis-induced ALI, the polarization of M1 AMs is reported to contribute to ALI development in sepsis. One study has reported that serum exosomes from mice with sepsis-related ALI can deliver miR-155 to macrophages to promote their M1 polarization, stimulate NF-κB activation, induce the production of TNF-α and IL-6, and eventually cause ALI[89]. Evidence also suggests that activated or M1-polarized macrophages isolated from animals with sepsis-related inflammation can act as important factors in the renal injury of AKI animal models[44]. M1 macrophages were found to be activated by heparin-binding protein (HBP) in sepsis-induced AKI, and heparin-induced suppression of HBP reduced M1 macrophage activation, TNF-α and IL-6 secretion, and the severity of renal injury[90]. However, to date no studies appear to have reported what signals or factors activate kidney macrophages, although EVs are promising candidates for this mechanism, and we anticipate that future studies will support this hypothesis.

3.4. *Inflammatory-related metabolic disease: Obesity and diabetes*

Obesity is associated with chronic activation of inflammatory pathways, and obesity-associated insulin resistance is a central mechanism in metabolic diseases such as type 2 diabetes[91,92]. Emerging evidence has shown that obesity-induced inflammation is a result of elevated levels of circulating pro-inflammatory

cytokines, such as TNF-α and IL-6, which negatively affect the insulin signaling cascade to promote insulin resistance[93]. Adipose tissue macrophages are the principal source of these inflammatory factors in obesity. Macrophages present in adipose tissue of lean individuals primarily exhibit an M2 phenotype, while macrophages in adipose tissue of obese individuals predominantly demonstrate an M1 phenotype[94]. An alternative M2 activation state maintained by STAT6 and PPARs has been detected in adipose tissue, in contrast to a classical M1 activation state driven by AP1, NF-κB, and other signal-dependent transcription factors[95]. Chemokines and cytokines secreted by adipose cells induce the recruitment and polarization of macrophages, which are important mediators of cell crosstalk leading to inflammation in adipose tissues.

EVs also play important roles in intercellular communication in adipose tissue, however, especially when one considers that exosome miRNAs have the ability to regulate TGF-β and WNT/β-catenin signaling in obesity-induced inflammation[96]. Human adipocyte derived EVs are also reported to have immunomodulatory effects on macrophages, and macrophages pre-treated with human adipocyte-derived EVs secrete small EVs that interfere with adipocyte insulin signaling[97]. This demonstrates that there is a reciprocal regulatory loop between adipocytes and macrophages that is mediated by EVs and which affects macrophage activation and adipocyte insulin signaling that leads to adipose tissue inflammation[98]. This behavior is demonstrated in animal models, where adipocytes can release EVs which act as "find me" signals to promote macrophage migration and infiltration[99]. Additionally, miR-155 in adipocyte-derived MVs induces M1 macrophage polarization, causing chronic inflammation and local insulin resistance[100]. Adipocyte-secreted exosomes also transport miR-34a into macrophages, thereby suppressing M2 polarization by repressing the expression of KLF4[101]. Adipocyte-derived small EVs can therefore regulate macrophage infiltration and the adipose tissue M1/M2 macrophage ratio to induce obesity-related inflammation. Alteration of the disease-promoting balance between M1 and M2 adipose tissue macrophages can also contribute to the regulation of adipose tissue inflammation and insulin resistance[102]. For example, Zhuge et al.[103] used linaglipin, a strong DPP-4 inhibitor, to treat obesity, and found that it could reduce M1-polarized macrophage migration while inducing an M2-dominant macrophage shift within white adipose and liver tissues, thereby attenuating obesity-induced inflammation and insulin resistance.

Complementary to adipocyte mechanisms to activate macrophages, activated macrophages not only secrete inflammatory cytokines that provoke systemic and local inflammation but also release EVs to influence adipocyte activity. Exosomes from LPS-activated macrophages upregulate some of the pathways and metabolic processes related to inflammation onset in adipocytes[20]. These exosomes also exhibit increased levels of miR-29a, which can be transferred into hepatocytes, myocytes and adipocytes to impair the insulin sensitivity of these target cells *in vitro* and *in vivo*, ultimately promoting obesity-induced insulin resistance and the development of type 2 diabetes[104]. These results indicate that activated macrophages participate in a variety of inflammation-related metabolic pathways by releasing small EVs that promote local and systemic chronic inflammation. Altering the polarization of macrophages and thus the cargoes and activities of their small EVs may improve macrophage-mediated inflammation. Melatonin serves as an example for the potential of such an approach, since it alleviates adipose inflammation both by direct actions on adipocytes and indirect activity on macrophages mediate by adipocyte-derived EVs[105].

Circulating EVs can thus polarize M1 macrophages to secrete small EVs that trigger pro-inflammatory responses (Fig. 3) associated with acute inflammatory responses (AP, AKI, ALI, and sepsis) that can resolve with supportive care or short-term interventions and non-resolving, chronic inflammatory conditions (IBD, obesity and diabetes) that are more difficult to attenuate and regress. Small EVs can, however, also carry protein and miRNA cargoes that limit attenuate acute inflammation and promote tissue repair or limit inflammation and tissue damage during chronic inflammation conditions. Better understanding of the roles that EVs play in regulating the polarization and exerting the regulatory effects of macrophages during the initiation and resolution of inflammatory responses and conditions may identify new therapeutic targets for intervention to limit excessive tissue injury. Approaches that target EV-mediated mechanisms involved in these processes may be more effective in limiting inflammation and tissue injury and have fewer side effects than approaches that attempt to attenuate macrophage accumulation or activity.

4. MSC EVs promote macrophages to an anti-inflammatory M2 phenotype

Given the ability of M2 macrophages to limit inflammation and tissue injury, several studies have examined the potential of different methods to stimulate M2 macrophages to release anti-inflammatory mediators *in vitro* and *in vivo*. Mesenchymal stromal cells (MSCs) have recently shown great potential as an alternative means to treat inflammatory disorders due to their immunomodulatory characteristics[106–108]. MSCs are mesodermal cells that can differentiate into chondrocytes, osteocytes, and adipocytes *in vitro*, and are present in multiple tissues including the umbilical cord, muscle, adipose and bone marrow[109,110]. While MSC-based products have been used in several clinical trials, various problems such as low transplanted cell viability, immunogenicity, poor homing to desired tissues, and safety issues have prevented MSCs from being used in clinical applications[107,111]. More recent research has therefore shifted from the use of MSCs to the use of MSC-derived EVs, which can effectively transfer MSC-derived functional molecules to target cells without the issues associated with direct MSC transfer. For example, the secreted apoptotic bodies from MSC carry the immunoregulatory factors of MSC, which are taken up by target cells by endocytosis or membrane fusion, and MVs or exosomes exert biological effects in a paracrine and endocrine manner[112]. Clinical trials have found that MSC-derived small EVs may offer specific advantages when compared to MSC cells due to their lower immunogenicity and greater safety profile, and MSC-EVs administration is also less likely to suffer from the pulmonary first pass effect[109,113]. More importantly, MSC-EVs have shown great potential in promoting M2 macrophage polarization in inflammatory related diseases[113].

4.1. *Bone marrow stromal cells (BMSCs)*

Bone marrow stromal cell (BMSC)-derived small EVs (BMSC-EVs) have been reported to reduce colonic inflammation by downregulating inflammatory responses and maintaining intestinal barrier integrity. The beneficial effects of BMSC EVs are blocked by macrophage depletion, indicating that BMSC EVs exert anti-

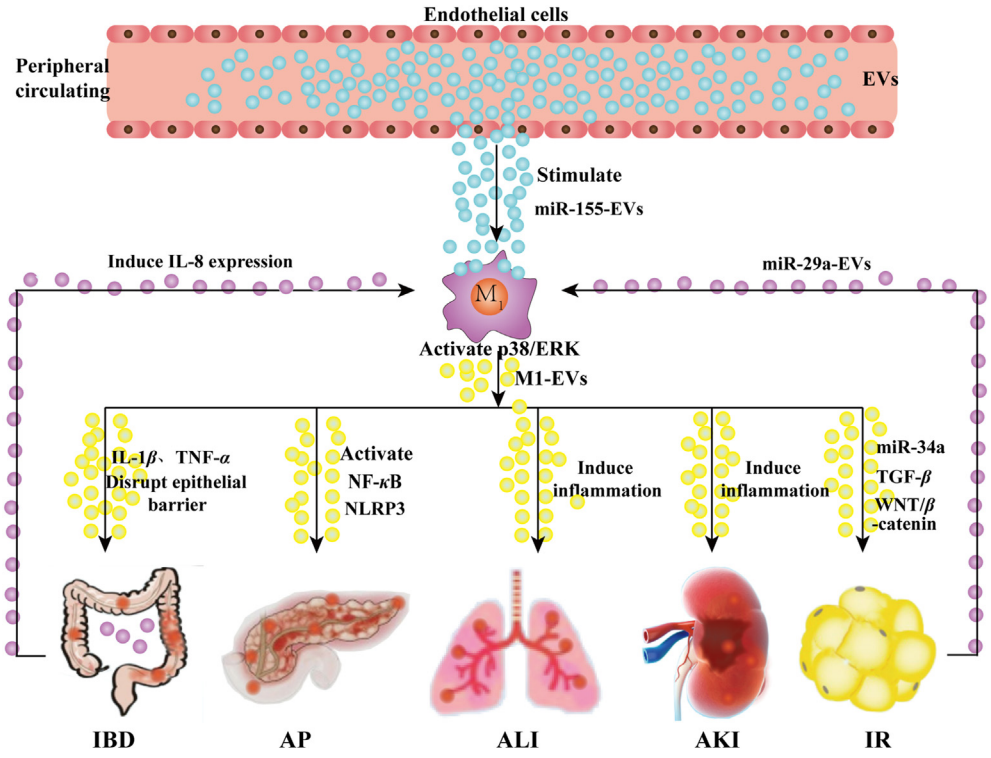

Figure 3 M1 macrophage EV interactions in inflammatory diseases affecting different tissues. EVs in the peripheral circulation carry miR-155 that activates p38/ERK signaling pathways and M1 macrophage polarization. M1 macrophage EVs (M1-EVs) demonstrate multiple pro-inflammatory effects, as they: induce proinflammatory IL-1β and TNF-α secretion in colon tissue, and endothelial dysfunction, to promote inflammatory bowel diseases (IBDs); activate NF-κB and NLRP3 signaling in acinar cells to induce acute pancreatitis (AP); target lung and kidney tissues to induce acute lung injury (ALI) and kidney injury (AKI); carry miR-34a, which upregulates TGF-β and WNT/β-catenin signaling in adipocytes to promote in insulin resistance (IR). Colon cells and adipocytes experiencing inflammatory conditions also release EVs that can promote macrophage polarization to pro-inflammatory phenotypes.

inflammatory through colonic macrophages[114]. Similarly, in an achilles tendon rupture model, Chamberlain et al.[115] found that exosomes derived from macrophages exposed to BSMC exosomes (BSMC-Exo) promoted more regenerative healing responses than BSMC-Exo. In a second tendon rupture model study, local administration of BMSC-Exo at the injury site was also found to promote the formation of fibrocartilage by increasing M2 macrophage polarization and decreasing M1 macrophage levels, with beneficial effects on tissue repair and biomechanical properties at the injury site[111]. Finally, in myocardial ischemia mouse model, intramyocardial injection of BMSC-Exo could reduce infarct size, and its effects have been confirmed by modifying the polarization of M1 macrophages to M2 macrophages[116,117]. These examples illustrate that BMSC-Exo and BMSC-EV have broad effects to attenuate inflammation and promote tissue repair through M2 macrophage-dependent mechanisms.

BMSCs possess some characteristics of immune cells and short-term memory of environmental signals, as one group has demonstrated by showing that BSMC exhibit improved therapeutic efficacy in promoting skin flap survival after being pre-treated with LPS[118]. This discovery poses question of whether small EVs secreted by LPS-treated BMSCs have improved anti-inflammatory properties. A study by Ti et al.[119] has reported that BSMC-Exo obtained after BSMCs were pre-conditioned with LPS (LPS-BSMC-Exo) were better than BMSC-Exo from untreated BSMCs at modulating the balance between macrophage phenotypes and promoting M2 macrophage activation due to their upregulation of anti-inflammatory cytokines, and that the let-7b/TLR4 pathway plays a critical role in this activity. Similarly, in an *in vitro* mouse model of myocardial injury, LPS-BSMC-Exo had superior therapeutic effects to attenuate post-infarction inflammation and cardiomyocyte apoptosis by regulating macrophage polarization than did BMSC-Exo from untreated cells[116]. These studies indicate that low concentration LPS-BMSC-Exo administration may be an alternative to BMSC therapy as cell-free treatment strategy to induce M2 macrophages.

BMSC-EV exposure should, in theory, be capable of inducing both M1 and M2 macrophage polarization. The literature we retrieved for this review indicated only that BMSC-EV promoted the polarization and activity of M2 macrophages. However, it is possible that pre-treatment of BSMCs with alternate stimuli could alter BSMC-Exo cargoes to promote pro-inflammatory responses in recipient cells, and is worth investigation.

4.2. *Adipose stem cells*

Although BMSC-derived small EVs are reported useful in tissue repair and disease treatment, the BSMC isolation procedure is complex and painful[120]. Adipose-derived stem cells (ADSCs), also known as adipose-derived stromal cells, are MSCs derived from the stromal vascular fraction of adipose tissue. Evidence suggests that adipose tissue can provide up to 500-fold more MSCs than an equivalent amount of bone marrow aspirate, and ADSCs have therefore emerged as a stable source of MSCs due to their greater relative abundance, high yield upon isolation, ease of collection *via* a less invasive procedure that has fewer ethical

Table 2 Macrophage polarization effects of EVs derived from stem cell from different tissue sources.

MSC source	Stimulus	Species; model	Macrophage polarization effect	Effects/mechanism	Therapeutic effect	Ref.
Bone marrow	No	Mouse; IBD (DSS & TNBS induced)	M2b macrophage polarization	Induction of IL-10 production	Suppressed inflammatory responses	114
Bone marrow	No	Mouse; Achilles tendon rupture	M2 macrophage polarization	Increase the number of endothelial cells and reduce type I collagen	Improved mechanical function & angiogenesis; reduced inflammation	115
Bone marrow	LPS	Mouse; myocardial infarction	M2 macrophage polarization	Depress NF-κB signaling pathway and activate AKT1/AKT2 signaling pathway	Reduced post-infarction cardiomyocyte apoptosis & inflammation	116
Bone marrow	No	Mouse; myocardial ischemia/reperfusion injury	M2 macrophage polarization	miR-183 targeting of the TLR4 pathway	Reduced infarct size & inflammation	117
Bone marrow	LPS	Rat; diabetic cutaneous wound healing	M2 macrophage activation	Let-7b regulation of the TLR4/NF-κB/STAT3/AKT signaling pathway	Reduced inflammation & enhance cutaneous wound healing	119
Adipose tissue	No	Mouse; Obesity	M2 macrophage polarization	Arginase-1 activation of STAT3 transcription.	Greater insulin sensitivity & reduced adipose inflammation & obesity	123
Adipose tissue	No	Mouse; *Aspergillus* protease-treated MLE12 epithelial cells	M2 macrophage activation	Reduce eotaxin and IL-25, increase TGF-β and IL-10	Reduced Th2-mediated inflammation	125
Adipose tissue	miR-30d-5p mimic	Rat; acute ischemic stroke	Promote M2 microglia/macrophage polarization	Suppress autophagy	Reduced cerebral injury area following infarction	126
Adipose tissue	LPS and D-galactosamine	Macrophages	Suppress M1 macrophage activation	miR-17 suppression of TXNIP/NLRP3 pathway	Reduce inflammatory factor secretion	127
Adipose tissue	No	Mouse; ovalbumin-induced asthma	M1 to M2 macrophage conversion	miR-183-5p sponging to enhance FoxO1-mediated M2 macrophage activation	Reduced airway remodeling	128
Human umbilical cord tissue	No	Mouse; spinal cord injury	Drive BMDM from M1 to M2 polarization	Inflammatory cytokine downregulation	Improved resolution of spinal cord injury	135
Human umbilical cord tissue	No	Rat; burn healing	Inhibit secretion of pro-inflammatory factors	miR-181c downregulation of TLR4 signaling pathway	Reduced burn-induced inflammation	136
Human umbilical cord tissue	IL-6	Mouse; chemically induced liver injury	Inhibit macrophage activation and cytokine production	miR-455-3p targeting of PI3K signaling	Reduced macrophage infiltration & improved liver histology	137
Human umbilical cord tissue	No	Mouse; acute liver injury	ND	Reduced inflammation	Accelerated resolution of acute liver injury	138
Human umbilical cord tissue	No	Mouse; acute liver injury & LPS-stimulated	Inhibit activation of the NLRP3 pathway	Reduced expression of the NLRP3	Enhanced liver tissue repair	139

		RAW264.7 macrophages		inflammasome and its regulated inflammatory factors		
Human umbilical cord tissue	TNF-α	Mouse; acute liver injury & LPS-stimulated RAW264.7 macrophages	Inhibit activation of the NLRP3 pathway	miRNA-299-3p reduction of proinflammatory cytokines and inhibition of the NLRP3 pathway	Reduced liver damage	140
Periodontal ligament	LPS	THP-1 macrophages	M1 macrophage polarization	Increased expression of M1-associated cytokines	M1 macrophage polarization	141

IBD, Inflammatory bowel diseases; DSS, dextran sodium sulfate; TNBS, 2,4,6-trinitrobenenesulfonic acid solution; LPS, lipopolysaccharide; ND, not described; BMDMs, bone marrow derived macrophages.

issues[120,121]. Due to the relatively simple procedure involved in ASDC isolation their ability of SADCs to differentiate into other tissue types of the mesoderm (*e.g.*, bone, cartilage and muscle), ADSCs have been studied in and proven useful in a wide variety of applications[122], such as potential therapies for arthritis, colitis, and autoimmune diabetes[123]. ADSCs have shown the same immunoregulation and regeneration capabilities as other MSCs, and small EVs secreted by ADSCs (ADSC-EV) appear to exert the same functions as ADSCs.

ADSC-EV, like BMSC-EV, has been found to promote the polarization and activation of M2 macrophages. Exosomes derived from ADSCs reduce adipose inflammation and obesity by interacting with macrophages to induce anti-inflammatory M2 phenotypes, in which activated M2 macrophages exert immunosuppressive and anti-inflammatory effects[123,124]. ADSC-EVs have also been shown to promote the polarization and activation of M2 macrophages to ameliorate inflammation in lung epithelial cells treated with an inflammatory factor[125]. While the mechanisms responsible for the activity of ADSC-EVs are not fully understood, one study indicates that the ability of these EVs to induce M2 macrophage polarization and limit inflammatory responses is mediated by inhibiting autophagy, a process that may shift macrophage polarization toward the M1 phenotype[126].

Several proteins and miRNAs in ADSC-derived exosomes can also activate M2 macrophage signaling pathways. For example, miR-17-enriched EVs secreted by ADSCs can suppress NLRP3 inflammasome activation by targeting TXNIP to contribute to the reduction in inflammatory factors secreted by macrophages[127]. Further, reduced expression of regulatory factors in target cells and tissue that leads to inflammation can be countered by the administration of modified ADSC-EVs that carry these factors. Lung tissue of asthmatic mice exhibits a marked downregulation of the circular RNA mmu_circ_0001359 and treatment of asthmatic mice with ADSC-EVs loaded with this RNA has been reported to reduce airway remodeling and promote the polarization of AM to an M2 phenotype to reduce the release of inflammatory factors[128]. In another example, tissue from the brains of patients and animals exposed to acute ischemic shock was found to exhibit reduced expression of microRNA miR-30d-5p, while treatment with ADSC-EVs produced by ADSCs expressing an miR-30d-5p mimic vector could effectively promote the M2 polarization of microglia and reduce the cerebral injury area at the site of infarction[126]. These studies indicate that ADSC can be readily modified *in vitro* to express small RNAs to produce EVs with therapeutic effects. Similar direct therapeutic approaches may also be possible for other inflammatory conditions where the downregulation of specific factors lead to enhanced, aberrant or dysregulated inflammation. Due to the high abundance of ADSCs in their source tissue relative to other MSCs, and the relative simplicity of the isolation and extraction processes required to produce ADSC isolates, ADSCs are likely to become the primary source of anti-inflammatory EVs employed to promote M2 macrophage polarization in the treatment of inflammatory disease when and such applications are approved for therapeutic applications.

4.3. Human umbilical cord mesenchymal stem cells (HUCMSCs)

MSCs derived from the human umbilical cord (HUCMSCs) can be obtained from the amniotic membrane, cord-lining, Wharton's jelly, or peri-vascular region[129]. HUCMSCs are isolated using a

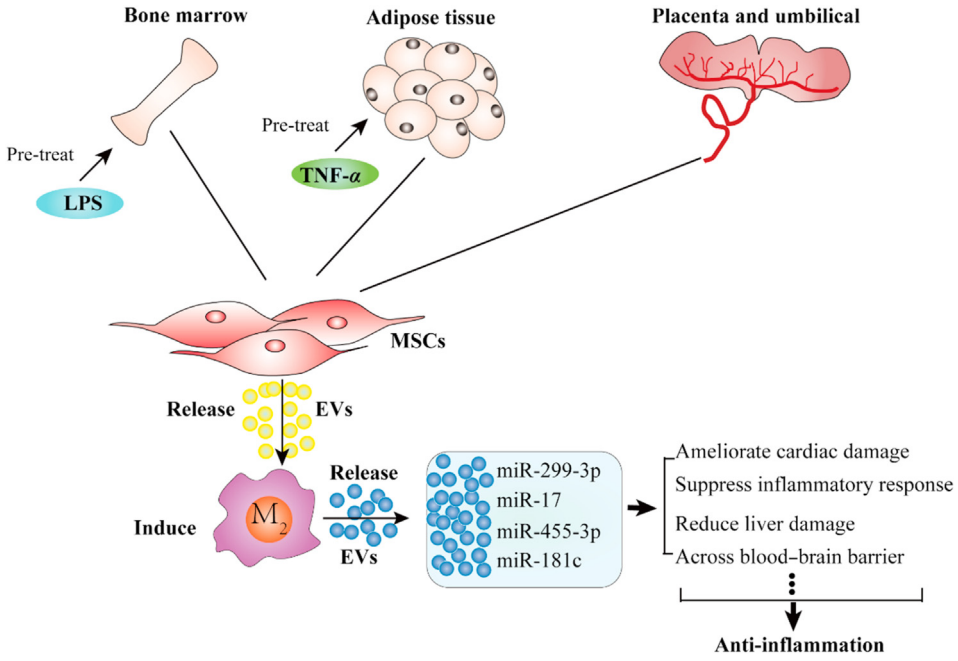

Figure 4 Extracellular vesicles (EVs) secreted by mesenchymal stromal cells (MSCs) from bone marrow, and adipose and umbilical cord tissue induce M2 macrophage polarization *via* specific miRNA cargoes to regulate an anti-inflammatory effects.

painless collection procedure and have rapid self-renewal properties, unlike BMSCs[129]. In addition, pre-clinical trials have found that HUCMSCs applications have fewer side effects and are generally safer than other MSC treatments[129]. Thus, HUCMSCs are a promising source of MSCs due to their relative abundance, non-invasive collection, convenient and inexpensive isolation procedure, low immunogenicity and high gene transfection efficiency[130]. Evidence shows that HUCMSCs exert their therapeutic effects primarily through EV-mediated paracrine effects. In many cases of inflammation-related diseases and tissue damage repair[110,131], HUCMSC EVs exhibit all of the advantages of HUCMSCs without their disadvantages, and are easier to maintain in a bioactive state during storage and transport[132]. HUCMSC EVs are acquired by extensive *in vitro* expanding HUCMSCs; are convenient to extract, store and transport; and exhibit lower immunogenicity and better biocompatibility than other macrophage-polarizing exosomes[133]. Multiple studies have now shown that HUCMSC EVs exhibit strong potential for use in "cell-free therapy" approaches[110].

HUCMSC EVs are reported to reduce inflammation by targeting macrophages, promoting M2 macrophage polarization, and enhancing the recruitment of M2 macrophages to several organs and tissues, including the islets, liver, fat, and muscle[134]. HUCMSC exosomes have been shown to effectively trigger bone marrow-derived macrophages to undergo a shift from M1 to M2 polarization *in vitro*, to possess characteristic anti-inflammatory effects, and to improve functional recovery in mice with spinal cord injuries by downregulating inflammatory cytokines (*e.g.*, TNF-α, MIP-1α, IL-6 and IFN-γ[135]). Systemic administration of HUCMSC exosomes has also been shown to reverse inflammation related to burn injuries in rats, by limiting macrophage-based inflammatory reactions[136]. HUCMSC exosome miR-181c played a pivotal role in attenuating burn-associated inflammation in this study, since HUCMSC exosomes that overexpressed miR-181c were more effective at suppressing TLR4 signaling in burned rats to reduce inflammation[136]. Similarly, another group examined the ability of HUCMSC exosome miR-455-3p, which was induced by IL-6 treatment, to inhibit IL-6 signaling by suppressing PI3K expression[137], and found that miR-455-3p-enriched exosomes inhibited LPS-challenged macrophage activation and cytokine production. Thus, like ADSCs cells, it is feasible to modify HUCMSCs *in vitro* to obtain EVs enriched in specific regulatory miRNAs for targeted treatment of selected inflammatory conditions.

HUCMSCs, unlike BMSCs and ADSCs, have innate hepatogenic properties, however, and HUCMSC therapy can be used to directly treat acute liver injury without requiring that HUCSMCs undergo differentiation or manipulation prior to their administeration[138], raising the question as to whether or not unmodified HUCMSC EVs exhibit similar tissue repair activity. One study has reported that unmodified HUCMSC exosomes can indeed efficiently accumulate in the liver to alleviate acute liver failure by reducing macrophage NLRP3 inflammasome activity[139]. However, a second study has shown that exosomes derived from HUCMSC treated with TNF-α exhibit enhanced therapeutic effects to attenuate liver damage[140], finding that miRNA-299-3p is upregulated in TNF-α-stimulated HUCMSCs and selectively packaged into exosomes to exert anti-inflammatory functions in exosome treatments[140]. Thus, HUCMSC-EVs appear to have a unique advantage for the treatment of liver inflammation and injury.

HUCMSC exosomes have also been reported to penetrate the blood−brain barrier and modulate the activation of microglia in mouse brains to alleviate neuroinflammation[131], which is a critical advantage for exosome-based applications intended to treat neurodegenerative conditions, such as Alzheimer's disease, and other neurological pathologies, since the blood−brain barrier poses a formidable barrier for most therapeutic approaches.

Most stem cells appear to produce EVs that can promote anti-inflammatory M2 macrophage polarization, regardless of whether the stem cells derive from bone marrow, adipose, or umbilical cord tissue, and LPS-pretreatment of many of these cells has been

reported to enhance the anti-inflammatory activity of their EVs. However, one report indicates that LPS-pretreated periodontal ligament stem cells secrete small EVs that can induce M1 macrophage polarization[141]. Various therapeutic effects mediated by stem cell derived EVs on macrophage polarization are listed in the following Table 2[114–117,119,123,125–128,135–141]. The ability of stem cells and their EVs to induce M1 or M2 macrophage polarization may thus depend on the source tissue and original microenvironment of the stem cells, which may affect their relative differentiation state, as well as conditions and stimuli these cells encounter during isolation and cell culture expansion. The ability of MSC EVs to promote M2 macrophage polarization appears to have significant potential utility for applications designed to attenuate inflammatory conditions and promote tissue repair, however, there is also a concern for potential negative consequences due to off-target treatment effects. For example, M2 macrophages present within a tumor microenvironment can accelerate tumor progression by promoting cell proliferation and metastasis, and MSC EV administration during non-small cell lung cancer has been shown promoting the growth and mobility of cancer cells by polarizing M2 macrophages[142].

Strategies that employ EVs isolated from different MSCs to induce M2 macrophage polarization to limit tissue inflammation and promote tissue repair (Fig. 4) have been attempted for various inflammatory disease conditions. MSC-derived EVs can promote AMs to adopt M2 phenotypes and attenuate tissue damage in clinically relevant lung injury models[143], and to reduce hypoxia-ischemic injury in neonatal mice by modulating microglia/macrophage polarization[144]. HUCMSCs seem to be the best overall source of anti-inflammatory EVs among the MSCs discussed herein for the reasons listed above. EVs of each of these MSC types, however, have shown considerable ability to inhibit tissue inflammation and injury, and their relative merits as therapeutic approaches designed to treat specific inflammatory conditions should be carefully considered in future studies.

5. Potential application of macrophage-derived EVs for inflammatory disease treatment

Conventional drug-loaded nanoparticles have, to a degree, demonstrated potential utility for infectious disease treatment, but such nanoparticles are often difficult to manufacture, can be rapidly cleared from the circulation by the mononuclear phagocyte system, and may cause undesirable side effects such as organ toxicity and the activation of immune responses[145–147]. Decorating nanoparticles with a polyethylene glycol (PEG) corona is thus sometimes used to reduce the recognition of nanoparticles by the mononuclear phagocyte system to avoid their rapid clearance from the circulation. However, small EVs have emerged as a better means for drug delivery than PEGylated nanoformulations, due to the low immunogenicity, high biocompatibility, and high efficacy of these EVs[22], although exosome PEGylation can also reduce mononuclear phagocyte system recognition after administration to mice to improve their time in circulation[147]. Small EVs contain a variety of adhesion molecules with their lipid bilayer that can interact with cell membrane factors to promote their interaction and uptake by specific cell types, and thus small EVs can have specific cell tropisms according to their surface display of specific adhesion molecules, which is reflective of their specific cell origin[145]. Nanoparticles are usually of abiotic origin, however, and thus lack these cell interaction mechanisms. Studies have also indicated that small EVs are taken up by their recipient cells about 30 times more efficiently than synthetic nanoparticles[145]. This suggests that drugs loaded into small EVs can be more efficiently delivered to target cells at therapeutic quantities, demonstrating a major advantage of small EVs-based drug delivery systems over common synthetic nanocarriers. Taken together, these characteristics imply that small EVs-based treatment approaches are likely to be more effective than those that employ synthetic nanoparticles.

Many challenges still need to be solved to allow the clinical translation of approaches that employ small EVs for therapeutic applications, especially for infectious disease applications. First, standard procedures for small EV isolation must be established to allow the consistent isolation of high purity small EV preparations. Several methods are currently used for small EV isolation, including immunoaffinity, density gradient, and ultracentrifugation isolation approaches, but each method has drawbacks that limit utility for clinical applications[19]. For example, immunoaffinity separations have low recovery rates; density gradient procedures are labor-intensive and are not amenable to automation; and ultracentrifugation approaches are time-consuming[19,22], while the performance of commercial kits requires further improvement. Small EV isolation methods thus require additional improvement to allow consistent large-scale isolation of high-purity EVs that will be required for clinical applications.

Second, additional studies are required to determine which cells (*e.g.*, macrophages or MSCs) are the most appropriate source for the small EVs used in a therapeutic application, which may vary by the specific application. Small EVs tend to have characteristics of their parent cells. EVs derived from M1 macrophages have elevated potential to activate inflammatory responses *via* boosting a Th1 and Th17 response, while EVs derived from M2 macrophages are generally able to attenuate inflammation *via* a mechanism bypassing a Th1 and Th17 response[148], while small EVs secreted by MSCs have been shown to suppress pro-inflammatory responses and stimulate cell proliferation in clinical trials for several diseases[149]. Approaches employing all these small EVs have lower therapeutic risk and superior safety profiles than cell-based therapy approaches employing their parental cells[150,151]. Beyond their ability to mediate pro- or anti-inflammatory responses, small EVs derived from these different cell types may exhibit distinct cell targeting capacities or other functionalities. Macrophages secrete exosomes that express CD47 receptors that interact with signal regulatory protein α (SIRPα) to produce a “don’t eat me” signal upon their interaction with phagocytes[147]. Macrophage-derived exosomes express intercellular adhesion molecule 1 (ICAM-1), enhancing their interaction with blood–brain barrier endothelial cells[152] and transit through the blood–brain barrier. Similarly, the innate ability of HUCMSC EVs to accumulate in the liver and attenuate hepatic tissue inflammation may make them the best candidates for small EV therapeutics when designing applications to treat liver inflammation and injury.

Finally, the ability to modify the cargoes of small therapeutic EVs by loading small molecules may represent an additional consideration. Many different approaches have been described to load small molecules into EVs, either directly or indirectly, including electroporation, sonication, incubation[19], and these exhibit different loading efficiencies, and may cause off-target effects[22]. Go et al.[153] have achieved remarkable results in the treatment of inflammation induced by Gram-negative bacteria by loading dexamethasone into monocyte-derived EVs by sonication, illustrating that it is feasible to directly load antibiotics directly into macrophages-derived EVs to produce effective therapeutics

for infectious disease treatment. In a related study, Yang et al.[154] used an incubation approach to load the antibiotic linezolid into exosomes derived from mouse RAW264.7 macrophages, and found that this exosome-encapsulated linezolid formulation had a better inhibitory effect on intracellular methicillin-resistant *Staphylococcus aureus* infections *in vitro* and *in vivo* that direct linezolid treatment, without any sign of macrophage cytotoxicity. These studies indicate that antibiotic-loaded macrophage-derived small EVs may be more effective therapeutics than direct treatment, particularly for intracellular microbial infections. Macrophage-derived EVs may be particularly useful for such applications, since macrophage cell lines can serve as stable sources of small EV preparations and are amenable to manipulations that promote the selective delivery of specific therapeutic molecules to desired target cells or tissues, while the inherent anti-inflammatory properties of such EVs may amplify desired therapeutic effects.

Finally, differences in stimuli encountered during cell isolation or culture or EV processing can alter the characteristics and function of small EV preparations produced from the same tissues or cells. Strictly controlling the consistency of small EVs therefore requires considerable attention to detail, and may be especially challenging when using stem cell populations. Additional studies are required to establish standardized procedures for EV isolation and manipulation for the various cell types under investigation. Further experiments are also needed to verify the roles of these EVs in specific processes pertaining to various categories of inflammatory disease.

6. Conclusion and perspectives

This review summarizes the specific effects of macrophages, MSCs and their small EVs in several inflammatory disease conditions, particularly in regard to inflammatory responses that lead to local or remote tissue damage, and how EV-based interaction loops between pro-inflammatory macrophages and tissues play a vital role in disease progression. Evidence now indicates that altering the interactions between macrophages and their target cells in tissues affected by inflammatory conditions *via* administration of native or exogenously modified EVs preparations can be a promising approach to attenuate tissue inflammation and injury. Consideration must be paid to the relative stage of the tissue injury/repair process or the severity of the inflammatory response, however, since both pro- and anti-inflammatory macrophage activities are required at different stages of the resolution of infection or tissue injury. In the early stages of some inflammatory responses, M1 macrophages release regulatory factors and recruit cells required to initiate innate and adaptive immune responses to limit and resolve local infections, while later in such infections or during injury responses M2 macrophages are needed promote tissue repair by limiting inflammation, clearing cell debris, and promoting the cell proliferation to replace cell deficits. Small EVs secreted by MSCs mentioned in this paper can polarize M2 macrophages, and EVs released by these cells have been shown to have important actions in the resolution of inflammatory responses and tissue repair, and may serve as therapeutics to improve the control of dysregulated or excessive inflammation that may cause tissue injury.

Several questions remain to be answered about the potential small EV therapeutics, including the best time to employ them and what their side effects may be when used in different situations.

Over-activation of M1 macrophages is expected to promote systemic inflammatory responses, potentially including atherosclerosis[155]. M2 macrophages appear to be the “default” form for most tissue-resident macrophages, and appear to be required to control systemic inflammation. However, M2 macrophage EVs can also facilitate tumor progression by promoting tumor cell proliferation and invasion[156]. Thus, the decision to use potential future EV applications to resolve inflammatory conditions or treat certain infections will need to consider multiple factors to achieve maximum efficacy and reduce potential side effects.

In addition to these considerations, further studies are also required to establish standardized conditions for EV isolation and modification procedures, since such differences may alter the characteristics and innate functionality of therapeutic small EV preparations. Macrophage-derived EVs appear to have potential as drug carriers for the treatment of infectious disease. However, similar studies have not yet demonstrated that such EVs can be used to deliver anti-inflammatory drugs to attenuate inflammatory disease conditions, although this is likely to be future applications, particularly when considering the innate anti-inflammatory characteristics of M2 macrophages within the topic aseptic inflammation.

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Author contributions

Qian Hu and Jesse K. Fletcher drafted the main part of the manuscript. Wenfu Tang and Christopher Lyon edited the manuscript and contributed by iteratively reviewing and improving the manuscript. Meihua Wan and Tony Y. Hu provided oversight of drafting the manuscript, and made substantive intellectual contributions and improvements. All authors read and approved the final manuscript.

Conflicts of interest

The authors have no conflicts of interest to declare.

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