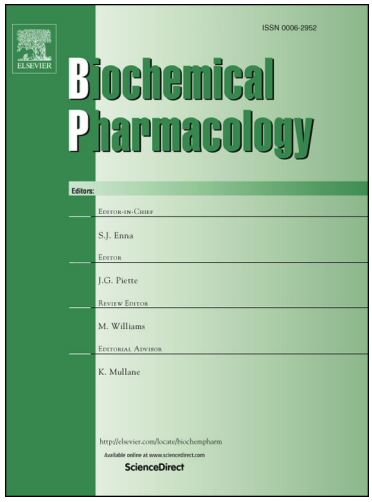

Review

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Extracellular vesicles: a new therapeutic strategy for joint conditions

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Abstract

Extracellular vesicles (EVs) are attracting increasing interest since they might represent a more convenient therapeutic tool with respect to their cells of origin. In the last years much time and effort have been expended to determine the biological properties of EVs from mesenchymal stem cells (MSCs) and other sources. The immunoregulatory, anti-inflammatory and regenerative properties of MSC EVs have been demonstrated in *in vitro* studies and animal models of rheumatoid arthritis or osteoarthritis. This cell-free approach has been proposed as a possible better alternative to MSC therapy in autoimmune conditions and tissue regeneration. In addition, EVs show great potential as biomarkers of disease or delivery systems for active molecules. The standardization of isolation and characterization methods is a key step for the development of EV research. A better understanding of EV mechanisms of action and efficacy is required to establish the potential therapeutic applications of this new approach in joint conditions.

Keywords: extracellular vesicles, mesenchymal stem cells, inflammation, immunomodulation, rheumatoid arthritis, osteoarthritis

1. Introduction

Extracellular vesicles (EVs) are actively secreted by cells and represent a mechanism for cell-to-cell signaling in physiological and pathophysiological responses [1,2]. These microparticles are usually classified based on the mode of biogenesis as microvesicles, exosomes, and apoptotic bodies [3]. Microvesicles and exosomes are both commonly found in extracellular fluids and represent the most described classes of EVs. Microvesicles are shedding vesicles

between 50 nm and 1 μm in diameter generated by plasma membrane protrusions followed by fission of their membrane stalk [3,4]. Exosomes are formed as intraluminal vesicles in endosomal compartments called multivesicular bodies and they are released in an exocytic manner by fusion of these multivesicular endosomes with the plasma membrane. These EVs show a mean size of 40 to 100 nm in diameter and are enriched in endosome related proteins [4,5] whereas apoptotic bodies (50–5,000 nm in diameter) are released from fragmented apoptotic cells [3].

Joint conditions represent an important public health problem as they are a major cause of pain, functional limitation and physical disability. As a main example, rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by synovial angiogenesis, hyperplasia of the synovial membrane and infiltration of immune cells besides cartilage damage and bone resorption [6]. RA is a systemic disease which can affect organs such as the lungs, heart and eyes and is associated with an increased risk of cardiovascular disease, infection, lymphoma, and reduced life expectancy [7,8]. The cause of RA is not yet fully understood, although autoimmune dysfunction plays a leading role in inflammation and joint damage, with a pre-rheumatoid phase preceding the onset of articular disease followed by established RA. Modification of the abnormal immune response by immune modulatory cells and other novel approaches represents an attractive possibility to achieve long-term tolerance and control of chronic inflammation [9].

Osteoarthritis (OA), the most prevalent joint condition in the elderly, is associated with progressive articular cartilage loss, low-grade synovitis and alterations in subchondral bone and periarticular tissues. There is an imbalance between anabolic and catabolic processes in the joint as well as a relevant contribution of mechanical stress and inflammatory mediators [10]. A number of risk factors are associated with OA, such as advancing age, obesity, and trauma that determine the progression of pathophysiological events in joint tissues [11]. There is no effective

treatment for OA although new therapies to stop disease progression or repair tissue damage are been investigated.

In the last years, the interest for the therapeutic applications of EVs has exponentially increased as these microparticles may reproduce the effects of parent cells with some advantages. In particular, EVs from mesenchymal stem cells (MSCs) provide a promising approach for immunomodulation and tissue regeneration. How these effects are mediated are not yet clear but EVs represent a novel strategy for future cell-free therapy of joint pathologies. Although these studies are at an early stage, the possible activity of EVs in joint conditions is of great interest and will be the focus of this review.

2. Isolation and characterization of extracellular vesicles

Current methods for isolating exosomes from biologic fluids include differential ultracentrifugation, density gradient centrifugation, size exclusion chromatography, polymer-based precipitation, filtration and immunoaffinity capture, as summarized in table 1. All of them have limitations such as co-isolation of contaminating materials, loss of EV components due to damaged membrane integrity during isolation or failure to completely isolate EV fractions. In particular, removal of serum proteins and lipoproteins is problematic [12]. Depletion of the most abundant serum proteins such as albumin or immunoglobulins seems necessary to avoid biasing downstream analysis. Sample collection from different biological fluids should take into consideration possible sources of artefacts and variability [13]. Platelet removal, for example, is mandatory when working with blood, plasma or serum, as platelets release EVs upon activation in freeze-thaw cycles [14]. Additionally, serum used to supplement culture media must be previously EV depleted [15]. Therefore the presence of contaminants may influence the behavior

of EV preparations leading to confusing effects on target cells. In addition, EVs from different sources can exhibit differences in composition or in non-specific component aggregation to their surface which can alter their physicochemical properties [16] and diverse EV subpopulations can be secreted by the same cell [17]. Consistency of pre-analytical procedures and report of complete experimental details have been recommended in order to get reproducible results [12].

There has been a great improvement of detection technologies during the past 20 years [18]. Quantification of EVs is usually performed by nanoparticle tracking analysis, tuneable resistive pulse sensing [19] or dynamic light scattering, and morphology confirmed by transmission electron microscopy, cryo-electron microscopy or atomic force microscopy [12]. Determinations of protein to lipid ratio, lipid bilayer order, and lipid composition may prove useful for quality control of EVs [20]. Western blotting or flow cytometry with fluorescent counting beads are normally used to detect EV protein markers [21]. The detection of specific markers would include CD63, CD9, and CD81 tetraspanins and endosome markers such as syntenin-1, ALG-2-interacting protein X (Alix) and tumor susceptibility gene 101 protein (TSG101), for exosomes [4,5,22]. Microvesicles can include cytoskeletal components (actin, actin-binding proteins (profilin-1, cofilin-1), myosin, tubulin), enzymes (alpha-enolase, pyruvate kinase, triosephosphate isomerase), membrane molecules (HLA-I, HLA-II antigens, Na^+/K^+ ATPase), proteins involved in vesicle biogenesis and trafficking (e.g. Ras-related proteins), lactadherin that binds to the phosphatidyl-serine surface of microvesicles, or clusterin (ApoJ), a protein involved in the clearance of apoptotic bodies and cell debris [21]. In addition, a set of components is cell-specific. Therefore, EVs from MSCs express on their surface MSC markers CD29, CD73, CD44 and CD105, as well as cell adhesion molecules and growth factor receptors. Inside EVs, a wide range of active molecules can be found such as cytokines, enzymes, nuclear

receptors, miRNAs and other RNAs such as transcription factor CP2/clock homolog, retinoblastoma-like-1, ubiquitin-related modifier-1 and interleukin-1 receptor antagonist [23].

Studies in 3T3-L1 mature adipocytes have shown a role for protein and lipid content in the characterization of large EVs (probably including microvesicles, with expression of β-actin and enrichment in endoplasmin and α-actinin-4), and small EV populations (sEVs, with expression of exosomal markers Alix, TSG101 and tetraspanins). The lipidomic analysis indicated cholesterol enrichment of sEVs, whereas large EVs were characterized by high amounts of externalized phosphatidylserine [24]. It has also been demonstrated the presence of two distinct subpopulations of exosomes (low density fractions exosomes and high density fractions exosomes). Both types express the exosomal markers Alix and TSG101 but differed in the presence of α-actinin-4, cyclin-Y (enriched in low density fraction exosomes) and ephrin type-A receptor 2 proteins (enriched in high density fractions exosomes) as well as in their RNA content [17]. As isolation methods based on different biogenesis pathways are still lacking, an universal nomenclature has been proposed based exclusively on size: large EVs pelleted at low speed, medium-sized EVs pelleted at intermediate speed, and sEVs pelleted at high speed. Among sEVs, further subcategories may be distinguished based on the presence or absence of different markers: a, enriched in CD63, CD9 and CD81 tetraspanins and endosome markers; b, devoid of CD63 and CD81 but enriched in CD9; and two groups not associated to the endosomal pathway: c, devoid of CD63/CD9/CD81; and d, enriched in extracellular matrix or serum-derived factors [22].

There is an increasing interest in the structural and functional biology of EVs. In addition to common components [25], these microparticles contain markers from the parent cells and therefore cell type specific protein, mRNA, miRNA, and lipid subsets have been identified which can be useful for diagnostic and therapeutic purposes. Interestingly, stress conditions or activation of intracellular signaling by mediators such as cytokines change EV composition and

therefore the response of recipient cells [26-28]. The content of proteins, RNA and lipids has been investigated by high-throughput methods. Genomic DNA has also been detected in EVs although its function is unknown [28]. In addition to classical techniques, proteomic analyses of EVs can be performed by high-resolution and high-sensitivity mass spectrometry and high-resolution liquid chromatography mass-spectrometry-based approaches [29]. These techniques and gas chromatography coupled to mass spectrometry, provide information on the presence of lipid species and metabolites in EVs [30]. EVs contain lipids in a bilayer membrane and also transport bioactive lipids and lipid related enzymes such as phospholipase A_2 and other enzymes involved in eicosanoid synthesis. Besides, EVs are enriched in cholesterol and sphingomyelin which can accumulate in recipient cells [31] as EVs may transfer lipids between cells for metabolism into bioactive mediators [32]. Metabolomic strategies have recently provided the characterization of EV metabolic activity [30]. Interestingly, high throughput transcriptomic studies have identified a wide range of mRNA and miRNA data sets based on microarray and next-generation sequencing analyses leading to a comprehensive data classification [33,34]. These EV components can be functional after transfer to cells [35]. Other RNA species within EVs include viral RNSs, Y-RNAs, fragments of tRNAs, small nuclear RNA, small nucleolar RNA, piwi-interacting RNAs, long non-coding RNAs and circular RNAs [28,36]. There are a wide range of studies on EV composition which are collected by three curated data repositories: ExoCarta [37], Vesiclepedia [3] and EVpedia [38], and functional enrichment analysis tools are also available [39].

3. Immunomodulatory effects of extracellular vesicles

A wide range of evidence indicates that EVs produced by both immune and non-immune cells can play an important role in the regulation of immunity (reviewed in [40,41]). Circulating endogenous EVs produced by different cell types contribute to the suppression of immune responses, either in an antigen-specific or a nonspecific manner. For instance, platelet-derived EVs can inhibit inflammatory responses due to the presence of 12-lipoxygenase which is transferred to mast cells to synthesize the pro-resolving mediator lipoxin A_4 [42]. Endothelial cell-derived EVs can suppress monocyte activation due to the transfer of miRNAs such as miR-10a able to target several components of the nuclear factor-κB (NF-κB) pathway, including interleukin-1 receptor-associated kinase 4 [43]. Some studies have suggested that antigen-specific immunosuppressive EVs from autologous plasma may be used to inhibit inflammation. Interestingly, intraarticular injection of exosomes obtained from autologous conditioned serum was safe and reduced pain and inflammatory markers in RA patients who do not respond well to conventional therapy [44]. In addition, blood-derived exosomes may be negative regulators of osteoclast formation in RA [45].

Oxidative stress plays an important role in the regulation of the immune response in arthritis [46]. In RA patients there is a significant elevation of surface thiols on circulating monocytes while the newly released EVs of isolated CD14+ cells from these patients have decreased thiol levels and enhanced peroxyredoxin 1 expression compared with healthy subjects. These results suggest that production of EVs by human monocytes may regulate oxidative stress in these cells [47]. It has been reported that macrophages release EVs containing Gla-rich protein which is a calcification inhibitor in articular tissues and a possible anti-inflammatory agent in chondrocytes, synoviocytes and monocytes/macrophages. This protein may link inflammation and calcification events in the joint and is able to inhibit the production of pro-inflammatory cytokines in macrophages [48].

On the other hand, human neutrophils release EVs able to block inflammatory responses of macrophages and induce the release of transforming growth factor-β1 (TGF-β1) which can promote the resolution of the inflammatory response [49]. Neutrophil-derived microvesicles also exert chondroprotective actions *in vitro* and in murine models of inflammatory arthritis. It has been demonstrated that neutrophils migrate into inflamed joints to release microvesicles which penetrate into the cartilage. Neutrophil microvesicles require annexin A1 and its receptor formyl peptide receptor 2 to exert protective effects on chondrocytes which are mediated by TGF-β1 production, extracellular matrix deposition and inhibition of chondrocyte apoptosis. Interestingly, RA synovial fluids contain abundant neutrophil-derived microvesicles with a possible cartilage protecting role [50].

Dendritic cell (DC)-derived EVs deliver their content into the cytoplasm of acceptor DCs which could be a mechanism involved in fine-tuning of the immune response [51]. Nevertheless, These EVs are not only vehicles to deliver immunosuppressive factors from their parent cells as they exert antigen-specific effects which depend on the presence of molecules such as MHC class II and B7. Distal therapeutic effects were also observed after local administration of DC EVs suggesting that they may act by interacting with endogenous immune cells at the membrane level or by transfer of proteins and RNAs leading to an immunosuppressive and anti-inflammatory behavior of these cells [44]. Exosomes from immature DC may be partially immunosuppressive [52] and they can be modified to enhance this property. It has been suggested that IDO expression in DCs modifies exosomes to render them tolerogenic. Therefore, exosomes derived from DCs overexpressing indoleamine 2,3-dioxygenase (IDO) have an anti-inflammatory effect in collagen-induced arthritis (CIA) and delayed-type hypersensitivity murine models. These exosomes may directly interact with T cells and other antigen-presenting cells (APCs) to alter their function which was partially dependent on B7 costimulatory molecules [53]. In the CIA

model, intravenous administration of exosomes derived from DCs expressing interleukin(IL)-10, DCs expressing IL-4 or DCs expressing FasL-effectively inhibited arthritis [54,55]. Injected exosomes are internalized by CD11c+ cells at the site of injection and in the draining lymph node. Local administration of exosomes was also able to inhibit the inflammation of murine delayed-type hypersensitivity in both the treated and the untreated distal paws in a MHC class II dependent and MHC class I independent manner [55].

The ability of Treg cells to release exosomes is required to inhibit Th1 cell proliferation *in vivo* and prevent systemic disease. It has been reported that the miRNA content of exosomes play an important role in this inhibitory effect. Therefore, let-7d is transferred to Th1 cells and mediates the suppression of Th1 cell proliferation and interferon-γ (IFN-γ) secretion [56]. The transfer of the dominant negative form of inhibitor of NF-κB kinase 2 (IKK2) has been used to give rise to immature CD4+CD25-Treg cells (dnIKK2-Treg). These cells release EVs containing specific miRNAs and inducible nitric oxide (NO) synthase which are delivered into target cells leading to block of cell cycle progression and induction of apoptosis. In addition, dnIKK2-Treg-EV-exposed T cells can be converted into regulatory cells [57].

Cell infiltration and the imbalance between cell proliferation and cell death contribute to pathological changes in RA. In particular, the resistance of synovial lymphocytes, macrophages and fibroblasts to apoptosis may play a role in the chronification of arthritis [58]. The bioactive death ligands FasL and APO2L/TRAIL are stored inside human T cells and secreted associated with EVs upon cell activation [59]. Interestingly, the number of EVs containing APO2L/TRAIL in synovial fluid is very low in RA patients and the persistence of activated T lymphocytes has been related to the resistance to Fas/CD95 and the inefficient secretion of EVs containing bioactive FasL and APO2L/TRAIL [60]. Therefore, EVs expressing APO2L/TRAIL may be a therapeutic approach for RA which has been explored in preclinical models. Intraarticular

injection of artificial lipid vesicles resembling natural EVs with bound APO2L/TRAIL exerted anti-inflammatory effects and inhibited synovial hyperplasia in a model of antigen-induced arthritis in rabbits [61].

EVs from other sources may be of interest for their immunoregulatory properties. For instance, bovine milk contains EVs expressing CD63 and immunoregulatory miRNAs (miR-30a, -223, -92a). Oral administration of bovine milk derived EVs delayed the onset of CIA and diminished cartilage pathology, bone marrow inflammation and serum monocyte chemoattractant protein-1, IL-6 and anticollagen IgG2a levels, accompanied by reduced splenic Th1 (Tbet) and Th17 (RORγt) mRNA [62].

MSC EVs have been shown to reduce inflammation, regulate immune responses and facilitate tissue regeneration [63]. There are complex interactions between MSCs and immune cells that may help to understand their immunomodulatory properties. The effects of MSCs are mediated by cell-to-cell contact and paracrine mechanisms due to the production of soluble molecules and EVs released into the extracellular milieu. The importance of cell-to-cell contact in immunosuppression by MSCs has been shown in different studies as well as the role of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 [64,65]. MSCs have been reported to secrete a wide range of molecules such as purines, bone morphogenetic proteins (BMPs), CD274, CCL2, connexin 43, IDO [66], prostaglandin E_2 [67], IL-6, IL-10, NO [68], heme oxygenase-1 [69], tumor necrosis factor-inducible gene-6 (TSG-6) [70], leukemia inhibitory factor (LIF), CD95/CD95 ligand, galectins, human leukocyte antigen-G5 (HLA-G5) [71], and growth factors such as TGF-β1 [68], hepatic growth factor (HGF) [72], vascular endothelial growth factor (VEGF), platelet-derived growth factor, fibroblast growth factor (FGF), etc. [73]. Other ways of cellular communication between

MSCs and immune cells include the bidirectional exchange of cytoplasmic components mediated by tunneling nanotubes derived from human T cells [74] and the transfer of EVs.

Cellular therapy with stem cells showed a low engraftment and poor survival leading to the demonstration that MSCs act through paracrine effects in animal models of ischemic heart disease and acute kidney injury [75-79]. Therefore, it was demonstrated that only the fraction of the conditioned medium (CM) containing products >1000 kDa (100-220 nm) provided cardioprotection in a mouse model of ischemia and reperfusion injury [80] leading to the confirmation that protective effects of MSC secretome depended on the presence of EVs [80-82]. In addition, EVs exert a modulating role on the effects of soluble mediators [83].

The beneficial effects of MSCs in glucocorticoid-refractory graft-versus-host disease in human patients have been related to immune response-modulating factors secreted by these cells and identified as EVs [84]. Similarly, EVs from umbilical cord (UC)-MSCs ameliorated the inflammatory immune reaction and kidney function in grade III-IV chronic kidney disease patients [85]. Phase I clinical studies with EVs have revealed a low toxicity and stability in plasma and different clinical studies have tested their potential in wound healing [86], hair regeneration [87], acne scars and skin rejuvenation [JSPH2012-082], type-1 diabetes [NCT02138331], the development of vaccines for different types of cancer or as vehicles for drug delivery to cells [88].

The degree of EV-mediated immunomodulation seems to be proportional to the ability of different immune cells to uptake these microparticles [89,90] leading to the inhibition of proliferation and differentiation processes [91]. EVs from MSCs may exert the strongest immunomodulatory effects on B cells compared with other lymphocyte subsets which may depend on the ability of B cells to incorporate EVs. Therefore, EVs from MSCs inhibit the proliferation of B cells and also of NK cells [89]. Nevertheless, the role on T cells has not been

clearly demonstrated. It has been reported that exosomes from adipose-derived MSCs (AMSCs) exert an inhibitory effect on proliferation, differentiation and activation of T cells [92]. In contrast, microvesicles from bone marrow (BM)-MSCs have been shown to exert a lower immunomodulatory effect on T-cell proliferation compared with the parent cells [93]. Other reports failed to demonstrate any effect on lymphocyte proliferation by EVs from MSCs [74]. In another study, BM-MSC EVs exhibited *in vitro* immunomodulatory effects on T cells but they were different from those of their parent cells [94]. Immunosuppressive effects of BM-MSCs can be enhanced by priming with IFN-γ and tumor necrosis factor-α (TNFα) which leads to higher ICAM-1 expression and internalization of EVs by immune cells. In addition, primed EVs enhance the immunosuppressive ability of resting BM-MSCs towards T cells, which may be mediated by IDO increase [89]. Another report has indicated the possible contribution of cyclooxygenase-2 and different miRNAs to the immunosuppressive effects of cytokine-stimulated BM-MSCs [95]. Microvesicles and exosomes from murine MSCs have been shown to inhibit the proliferation of CD8+ T cells and the proliferation and activation of B cells. In addition, both types of EVs increased the Treg population but were without effect on CD4+ IFNγ+ T cells [96].

EVs released by MSCs are efficiently internalized by macrophages and induce proliferation and the transition of pro-inflammatory macrophages to an anti-inflammatory and pro-resolving M2 phenotype [97]. It was demonstrated that microvesicles from murine AMSCs were quickly incorporated into the intracytoplasmic region of M1-macrophages and promoted a M2-like phenotype and the reduction of pro-inflammatory miR-21 and miR-155. These results were confirmed *in vivo* in an experimental model of acute peritonitis [98]. Also, M2 polarization was induced by MSC EVs in mouse or human monocytes which in turn polarized activated

CD4+ T cells to CD4+CD25+FoxP3+ Treg cells [99]. In contrast, EVs from UC-MSCs did not polarize monocytes [100]. Figure 1 shows a summary of the immunosuppressive effects of EVs.

Few studies on the *in vivo* effects of EVs in arthritis models have been published. It has been reported that administration of EVs from murine MSCs ameliorated the symptoms in the mouse CIA model of RA [96]. In bovine serum albumin-induced synovitis in pigs, intraarticular administration of EVs from porcine BM-MSCs exerted anti-inflammatory effects with reductions in synovial lymphocytes count and TNFα expression. These EVs efficiently counteracted the antigen-driven T cell response and may represent a therapeutic strategy for the treatment of T cell mediated diseases such as RA [101].

Transfer of EVs components can play an important role in the effects of these microparticles. MSC EVs include a cargo of immunomodulatory proteins which may act in a synergistic manner [102]. These microparticles thus induce high levels of anti-inflammatory TGF-β1 and IL-10 [103,104], and inhibit pro-inflammatory IL-1β, IL-6, TNF-α and IL-12p40 [99]. Microvesicles derived from mouse BM-MSCs express regulatory molecules present in parent cells such as programmed death-ligand 1 (PD-L1), galectin-1 (Gal-1) and TGF-β1 which confer tolerogenic properties to these microparticles [105]. Interestingly, PD-L1 contributes to the development of inducible T regulatory (iTreg) cells [106] while Gal-1 has been shown to induce growth arrest and apoptosis of activated T cells and contribute to the promotion and generation of Treg cells [107,108]. Therefore, Gal-1 gene therapy or protein administration to DBA/1 mice inhibited clinical and histological manifestations of arthritis in the CIA model [109]. The results of these studies support the interest of EVs in the treatment of chronic inflammatory and autoimmune disorders. In addition, microvesicles from MSCs contain ribonucleoproteins involved in the intracellular traffic of RNAs as well as selective miRNAs which may be transferred to target cells [110] and likely exert immunomodulatory effects in arthritic diseases

(reviewed in [111]). Apart from the presence and properties of endogenous miRNAs, loading of EVs with miRNAs or anti-miRs may be an interesting approach to enhance the immunoregulatory activity of these microparticles in chronic inflammatory conditions [42].

4. Regenerative properties of extracellular vesicles in joint conditions

Joint conditions are important targets of MSC therapy mainly to treat chondral and/or bone lesions and defects resulting from injury or trauma, or in OA. In the last years, the possible applications of MSCs in cartilage repair used alone or combined with biomaterials have been extensively explored. MSCs are injected into the joint space, or implanted in a scaffold matrix or as tissue engineered constructs in order to create a favorable microenvironment for tissue repair (for review see ref. [112]). Stem cells are capable of selectively homing to injured tissues and differentiating into several types of cells to repair the lesion and improve the affected function. Humoral mediators produced by injured tissue would be chemotactic for stem cells, they also stimulate local proliferation of endogenous or exogenous stem cells or could be a signaling mechanism to expand the pool of bone marrow progenitor cells in response to tissue injury [113]. Nevertheless, it has been reported that chondrogenesis in 3D culture generates constructs whose mechanical properties are inferior to constructs formed with chondrocytes [114] leading to studies on different strategies to improve the chondrogenic potential of MSCs [115].

Cell differentiation and engraftment would not be the sole mechanisms for tissue regeneration as transplanted cells become fewer and disappear soon after transplantation [115]. Additionally, MSCs exhibit a variety of trophic activities relevant to musculoskeletal therapy and can promote chondrogenesis, osteogenesis, musculogenesis, tenogenesis, angiogenesis and

neurogenesis (reviewed in [73]). The efficacy of MSC therapies in joint repair has been demonstrated in many animal models and clinical studies [116-126].

The effectiveness of many MSC-based therapies in tissue repair has been attributed to the paracrine secretion of these cells as only a small percentage of the MSC populations injected into the joint actually remain at the site of injury (reviewed in [8]). The MSC secretome would promote tissue repair by modulating the local microenvironment and supporting growth and activity of local cells. Nevertheless, the composition of MSC secretome is quite complex and varies depending on the microenvironment of cells [127]. For instance, MSCs have a differential response to synovial fluid from early- versus late-stage OA, with a higher secretion of CXCL8, IL6 and CCL2 in the first case [128]. Accordingly, pretreatment of MSCs with different factors can improve the release of immunomodulating or regenerating mediators [129] as it has been shown by priming the parent MSCs with lipopolysaccharide [130].

The CM of MSCs contains a wide range of cytokines, chemokines, hormones, lipid mediators, cytokines, growth factors and extracellular matrix components which can mediate tissue healing. The regenerative properties of CM from MSCs have been explored in many different tissues (reviewed in [88]). Therefore, MSC CM can regenerate bone through mobilization of endogenous stem cells, angiogenesis and osteogenesis [131] and promote periodontal tissue regeneration [132] and healing of bisphosphonate-related osteonecrosis of the jaw in rats [133]. Interestingly, the therapeutic efficacy of human BM-MSCs CM was demonstrated in a human clinical study. This CM containing insulin-like growth factor-1, VEGF, TGF-β1 and HGF, in beta-tricalcium phosphate or an atelocollagen sponge, regenerated alveolar bone [134].

Treatment of OA chondrocytes or synovial cells with CM from BM-MSCs or AMSCs in an inflammatory milieu inhibits the production of inflammatory and catabolic agents [9,10]. In

this respect, we have reported that CM from AMSCs exert protective effects in OA chondrocytes [20,21]. Therefore, factors produced by MSCs can enhance the anabolic properties of a wide range of cells such as chondrocytes, chondrocyte progenitor cells, cartilage-derived stem/progenitor cells, synovium-resident multipotent progenitor cells, osteoblasts/osteoclasts/resident MSCs in subchondral bone and chondrogenic cells within the infrapatellar fat pad [73].

The EVs present in CM show a great potential in the regeneration of joint tissues to replace stem cell-based therapy. EVs secreted by hMSC carry hyaluronan on their surface which is able to interact with proteins and proteoglycans of extracellular matrix to maintain tissue homeostasis, and contribute to extracellular matrix remodeling and tissue healing [135,136]. EVs express adhesion molecules to bind to and interact with cells, but they are also able to bind to extracellular matrix components. EVs from some cell types contain extracellular matrix regulatory proteins involved in re-structuring, cytokine release, angiogenesis and cell migration as well as lysyl oxidases which crosslink collagens and elastin [137,138]. In particular, exogenous lysyl oxidases have been shown to be useful in cartilage integration problems [139]. Interestingly, EVs from different cell types are able to transfer the mRNA of growth factors and their receptors to tissue cells to initiate tissue repair responses [140,141].

Treatments with MSC EVs are able to reproduce the main actions of CM suggesting that these microparticles are relevant mediators. EVs from naïve or genetically modified MSCs may be used to improve the regenerative properties of these cells as they can modulate the microenvironment of damaged cartilage to promote repair or to enhance the chondrogenic ability of these cells [124]. Different scaffolds have been investigated to retain MSC EVs and promote cartilage repair. One of them is a photoinduced imine crosslinking hydrogel glue with excellent

biocompatibility and integration with cartilage matrix which has been tested to prepare an acellular tissue patch for cartilage regeneration [142].

Interestingly, OA chondrocytes internalize EVs from BM-MSCs leading to the upregulation of aggrecan and type II collagen. In addition, gene expression of IL-1, IL-6, IL-8 and IL-17 as well as collagenase activity induced by TNFα were significantly reduced [143]. It has also been reported that EVs from mouse BM-MSC exert anti-apoptotic effects in chondrocytes and immunosuppressive effects in macrophages. *In vivo* administration of these EVs partly protected cartilage and bone in the murine collagenase model of OA [144]. We have provided evidence that microvesicles and exosomes from human AMSC CM exert anti-inflammatory and protective effects in OA osteoblasts [145] and chondrocytes (our unpublished results) *in vitro*. Anti-inflammatory and chondroprotective effects of EVs derived from AMSCs have also been described in murine cells [146].

The release of pro-inflammatory mediators and reactive oxygen species can result in mitochondrial changes, inflammation, oxidative stress and DNA alterations which can induce premature senescence [37]. We have recently shown that microvesicles and exosomes from human AMSCs reduce the production of inflammatory mediators, mitochondrial membrane alterations and oxidative stress in OA osteoblasts which results in the down-regulation of cell senescence [145] (Figure 2).

Different studies have demonstrated that EVs enhance skeletal muscle [147], bone [148] and cartilage [142] regeneration. Administration of AMSC EVs in a model of skeletal muscle injury reduced the inflammatory response and accelerated the muscle regeneration process [97]. BM-MSC-derived EVs led to bone formation in calvarial bone defects with an essential role for miR-196a in the regulation of osteoblastic differentiation [148]. Injection of exosomes from human induced pluripotent stem cell-derived MSCs (iPS-MSCs) by intravenous route prevented

osteonecrosis induced by steroid in rats. This treatment activated the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway on endothelial cells leading to local angiogenesis [149]. Protective effects of these type of exosomes were also observed in an ovariectomized rat model [150] and, in combination with a tricalcium phosphate scaffold, in rat calvarial bone defect. *In vitro* studies showed that these exosomes can be internalized into BM-MSCs and enhanced the proliferation, migration, and osteogenic differentiation of these cells. Activation of the PI3K/Akt signaling pathway by exosomes likely plays an important role in these effects [151].

Intraarticular injection of exosomes obtained from human ESCs has been shown to completely restore cartilage and subchondral bone in a model of osteochondral defects in rats [152]. In another study, exosomes secreted by human synovial MSCs were internalized by human chondrocytes *in vitro* and induced proliferation and migration but reduced extracellular matrix production. These effects were due to the high Wnt5a and Wnt5b expression in these cells which activated YAP and led to the suppression of SOX9 expression. *In vivo* intraarticular injection of exosomes resulted in a weak protective effect in the rat OA model established by transecting the medial collateral ligament and the medial meniscus [153]. In another OA model in mice injected with collagenase, intraarticular administration of human synovial MSC exosomes significantly attenuated OA progression. In the same model, injection of exosomes from human iPS-MSCs had a superior therapeutic effect. Therefore, these cells may be a better source of exosomes for cartilage repair with other advantages as they can be induced from patient-specific adult somatic cells such as peripheral blood cells without an invasive harvesting and with a high yield. In addition, autologous cells can be used overcoming ethical issues and immune activation [154].

Some treatments using EVs from other sources may also be useful in OA. As an example, EVs from autologous conditioned serum have been shown to protect human OA cartilage from glycosaminoglycan loss in basal conditions and in the presence of IL-1β [155]. There is an

ongoing observational study that evaluates the characteristics of autologous platelet-rich plasma (PRP) in the therapy and treatment of musculoskeletal pain and OA. This study will test the hypothesis that PRP characteristics, such as platelet and microparticle content and composition can be predictive for clinical outcome for PRP treatments (NCT02726464).

Further studies are necessary to establish the mechanisms underlying the regenerative effects of EVs but these microparticles contain many regulatory molecules that may be transferred to target cells and contribute to their biological effects. It has been suggested that EVs delivery to damaged tissue may contribute to epigenetic reprogramming of target cells [156,157]. MSC EVs repair ability can depend on the restoration of cartilage homeostasis. In OA, there is chondrocyte loss or cellular senescence induced by abnormal mechanical stress, inflammation, oxidative stress and mitochondrial dysfunction [158,159]. EVs may transfer to chondrocytes glycolytic enzymes such as phosphoglucokinase and pyruvate kinase, and ATP generating enzymes such as adenylate kinase and nucleoside-diphosphate kinase that may compensate the reduced mitochondrial ATP production in OA chondrocytes. In addition, MSC EVs contain CD73 which is able to convert the extracellular ATP released by injured tissues to adenosine. It is known that EVs induce cell proliferation through adenosine-mediated phosphorylation of extracellular signal-regulated kinase (ERK)-1/2 and Akt [160].

On the other hand, the regulation of immune cells and the pro-inflammatory environment plays an important role in tissue regeneration. M1 polarized synovial macrophages from OA patients secrete factors inhibiting MSC chondrogenesis [161] whereas MSCs can induce M2 polarization which reduce inflammation and improve cartilaginous tissue regeneration [162]. As EVs can exert immunomodulatory and anti-inflammatory effects, it is likely that these properties contribute to joint protection and repair.

MSC-derived EVs contain a wide range of miRNAs which may facilitate intercellular communication and contribute to different phases of the healing process [97,163]. Many of these miRNAs are involved in signal transduction, cartilage metabolism and OA progression. For instance, mir-23b is a mediator of chondrocyte differentiation of human MSCs [164] and miR-221 and miR-92a may contribute to the regulation of cell proliferation and differentiation of chondroprogenitor cells [165-167]. It is also known that key catabolic enzymes such as aggrecanase-2 and metalloproteinase-13 are the target of miR-125b and miR-320, respectively [168,169]. The expression of miR-320 is reduced in OA cartilage compared with normal cartilage. This miRNA regulates chondrogenesis and IL-1β-stimulated catabolic effects in mouse chondrocytes [169]. Another miRNA with therapeutic potential in OA is miR-140, which regulates cartilage homeostasis and development [170]. Interestingly, modification of synovial MSCs to overexpress miR-140-5p improved EV properties *in vitro* and *in vivo* leading to a significant inhibition of cartilage degradation in a surgical model of knee OA in rats [153]. All together these data support the interest of EVs containing miRNAs with beneficial effects on joint metabolism to develop potential therapeutic approaches in OA.

5. Extracellular vesicles in the physiopathology of joint conditions

EVs mediate cell communication in pathological states and may act as signaling structures involved in the induction and amplification of immunity and inflammation. Therefore, some EVs can play a pathogenic role in joint conditions (for extensive reviews see refs. [171-176]). EVs may exert different roles in inflammation depending on the cell source, cell target and the environment where they can be influenced by multiple factors. Some studies have revealed

that EVs are a component for autocrine and/or paracrine stimulation although they may also initiate counter-regulatory mechanisms which potentially contribute to the resolution of inflammation. In this context, EVs released by activated human monocytes have been shown to activate NF-κB and production of cytokines and oxygen radicals which may lead to the amplification of the inflammatory response. At the same time, these EVs are able to enhance PPAR-γ expression which is involved in the control of inflammation [177]. It is also known that EVs from T cells can modulate the effects of TNFα, suggesting a cross-talk between cytokines and EVs. Besides promoting the induction of inflammation, treatment with EVs significantly upregulated a number of anti-inflammatory genes [83].

The stimulation of immune responses by EVs in certain situations can initiate or exacerbate autoimmune diseases. EVs may exert immunostimulatory effects by a number of mechanisms. EVs express different molecules of the parent cells and may transfer antigens, MHC molecules and costimulatory molecules to immune cells. As an example, DCs produce EV-associated MHC class I complexes which are transferred to other naive DCs for efficient CD8+ T cell priming which can be viewed as an amplification process for DC-mediated CTL responses [178]. Similarly, EVs from both human and murine B lymphocytes are able to induce antigen-specific MHC class II-restricted T cell responses. These studies support the view that EVs produced by APCs may act as vehicles for MHC class II-peptide complexes involved in maintenance of long-term T cell memory or T cell tolerance [179].

Synovial EVs formed in an inflammatory environment may stimulate articular cells to release more inflammatory mediators and degradative enzymes and thus contribute to articular damage [27,180]. Some EVs can also contain degradative enzymes and therefore EVs released by rheumatoid synovial fibroblasts degrade aggrecan in a tissue inhibitor of metalloproteinase-3-sensitive manner which may facilitate cell invasion through aggrecan-rich extracellular matrices

[181]. Similarly, hexosaminidase activity is found to be associated with RA synovial fibroblast-derived EVs [180]. On the other hand, it has been reported that EVs released by chondrocytes contribute to pathologic mineralization of cartilage in musculoskeletal pathologies such as OA [182] and their secretion is regulated by autophagy [183].

More studies are necessary to dissect EV signaling pathways and molecular mechanisms in the physiopathology of joint conditions. As EVs produced by some cell types may be mediators of the pathophysiological changes that occur in the joint environment, a therapeutic strategy may be the inhibition of production and release, modification of harmful content or elimination of microparticles contributing to pathological processes [184].

6. Extracellular vesicles as biomarkers of joint disease

There is a considerable interest in identifying noninvasive specific biomarkers which may reflect the alterations in joint tissues. At present, prognostic tools especially for OA and spondyloarthritis are still lacking. Early identification of predictive markers is crucial to address the risk, the presence, the evolution and the response to treatment in chronic joint conditions [185]. The release of EVs into the extracellular space allows to examine them in body fluids as novel candidates for disease biomarkers to use in diagnosis, prognosis and treatment. Of note, in situations where the same biomarker molecules can be indicative of more than one condition, EVs would be the method of choice to trace the cell type causing the alteration. These microparticles can be immuno-isolated based on recognition of a significantly enriched protein on the membrane surface [186].

Serum EVs are enhanced in RA. In particular, endothelial EVs have a deleterious effect on endothelial cell function and may be a marker of vascular damage [187] while platelet-derived EVs levels may be related to disease activity [188]. Nevertheless, circulating EVs exposing complement components, C reactive protein or serum amyloid-P are elevated in early active RA although effective drug treatments do not decrease their levels suggesting a limited value as biomarkers [189].

Other reports indicate that serum EVs may be useful as additional markers of disease activity in patients with RA. For instance, differences in EV levels of amyloid A and lymphatic vessel endothelial hyaluronic acid receptor-1 have been found between the clinical remission and non-clinical remission groups [190]. In addition, high expression of Hotair has been demonstrated in blood mononuclear cells and serum EVs of RA patients whereas a lower level of Hotair was detected in differentiated osteoclasts and rheumatoid synoviocytes [191]. Platelet EVs are also elevated in RA and other inflammatory arthritis synovial fluid compared with OA and may play a role in the amplification of the inflammatory process. In this respect, collagen receptor glycoprotein VI has been identified as a key trigger for platelet EV generation in arthritis [192].

Synovial EVs contain citrullinated proteins, which are known autoantigens and biomarkers in RA [193]. In synovial fluid from RA patients, the number of microvesicles positive for receptor activator of NF-κB and its ligand are increased as well as CD3+ and CD8+ microvesicles which might reflect a locally enhanced activation of CD8+ T cells [21]. In addition, CD4+ T-cell-derived CD161+CD39+ and CD39+CD73+ EVs in synovial fluid have been recently proposed as reciprocal biomarkers for RA [194].

Differences in miRNA expression in EVs may lead to propose new biomarkers in joint conditions [174,195]. In the last years many studies have focused on circulating miRNAs as

biomarkers of disease which represent an important part of EV composition (reviewed in [175]). The changes in synovial fluid-derived EV miRNA with joint alterations provide a unique opportunity to discover candidate biomarkers. Interestingly, studies of miRNA expression in synovial fluid EVs from OA patients have shown sex specific changes. Therefore, in female patients, miR16-2-3p was upregulated and miR26a-5p, miR146a-5p and miR-6821-5p were downregulated while in male patients, miR-6878-3p was downregulated and miR-210-5p was upregulated. These results also suggested that estrogen might play an important role in EV derived miRNA [196]. Therefore, a gender dimension should be considered in the investigation of specific biomarkers for joint conditions.

7. Challenges in EV research

Several nomenclature and methodological challenges have raised concerns among the community about the reproducibility and comparability of the different reports published in recent years. In particular, the disparity of isolation and characterization approaches, and the lack of unified nomenclature and handling criteria are hindering the understanding of EVs biological functions [18]. In that regard, organizations such as the International Society for Extracellular Vesicles (ISEV) have published guidelines in an increasing effort to integrate the currently accepted isolation and characterization methods [18,197].

Vesicles shed from the cell plasma membrane are often called microvesicles, microparticles or ectosomes, with a sized ranged between 50-100 nm to even few micrometers depending on the author's criteria and the isolation method. Small vesicles secreted from multivesicular endosomal bodies are usually called exosomes and classically considered to be

under 150 nm in diameter, but most common isolation procedures based on the use of 200-nm pore filters and ultracentrifugation, isolate mixed EV populations. In practice, EV classification is not clear-cut as microparticles exhibit overlapping similarities in size, morphology, density and protein markers of both endosome and plasma membrane [16] and even the presence of different subpopulations within the same EVs class has been demonstrated [17,22]. In addition, a single cell can release EVs with differences in size, biogenesis and content which can vary depending on the cell type and its physiologic state [198].

From upstream sample handling to isolation and characterization, there is presently no single standardized method to universally obtain pure EV products. Generally, a highly pure EV isolate is obtained at the expense of therapeutic potency, yield, cost and/or scalability. These considerations are of critical importance when dealing with EVs as therapeutic agents, as industrial scale production must deliver an acceptable compromise between purity, activity and cost [199]. Currently, characterization efforts have focused on physical properties such as size and concentration, and vesicular content in terms of protein, lipid and nucleic acid composition. As the smallest EVs reach sizes of 50 nm or even less, current size analysis methods struggle to reach this detection limit, making comparisons of different concentrations difficult and statistically compromised.

Vesicular cargo includes proteins, RNA, DNA, lipids and metabolites, and may be inside EVs or on their surface. Subvesicular localization must be considered during characterization procedures to avoid artefacts and false positives. Importantly, the isolation method severely impacts the purity of EVs and therefore the omics profiles [200] and possible EV applications. However, as EV isolates contain disparate populations, current data should be considered as an average of the RNA content of all EV subpopulations. Additionally, most biofluids contain

potential contaminants such as RNA- and miRNA-carrying proteins, making analyses difficult to decipher even after treatment with RNAses [201].

It is crucial to improve the methods to isolate and characterize the different EV types. This issue is a source of confusion leading to contradictory results but it is also the first necessary step for studies of pharmacological activity and therapeutic efficacy. As many factors can influence the reproducibility of effects, different steps need to be taken to assure homogenous EV preparations and guarantee their efficacy and safety. In the last years, the International Society for Extracellular Vesicles (ISEV) has released position papers and the Minimal Information for Studies on EVs (MISEV) to help researchers overcome these problems. Furthermore, to increase reproducibility and transparency of EV methodologies, the EV-TRACK knowledgebase has been recently developed [202]. Strict standardized protocols must be implemented to effectively control all aspects of EV production and application, from culture of source cells to medicinal product preparation and administration. Qualitative and quantitative EV technologies need to be thoroughly validated. New technologies may help to advance this research field. For instance, EV uptake can be determined at single cell level using the Cre reporter methodology or bioluminescence methods can be employed to determine EV release and uptake and new-omics approaches have been incorporated to improve the knowledge of molecular EV components [30]. In addition, there is a need for normalization and control in sample collection and methods for keeping and transporting EV samples. All these points are essential to detect relevant differences between health and disease in clinical studies. It is not surprising that results found in the literature showed EV clinical studies of small populations with small portion of large effect size. Improved methodologies and study design are needed including larger numbers of samples in order to determine whether there is an effect at the population level [203].

Human EV-based therapeutics is subjected to the regulatory frameworks of biological medicinal products covering preclinical development, quality aspects, non-clinical safety requirements and the clinical testing. In the context of EV-related therapies and their approval, a complete *in vitro* and *in vivo* testing must be outlined. This should at least include assays to identify and characterize the components of the EV isolate (molecular fingerprinting), potency assays to quantify the EV-mediated therapeutic effect, and functional tests to determine their mechanisms of action as well as pharmacokinetic and toxicology studies. In the particular case of EV research, issues such as localization of molecules —inside the vesicle, embedded in the membrane, or associated outside— and mechanisms of cell-EV interaction —mainly vesicle internalization or plasma membrane receptor signaling— must be carefully taken into consideration for a thorough pharmacological validation [199].

For clinical application, compliance with safety standards related to inadvertent microbial and viral contamination and GxP standards (Good Manufacturing/Good Laboratory/Good Distribution/Good Clinical/Good Scientific Practice or GMP/GLP/GDP/GCP/GSP) is necessary for the production and quality control [1]. As a further step, one important hurdle is the ability to produce consistent products on a large scale. There is a need of developing clinical-grade robust and stable manufacturing processes.

8. Perspectives

Interest in cell-derived EVs has exponentially increased due to their proposed contribution to homeostasis and disease, and their potential as future therapeutic and diagnostic tools. In particular, EVs have recently received a great deal of attention as a possible better alternative to

MSC therapy in autoimmune conditions and tissue regeneration. The induction of immunological reset by MSC EVs has become an attractive possibility in RA and other autoimmune conditions. while the use of EVs for joint repair and OA could potentially be a better cost-effective therapy compared with MSC administration [160].

EVs offer the possibility to develop cell-free therapeutic approaches with less regulatory obstacles and clinical risks associated to cell therapies. Besides, they may have potential advantages in biomanufacturing, storing and distribution and may represent a more reproducible therapeutic tool [90,94]. EVs contain many biomolecules from the parent cells and can have advantages compared with cell therapy, as injected cells may die or fail to fully home into the lesion while EVs injection allows for a more precise dosing schedule and a better control of treatment or suspension of administration. The use of EVs may also eliminate problems such as blood vessels occlusion and generation of altered cell phenotypes [160,204].

Compared with MSCs, which produce different molecules according to the microenvironment leading to complex interactions or can exhibit opposite effects depending on the stimulus used to trigger immune cells [205], EVs may lead to results less dependent on the environment and more predictable. The content of these microparticles is protected from enzyme degradation, and this natural mechanism can be used to deliver active molecules to cells. In this respect, EVs are less likely to alter target cells than artificial nanoparticles. The small size may be an advantage in relation with the selection of administration routes in comparison with cell therapy. In addition, their bi-lipid layer vesicular structure is membrane permeable and their surface proteins may confer targeting ability due to their affinity for specific cell membranes or extracellular matrix in diseased tissues [79,157,206].

EVs have a lower immunogenic potential compared with cells [204] and thus allogeneic EVs have been reported to be safe and may be an appropriate source for large-scale production

[160] in preclinical studies and clinical applications. In this respect, technological advances can improve large-scale preparation of EVs. For instance, recent studies have demonstrated that microvesicle production by MSCs can be amplified using a 3-D bioprocessing method keeping the biological activity of these microparticles [207].

Modification of EVs may improve their properties to regulate different processes. Therefore, the parent cells could be primed or genetically modified and then expanded in order to produce modified EVs e.g. without histocompatibility antigens to minimize the possibility of immune reactions, expressing relevant proteins, lipids or RNA to maximize the pharmacological effects, or molecules that facilitate their tropism and retention in damaged tissues or recognition by target cells thus improving treatment selectivity. In addition, different approaches e.g. integration in a hydrogel-scaffold or chondroitin sulfate sponge are in development to facilitate stable long-term delivery to joint tissues [174].

On the other hand, the determination of EVs can be useful as biomarkers of joint diseases as the content of microparticles is related to the parent cell and its microenvironment. In this respect, miRNA and proteome analyses represent promising approaches.

The standardization of isolation and characterization methods is crucial for the development of this novel tool. It is apparent that much work both *in vitro* and *in vivo* is needed in order to better understand the biogenesis, composition, appropriate delivery technique, *in vivo* stability and distribution, internalization, mechanisms of action, efficacy, long-term actions and safety of EVs.

Although we only focus on limited aspects of EVs, there are new mechanisms to be identified which may lead to other potential applications of these microparticles. Taken as a whole, the studies outlined in this review reinforce the increasing interest in the field and the efforts devoted to understand EV biology. Nevertheless, the complexity of the topic has raised a

number of important questions which need to be answered before this novel approach can progress to clinical applications in joint conditions.

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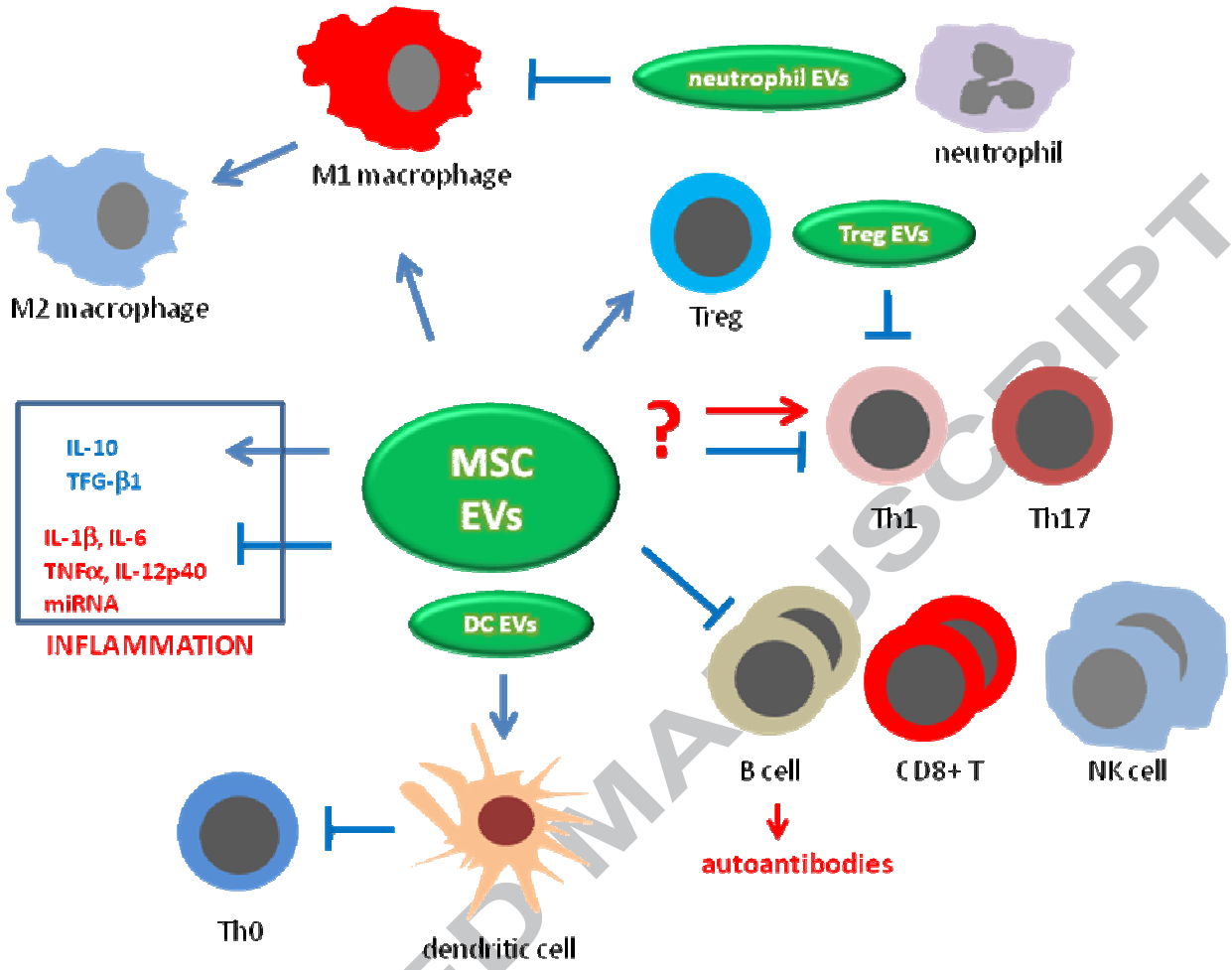
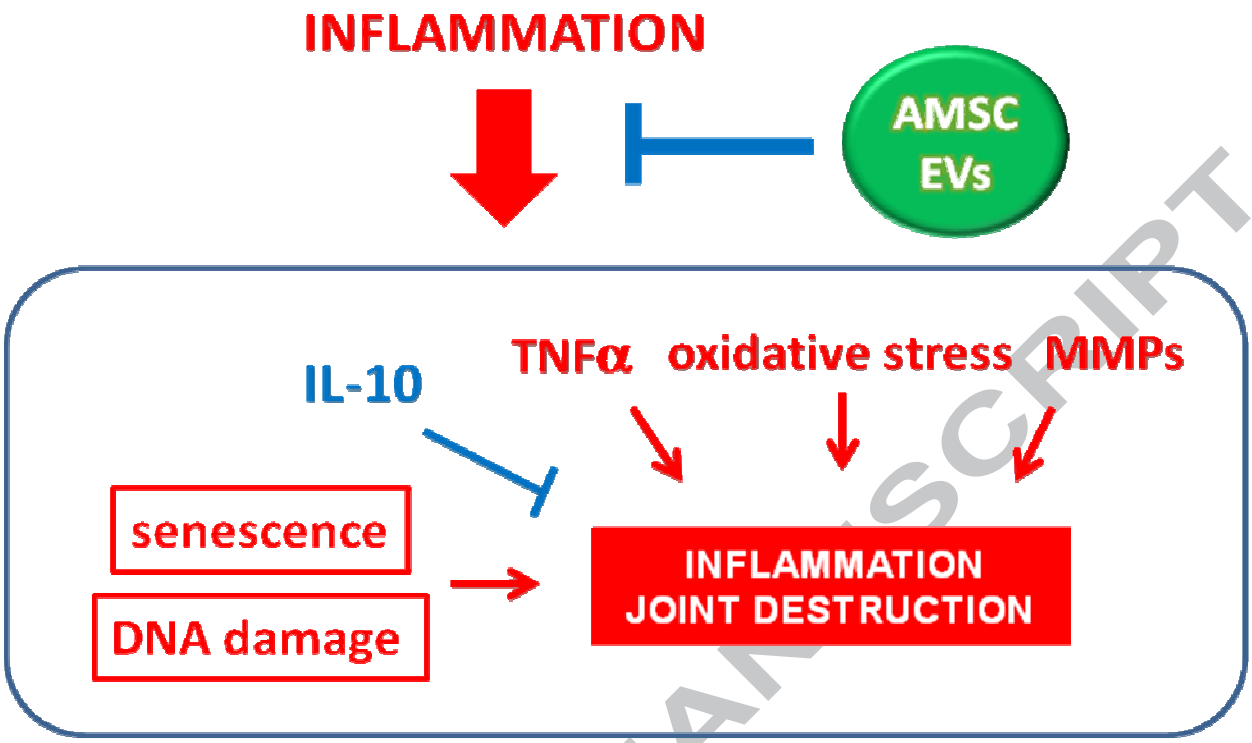
LEGENDS TO FIGURES

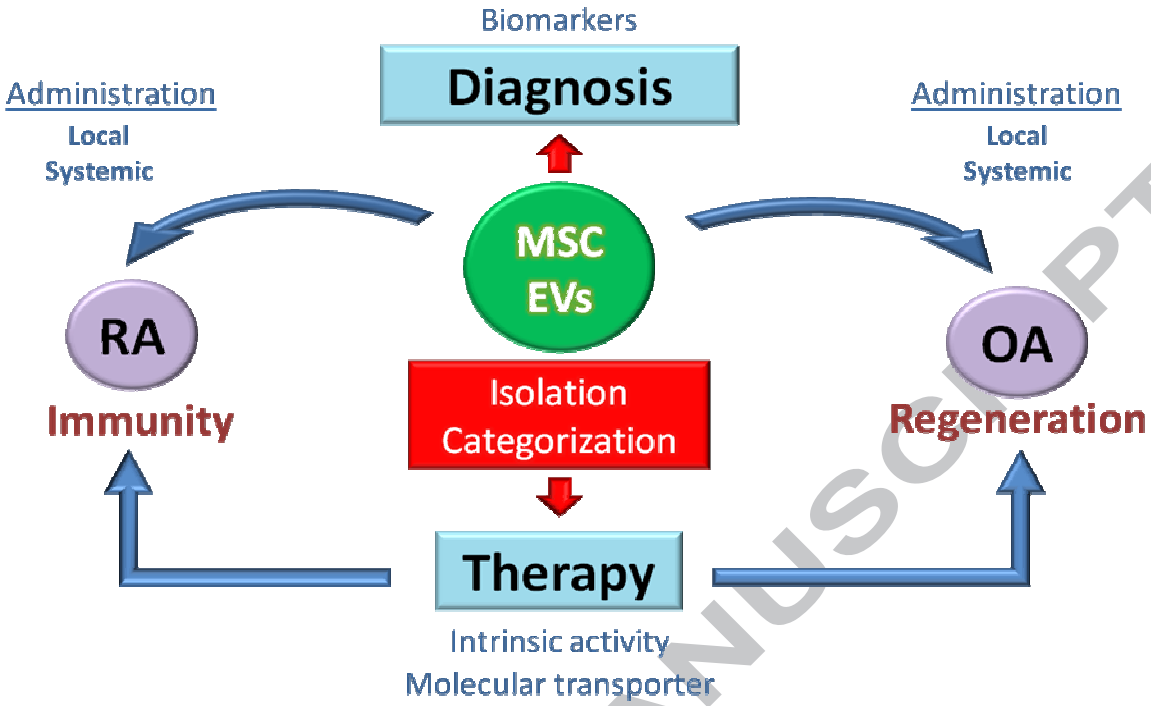
Figure 1. Immunosuppressive effects of EVs from MSCs and other cell types. EVs from MSCs reduce the proliferation and differentiation of CD8+ T cells, B cells and NK cells while favor the differentiation of Treg cells and the switch of pro-inflammatory monocytes and macrophages (M1) to an anti-inflammatory phenotype (M2). The effect on CD4+ T cells has not been clearly demonstrated. Neutrophil EVs exhibit anti-inflammatory actions on macrophages. EVs from Treg cells inhibit Th1 cell proliferation. DC EVs can interact with T cells and APCs to alter their function. In inflammatory conditions, EVs inhibit the production of pro-inflammatory mediators and enhance that of anti-inflammatory and pro-resolution mediators in different cell types.

Figure 2. Joint protective effects of AMSC EVs. In OA osteoblasts or chondrocytes subjected to inflammatory conditions, EVs reduce the production of pro-inflammatory and catabolic mediators as well as the induction of DNA damage and cell senescence while the production of the anti-inflammatory cytokine IL-10 is enhanced.





Graphical abstract



Method	Principle	Scalability	Advantages	Challenges
Precipitation	Precipitant agent	Yes	Fast	Low purity
			Simple	Protein contaminations
				Precipitant interferences
Differential ultracentrifugation	Size	No	Common	EV aggregation
			EV subfractioning	Possible loss of function
Density gradient ultracentrifugation	Density	No	Common	Gradients may interfere with EVs' activity
			EV subfractioning	Possible loss of function
			High purity	Time consuming
Ultrafiltration	Size	Yes	High concentration	Aggressive
				Bias towards pressure-resisting EVs
Size exclusion chromatography	Size	Yes	High purity	Low yield
			Removal of soluble proteins	Need of further concentration steps
Immunoaffinity	EV phenotype	No	Fast	Low yield
			High purity	Expensive
				Bias towards known markers-containing EVs

Table 1. Best established methods for the isolation of EVs [12-18].