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Therapeutic potential in rheumatic diseases of extracellular vesicles derived from mesenchymal stromal cells

Giuliana Minani Bertolino¹ , Marie Maumus¹, Christian Jorgensen^{1,2,3}  & Danièle Noël^{1,2,3} 

Abstract

The incidence of rheumatic diseases such as rheumatoid arthritis and osteoarthritis and injuries to articular cartilage that lead to osteochondral defects is predicted to rise as a result of population ageing and the increase in high-intensity physical activities among young and middle-aged people. Current treatments focus on the management of pain and joint functionality to improve the patient's quality of life, but curative strategies are greatly desired. In the past two decades, the therapeutic value of mesenchymal stromal cells (MSCs) has been evaluated because of their regenerative potential, which is mainly attributed to the secretion of paracrine factors. Many of these factors are enclosed in extracellular vesicles (EVs) that reproduce the main functions of parental cells. MSC-derived EVs have anti-inflammatory, anti-apoptotic as well as pro-regenerative activities. Research on EVs has gained considerable attention as they are a potential cell-free therapy with lower immunogenicity and easier management than whole cells. MSC-derived EVs can rescue the pathogenetic phenotypes of chondrocytes and exert a protective effect in animal models of rheumatic disease. To facilitate the therapeutic use of EVs, appropriate cell sources for the production of EVs with the desired biological effects in each disease should be identified. Production and isolation of EVs should be optimized, and pre-isolation and post-isolation modifications should be considered to maximize the disease-modifying potential of the EVs.

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Key points

- Extracellular vesicles (EVs) have important roles in intercellular communication and convey many components at their surface or in their cargo that reproduce the functions of parental cells.
- Mesenchymal stromal cell (MSC)-derived EVs can regulate and prevent symptoms in non-clinical models of osteoarthritis and rheumatoid arthritis, notably by targeting local and systemic inflammation, and restoring a healthy chondrocyte phenotype.
- Improvement of isolation yields of MSC-EVs and functionality optimization by pre-isolation or post-isolation engineering are still required.
- Clinical translation of MSC-EVs for treatment of rheumatic diseases is ongoing, suggesting future potential for the treatment of these painful and disabling diseases.

Introduction

Rheumatic diseases are a heterogeneous group of disorders affecting joints, bones, muscles and connective tissues. They include diseases such as osteoarthritis (OA) and rheumatoid arthritis (RA), which are among the leading causes of severe disability worldwide. The economic burden from rheumatic diseases is substantial.

Surgical interventions are sometimes necessary for severe instances of rheumatic disease, particularly when joint damage is extensive. However, factors such as disease complexity, systemic involvement and complications can lead to poor surgical outcomes, as described for OA¹. Many therapeutic strategies focus on symptom management rather than altering the progression of the underlying disease. Medications such as NSAIDs are commonly prescribed to control inflammation and pain. Physical therapy, occupational therapy and assistive devices are also used for improvement of joint mobility and function. Although these approaches can provide symptomatic relief, they do not necessarily slow down or halt disease progression.

The use of mesenchymal stromal cells (MSCs) in cell-based therapies is a potential strategy for the treatment of rheumatic disease. The initial evidence that MSCs have potent immunosuppressive and regenerative properties prompted many studies on their potential use in rheumatic diseases². Despite promising clinical results from randomized clinical trials, MSC therapeutic benefit is rather unpredictable, probably because of the substantial heterogeneity between donors, as well as batch-to-batch variation³. Strategies aiming to use MSC-derived extracellular vesicles (MSC-EVs) are being developed to overcome some of the problems related to cell-based therapy. The interest in EVs has progressively grown since the discovery that they have biological content, with results indicating that MSC-EVs are potent mediators of MSC effects locally and systemically, notably in OA⁴.

In this Review, we describe the sources of heterogeneity in MSC-EV production, as well as the extensive work on the use of MSC-EVs for the treatment of rheumatic diseases, focusing on OA and RA, with some insights on other degenerative disorders. Furthermore, we elaborate on the need for optimization of EV production, which could enhance the isolation yield and their potency as biotherapeutics, and we discuss the preclinical data conveying the promise of therapeutic applications of MSC-EVs in rheumatic diseases.

EV heterogeneity: biogenesis, cargo and interactions with target cells

In addition to the molecular secretome, large numbers of EVs are secreted by cells. EV is “the generic term for particles naturally released from the cell that are delimited by a lipid bilayer and cannot replicate, i.e. do not contain a functional nucleus” as endorsed by the International Society for Extracellular Vesicles⁵. EVs are secreted by all cell types, are abundant in all biofluids and contain lipids, nucleic acids, proteins and even organelles (mainly mitochondria)⁶. EVs were initially thought of as contributors to protein quality control through the elimination of misfolded cellular compounds⁷. Over the years, further studies showing their composition and the functional aspects of their cargo have led to recognition of EVs as important mediators of intercellular communication⁸. Several studies have shown that EVs have important roles in bioactive-component exchange between cells in normal homeostatic processes, and that they contribute to pathological developments. Therefore, understanding the mechanisms underlying the factors that influence EV cargo composition is crucial for comprehending their roles in intercellular communication and their potential applications in diagnostics and therapeutics.

EV biogenesis

Based on their biogenesis, EVs are now broadly divided into two main categories (Fig. 1). Vesicles of endosomal origin are traditionally called exosomes and are formed by the inward budding of the endosomal membrane during the maturation of multivesicular endosomes, resulting in secretion upon fusion of multivesicular endosomes with the cell surface⁸. Data published in 2022 provide evidence of the participation of further endomembranes in exosome biogenesis, such as the endoplasmic reticulum and nuclear envelope^{9,10}. Another EV category, referred to as ectosomes, is generated by outward budding or blebbing and subsequent fission of the plasma membrane, with EV release into the extracellular space. Classification of ectosomes into microvesicles, exophers, oncosomes, migrasomes or apoptotic bodies depends on their cargo or the producer cells, as reviewed elsewhere¹¹.

Cells can secrete extracellular nanoparticles that are not classified as EVs, but are instead known as non-vesicular extracellular nanoparticles. Exomeres and supermeres are small non-vesicular extracellular nanoparticles identified as a distinct subpopulation of particles released by cells¹¹. They are smaller than typical EVs, ranging from 22 nm to 50 nm in diameter (Fig. 1). They are thought to be derived from the endosomal pathway and carry various molecules such as RNA, proteins and lipids, but are distinct from each other in terms of size, morphology, composition, cellular-uptake dynamics and tissue distribution¹². The study of their specific functions and mechanisms is still at an early stage but is likely to identify these vesicles as potential circulating biomarkers and therapeutic agents.

Because current isolation methods cannot segregate EVs from different origins, and consensus has not yet emerged on specific markers of EV subtypes, assigning an EV to its biogenetic pathway is extremely difficult. EV size is very heterogeneous and is not specific to particular biogenetic pathways (Fig. 1). However, different isolation methods can enhance specific size ranges in EV samples, so EVs are referred to in some studies as small (usually 50–150 nm in diameter), medium (200–800 nm in diameter) or large (usually >1 µm in diameter)¹³. Results have shown that large and small MSC-derived EVs can differ in cargo composition and functions⁸. Functional analysis has shown that samples enriched with small MSC-EVs have greater activity in promoting renal protection from injury *in vitro* than samples enriched with large EVs¹⁴.

Similarly, small EVs from untreated MSCs, but not large EVs, are able to reduce signs of arthritis in a mouse model¹⁵. These studies highlight the functional heterogeneity of MSC-EVs and suggest that the enrichment of specific EV fractions can be beneficial for clinical applications.

Cell source

A single cell can produce different types of EVs that have distinct structural and biochemical properties. Many mechanisms could potentially

be involved in the cargo formation process, as reviewed elsewhere¹⁶. A conserved range of proteins is enriched in EVs compared with their parental cells, including CD9, CD63, CD81, tumour susceptibility gene 101 protein (TSG101) and specific lipids. However, other repertoires (proteins, RNA, DNA, lipids and even organelles such as mitochondria) that compose the EV cargo, membrane and corona can vary drastically according to the cell source, cell viability, activation state and biogenesis^{17–19}. Some cell sources can produce particularly large

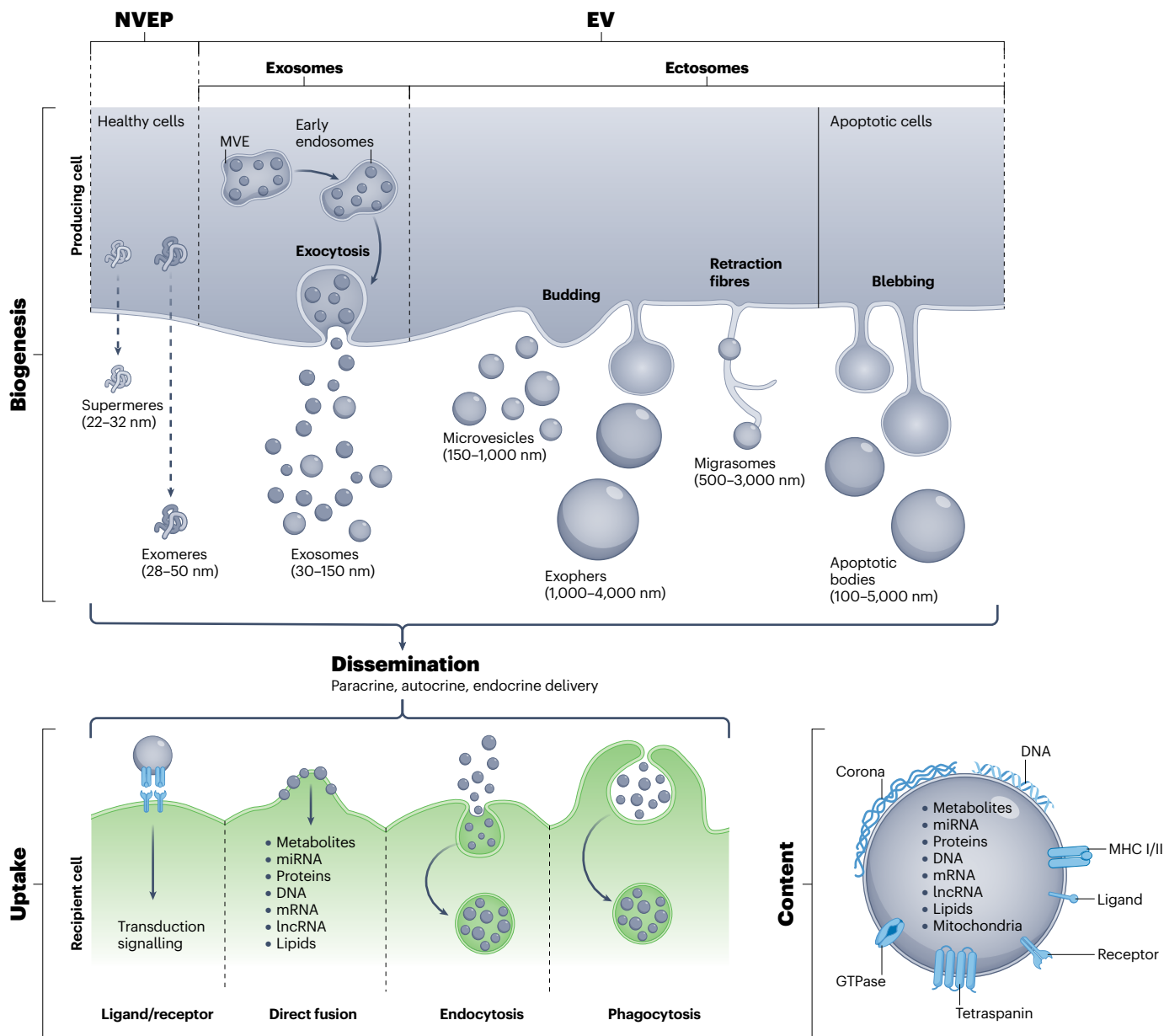


Fig. 1 | Biogenesis, uptake and content of extracellular vesicles. Cells release heterogeneous extracellular vesicles (EVs) and non-vesicular extracellular particles (NVEPs, such as supermeres and exomeres) into the extracellular environment. EVs encompass exosomes, which are released by exocytosis, and ectosomes (including microvesicles, exophers, migrasomes and apoptotic bodies), which are produced by budding or blebbing. EVs are delimited by a lipid bilayer enriched in phosphatidylserine, sphingomyelin and cholesterol as well as GTPase, tetraspanins

(CD9, CD63 and CD81), MHC class I and class II molecules, and several receptors and ligands. EVs act in paracrine, autocrine or endocrine manners on recipient cells, by inducing cell responses through ligand–receptor interactions at the cell membrane or by delivering their cargo by direct fusion, endocytosis or phagocytosis. The EV cargo contains RNA, DNA, proteins, lipids, metabolites and mitochondria. EVs also possess a corona, which is a layer of biomolecules that becomes associated with the EV surface. lncRNA, long non-coding RNA; miRNA, microRNA; MVE, multivesicular endosome.

quantities of EVs, whereas others can have efficient mechanisms for packaging specific bioactive molecules within the EVs. For example, bone-marrow-derived MSCs (BM-MSCs) exhibit greater EV production capacity and secretory profiles than adipose-derived-MSCs (ASCs), suggesting distinct activities of EVs derived from different MSC sources²⁰. EVs from different sources can therefore potentially have different clinical uses.

Cell viability and ageing

During the process of apoptosis, cells release a distinct set of EVs that comprises, in addition to exosomes and ectosomes, apoptotic bodies. During apoptosis, the dying cell undergoes various changes, such as DNA fragmentation and proteolytic cleavage of proteins. Consequently, EVs from apoptotic cells contain unique protein fragments and modified DNA compared with other EV types²¹. Understanding the role of EVs from apoptotic cells in intercellular communication is an active area of research in the field of MSC-EV biology. For example, in a study from 2022 comparing apoptotic vesicles and exosomes derived from MSCs, distinct protein cargo and surface markers were identified in apoptotic vesicles, and the therapeutic potential of these vesicles was demonstrated in the treatment of haemophilia A by leveraging the Fas–FasL linkage to activate platelet functions and improve blood clotting in mice lacking factor VIII²². MSC-released apoptotic bodies also promote cutaneous wound healing by triggering the polarization of macrophages towards the M2 phenotype²³. Therefore, the apoptosis state of MSCs should be taken into account as it can affect the biological functions of EV preparations.

Aspects of cell ageing can also influence EV content and yield. For example, senescent MSCs secrete more EVs than non-senescent MSCs²⁴. EVs from senescent MSCs also differ in their functional capacities. EVs derived from ageing MSCs can lack the protective properties that are observed with EVs from young MSCs in disease models that include models of rheumatic diseases, as reviewed elsewhere²⁵. Consequently, the production of MSC-EVs should incorporate quality controls to assess the proportion of senescent cells and evaluate their influence on the functionality of the EV batches.

Corona formation

When EVs are released into the surrounding environment, they can interact with various soluble components that participate in the formation of a corona, which is a layer of biomolecules that becomes associated with the EV surface²⁶. Corona formation is a common phenomenon in various biofluids, and the composition of the corona reflects the specific cell type, the cellular environment and the conditions under which the EVs are produced, as reviewed elsewhere²⁷. The process of corona organization is supposed to involve protein aggregation and electrostatic interactions. In addition to the non-specific adsorption of proteins onto the surface of EVs, specific interactions between receptors and ligands can also occur²⁸. The corona can influence the interactions of EVs with target cells or tissues²⁶. Therefore, corona content is another source of heterogeneity in EV preparations, and manipulating the composition and characteristics of the corona could pave the way to improvements in the therapeutic applications of EVs.

EV isolation methods

The technique used for EV isolation can affect the composition and characteristics of the isolated population. Different isolation methods, such as size-exclusion chromatography (SEC), ultracentrifugation, ultrafiltration, precipitation and affinity-based isolation, each has its

strengths and limitations. Studies that have compared isolation techniques for MSC-EVs have assessed factors such as concentration, size distribution, surface markers and functional activity^{29,30}. Data indicate that SEC yields purer and more functional MSC-EVs than ultracentrifugation. SEC-based isolation is gentle and it preserves the integrity of EVs, but it can have lower recovery rates than other methods, making it less efficient for large-scale isolation³⁰. Phosphatidylserine-based affinity isolation results in the highest purity of EVs, but suffers from EV loss²⁹. Isolating MSC-EVs with high yield and purity remains challenging because of their small size and heterogeneity, meaning that further technological advances are required to address these issues.

Interaction with target cells

As a mechanism of cell–cell communication, EVs transmit signals to target cells. EVs can transport molecules that are associated with the corona or the lipid bilayer, or that are contained in the cargo that is protected from enzymatic degradation during transit to adjacent cells or over longer distances. Following molecular interactions involving membrane-exposed compounds from both cells and vesicles, EVs can deliver fully functional content or activate molecular signalling through membrane-based communication³¹ (Fig. 1). EVs can transmit information by acting at the cell surface, without content delivery^{32,33}. Alternatively, once docked, EVs can deliver their cargo by direct fusion with the plasma membrane, although such events are likely to be rare³⁴. Most of the experimental evidence suggests that EVs are usually incorporated via endocytosis, using clathrin-dependent or clathrin-independent pathways. EVs can also be internalized by phagocytosis, with incorporation into phagosomes and sorting into phagolysosomes in a cytoskeleton-dependent way^{35–37}. In the past few years, the capacity of MSC-EVs to transfer their cargo to recipient cells has gained much attention in the fields of regenerative medicine and drug delivery. EVs possess interesting features such as low immunogenicity and low toxicity, the ability to transfer cargo to the cytosol of recipient cells, and the ability to cross natural boundaries, such as the blood–brain barrier³⁸. In this context, MSC-EVs have proved their therapeutic efficacy in non-clinical models for various pathological conditions and are currently being evaluated clinically³⁹.

The therapeutic benefits of MSC-EVs in OA

OA is a chronic degenerative rheumatic disease that affects the articular cartilage, subchondral bone, ligaments, capsule and synovium⁴⁰. In a patient with OA, one or more diarthrodial joints are affected, including small joints such as those in the hand and larger joints such as the knee and hip joints. OA is characterized by the slow destruction of articular cartilage associated with low-grade inflammation of joint tissues, changes to the subchondral bone and osteophyte formation. Primary OA arises from a convergence of risk factors, in particular advancing age and obesity. Additional factors that contribute to the risk include knee malalignment, heightened biomechanical stress on joints and genetic predisposition⁴¹. Currently, no therapeutic agent has demonstrated effectiveness in modifying the disease progression or providing substantial benefits for patients with OA. Instead, OA management typically focuses on pain relief, improvement of joint function and lifestyle modifications such as exercise and weight management⁴². In the past decade, the use of MSC-EVs has emerged as a promising therapeutic tool to regulate OA joint pathogenesis. Evidence now suggests that MSC-EVs can slow down inflammation in synoviocytes and counterbalance changes in chondrocyte viability and metabolism.

Effects of EVs on chondrocytes and synoviocytes

BM-MS-C-EVs can promote the proliferation and migration of OA chondrocytes stimulated with TNF⁴³. Various microRNAs (miRNAs) and other non-coding RNAs in MSC-EV cargoes can regulate the proliferation and migration of chondrocytes. The long non-coding RNA (lncRNA) H19 that is present in umbilical cord MSC-EVs (UC-MS-C-EVs) acts as a competing endogenous binding agent (or 'sponge') for miR-29b-3p, thereby increasing FOXO3A expression and enhancing chondrocyte migration⁴⁴. The lncRNA H19 is also packaged in EVs from fibroblast-like synoviocytes (FLS) and is reported to promote chondrocyte survival and migration by targeting the axis of miR-106b-5p and TIMP2 (ref. 45). Another lncRNA (KLF3-AS1) that is packaged in BM-MS-C-EVs acts as a sponge for miR-206, thereby facilitating expression of GIT1, which induces the proliferation of chondrocytes⁴⁶. The lncRNA KLF3-AS1 also contributes to an increase in the number of proliferative PCNA-positive chondrocytes in a rat model of OA⁴⁷. The non-coding circular RNA circHIPK3, which is abundant in MSC-EVs, stimulates chondrocyte proliferation and migration and inhibits apoptosis⁴⁸. When transferred into chondrocytes, circHIPK3 might act as a sponge for miR-124-3p to promote myosin heavy chain 9 expression, thereby contributing to cartilage protection.

The imbalance between cartilage extracellular matrix (ECM) synthesis and degradation in favour of catabolism in OA can be corrected by MSC-EVs, which can upregulate cartilage markers in vitro^{43,49–51}. MSC-EVs enhance ECM synthesis through expression of glycosaminoglycan, type 2 collagen and aggrecan. They also reduce ECM degradation by decreasing expression of catabolic factors such as matrix metalloproteinase (MMP)-1, MMP-3, MMP-9, MMP-13 and ADAMTS-5. ASC-EVs can regulate the catabolic environment of OA by decreasing NF- κ B pathway activity⁵². This beneficial effect on ECM catabolism can be mediated by miR-136-5p, which is enriched in MSC-EVs and which regulates the catabolic activity of OA chondrocytes by targeting ELF3 and decreasing MMP-13 expression⁵³. Another miRNA, miR-125a-5p, is enriched in BM-MS-C-EVs and interacts with E2F transcription factor, leading to downregulation of MMP-13 expression, which limits cartilage degradation in vivo⁵⁴. Besides their effects on chondrocytes, EVs from MSCs over-expressing miRNAs, notably miR-92a-3p and miR320c, can enhance the chondrogenic differentiation of mesenchymal progenitors^{55,56}. These findings suggest that intra-articular injections of MSC-EVs could favour the generation of chondrocytes from mesenchymal progenitors and restore the physiological functions of mature chondrocytes exposed to a pathological environment.

MSC-EVs can improve chondrocyte survival and reverse mitochondrial dysfunction. BM-MS-C-EVs can protect OA chondrocytes against apoptosis induced by IL-1 β treatment^{49,57}. After uptake by chondrocytes, BM-MS-C-EVs inhibit mitochondria-induced apoptosis via inhibition of p38 and ERK phosphorylation, along with induction of AKT phosphorylation. Oxidative stress-induced apoptosis is inhibited by UC-MS-C-EVs via the release of miR-100-5p, which targets NADPH oxidase 4, thereby blocking the production of reactive oxygen species (ROS)⁵⁸. Overexpression of miR-140-5p in EVs from dental pulp stem cells inhibits chondrocyte apoptosis in a rat model of OA by regulating the expression of apoptosis-related proteins⁵⁹. In addition, the upregulation of autophagy-related proteins LC3 and Beclin-1, notably via peroxiredoxin 6 expression, reduces oxidative stress and promotes mitophagy mediated by Drp1 in chondrocytes^{60,61}. Notably, autophagy is modulated by miR-100-5p transported in ASC-EVs via the inhibition of mTOR, which is deregulated in OA⁶². Other results indicate that lncRNA KLF3-AS1 reduces apoptosis and senescence via activation of

the PI3K–Akt–mTOR signalling pathway, and lncRNA MEG-3 regulates IL-1 β -induced senescence and apoptosis in chondrocytes^{63,64}. More recently, BM-MS-C-EVs were shown to inhibit pyroptosis (a cell-death programme that is induced by inflammation) via the delivery of miR-326 to OA chondrocytes, thereby inhibiting histone deacetylase 3 (HDAC3) and activating the STAT1–NF- κ B p65 signalling pathway⁶⁵. Finally, MSC-EVs can increase glutamine metabolism in OA chondrocytes and alleviate OA progression⁶⁶. These results support a pro-survival role of MSC-EVs on chondrocytes exposed to an apoptotic environment.

Effects of EVs on immune cells

MSC-EVs can inhibit the production of pro-inflammatory factors (notably TNF, IL-1 β , IL-6, IL-8 and nitric oxide) by OA chondrocytes and synoviocytes^{43,49,67}. MSC-EVs can also act directly on macrophages to reduce inflammatory macrophage infiltration and induce macrophage polarization towards an anti-inflammatory M2 phenotype, resulting in the reduction of synovial membrane hyperplasia⁶⁸. In a rat model of OA, intra-articular injection of BM-MS-C-EVs resulted in reduction of synovial-fluid concentrations of pro-inflammatory cytokines IL-1 β and TNF, and elevation of anti-inflammatory IL-10 (ref. 69). The beneficial effect of BM-MS-C-EVs in reducing chondrocyte inflammation and cartilage degradation in a rat model of OA might be mediated by the lncRNA malat-1 (ref. 70). Interestingly, EV internalization in FLS is mediated in part by the hyaluronan receptor CD44 that is present on the surface of EVs and by hyaluronan that is expressed on FLS⁷¹. Accumulated in vitro data argue for an anti-inflammatory and anabolic effect of MSC-EVs on both OA synoviocytes and chondrocytes. In vivo results point to an efficient protective effect of MSC-EVs in various joint compartments, but a curative effect remains to be demonstrated. The next efforts should be aimed at improving the function of MSC-EVs for better efficacy.

The therapeutic effect of MSC-EVs in RA

RA is a chronic systemic autoimmune disease that is characterized by persistent synovial inflammation, which results in cartilage and bone degradation, leading ultimately to joint deformities. RA primarily affects the small joints of the hands and feet symmetrically. However, it can also involve other joints, such as wrists, elbows, knees and ankles. RA is characterized by joint pain, swelling, stiffness and tenderness, and can also cause systemic symptoms, including fatigue, fever, weight loss and general malaise⁷². Genetic and environmental risk factors as well as systemic immune-cell dysregulation have key roles in the pathogenesis of the disease and the loss of immune tolerance⁷³. The management of RA typically involves a combination of pharmacological and non-pharmacological interventions. Current drug treatments include DMARDs, glucocorticoids and NSAIDs. DMARDs, such as Janus kinase inhibitors and antibodies targeting specific immune cells and cytokines, can delay RA progression, but 20–30% of patients either do not respond to treatments or exhibit major adverse effects⁷⁴.

MSCs from various sources have attracted attention for their capacity to alleviate or prevent inflammation, and results from pre-clinical studies and clinical trials are promising in terms of safety and efficacy, as reviewed elsewhere⁷⁵. Nevertheless, it is important to note that in autologous settings, MSCs from patients with RA have limited suppressive capacity, and in a more general fashion, patients with RA receive medication that can interfere with cell-based treatments^{76,77}. Therefore, EVs isolated from allogeneic MSCs might

be more therapeutically effective than autologous EVs, as assessed in various non-clinical models of RA.

Role of MSC-EVs in immune cells in RA

The first report of the effect of murine MSC-EVs in RA demonstrated immunosuppression via induction of regulatory T (T_{reg}) and B (B_{reg}) cell responses *in vitro* and in a mouse collagen-induced arthritis (CIA) model¹⁵. Interestingly, small EVs were more potent for *in vivo* induction of a B_{reg} cell response than larger vesicles. In the same mouse model, the arthritic score and concentrations of inflammatory markers were reduced after injection of human UC-MSC-EVs or gingival MSC-EVs that restored the ratio of T helper type 1 (T_H1) and T_H2 cells to T_{reg} cells in favour of the T_{reg} cell population^{78–80}. In addition, UC-MSC-EVs were shown to have immunomodulatory effects including regulation of the imbalance between T_{reg} cells and T_H17 cells and of inflammatory factor secretion by peripheral blood mononuclear cells obtained from patients with RA⁸¹. Rat BM-MSC-EVs also have an anti-inflammatory effect on macrophages *in vitro*, decreasing the expression of NLRP3 inflammasome-associated protein⁸². This effect is related to miR-223 carried by the EVs, which targets NLRP3. *In vivo*, subcutaneous injection of rat BM-MSC-EVs decreases the clinical score in the rat model of complete Freund's adjuvant-induced arthritis. EVs isolated from human BM-MSCs cultured in normoxic, hypoxic or pro-inflammatory conditions all have similar therapeutic effects in relation to knee-joint swelling in an antigen-induced arthritis murine model through modulation of the T_{reg} - T_H17 balance in the spleen and lymph nodes of the animals⁸³. These results suggest that hypoxic or inflammatory priming of MSCs does not enrich EV cargoes in anti-inflammatory molecules that could mediate a regulatory function in RA models.

Role of MSC-EVs in FLS in RA

EVs derived from MSCs that overexpress particular miRNAs can modulate inflammatory phenotypes of FLS obtained from human or murine synovia. For example, MSC-EVs with induced expression of miR-150-5p can reduce expression of inflammatory and catabolic markers in RA FLS *in vitro* and the arthritic score *in vivo* following intra-articular administration⁸⁴. miR-150-5p-expressing BM-MSC-EVs also downregulate angiogenesis and tube formation (which is dependent on cell migration and invasion) in human umbilical vein endothelial cells by targeting MMP-14 and VEGF. Through the inhibition of synovocyte hyperplasia and angiogenesis, MSC-EVs expressing miR-150-5p reduce the progression of arthritis in the CIA model. Addition of MSC-EVs to FLS cultures diminishes the secretion of pro-inflammatory cytokines, and this reduction is accentuated by the use of miR-320a-enriched MSC-EVs⁸⁵. In the CIA mouse model, intravenous administration of MSC-EVs containing miR-320a improves the arthritic index and reduces serum concentrations of pro-inflammatory cytokines. Other miRNAs or lncRNAs (such as miR-21 or HAND2-AS1, respectively) that are present in BM-MSC-EVs and taken up by FLS in culture also result in reduced production of inflammatory cytokines^{86,87}. HAND2-AS1 acts as a sponge for miR-143-3p, promoting the expression of TNF-induced protein 3 and inactivation of the NF- κ B pathway⁸⁶. Finally, human synovial MSC-EVs regulate FLS-supernatant-induced angiogenesis via the circEDIL3-miR-485-3p-PIAS3-STAT3-VEGF pathway⁸⁸.

MSC-EVs can restrain proliferation and increase apoptosis of RA FLS in preclinical models such as the CIA rat model⁸⁹. This effect is mediated by miR-140-5p, which targets SGK1, an essential regulator of immune modulation⁹⁰. Overexpression of miR-124a in BM-MSC-EVs also promotes *in vitro* apoptosis of rheumatoid FLS MH7A cells⁹¹. *In vivo*

in the CIA mouse model, miR-21 from BM-MSC-EVs suppresses production of the transcription factor KLF4 by targeting TET1, thereby reducing the proliferation of FLS and the production of pro-inflammatory mediators⁸⁶. MSC transfection with circFBXW7-expressing vectors produces circFBXW7-enriched MSC-EVs that suppress proliferation and migration and enhance apoptosis in an RA FLS cell line, while alleviating inflammation and arthritic score in a rat model of RA⁹². circFBXW7 can sequester miR-216a-3p, thereby increasing expression of HDAC4, and notably expression of circFBXW7 and HDAC4 is lower in clinical RA samples than in healthy samples. Expression of the lncRNA HAND2-AS1 is low in RA synovial tissues, but induced overexpression of HAND2-AS1 in BM-MSCs produces HAND2-AS1-enriched EVs that can increase apoptosis and reduce proliferation in an RA FLS cell line⁸⁷. Finally, miR-320a, miR124a and HAND2-AS1 expressed in MSC-EVs can reduce the migration of RA FLS^{85,87,91}. Altogether, the data argue for an immunosuppressive and pro-survival function of MSC-EV cargoes on RA synoviocytes.

Role of MSC-EVs in other cell types in joints in RA

MSC-EVs affect the course of arthritis in murine models primarily by reducing inflammation and increasing apoptosis in the synovium. Their role in chondrocytes has been far less frequently investigated. However, in the CIA rat model UC-MSC-EVs reduce the number of apoptotic chondrocytes and increase the ratio of anti-apoptotic Bcl2 to pro-apoptotic Bax markers⁸⁹. Therefore, MSC-EVs might also improve the survival of chondrocytes and their phenotype, which is altered by the inflammatory and catabolic environment in patients with RA.

Therapeutic effects of MSC-EVs in other relevant diseases

In addition to OA and RA, the effects of MSC-EVs have also been evaluated in other disease models, with outcomes that could be relevant to the development of EVs for rheumatological applications. Here, we briefly discuss some of the studies with preclinical results in other diseases of cartilage and bone.

Intervertebral disc degeneration

Intervertebral disc (IVD) degeneration is a common spinal condition characterized by the breakdown of the fibrocartilaginous discs between the vertebrae, leading to pain, stiffness and reduced mobility⁹³. Experimental and preclinical results are promising for therapeutic effects of MSC-EVs in IVD degeneration, as reviewed elsewhere⁹⁴. MSC-EVs can reduce apoptosis in IVD cells and promote the synthesis of ECM components, such as collagen and proteoglycans^{95–97}, helping to restore the structural integrity and mechanical properties of degenerated discs. Furthermore, MSC-EVs possess anti-inflammatory properties, reducing the production of pro-inflammatory molecules and modulating the immune response within the disc microenvironment^{64,98,99}. By suppressing inflammation, MSC-EVs can alleviate pain and inhibit the further degradation of IVDs.

Osteoporosis

Osteoporosis is a progressive bone disorder characterized by reduced bone density and increased risk of fractures¹⁰⁰. MSC-EVs can promote bone formation and regeneration by stimulating the proliferation and differentiation of osteoblasts, the cells responsible for bone synthesis^{101,102}. These EVs enhance the production of ECM components, such as collagen and osteocalcin, leading to increased bone mineralization and improved bone strength. By modulating the immune

system, MSC-EVs can also help to mitigate inflammation and reduce the excessive bone resorption that is associated with osteoporosis^{102–104}. Furthermore, MSC-EVs can stimulate angiogenesis, which is crucial for providing nutrients and oxygen to bone cells and promoting bone regeneration¹⁰⁵.

Perspectives on EV-based strategies to improve therapy in rheumatic diseases

Improving EV production and cargo loading

Despite enormous development in the field and demonstration of the concept of EVs as potential biotherapeutic products, large-scale clinical application still faces some challenges. First, the capacity for EV production by cells is limited, which might represent a drawback for disease treatments that require high EV dosages. Recent technological efforts have addressed key issues in EV biogenesis using various methods to increase EV release. Genomic manipulation, adjustment of culture conditions and the application of biophysical and mechanical forces have shown that cellular metabolic changes can increase EV yields. For instance, 3D culture of UC-MSCs has yielded a 7.5-fold increase in EV yield compared with 2D culture, along with improvement of EV biological activity in the promotion of osteochondral regeneration¹⁰⁶. Along with 3D culture, externally applied mechanical forces such as cyclic mechanical stretch can increase EV production and biological potential¹⁰⁷. Induction of hypoxia and oxidative stress can also increase release of EVs and interfere with their cargo, as reviewed elsewhere¹⁰⁸. Furthermore, EV release from human BM-MSCs can be boosted three-fold by treatment with a combination of N-methyl-dopamine and nor-epinephrine, without altering EV functionality¹⁰⁹. Another effective way to increase EV release is the modulation of key components of the EV biogenesis machinery, including overexpression of the tetraspanin CD9, upregulation of intracellular concentrations of calcium ions and overexpression of cortactin^{110–112}.

Other studies have focused on establishing strategies to generate EVs that contain a defined molecule either in the EV cargo or on the membrane surface, to modify EV biological properties, stability or ability to be incorporated by a specific target cell. The techniques associated with the optimization of EV cargo and corona include passive techniques, such as incubation of EV-producing cells with the drugs that are intended for delivery¹¹³, as well as genetic techniques, such as transfection of parental cells to enable specific molecules to be incorporated into the EV biogenesis pathway¹¹⁴ (Fig. 2).

In terms of drug loading following EV isolation, various strategies can be used, as reviewed elsewhere¹¹⁵. Passive incorporation of hydrophilic macromolecules (such as nucleic acids and proteins) might require temporary disruption of the EV membrane via techniques including sonication, electroporation, freeze-thawing, osmotic shock and saponin permeabilization. However, the cargo-loading method itself can alter the biophysical characteristics or biological function of EVs, and the loading efficiency can vary between different types of cargo¹¹⁶.

Engineering MSC-EVs for therapy of rheumatic diseases

Various strategies can potentially increase the efficacy of EVs, extend their presence at the site of action or their bioavailability, or increase their penetrance in target cells (Table 1). One strategy relies on modification of the surface charge of MSC-EVs. Reversal of EV surface charge by post-isolation incubation with a cationic amphiphilic macromolecule (ϵ -polylysine-polyethylene-distearyl phosphatidylethanolamine) can enhance cartilage uptake of MSC-EVs, their penetration

into cartilage and joint retention, leading to enhanced efficacy of OA treatment¹¹⁷. Metabolic engineering of EVs might enhance their targeting to inflamed joints¹¹⁸. In a test of this theory, the surface of parental MSCs was engineered by bio-orthogonal click chemistry preceding EV production to express dibenzocyclooctyne-conjugated dextran sulfate. Following systemic administration of the engineered MSC-EVs to mice with CIA, EVs accumulated in the inflamed joints, inducing a cascade of anti-inflammatory events via macrophage phenotype regulation. ROS-responsive EVs isolated from tolerogenic dendritic cells can be generated by introducing a thioketal linker-embedded polyethylene glycol (PEG) on the EV surface via hydrophobic insertion¹¹⁹. PEG-decorated EVs have prolonged circulation in the blood following intravenous administration, and the detachment of PEG following ROS overproduction by activated immune cells in inflamed joints facilitates EV uptake and a more efficient therapeutic effect in the CIA mouse model.

MSCs can be transfected with vectors that encode cargo molecules, resulting in the release of specifically enriched EVs. As described above, MSC-EVs enriched for HAND2-AS1 can modify the 'tumour-like' properties of RA FLS⁸⁷. Similarly, overexpression of miR-155-5p and miR-140-5p in synovial MSC-EVs increases the capacity of EVs to protect cartilage and prevent OA^{120,121}. EVs can also act as vehicles to deliver genome-editing therapy. In an example of this approach, dendritic cells were genetically modified to express Lamp2b, an exosomal membrane protein, fused to a surface-displayed chondrocyte-affinity peptide, leading to production of EVs that were then merged with liposomes encapsulating CRISPR/Cas9 plasmids to generate hybrid vesicles¹²². Following intra-articular administration in arthritic rats, these hybrid vesicles efficiently ablated the expression of *Mmp13* in chondrocytes and attenuated the degradation of ECM proteins in cartilage.

An additional approach to enhancing EV functionality relies on priming MSCs prior to EV production. For example, IL-1 β priming of ASCs can enhance EV penetration into cartilage and inhibition of the expression of inflammatory markers of OA chondrocytes^{123,124}. MSC priming with TGF β 1 causes miR-135b overexpression in EVs, which increases chondrocyte proliferation by the negative regulation of the transcription factor Sp1 (ref. 125). TGF β 3-primed MSC-EVs shift the phenotype of OA chondrocytes towards anabolism, inhibit apoptosis and favour the M2 polarization of macrophages in vitro, and protect against cartilage and bone degradation in an OA mouse model⁴⁹. Similar effects occur with curcumin-primed BM-MSC-EVs, which upregulate the expression of miR-126-3p in IL-1 β -treated OA chondrocytes, promoting chondrocyte proliferation, migration and anabolism, and decreasing oxidative stress and apoptosis^{126,127}. Kartogenin-primed BM-MSC-EVs also promote cartilage repair, and parathyroid hormone-stimulated MSCs produce EVs that downregulate the secretion of IL-2, IL-6 and TNF by OA chondrocytes, and increase their proliferation, migration and the expression of type II collagen^{128,129}. Finally, EVs produced after MSC preconditioning with hypoxia are more prone to stimulating chondrocyte proliferation, migration and survival than EVs produced under normoxic conditions, as a result of the delivery of miR-216a-5p^{130,131}.

Another strategy to control the release of EVs involves embedding them in a biomaterial. Implantation of a hydrogel composed of gelatin methacrylate and decellularized cartilage ECM in an osteochondral defect enables sustained release of MSC-EVs, leading to increased chondrocyte migration, chondrocyte mitochondrial function restoration and macrophage polarization towards an M2 phenotype¹³². Similar results have been obtained using a thermosensitive hydrogel¹³³.

Few studies have compared the efficacies of different sources of MSC-EVs. Nevertheless, evidence indicates that induced pluripotent

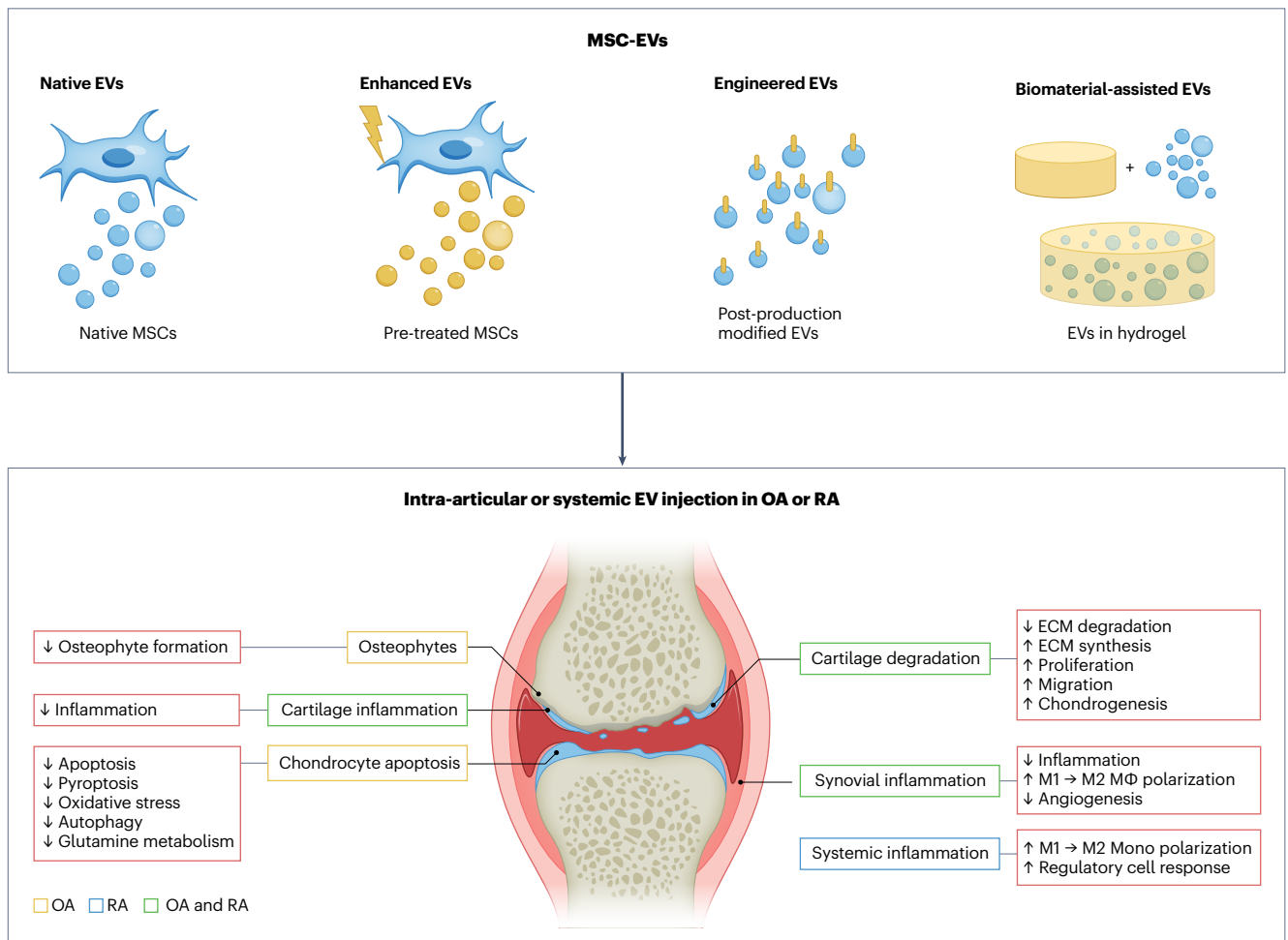


Fig. 2 | Applications of MSC-derived EVs in OA and RA. Mesenchymal stromal cell-derived extracellular vesicles (MSC-EVs) can be used in their native form, they can be optimized by pre-isolation or post-isolation modification, and they can be associated with biomaterials. EVs can be applied via intra-articular injection in patients with osteoarthritis (OA) or via systemic injection in patients

with rheumatoid arthritis (RA). Injection of EVs can improve disease features that are specific to OA (osteophytes and chondrocyte apoptosis) or to RA (systemic inflammation) or that are common to OA and RA (cartilage inflammation, cartilage degradation and synovial inflammation). ECM, extracellular matrix; MΦ, macrophage; Mono, monocyte.

stem cell-derived MSC-EVs are more effective than synovium-derived MSC-EVs for the alleviation of OA¹³⁴. Results also suggest that BM-MSC-EVs have greater therapeutic efficacy than ASC-EVs¹³⁵. Although beyond the primary focus of this Review, it is worth noting that sources of EVs other than MSCs have been successfully used for OA treatment in pre-clinical models, including urine-derived stem cells^{136,137}, platelet-rich plasma^{138,139}, bovine milk¹⁴⁰ and M2 macrophages¹⁴¹. By contrast, evidence suggests that EVs from endothelial cells increase chondrocyte apoptosis, whereas EVs isolated from IL-1β-treated synovial fibroblasts or OA subchondral bone increase cartilage degeneration^{142–145}. These findings highlight the need to identify the best sources of cells or to generate engineered cells or EVs to improve EV-based treatments.

EVs from a variety of cell sources can be produced and modified with increasing sophistication to enhance their homing and therapeutic potential, demonstrating the considerable potential of this versatile therapeutic platform. As previously discussed, MSC-EVs are the most frequently investigated source of EVs in many therapeutic

applications, and studies in non-clinical models have shown promising results, notably in rheumatic diseases for which curative treatments are still lacking.

Challenges in MSC-EV clinical translation

The utilization of EVs in rheumatic disease therapeutics holds considerable appeal because of the inherent complexity in their mechanism of action. These particles possess the ability to activate numerous interconnected signalling pathways and induce pro-anabolic or pro-angiogenic responses in various cell types that are essential for cartilage or bone formation. This ability confers a distinct advantage over methods based on individual growth factors, such as those involving BMP-2 or BMP-7, which frequently fall short in replicating the intricate nature of bone formation and can result in unfavourable consequences to patients, such as ectopic bone formation¹⁴⁶. Moreover, although not yet fully understood, the many mechanisms of action that complex EV populations (in a single medicinal product) can exert to fight against

several dysregulated pathways might be more effective than drugs that target individual pathways. Engineered EVs can also be used as vectors for different molecules or drugs with higher efficacy and safety than current pharmaceuticals or drugs in synthetic carriers. Polymeric micelles, liposomes and nanoscale polymer–drug conjugates form the foundation of nanoparticle-based drug delivery systems, which offer enhanced pharmacokinetics and biodistribution of both chemical and biological therapeutic agents. Nevertheless, their usage raises concerns regarding potential immunogenicity and cytotoxicity, as well as their clearance following clinical administration, as reviewed elsewhere¹⁴⁷.

The safety and efficacy of MSC-EVs as therapeutic products are currently being evaluated in clinical trials for the treatment of diverse

pathological conditions, in particular inflammation-related diseases and COVID-19 or acute respiratory distress syndrome³⁹. Two trials in musculoskeletal diseases (meniscus injury and OA) are currently recruiting patients. The phase II trial NCT05261360 is intended to evaluate and compare the efficiency of autologous synovial-fluid-derived MSCs and their corresponding EVs on knee injury. Patients in the experimental group will receive a single dose of MSCs (1×10^6 cells per kg) in the right knee and a single dose of their corresponding EV in the left knee by intra-articular injection. Thirty patients will be recruited and the readout on clinical efficacy will be assessed 1 year after the treatment. The other registered trial (NCT05060107) is a phase I study aiming to assess the safety and efficacy of a single intra-articular injection

Table 1 | Engineering of EVs using pre-isolation or post-modification techniques in preclinical models of rheumatic diseases

Cell source	Modification	Pre-isolation or post-isolation	Disease model	Outcome	Ref.
Human iPSC-derived MSCs	Reversal of EV surface charge	Post-isolation	OA	Enhanced cartilage uptake, cartilage penetration and joint retention capacities in vitro; improved intra-articular bioavailability in vivo	117
BM-MSCs	Implantation of EVs in a 3D-printed scaffold	Post-isolation	OA	Enhanced chondrocyte mitochondrial dysfunction and chondrocyte migration, polarization of the synovial macrophage response towards an M2 phenotype; cartilage regeneration in the rabbit model	132
hASCs	IL-1 β -primed cells	Pre-isolation	OA	Increased chondroprotective miRNA cargo; faster penetration of EV in collagen-rich areas of cartilage explant	123
hBM-MSCs	TGF- β 3-primed cells	Pre-isolation	OA	Induction of chondrocyte marker expression and decrease of catabolic and inflammatory marker expression, reduction of chondrocyte apoptosis in vitro; enhanced OA-induced damage in a mouse model	49
hBM-MSCs	IL-1 β -primed cells	Pre-isolation	OA	Inhibition of OA chondrocyte inflammatory marker expression in vitro via miR-147b from EV cargo	124
hBM-MSCs	Curcumin-primed cells	Pre-isolation	OA	Upregulation of miR-126-3p in OA chondrocytes leading to migration, proliferation, anabolism and lower oxidative stress and apoptosis	126
hBM-MSCs	PTH (1-34)-primed cells	Pre-isolation	OA	Increase in in vitro OA chondrocyte migration, proliferation and chondral matrix formation	128
mMSCs	TGF- β 1-primed cells	Pre-isolation	OA	Increase in miR-135b in EV cargo and in chondrocyte proliferation by negative regulation of the transcription factor Sp1 in vitro; reduction of cartilage degradation in vivo	125
mASCs	Curcumin-primed cells	Pre-isolation	OA	Decrease in chondrocyte oxidative stress and apoptosis in vitro and improved cartilage protection in vivo	127
mBM-MSCs	Kartogenin-primed cells	Pre-isolation	OA	Increased chondral matrix formation and diminution of tissue degradation in an OA model	129
mBM-MSCs	Hypoxia pre-conditioning	Pre-isolation	OA	Augmentation of in vitro chondrocyte proliferation, migration and survival via the miR-216a-5p–JAK2–STAT3 signalling pathway	130
Human synovial MSCs	miR-155-5p overexpression	Pre-isolation	OA	Enhanced articular chondrocyte proliferation and migration without damaging ECM secretion in vitro, and prevention of OA in vivo	120
Synovial MSCs	miR-155-5p overexpression	Pre-isolation	OA	Stimulation of OA chondrocyte proliferation, migration, ECM secretion and suppression of chondrocyte apoptosis in vitro, through Runx2 targeting; prevention of OA in a mouse model	121
hBM-MSCs	Hypoxia pre-conditioning	Pre-isolation	OA	Improved chondrocyte proliferation and migration and suppression of chondrocyte apoptosis in vitro through the miRNA-18-3P–JAK–STAT or miRNA-181c-5p–MAPK signalling pathways	131
hBM-MSCs	HAND2-AS1 overexpression	Pre-isolation	RA	Suppression of the proliferation, invasion, migration and inflammation and induction of apoptosis in RA-FLS in vitro and in vivo through the inactivation of the NF- κ B pathway via the miR-143-3p–TNFAIP3 axis	87
hASCs	Cell surface modification by click chemistry	Pre-isolation	RA	Enhanced targeting ability to inflamed joints; lower levels of inflammation and strong polarization effect towards the anti-inflammatory macrophage phenotype in vitro and in vivo	118

ASC, adipose-derived MSC; BM-MSC, bone-marrow-derived MSC; ECM, extracellular matrix; EV, extracellular vesicle; FLS, fibroblast-like synoviocytes; hASC, human ASC; hBM-MSC, human BM-MSC; iPSC, induced pluripotent stem cell; mASC, mouse ASC; mMSC, mouse MSC; mBM-MSC, mouse BM-MSC; MSC, mesenchymal stromal cell; OA, osteoarthritis; PTH, parathyroid hormone; RA, rheumatoid arthritis.

of MSC-derived exosomes in patients with moderate knee OA. A dosage of 3×10^{11} to 5×10^{11} EV particles from allogeneic UC-MSCs will be assessed in 10 patients, with a 1-year follow-up to record adverse events, pain, synovitis, disability and the percentage of responders. This study will be the first-in-human clinical trial using MSC-EVs for the treatment of a rheumatic disease.

Compared with parental cells, MSC-EVs have potential benefits in several domains: safety, because they cannot replicate and can be filtered through 0.22- μm membranes for sterilization; immunogenicity, as they have low expression of MHC molecules; and ease of use, as they can be stored at -80°C or even at room temperature as off-the-shelf lyophilized products²⁵. Nevertheless, there remain many challenges to address related to the scalable production, batch-to-batch standardization and characterization of EVs for clinical-grade production as biological products¹⁴⁸. More studies are needed to control factors that might interfere with the treatment scheme. Reducing variability between MSC sources and donors to enhance reproducibility in clinical outcomes could be achieved using universal MSC sources of either immortalized MSC lines or induced pluripotent stem cells, for unlimited access³⁹. The pharmacokinetics, toxicity and mechanism of action of MSC-EVs related to the intended application will have to be identified. The characterization of the active substance and the ‘excipient’ non-biologically active components of MSC-EVs will have to be documented before proceeding to the industrialization step. Boosting the biological efficacy of MSC-EVs by engineering parental cells or post-production EVs as well as improving EV delivery by encapsulation in hydrogels for a prolonged in vivo effect might help to bridge the gap towards industrialization. Nonetheless, the categorization of EV products is necessary according to the source of the EVs and whether they can be considered as native EVs or as vector EVs transferring biological information or drugs, to enable authorization by regulatory agencies before patients can be recruited for EV-based therapies.

Conclusions

In the past decade, developments in the study of EVs for clinical applications have identified MSC-EVs as potential innovative therapeutics. In this emerging field, sufficient evidence has been provided to indicate that rheumatic diseases, in particular (but not exclusively) OA and RA, could be treated using EV-based therapies. A rapidly growing body of evidence now indicates that MSC-EVs have key roles in the regulation and prevention of these diseases, notably by targeting both local and systemic inflammation, and by restoring a healthy chondrocyte phenotype. However, it has to be underlined that RA treatment is more challenging than OA because of the systemic nature of the disease and the occurrence of damage to multiple joints. The EV field has to take advantage of the vast amount of data gathered from the recent MSC-based clinical trials to better design future treatments. One possibility is to stratify patients who are better responding to treatments or to identify predictive markers of response to treatments. To date, a single phase I clinical trial is currently recruiting patients for MSC-EV-based OA treatment. As yet, the optimal dose and timing of treatments have not been investigated in large-animal models, which should be more relevant to the human condition than murine models. An interesting option, which is difficult to achieve using parental MSCs, would be to use repeated doses of allogeneic MSC-EVs or encapsulated MSC-EVs for more efficient and long-term treatments. MSC-EVs show exciting potential for reducing inflammation and improving survival and anabolic activity of cells in the different joint compartments, thereby providing a novel treatment approach to rheumatic diseases. Nevertheless, further

work is needed to deepen the understanding of their functionality and the way to improve their effectiveness in the context of regenerative medicine to help slow down disease progression.

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Author contributions

All authors researched data for the article and contributed substantially to discussion of the content, and to writing the article. D.N. and G.M.B. reviewed and edited the manuscript before submission.

Competing interests

The authors declare no competing interests.

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