


# Osteoimmune Interactions and Therapeutic Potential of Macrophage-Derived Small Extracellular Vesicles in Bone-Related Diseases

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**Abstract:** Due to the aging of the global population, the burden of bone-related diseases has increased sharply. Macrophage, as indispensable components of both innate immune responses and adaptive immunity, plays a considerable role in maintaining bone homeostasis and promoting bone establishment. Small extracellular vesicles (sEVs) have attracted increasing attention because they participate in cell cross-talk in pathological environments and can serve as drug delivery systems. In recent years, an increasing number of studies have expanded our knowledge about the effects of macrophage-derived sEVs (M-sEVs) in bone diseases via different forms of polarization and their biological functions. In this review, we comprehensively describe on the application and mechanisms of M-sEVs in various bone diseases and drug delivery, which may provide new perspectives for treating and diagnosing human bone disorders, especially osteoporosis, arthritis, osteolysis, and bone defects.

**Keywords:** small extracellular vesicles, macrophage, crosstalk, drug delivery, bone homeostasis

## Introduction

Extracellular vesicles (EVs) are nanovesicles with a lipid bilayer structure that are released by various cells.<sup>1</sup> In recent years, EVs have attracted extensive attention as promising alternative mediators of cell communication, and can directly affect a number of physiological and pathological processes in target cells.<sup>2–4</sup> As they have numerous advantages over conventional synthetic carriers, EVs have been considered as a next-generation drug delivery platform, opening new frontiers for modern drug delivery.<sup>5,6</sup> EVs can be categorized into multiple subtypes based on their origins and physical characteristics.<sup>7</sup> Among these different subtypes of EVs, small EVs (sEVs) have been one of the most widely studied subtypes in recent years.<sup>8</sup>

sEVs, with a diameter of 30–200 nm, are released by different cells, originate from the endocytic pathway<sup>7</sup> and widely distributed in a variety of body fluids.<sup>9,10</sup> All cells can release sEVs regardless of whether they are in a state of normal or pathological conditions. Due to their different sizes, levels, contents and origins, sEVs have great heterogeneity and target specific organs or cells, leading to different biological functions.<sup>11</sup> As natural endogenous nanocarriers, intercellular communication through sEVs is a crucial process for tissue progression.<sup>12</sup> sEVs, which are considered mediators of a novel mode of cell-to-cell communication, participate in intercellular signaling by transferring their encapsulated bioactive components such as RNAs, proteins, metabolites, nucleic acids, and lipids to regulate cellular behavior as a kind of signal, or by traveling to a distant body site to contribute to homeostasis and disease and are endocytosed by target cells, leading to changes in gene expression and cellular function in state of health and disease.<sup>1,13,14</sup>

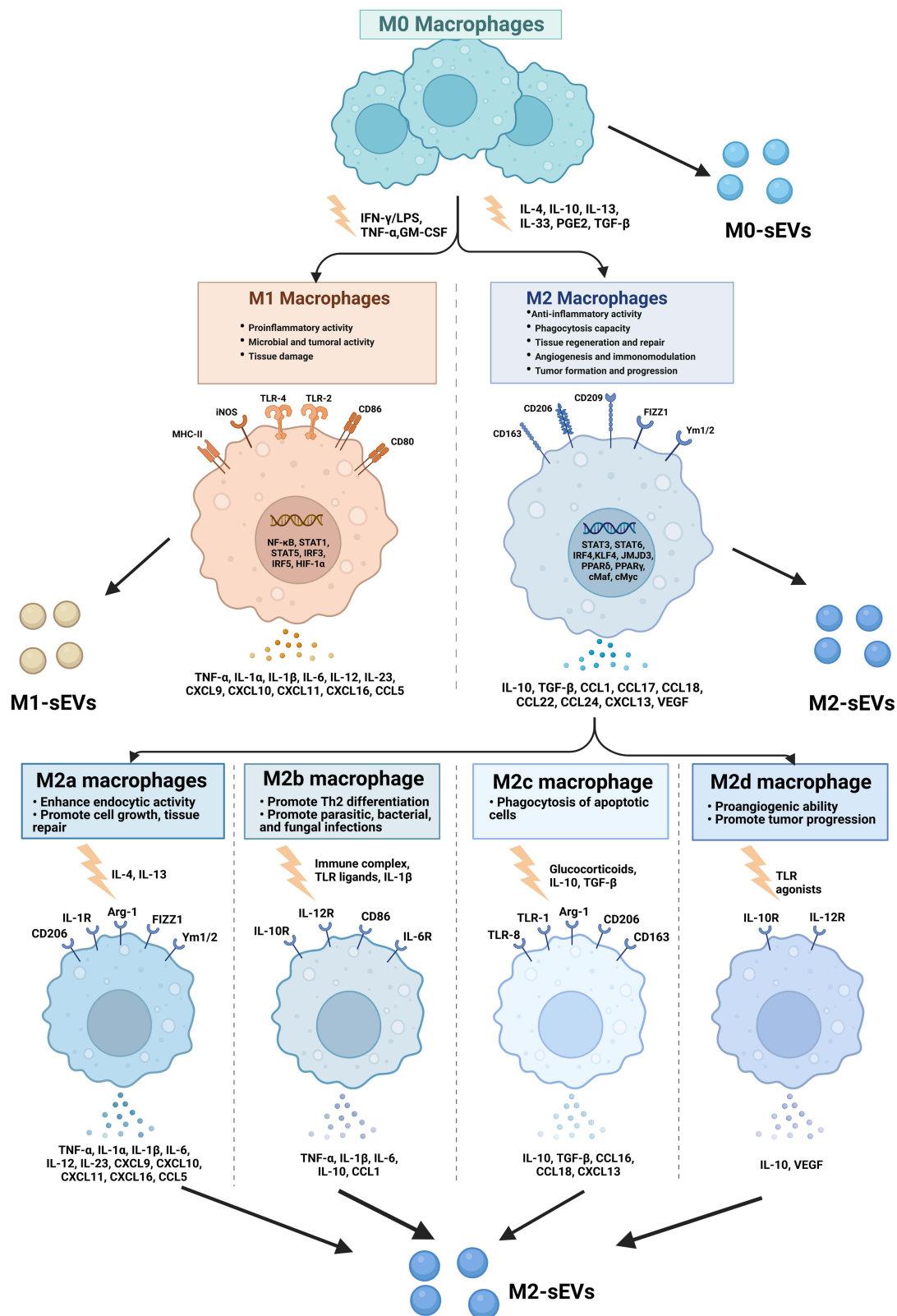
Macrophages are indispensable immune cells that contribute to both proinflammatory and anti-inflammatory processes and are involved in both tissue destruction and regeneration. Macrophages play an indispensable role in both innate immune responses and adaptive immunity and are distributed in all tissues of the body, with high plasticity and heterogeneity according to the tissue and organ in which they reside.<sup>15</sup> In response to various stimuli, macrophages can switch phenotypes from unpolarized (M0) to polarized (M1 and M2) states and play unique roles in different stages of disease status and tissue healing.<sup>4,16–18</sup> Moreover, the latest opinions tend to describe that M2 macrophages can be further divided into four subtypes (M2a, M2b, M2c and M2d) according to different stimulations.<sup>19,20</sup> (Figure 1) Macrophages perform functions that are usually related to their secretion of sEVs and microRNAs (miRNAs) carried by sEVs (Figure 2). Moreover, recent studies have demonstrated that sEVs derived from macrophages play crucial roles in different biological processes, such as the immune response,<sup>21</sup> signal transduction,<sup>22</sup> cell proliferation,<sup>23</sup> and angiogenesis,<sup>8</sup> and promote or inhibit a range of diseases, such as orthopedic disorders,<sup>24</sup> chronic inflammation,<sup>25</sup> cardiovascular disease,<sup>26</sup> metabolic disorders and tumors.<sup>1,11,27</sup> Based on the current knowledge, this article reviews the status of investigations of sEVs secreted from macrophages and their potential application to bone diseases and aims to describe novel approaches for bone disease treatment in clinical practice.

## Promotion of Osteogenesis

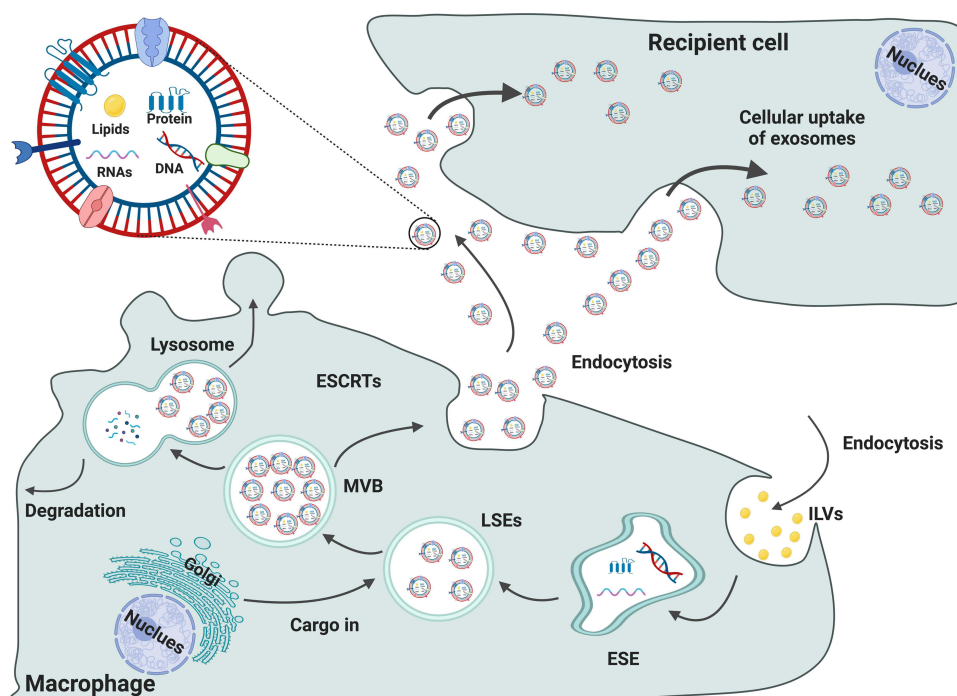
sEVs are lipid bilayer nanovesicles that are secreted by most cell types and control intercellular communication. Increasing data suggest that macrophages can establish an optimal microenvironment to reduce inflammation and promote osteogenesis through a paracrine mechanism.<sup>28</sup> sEVs derived from macrophages have been investigated as potential immunomodulatory agents in clinical therapy.<sup>29,30</sup> A large number of studies have demonstrated that sEVs can be used in cell-free therapy to effectively improve bone disease and solve the problems (immune rejection, insufficient bone mass, and poor stability) caused by traditional bone implants and stem cell therapy.<sup>31,32</sup>

In preclinical investigations, sEVs from macrophages with different polarization phenotypes were found to have different effects on bone metabolism.<sup>4</sup> Based on the observations that macrophages contribute to bone regeneration, macrophage polarization to the M1 phenotype may be essential during the early phases of osteoinduction and bone regeneration, and the M2 phenotype may foster continued bone regeneration.<sup>30</sup> Huang et al demonstrated that sEVs secreted by M1 macrophages promoted the proliferation and osteogenic and adipogenic differentiation of bone marrow mesenchymal stem cells (BMSCs). However, the sEVs secreted by M2 macrophages impaired the proliferation of BMSCs. M0-sEVs exhibit no considerable influence on the proliferation of BMSCs.<sup>33</sup> Another study indicated that sEVs from M1 macrophages attenuated cementoblast mineralization, while sEVs from M2 macrophages facilitated cementoblast mineralization.<sup>4</sup> During tissue healing and regeneration, biomimetic intrafibrillarly mineralized collagen (IMC) was found to regulate M2 macrophage polarization and secrete sEVs. Human BMSCs were cultured in osteogenic medium supplemented with IMC-sEVs. IMC-sEVs promoted BMSC osteogenic differentiation by increasing the expression levels of BMP2 and Smad5 (one of the classic proteins of the osteogenic pathway). Blockade of sEV secretion by GW4869 significantly impaired BMSC osteogenesis.<sup>26</sup>

In recent years, the function and potential application of miRNAs in osteogenic differentiation have attracted increasing attention. MiR-690 enriched in M2-sEVs could facilitate osteogenesis and reduce adipogenesis by upregulating the levels of IRS-1 and TAZ, suggesting a potential role of M2-sEVs as a therapeutic tool for bone loss.<sup>34</sup> Macrophage-derived sEVs containing miR-381 suppress the osteogenic differentiation of BMSCs by reducing the levels of ALP and Runx2, and the effect could be reversed by a miR-381 inhibitor. Mg<sup>2+</sup> is essential for the maintenance of physiological homeostasis in tissues and organs and has been verified to significantly decrease miR-381 expression and further facilitate the osteogenic differentiation of BMSCs.<sup>35</sup> IL-10 is considered to be a crucial regulator of bone homeostasis and inflammatory conditions, playing a prominent role in regulating osteoblast/osteoclast differentiation and function. M2-sEVs could upregulate IL-10 cytokine expression in BMSCs and bone marrow-derived macrophages (BMDMs) by delivering IL-10 mRNA to cells directly, leading to the activation of the cellular IL-10/IL-10R pathway to regulate cell differentiation and bone metabolism and preventing bone loss in murine periodontitis models.<sup>36</sup>



**Figure 1** Macrophage polarization. M $\phi$ s can be roughly divided into two subtypes (M1 & M2) depending on different microenvironmental stimuli. M2 M $\phi$ s can be roughly divided into four subtypes (M2a, M2b, M2c, and M2d) depending on different microenvironmental stimuli. M1 M $\phi$ s are typically induced by IFN- $\gamma$ /LPS while M2 M $\phi$ s are induced by IL-4/IL-10. M1 M $\phi$ -sEVs secrete high levels of proinflammatory cytokines, such as TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-12, and IL-23, promoting inflammatory and cytotoxic responses. M2 M $\phi$ -sEVs not only directly inhibit the expression of proinflammatory enzymes and cytokines, such as IL-12 and TNF- $\alpha$ , to achieve anti-inflammatory effects but also display higher levels of certain anti-inflammatory factors, such as IL-10 and TGF- $\beta$ , thereby resolving deleterious inflammatory conditions.



**Figure 2** The formation of small extracellular vesicles derived from macrophages. The cytoplasmic membrane of Mφs initially invaginates to form endocytic vesicles, and multiple endocytic vesicles fuse to form early-sorting endosomes (ESEs). The ESEs then invaginate, encapsulating intracellular material in the process and further transforming into late-sorting endosomes (LSEs), which are known as multivesicular bodies (MVBs). MVBs then fuse with the cytoplasmic membrane and release EVs into the extracellular space.

## Promotion of Osseointegration

Angiogenesis and osteogenesis are essential preconditions for achieving favorable osseointegration of the bone implants. Increasing evidence has demonstrated that osteoimmunomodulation mediated by macrophage-derived sEVs plays a pivotal role in angiogenesis and osteogenesis.<sup>37,38</sup> sEVs can be internalized by various cells participating in bone formation, such as endothelial cells and osteoblasts, to intervene in the osseointegration.

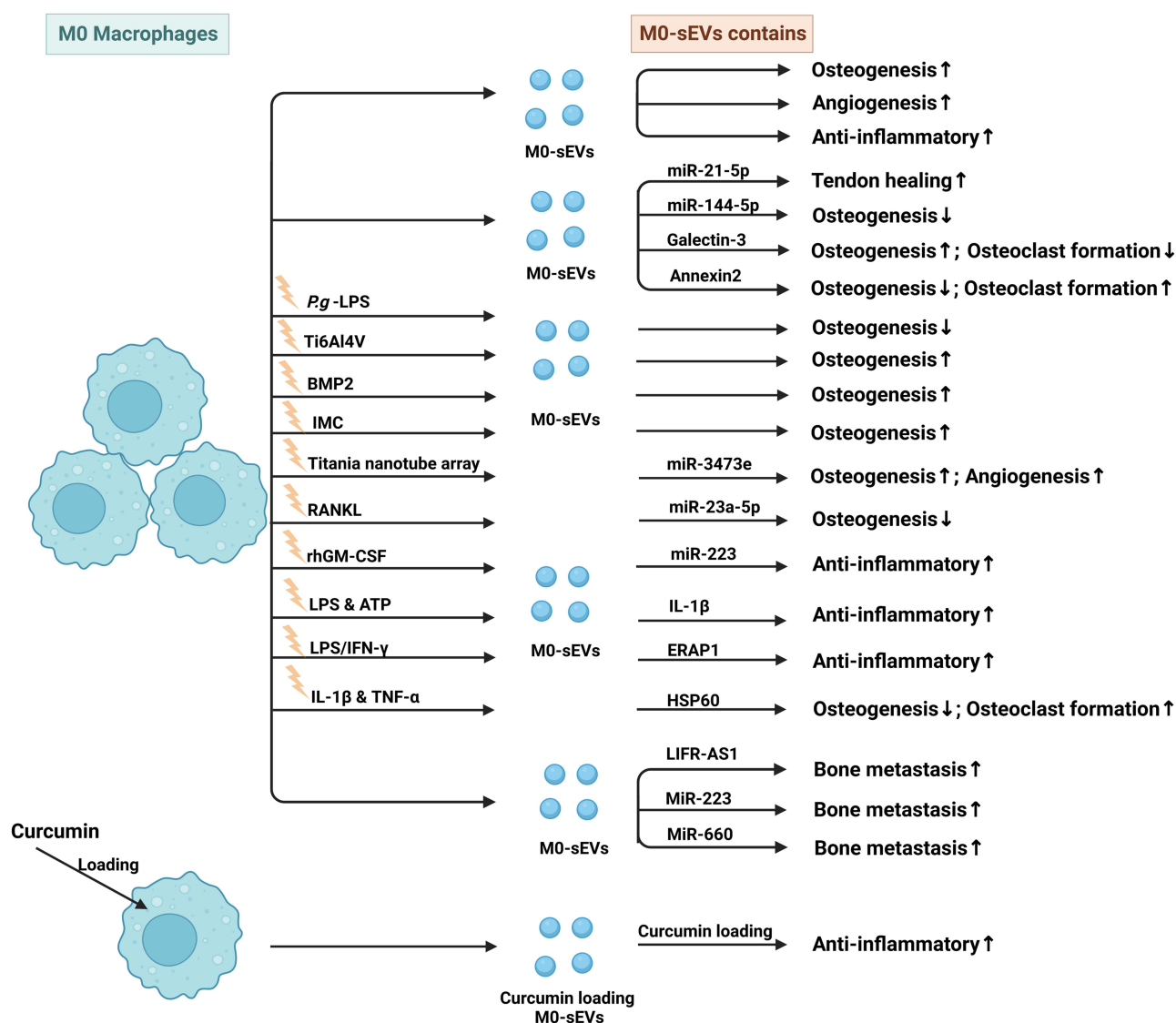
In one study, sEVs derived from bone morphogenetic protein 2 (BMP2)-stimulated macrophages were used to modify titanium nanotube implants. The incorporation dramatically increased the expression of early osteoblastic differentiation markers, ALP, Runx2 and BMP2; promoted the osteogenic differentiation of BMSCs; activated autophagy of hBMSCs; and altered the secretion of cytokines associated with bone remodeling, indicating the important effects of macrophage-sEVs in osseointegration.<sup>39</sup> Titanium and titanium alloys are the most widely used implant materials in clinics due to their good biocompatibility and mechanical properties and have an important influence on osseointegration. Mouse RAW264.7 cells stimulated with titanium disks (Ti6Al4V) were found to polarize into the anti-inflammatory M2 phenotype. sEVs derived from M2 macrophages induced upregulation of osteoblast differentiation marker genes Runx2 and Col-1 in MC3T3-E1 cells. Moreover, several miRNAs involved in the progress of osseointegration were upregulated or downregulated, indicating that M2-sEVs played an important role in the process of osteoimmunity-promoting osseointegration.<sup>40</sup> Another study confirmed that sEVs extracted from macrophages cultured with titania nanotube arrays anodized with a voltage of 40 V (TNA-40) can significantly upregulate the ALP activity and osteogenic-related gene expression of BMSCs, promote endothelial cell (EC) migration, and enhance angiogenic capacity through the interaction between miR-3473e and the target gene Akt1.<sup>41</sup> The immunosuppressive and anti-inflammatory molecule ADA2AR has been proven to enhance the proliferation and migration of vascular endothelial cells (VECs) and promote angiogenesis and the selection of M-sEVs during bone healing. However, the underlying mechanism warrants further research.<sup>16</sup> sEVs derived from alternatively activated M2 macrophages can induce in situ direct conversion of classic activated M1 macrophages into reprogrammed M2-like phenotype to promote cutaneous wound healing and repair by enhancing angiogenesis and re-epithelialization.<sup>42</sup>

## Promotion of Tissue Repair and Regeneration

Macrophage-derived sEVs may serve as an emerging functional tool in biomaterial-mediated endogenous bone regeneration. Macrophages with different polarization phenotypes influence cementoblast mineralization through sEVs, M1 macrophages attenuate mineralization, and M2 macrophages enhance mineralization.<sup>43</sup> In a diabetic wound rat model, macrophage-derived sEVs exerted anti-inflammatory effects by inhibiting the secretion of proinflammatory enzymes and cytokines, weakening the infiltration of inflammatory cells, and inducing endothelial cell proliferation and migration to improve angiogenesis and re-epithelialization to further promote diabetic wound repair.<sup>44</sup>

## M-sEVs and Bone Diseases

Macrophages have been recognized as essential regulators of bone homeostasis.<sup>45,46</sup> M-sEVs play a crucial role in bone diseases by interfering with osteoblast differentiation, osteogenesis, angiogenesis, cell proliferation, apoptosis, the immune response, and inflammatory factors. sEVs derived from different macrophage subtypes perform diverse functions (M0-sEVs in Figure 3 and Table 1, M1-sEVs in Figure 4 and Table 2, M2-sEVs in Figure 5 and Table 3).



**Figure 3** Published data on the biogenesis of sEVs derived from M0 macrophages in bone-related diseases.

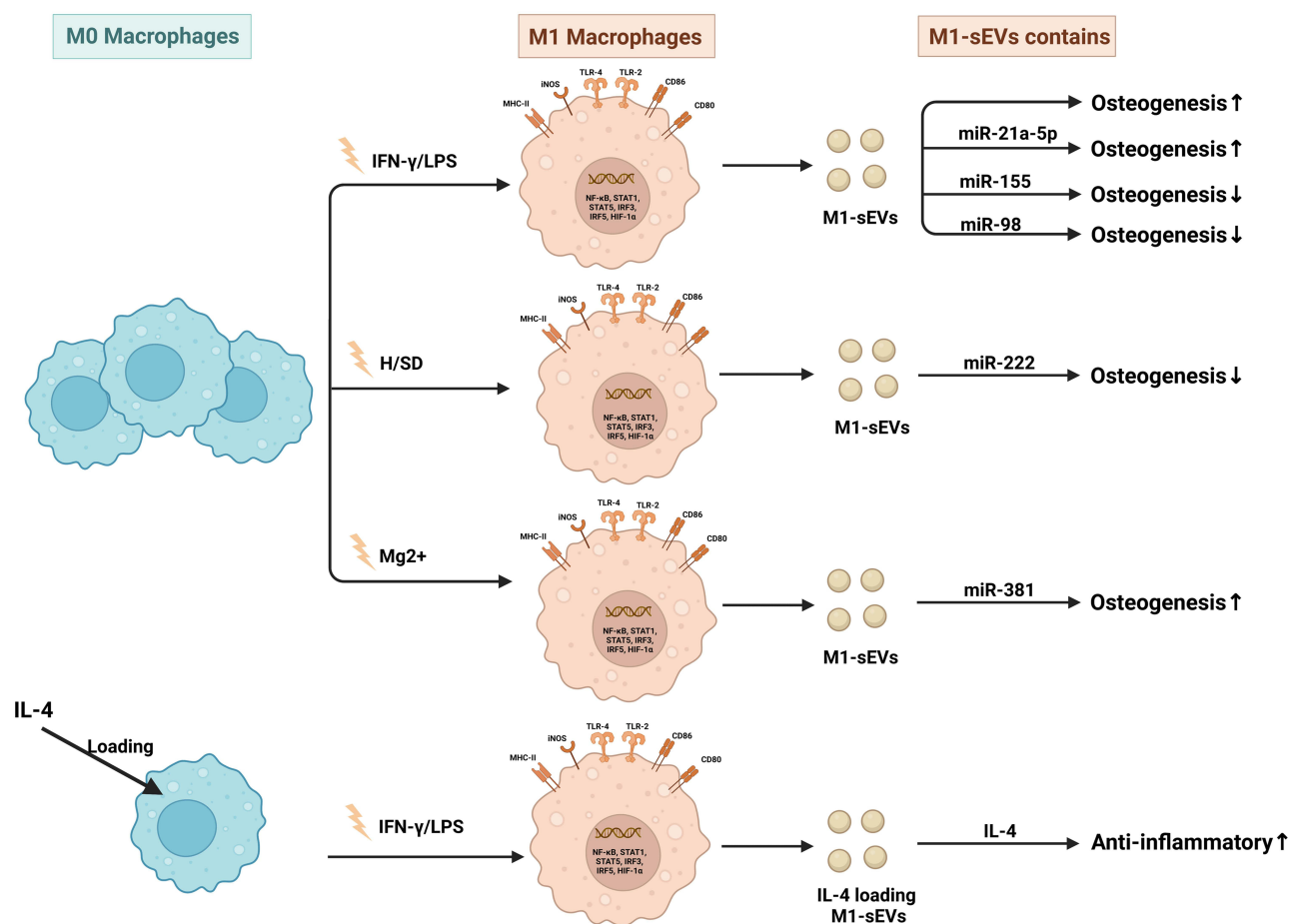
**Table I** Published Studies on Biogenesis of M0-Macrophage (M0-M $\phi$ ) Derived sEVs in Bone-Related Diseases

sEVs and Contains	Source of M $\phi$	Induction Methods	Functions & Pathway	Model System	Application	Ref.
M0-sEVs	C57BL/6 mMSCs	–	Promote repair/regeneration	Rat calvarial bone defect model	Bone defect	[4]
M0-sEVs	SD rats peripheral blood	–	Promote angiogenesis and VECs migration during bone healing	Rat femur fracture model	Bone fracture	[16]
M0-sEVs	RAW264.7	–	Promote HG-HUVECs migration and tube formation; promote cell migration and proliferation in HG-HUVECs via anti-inflammation; promote wound healing in type I diabetic rats	Diabetic rat wound model; HG-HUVECs	Diabetic bone disease	[44]
M0-sEVs containing miR-21-5p	C57BL/6 mice	–	Increase proliferation, migration, and fibrotic activity of tendon cells, induce peritendinous fibrosis after tendon injury via the miR-21-5p/Smad7 pathway	Mice underwent complete transection and repair of the FDL tendon; NIH3T3 fibroblast cells	Tendon injury	[58]
M0-sEVs containing miR-144-5p	Diabetic SD rat BMDMs	–	Reduce the bone repair and regeneration by suppressing Smad1 expression; Osteogenesis $\uparrow$	Rat femoral fracture model	Bone fracture	[55]
M0-sEVs containing Galectin-3	SD rat BMDMs	–	Inhibit osteoclast formation in a rat bone marrow culture system; inhibit osteoclast differentiation by suppressing NFATc1 expression	Rat AA model	Osteoporosis	[70]
M0-sEVs containing Annexin2	–	–	Increase RANKL expression in adherent hBMCs by MAPK pathway; promote osteoclast formation by stimulating the proliferation and differentiation of OCL precursors	BMSCs	Bone loss	[69]
M0-sEVs	RAW264.7	Pg -LPS	Inhibit BMSCs osteogenic differentiation by mediating inflammatory stimulation via upregulating IL-6 and TNF- $\alpha$ expression in BMSCs	Mouse BMSCs	Bone loss	[97]
M0-sEVs	RAW 264.7	Ti6Al4V disks	Promote osteogenic differentiation and mineralization of MC3T3-E1 cells; Osteogenesis $\uparrow$	MC3T3-E1 cells	Bone loss	[40]
M0-sEVs	RAW 264.7	BMP2	Promote hBMSCs osteogenic differentiation by increasing the expression of early osteoblastic differentiation markers, ALP and BMP2; Osteogenesis $\uparrow$	hBMSCs	Bone fracture	[39]
M0-sEVs	SD rat BMDMs	IMC	Facilitate MSC osteogenesis via the BMP2/Smad5 pathway; Osteogenesis $\uparrow$	Rat mandibles defect model	Bone defect	[28]
M0-sEVs containing miR-3473e	RAW 264.7	Titanium nanotube arrays	Induce osteogenic differentiation of BMSCs, and promote ECs migration and enhance the angiogenesis via upregulating Akt1 gene	Mouse BMSCs/hECs	Bone loss	[41]
M0-sEVs containing miR-23a-5p	RAW264.7	RANKL	Suppress osteogenic differentiation by inhibiting Runx2 and promoting YAP1-mediated MTIDP	hFOB 1.19 and MC3T3-E1 cells	Bone loss	[95]
M0-sEVs containing miR-223	THP-1	rhGM-CSF	Activate monocytes differentiation, hematopoietic cell production in the marrow and release of more sEVs as a mediator of inflammation response	THP-1	Inflammation-associated disease	[87]



M0-sEVs containing IL-1 $\beta$	THP-1	LPS & ATP	Initiate synovitis during TMJOA; induce cartilage degradation by promoting the differentiation of osteoclasts	Rat TMJOA model	Inflammation-associated disease	[73]
M0-sEVs containing ERAP1	RAW264.7	LPS/IFN- $\gamma$	Enhance the phagocytic and nitric oxide synthetic activities of macrophages, and modifies inflammatory reactions	RAW264.7 and Murine peritoneal M $\phi$	Inflammation-associated disease	[74]
M0-sEVs containing HSP60	ICR mice BMSCs	IL-1 $\beta$ and TNF- $\alpha$	Promote RANKL-induced osteoclast formation and bone resorption by upregulating TLR-2 expression in osteoclast precursors	Mice BMSCs	Osteoporosis	[71]
Curcumin loading M0-sEVs	RAW264.7	–	Promote anti-inflammatory activity of M0-sEVs	LPS-simulated septic shock mouse model	Inflammation-associated disease	[106]
M0-sEVs containing LIFR-ASI	THP-1	–	Promote osteosarcoma cell proliferation, migration and invasion abilities via miR-29a/NFIA pathway	Tumor xenograft mouse model; osteosarcoma cells	Osteosarcoma	[99]
M0-sEVs containing MiR-223	TAM	IL-4	Promote breast cancer cells invasion through miR-223/Mef2c/ $\beta$ -catenin pathway	Breast cancer cell lines, SKBR3 and MDA-MB-231	Tumor bone metastasis	[101]
M0-sEVs containing MiR-660	TAM	–	Promote invasion and migration of breast cancer cells by inhibiting KLHL21, and increase the bone metastasis	BALB/c female athymic mice; breast cancer cells	Tumor bone metastasis	[102]

**Abbreviations:** AA, adjuvant-induced arthritis; BMDM, bone marrow derived-macrophages; BMSCs, bone marrow mesenchymal stem cells; CIA, collagen-induced arthritis; HAL, hexyl 5-aminolevulinate hydrochloride; KOA, knee osteoarthritis; sEVs, small extracellular vesicles; TAM, tumor-associated macrophages; TMJOA, temporomandibular joint osteoarthritis; OA, osteoarthritis; RA, rheumatoid arthritis; VECs, vascular endothelial cells.



**Figure 4** Published data on the biogenesis of sEVs derived from M1 macrophages in bone-related diseases.

## Bone Fracture or Defect

Bone fracture is a common clinical challenge and easily causes compromised physical activity, loss of productivity, and decreased quality of life. Osteoblast differentiation of MSCs is an important process during fracture healing. Studies have shown that during the early stage of the proinflammatory response, miRNA-21a-5p, which is overexpressed by M1-sEVs can promote the osteogenic differentiation of BMSCs.<sup>47</sup> During the anti-inflammatory response that occurs during the late stages of the disease, M2-sEVs overexpressing miRNA-5106 were found to inhibit the expression of salt-induced kinases 2 and 3 (SIK2 and SIK3) to promote osteoblast differentiation and bone mineral deposition, and accelerate fracture healing.<sup>48</sup> MiR-26a-5p carried by sEVs from M2 macrophages actively promoted the expression of the bone differentiation-related proteins ALP, RUNX-2, OPN, and CoL-2 in BMSCs, and the effect could be attenuated by the inhibitor GW4689. MiR-26a-5p in M2-sEVs can promote osteogenic differentiation and inhibit adipogenic differentiation, indicating a positive role during bone fracture healing.<sup>49</sup>

Bone defects resulting from fractures and disease are a medical concern, and are often unable to heal spontaneously by the body's repair mechanisms. Macrophage-derived sEVs may serve as an emerging functional tool in biomaterial-mediated endogenous bone regeneration. MiR-155-enriched M1-sEVs were found to impair osteoblastic differentiation by reducing the expression of BMP2, BMP9 and RUNX2. MiR-378a contained in M2-sEVs acts as a positive regulator of osteogenesis by specifically activating upon the BMP2 signal transduction pathway. The expression of BMP2 and BMP9 in MSCs increased after miR-378a was transferred into calvaria defect rats.<sup>4</sup>



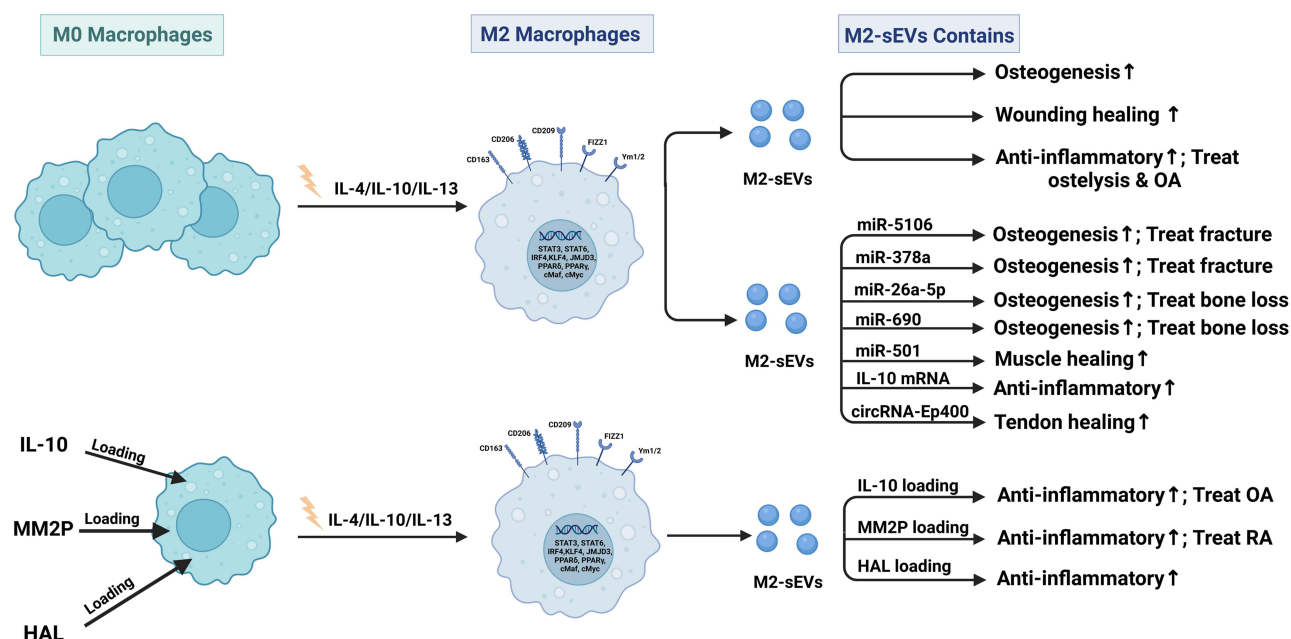
**Table 2** Published Studies on Biogenesis of sEVs Derived from M1-Macrophage (M1-M $\phi$ ) in Bone-Related Diseases

sEVs and Contains	Source of M $\phi$	Induction Methods	Functions & Pathway	Model System	Application	Ref.
M1-sEVs	RAW264.7	IFN- $\gamma$ and LPS	Attenuate cementoblast mineralization; Osteogenesis↓	Rats orthodontic tooth movement model	Inflammation-associated disease	[43]
M1-sEVs containing miR-21a-5p	RAW 264.7	IFN- $\gamma$ and LPS	Promote osteogenesis of BMSCs; Osteogenesis↑	Mouse BMSCs	Bone loss	[47]
M1-sEVs containing miR-155	C57BL/6 mMSCs	IFN- $\gamma$ and LPS	Reduce MSCs osteogenic differentiation by reducing BMP2, BMP9 and RUNX2 expression, and inhibit bone repair; Osteogenesis↓	Rat femur fracture model	Bone fracture	[4]
M1-sEVs containing miR-98	RAW264.7	IFN- $\gamma$ and LPS	Inhibit osteogenic differentiation and enhance miR-98 expression in MC3T3-E1 cells; exacerbate bone loss and osteoporosis by downregulating DUSP1 and activating the JNK signaling pathway	Mouse PO model	Osteoporosis	[57]
M1-sEVs containing miR-222	THP-1	H/SD	Inhibit BMSC viability and migration, and induce BMSC apoptosis by delivering miR-222 to BMSC to inhibit Bcl-2 expression	BMSCs	Osteoporosis	[75]
M1-sEVs containing miR-381	SD rat BMDMs	Mg <sup>2+</sup>	Promote BMSCs osteogenic differentiation via increasing Wnt5a and FZD3 expression; Osteogenesis↑	Rat BMSCs	Bone loss	[35]
IL4-sEVs loading M1-sEVs	RAW264.7	LPS/IFN- $\gamma$	Suppress pro-inflammatory mediator expressions in M1-M $\phi$ and enhance M2 marker genes expression; promote tissue repair and regeneration following joint damage in RA by modulating the M $\phi$ polarization	DBA/1J CIA model	RA	[103]

**Abbreviations:** BMDM, Bone marrow derived-macrophages; BMSCs, bone marrow mesenchymal stem cells; CIA, collagen-induced arthritis; sEVs, small extracellular vesicles; H/SD, hypoxia/serum deprivation; PO, postmenopausal osteoporosis; RA, Rheumatoid arthritis; SD, Sprague-Dawley.

## Osteoporosis

Osteoporosis is considered as the most prevalent bone disorder in elderly people worldwide and is characterized by low bone mass, deterioration of bone tissue and decreased bone strength. When people are experience estrogen deficiency, aging, high levels of glucocorticoids, inflammation and hyperparathyroidism, the balance between the activities of bone-forming osteoblasts and bone-resorbing osteoclasts is disrupted, causing the occurrence of osteoporosis.<sup>14,50–52</sup> Osteoporosis is more common in women than in men, and is a strong risk factor for fragility fractures in untreated people. It is estimated that 40% of postmenopausal women and 30% of older men (over 70 years of age) are at risk of experiencing osteoporotic fractures and the number continues to increase, posing tremendous medical and economic challenges to patients and society.<sup>53</sup> Type 2 diabetes mellitus (T2DM) is a common cause of secondary osteoporosis. Microvascular complications of T2DM lead to reduced blood flow to bone and may contribute to bone loss and its fragility, leading to an increased risk of bone fracture.<sup>54</sup> MiR-144-5p, which was derived from diabetic bone marrow-derived macrophage (dBMDM)-sEVs, could be transferred into BMSCs to suppress bone regeneration by targeting Smad1 and impair bone repair in vitro and vivo, increasing the risk of delayed bone union and nonunion and doubling the time of fracture healing associated with diabetes.<sup>55</sup> The osteogenic differentiation of BMSCs is impaired under high-glucose and high-insulin (HGI) conditions in vitro. The effect is partially relieved by the intervention of M2 macrophage-derived sEVs through activating the Hedgehog signaling pathway. The ALP activity of BMSCs in the HGI group was found to be lower than that in the control group. After intervention with M2-sEVs, ALP activity was significantly higher than that in the HGI group. In addition, the expression of the osteogenesis-related proteins COL1A1 and GLI1 is upregulated after M2-sEV treatment, suggesting that M2-sEVs have therapeutic potential for the treatment of diabetic bone disease.<sup>56</sup> In a mouse model of postmenopausal osteoporosis, miR-98 enriched in M1-sEVs could exacerbate bone loss by downregulating the expression of dual specificity phosphatase 1 (DUSP1) activating the JNK signaling pathway.<sup>57</sup>



**Figure 5** Published data on the biogenesis of sEVs derived from M2 macrophages in bone-related diseases.

## Muscle Injury and Tendinopathy

Muscle injuries such as tendon tears and tendinopathies are common and easily cause considerable disability, pain, health care costs, and losses productivity. Following tendon injury, the development of fibrotic healing response impairs tendon function and restricts tendon motion. Peritendinous tissue fibrosis poses a major clinical problem in hand surgery. sEVs from macrophages are crucial factors in tissue microenvironment regulation following tissue injury. Macrophage-derived miR-21-5p-enriched sEVs lead to increased proliferation, migration, and fibrotic activity of tendon cells by inhibiting the expression of Smad7, providing potential targets for the prevention and treatment of tendon adhesion.<sup>58</sup> MiR-501 overexpressed in M2-sEVs can be transferred into myoblasts, downregulate the expression of the transcription factor YY1, affect myoblast differentiation and promote the formation of myotubes, thus leading to regeneration of injured pubococcygeal muscle.<sup>59</sup> M2 macrophage-derived circRNA-Ep400-containing sEVs inhibited miR-15b-5p but promoted the expression of Col-I, Col-III,  $\alpha$ -SMA and FGF1/7/9 in both fibroblasts and tenocytes, thus promoting peritendinous fibrosis and providing novel therapeutics for tendon injury treatment.<sup>60</sup>

## Inflammation and Bone Osteolytic Diseases

Inflammatory-mediated pathological bone osteolytic diseases including osteoarthritis (OA), rheumatoid arthritis (RA), immunoinflammatory osteolysis, aseptic prosthesis loosening, and periodontitis are major orthopedic diseases that cause disability and impose a substantial economic burden worldwide.<sup>61,62</sup> Bone remodeling is a process involving osteoblast formation and osteoclast resorption to maintain bone architecture balance and systemic mineral homeostasis.<sup>63</sup> Chronic inflammation caused by various factors can disrupt this delicate balance between osteoblasts and osteoclasts and disturb bone metabolism, causing osteolytic disorders.<sup>64</sup> Macrophages play a substantial role in bone diseases caused by chronic inflammation.<sup>64</sup> Proteomic studies have shown that macrophage-derived sEVs contain a large number of proteins, such as alarmins, chemokines, and inflammatory cytokines and play a critical role in bone remodeling as vehicles.<sup>65</sup> Alarmins are endogenous molecules often detected in macrophage-sEVs that include annexins, galectins, heat-shock proteins and S100-alarmins, which are released upon cellular stress and activate the immune system.<sup>66–68</sup> Alarmins play wide roles in osteocyte, osteoblast and osteoclast differentiation and function. Annexin2 in macrophage sEVs can stimulate bone resorption by promoting the overexpression of the pro-osteoclastogenic factors GM-CSF and RANKL, and facilitating osteoclast formation.<sup>69</sup> Galectin-3 as a regulator of the osteoblast-osteoclast interaction inhibits osteoclastogenesis and

**Table 3** Published Studies on Biogenesis of sEVs Derived from M2-Macrophage (M2-M $\phi$ ) in Bone-Related Diseases

sEVs and Contains	Source of M $\phi$	Induction Methods	Functions & Pathway	Model System	Application	Ref.
M2-sEVs	RAW 264.7	IL-4	Promote osteogenesis of BMSCs; Osteogenesis $\uparrow$	Mouse BMSCs	Bone loss	[47]
M2-sEVs	RAW 264.7	IL-4	Promote osteogenic differentiation of BMSC via activating Hedgehog signaling pathway; Osteogenesis $\uparrow$	Mouse BMSCs	Diabetic bone disease	[56]
M2-sEVs	BALB/c mice BMDMs	IL-4 and IL-10	Enhance angiogenesis, re-epithelialization, collagen deposition, fibroblast migration and endothelial cell tube formation by the reprogrammed M2-M $\phi$ s and the direct conversion of M1 to M2 M $\phi$ s	Balb/c mice wound healing model	Wound healing	[42]
M2-sEVs	RAW264.7	IL-4	Facilitate cementoblast mineralization; Osteogenesis $\uparrow$	Rat orthodontic tooth movement model	Inflammation-associated disease	[43]
M2-sEVs	SD rat BMDMs	IL-4	Regulate the production of proinflammatory factors and the level of osteogenesis-related proteins via inhibition of the PI3K/AKT/mTOR pathway, and treat cartilage damage; Osteogenesis $\uparrow$	Rat KOA model	OA	[80]
M2-sEVs containing miR-5106	C57BL/6 mice BMDMs	IL-4	Promote osteogenic differentiation of BMSC via suppressing the expression of SIK2 and SIK3; Osteogenesis $\uparrow$	Mouse femoral fracture model	Bone fracture	[48]
M2-sEVs containing miR-378a	C57BL/6 mMSCs	IL-4	Increase MSCs osteoinductive gene expression, and promote repair/regeneration; Osteogenesis $\uparrow$	Rat femur fracture model	Bone fracture	[4]
M2-sEVs containing miR-26a-5p	SD rat BMDMs	IL-4	Induce osteogenic differentiation of BMSCs to inhibit lipogenic differentiation by promoting the expression of osteogenic differentiation-related proteins ALP, RUNX-2, OPN, and Col-2; Osteogenesis $\uparrow$	Rat BMSCs	Bone loss	[49]
M2-sEVs containing miR-690	RAW 264.7	IL-4	Facilitate osteogenesis and reduce adipogenesis of BMSCs; Osteogenesis $\uparrow$	Mouse BMSCs	Bone loss	[34]
M2-sEVs containing miR-501	RAW264.7	IL-4	Promote regeneration of experimentally injured pubococcygeal muscle; promote C2C12 myoblast differentiation via targeting YY1	Mouse SUI model; C2C12 Myoblasts	Muscle regeneration	[59]
M2-sEVs containing IL-10 mRNA	C57BL/6 mice BMDMs	IL-4	Promote BMSCs osteogenic differentiation while suppressing BMDM osteoclast formation, and prohibit pathological alveolar bone resorption by upregulating IL-10 expression of BMSCs and BMDM via delivering IL-10 mRNA to cells, and activating IL-10/IL-10R pathway	Murine periodontitis model	Inflammation-associated disease	[36]
M2-sEVs containing circRNA-Ep400	C57BL/6 mice BMDMs	IL-4 and IL-13	Promote fibrosis, proliferation, and migration in both fibroblasts and tenocytes; promote peritendinous fibrosis after tendon injury via the miR-15b-5p/FGF-1/7/9 pathway	Mice underwent complete transection and repair of the FDL tendon; NIH3T3 fibroblast cells and tenocytes	Tendon injury	[60]
IL-10 pDNA/BSP loading M2-sEVs	RAW264.7	IL-4	Attenuate the production of pro-inflammatory cytokines and promote macrophage polarization to an anti-inflammatory state	DBA/1J CIA model	RA	[105]
MM2P loading M2-sEVs	RAW 264.7 and C57BL/6 mice BMDMs	IL-4 and IL-13	Facilitate M2 polarization by facilitating transactivation of SOX9 through STAT3, and prevent the SHP2-mediated dephosphorylation of STAT3; promote chondrocyte-specific protein and cartilage repair through exosome-derived SOX9 mRNA stabilized by the interaction of MM2P and FUS.	Mouse primary chondrocytes	OA	[80]
HAL loading M2-sEVs	RAW264.7	IL-4	Exert the superiority of anti-inflammatory M2-M $\phi$ for simultaneously targeting and alleviating inflammation by surface-bonded chemokine receptors and secreted anti-inflammatory cytokines	BALB/c mice acute peritonitis	Inflammation-associated disease	[106]

**Abbreviations:** BMDM, bone marrow derived-macrophages; BMSCs, bone marrow mesenchymal stem cell; CIA, collagen-induced arthritis; HAL, hexyl 5-aminolevulinate hydrochloride; KOA, knee osteoarthritis; sEVs, small extracellular vesicles; SUI, Stress urinary incontinence; OA, osteoarthritis; RA, rheumatoid arthritis.

regulates bone homeostasis.<sup>70</sup> Increased HSP60 levels stimulated by IL-1 $\beta$  and TNF- $\alpha$  can promote osteoclast formation and activity by activating RANK-RANKL signaling by binding to TLR2.<sup>71</sup> IL-1 $\beta$ , a critical inflammatory cytokine in the inflammatory cascade response, is also contained in macrophage-sEVs. It stimulates the production of various proinflammatory mediators, such as cytokines, chemokines, and matrix metalloproteinases (MMPs), leading to the continued progression of chronic inflammation.<sup>72,73</sup> In inflammatory conditions, LPS/IFN- $\gamma$  can stimulate macrophages to secrete endoplasmic reticulum aminopeptidase 1 (ERAP1) via sEVs to enhance the phagocytic and nitric oxide (NO) synthetic activities of macrophages and modify inflammatory reactions.<sup>74</sup> Similarly, macrophages secrete pro- and anti-inflammatory mediators into the micro milieu to regulate bone homeostasis in inflammatory conditions. During the inflammatory phase, the production and release of sEVs are abundant and easily cause tissue cell hypoxia due to the increase in cell metabolism and the reduction in the levels of substrates. Under hypoxic conditions, sEVs enriched with miR-222 derived from M1 macrophages can be released to target BMSCs, inducing cell apoptosis and thereby regulating bone formation.<sup>75</sup> MiR-222 has also been reported to inhibit endothelial cell migration, proliferation, and angiogenesis by

suppressing the production of nitric oxide synthase.<sup>76</sup> Under inflammatory conditions, chemokines and proinflammatory cytokines (IFN- $\gamma$ , LPS, and IL-1 $\beta$ ) are produced and induce polarization of M1 macrophages. MiR-155 is derived from M1 macrophage sEVs and transferred into endothelial cells, controlling endothelial cell migration and cell-cell connections, thereby regulating vascular permeability.<sup>38,77</sup>

## Osteoarthritis

Osteoarthritis is the most common form of joint disease and is characterized by pain, cartilage destruction, synovial fibrosis osteophyte formation, and sometimes the swelling of certain joints such as the hand, spine, hip and knee, and is frequently accompanied by synovitis.<sup>78</sup> Multiple studies have confirmed that macrophages play an important role in the occurrence of OA through inflammatory factors, cytokines, miRNA and proteins.<sup>79</sup> The long noncoding RNA (lncRNA) MM2P induces M2 macrophage polarization, and promotes the spread of SOX9 enriched in M2-sEVs into chondrocytes, significantly enhancing the differentiation and function of chondrocytes, which promotes cartilage repair and supports a novel therapeutic strategy for OA.<sup>80</sup> Knee OA (KOA) is a degenerative disease with the main symptoms of cartilage destruction and knee joint pain, resulting in the activation of the PI3K/Akt/mTOR signaling pathway in the body. sEVs derived from M2 macrophage intervention can inhibit the protein and mRNA expression of PI3K, Akt, and mTOR in KOA model rats and reduce cartilage injury, indicating that M2-sEVs may exert a therapeutic effect on KOA, but the mechanism is still unclear.<sup>81</sup>

## Rheumatoid Arthritis

RA is one of the most widespread chronic inflammatory joint diseases with strong genetic predisposition factors and is characterized by synovitis, cartilage erosion and bone loss accompanied by pain, swelling, stiffness, and joint deformity, and can also damage extra-articular organs, such as the heart, kidney, lung and nervous system.<sup>82,83</sup> MiR-223 is enriched in macrophage-sEVs under inflammatory conditions.<sup>84</sup> As an innate immune regulatory factor, the expression of miR-223 is highest in the bone marrow cavity, playing an important role in the differentiation of granulocytes and macrophages during the differentiation of bone marrow. MiR-223 activates the immune system, promotes macrophage polarization to the anti-inflammatory M2 type, and induces the production of more sEVs.<sup>38,85</sup> Another study revealed the multiple roles that miR-223 plays in bone metabolism by regulating osteoclast differentiation, representing a promising diagnostic tool for the treatment of bone diseases related to aberrant bone metabolism.<sup>86</sup> Another study also showed that miR-223 derived from macrophages can induce the differentiation of monocytes, activate hematopoietic cell production in the marrow, and promote the release of more sEVs. Thus, this feedback mechanism may be an innate response element that regulates host defense and inflammation.<sup>87</sup>

## Immunoinflammatory Osteolysis and Aseptic Loosening

Aseptic loosening is a common complication following total joint arthroplasty due to periprosthetic inflammatory osteolysis and is the leading cause of revision procedures.<sup>88,89</sup> Wear particles released from the sliding surfaces of prosthetic materials induce a persistent local inflammatory response, thereby stimulating bone resorbing-osteoclast activities and bone loss around the implant.<sup>90,91</sup> Macrophages activated by wear particulate debris release an array of cytokines and proinflammatory mediators in the joint fluid and facilitate the recruitment of inflammatory cells.<sup>92,93</sup> MiR-155 can be stimulated by activators of proinflammatory M1 phenotype macrophages (LPS, IFN- $\alpha$ ) and released to promote the secretion of inflammatory cytokines such as TNF- $\alpha$  and IL-12, thereby aggravating the inflammatory response.<sup>94</sup> The production of miR-23a-5p-containing sEVs induced by RANKL effectively inhibits the expression of Runx2 and suppresses osteoblast differentiation.<sup>95</sup>

## Periodontitis

Chronic periodontitis is one of the most common diseases of the oral and maxillofacial regions and is caused by bacterial infection and the host immune-inflammatory response, leading to alveolar bone absorption and tooth loosening and shedding.<sup>96</sup> *Porphyromonas gingivalis* (*P.g*) is the major pathogenic bacterium involved in chronic periodontitis. A recent study revealed that *P.g*-LPS-activated macrophage-derived sEVs can inhibit BMSC osteogenic differentiation by

mediating inflammatory stimulation. *P.g* LPS-stimulated macrophages produced an inflammatory response and released sEVs. After BMSCs had taken up inflammatory sEVs, the gene and protein expression levels of TNF- $\alpha$  and IL-6 were significantly unregulated, but the expression of ALP, RUNX2, and COL-1 decreased, indicating an inhibitory effect on BMSCs in inflammatory states.<sup>97</sup>

## Osteosarcoma and Tumor Bone Metastasis

Osteosarcoma (OS) is one of the most common primary malignant bone tumors in adolescents, and in general, it occurs in males more frequently than in females.<sup>98</sup> sEVs from macrophages have been reported to play critical roles in regulating the proliferation, metastasis, and angiogenesis of osteosarcoma by participating in intercellular contacts and controlling cellular signaling.<sup>99</sup> LIFR-AS1 as a newly described tumor-related lncRNA, has been reported to be released from macrophages to osteosarcoma cells via sEVs and further promote tumor progression by acting as a sponge in the miR-29a/NFIA pathway.<sup>100</sup> Bone is one of the most common metastatic sites of breast cancer. sEVs enriched in miR-223 secreted from IL-4-activated macrophages were found to promote breast cancer cell invasion through the miR-223/Mef2c/ $\beta$ -catenin pathway.<sup>101</sup> sEVs containing miR-660 derived from tumor-associated macrophages promoted the invasion and migration of breast cancer cells by inhibiting KLHL21 activity and then increased bone and lung lymph node metastasis.<sup>102</sup>

## Drug Delivery

sEVs have been demonstrated to be endogenous delivery carriers with excellent biocompatibility, low cytotoxicity, and immunological inertness and are an ideal potential candidate for the treatment of many diseases. Numerous studies have shown that sEVs derived from macrophages can carry various drugs, such as protein, DNA and small molecular drugs, and play a role in inflammatory regulation. IL4-loaded sEVs exhibit high anti-inflammatory effects by modulating macrophage polarization. IL4-sEVs are absorbed by macrophages via endocytosis and regulate the M1/M2 status and suppress downstream fibroblast proliferation, pannus formation, and bone destruction in CIA mice. In the synovium of RA joints, IL4-sEV-infiltrated macrophages predominantly secrete proinflammatory mediators, which are expected to contribute to the in vivo anti-inflammatory effects and are considered for the treatment of chronic inflammatory rheumatoid arthritis.<sup>103,104</sup> Another study showed M2 sEVs/pDNA/BSP nanoparticles using M2-sEVs as a platform for the codelivery of IL-10 pDNA and the chemotherapeutic drug betamethasone sodium phosphate (BSP), which can effectively increase IL-10 secretion, attenuate the production of proinflammatory cytokines (IL-1 $\beta$ , TNF- $\alpha$ ), promote macrophage polarization from a proinflammatory to an anti-inflammatory state, and efficiently reduce joint damage for RA treatment.<sup>105</sup> Engineered M2-sEVs loaded with FDA-approved hexyl 5-aminolevulinate hydrochloride (HAL) exhibited excellent inflammatory-tropism and anti-inflammatory effects via surface-bonded chemokine receptors and anti-inflammatory cytokines derived from M2 macrophages. The encapsulated HAL can facilitate the biosynthesis of anti-inflammatory carbon monoxide and bilirubin and further enhance anti-inflammatory effects.<sup>106</sup> Curcumin is a natural polyphenol with anti-inflammatory, antineoplastic and antioxidant activities. Curcumins complexed with sEVs (Cur-sEVs) have been found to have increased stability and bioavailability. Macrophages treated with Cur-sEVs produced significantly lower levels of IL-6 and TNF- $\alpha$  than macrophages treated with curcumin or sEV treatment alone, indicating a higher anti-inflammatory activity.<sup>107</sup> Dexamethasone sodium phosphate encapsulated in M-sEVs (Dex/sEVs) showed an anti-inflammatory effect on macrophages by suppressing the expression of proinflammatory cytokines (IL-1 $\beta$ , TNF- $\alpha$ , and IL-6) and increasing the levels of anti-inflammatory cytokines (IL-10), promoting the bone and cartilage repair and significantly reducing the incidence of inflamed joints in CIA mice.<sup>108</sup> For the application of sEVs to achieve good therapeutic effects, safe manufacturing, quality control, and scalable mass production solutions are needed. Thus, optimized technologies need to be developed in the future to facilitate the clinical use of sEV delivery.

## Discussion and Future Perspectives

Orthopedic diseases constitute one of the major causes of disability and impose a considerable economic burden worldwide. Macrophages play an important role in maintaining bone homeostasis and promoting bone establishment. sEVs derived from macrophages, which act as mediators of intercellular communication are potential



immunomodulatory agents investigated in the context of basic research and clinical therapy. This paper describes the contribution of sEVs derived from macrophages in bone diseases and provides a theoretical basis for further research and the clinical application of sEVs as ideal candidates for drug delivery systems in the treatment of orthopedic diseases in the future. sEVs, as an important medium of communication between cells, can mediate bone metabolism through the paracrine transmission of various bioactive substances.<sup>30,109</sup> We focused on miRNAs and intracellular proteins as key mediators of sEV effects on bone homeostasis. In addition, due to the advantages of lower toxicity and immunogenicity, higher biocompatibility, natural targeting ability, and good biological barrier permeability, their carrier functions can be used for more complex scenarios than lipid nanoparticles. M-sEVs have been used as a vehicle for the delivery of drugs with remarkable inflammatory tropism and anti-inflammatory effects.<sup>110</sup> However, it is difficult to determine the exact dose and timing for application, and basic research and preclinical exploration of the specific mechanism of sEVs for intercellular communication under physiological and pathological conditions are still an immature stage. The contents of sEVs are complex, which hinders further exploration of their mechanisms of action. The exact mechanisms of sEVs still need to be further investigated and the application of engineered sEVs for clinical regeneration requires continued study of safety, feasibility and mass productivity.

## Conclusion

M-sEVs play important roles in the mechanism of bone disease by interfering with osteogenesis, angiogenesis, epithelialization, cell proliferation and migration, differentiation, the immune response, inflammatory factors and others. They participate in a variety of pathways associated with bone homeostasis and can be used as drug carriers. It is believed that M-sEVs have great therapeutic potential in all aspects of medical treatment in the future development of biotechnology.

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The authors report no conflicts of interest in this work.

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