Enhancing Extracellular Vesicles for Therapeutic Treatment of Arthritic Joints.

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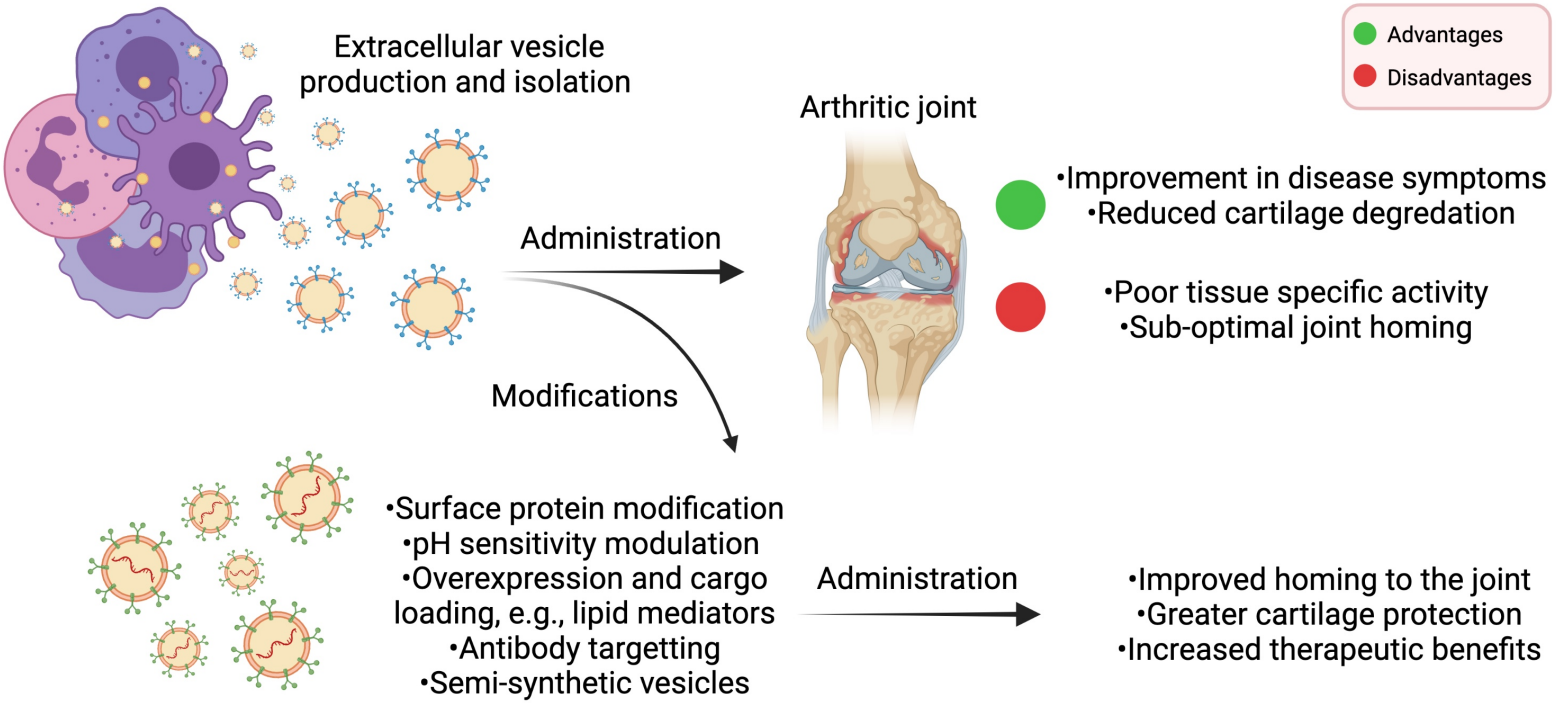
Abstract

Extracellular vesicles are small membrane-derived packages of information that are released from virtually all cell types. These nano-packages contain regulatory material including proteins, lipids, mRNA and microRNA and are a key mechanism of paracellular communication within a given microenvironment. Encompassed with a lipid bilayer, these organelles have been attributed numerous roles in regulating both physiological and pathological functions. Herein, we describe the role of EVs in the context of Rheumatoid and Osteoarthritis and explore how they could be harnessed to treat inflammatory and degenerative joint conditions. These structures offer a promising therapeutic strategy for treating musculoskeletal diseases due to their bioactive content, stability, small size and intrinsic ability to enter the avascular cartilage, a notoriously challenging tissue to target. We also discuss how EVs can be manipulated to load therapeutic cargo or present additional targeting moieties to enhance their beneficial actions and tissue regenerative properties.


Extracellular vesicle production and isolation
Administration
Modifications
Arthritic joint
Advantages
Disadvantages
•Improvement in disease symptoms
•Reduced cartilage degredation
•Poor tissue specific activity
•Sub-optimal joint homing
•Surface protein modification
•pH sensitivity modulation
•Overexpression and cargo loading, e.g., lipid mediators
•Antibody targetting
•Semi-synthetic vesicles
Administration
•Improved homing to the joint
•Greater cartilage protection
•Increased therapeutic benefits

Highlights:

- In RA and OA there is an unmet clinical need for improved therapeutics
- MSC, DC and PMN-EVs display cartilage protective and tissue regenerative properties
- EVs can be manipulated to overexpress miRNAs, cytokines or enzymes
- EVs contain natural targeting moieties and can be modified for enhanced homing
- EVs offer a stealth paracellular mechanism for delivering therapeutic cargo

Introduction

Musculoskeletal conditions are the number one cause of disability worldwide, affecting more than 10 million people in the UK alone and more than a billion people globally. Rheumatoid arthritis (RA) is the most common inflammatory arthropathy and represents a major socioeconomic and healthcare burden. Moreover, the quality of life for people with arthritis is dramatically reduced. Osteoarthritis (OA) is a highly prevalent degenerative condition affecting the joints and that remains incurable (Hunter & Bierma-Zeinstra, 2019). Interventions to alleviate symptoms are currently utilised which include changes in lifestyle as well as pharmacological and surgical strategies (Kloppenburg & Berenbaum, 2020; Onishi et al., 2012). Therefore, there is an essential need to develop new and more effective treatments for these conditions.

Synovial Joint Biology

In order to effectively target arthritic joints, we must first consider the architecture of the synovial joint and the difficulties that arise when targeting therapeutics to this anatomical location. Synovial joints are load-bearing joints, found between opposing bone surfaces. Their architecture prevents friction and wear whilst facilitating movement. These joints consist of a thin fibrous membrane called the synovium which surrounds the joint, forming a cavity. The synovium is composed of two functionally distinct layers, the intimal lining layer and the sub-lining layer. The latter is the outer-most layer of the synovial membrane containing extracellular matrix (ECM) proteins such as collagen and a loose connection of cells. Whilst the intima lining layer is a thin superficial inner-facing layer, composed of a ratio of type A cells: macrophage-like synovial cells and type B cells; fibroblast-like synoviocytes (FLS) (Bartok & Firestein, 2010). Inside, the joint is bathed in synovial fluid, a rich, viscous lubricating fluid requiring the presence of hyaluronan and lubricin (Jay et al., 2000). Within the synovial cavity is a layer of articular cartilage that covers the epiphyseal ends of bone. Articular cartilage is a dense ECM, imperative for mechanical load bearing and friction resistance. The most abundant macromolecule found in the cartilage is type II collagen, water and a mix of proteoglycans including hyaluronan, aggrecan and decorin. The chondrocyte is the only cell type found within the cartilage under normal conditions and maintains the cartilage by synthesising and secreting the aforementioned matrix proteins that give the cartilage its shock-absorbing and gliding properties. Chondrocytes in healthy cartilage do not normally replicate but maintain the ECM by low turnover replacement of specific matrix proteins. However, damage caused by different forms of arthritis or joint trauma, coupled with the limited healing capacity of cartilage, makes this tissue a challenge to repair and even more difficult to regenerate.

Osteoarthritis

OA is a disease associated with aging, during which gross changes occur to the joint architecture as a result of cartilage degradation, subchondral bone thickening, osteophyte formation and joint hypertrophy. Ultimately, these changes lead to significant pain and reduced mobility, decreasing the patient's quality of life (Hunter et al., 2013). Past joint trauma is one of the *key* aetiological factors driving disease, increasing OA risk by as much as four times. Indeed, up to half of individuals that had a previous traumatic knee injury presented radiographic signs of OA many years on (Roos, 2005). Inflammation as a result of traumatic joint injury, in combination with the low-grade chronic inflammation that exists during ageing, are thought to be responsible for the inflammatory processes carried out during OA progression (Chen et al., 2017).

Cartilage matrix molecules, such as fibronectin and hyaluronic acid, are released into the synovium following joint injury; these have been found at elevated levels in synovial fluid during OA in both humans and mice (Belcher et al., 1997; Lasarte et al., 2007; Scheibner et al., 2006). These matrix components are potent damage associated molecular patterns (DAMPs) which can efficiently activate toll-like receptors (TLR), such as TLR-2 and 4. The expression of these TLRs are reportedly increased on chondrocytes within lesions in the OA joint (H. A. Kim et al., 2006). In addition, other damage released matrix components such as fibromodulin and osteoadherin, also results in the deposition and activation of complement components within the joint (Sjöberg et al., 2005; Sjöberg et al., 2009). Activation of complement and TLRs by matrix components can efficiently induce pro-inflammatory signalling and phenotype acquisition (Barreto et al., 2017; Wang et al., 2020). Damage associated pro-inflammatory signalling within the joint initiates the aberrant expression of matrix degrading enzymes like matrix metalloproteinases (MMPs) and aggrecanases (Troeberg & Nagase, 2012). Elevations in synovial MMP-3 and -13 have been shown to directly increase OA cartilage degradation (Li et al., 2017). The resultant inflammatory milieu within the OA joint, amplifies further pro-inflammatory signalling and immune cell recruitment in a self-perpetuating cycle (Scanzello & Goldring, 2012).

Inflammatory cytokines such as interleukin (IL)-1β and tumour necrosis factor (TNF)α have established roles in synovitis and damage during OA (Wojdasiewicz et al., 2014). In the context of the joint, IL-1β production can be stimulated by mineral crystals activating the NLR family pyrin domain containing 3 (NLRP3) inflammasome in synoviocytes. The increased production and presence of calcium phosphate and hydroxyapatite crystals can be found in articular cartilage during OA and it is thought these are involved in cartilage mineralisation in addition to driving inflammatory processes (Corr et al., 2017).

T cells represent one of the major recruited immune cell types during OA. γ-chain cytokines such as IL-2 and IL-15 are shown to be elevated in the OA joint and facilitate the contribution of T cell joint damage by promoting infiltration and survival (Warner et al., 2020). In more recent years, the molecular pathogenesis of OA has been shown to be heavily correlated with the production and activity of growth factors. Growth factors such as Wingless-related integration site (Wnt3a); the hedgehog family of proteins and transforming growth factor beta (TGF-β) are traditionally involved in repair pathways within the joint

(Huang et al., 2018). Under homeostatic conditions, TGF-β is produced in ample amounts by chondrocytes and has been found to be stored in large reserves within the ECM. Following OA pathogenesis and importantly cartilage degradation, this TGF-β reserve has been shown to be heavily depleted and the dysregulation of TGF-β homeostasis, alongside other growth factors, denotes a disturbance in an important regulatory mechanism which contributes to joint hypertrophy in OA (Tang et al., 2018; Zhao et al., 2016).

Rheumatoid Arthritis

RA is a highly heterogenous, chronic autoimmune condition that can have debilitating effects on the joints with lasting deformity. It affects approximately 0.5 – 1.0 % of individuals in the western world and is most common in people aged 45 years and over (Smolen et al., 2018). RA is associated with a number of co-morbidities including atherosclerosis, cerebrovascular disease risk and osteoporosis (van Onna & Boonen, 2016). RA can affect any joint with synovial tissue: knees, shoulders, elbow, ankles and hips. Symptoms of RA may progress slowly, come on in waves, or be severe and unrelenting. In the latter case, the disease is associated with systemic inflammation and the appearance of nodules (Smolen et al., 2018). The clinical presentation of RA is chiefly chronic pain, typically around the small joints of the hands and feet, which become swollen and tender; patients characteristically display stiffness in joints which tends to be worse in the morning. In aggressive, longstanding or poorly-managed disease, deformities can occur resulting in a fixed loss of extension in some joints, limited movement or ligament rupture (Chinchalkar & Pitts, 2006).

Unfortunately, the aetiology of RA remains largely elusive, although it is thought to be heterogenous in nature, involving a number of initiating events which may indeed determine the success of different therapeutics. Genetic associations, especially within MHC alleles have been linked to susceptibility in RA. Environmental factors such as smoking, obesity and alcohol consumption have been shown to play a role in its development and are often interlinked; risk factors include exposure to silica (Stolt et al., 2005), oral and gut microbiota (Hajishengallis, 2015; Konig et al., 2016) obesity (Ljung & Rantapää-Dahlqvist, 2016), post-traumatic stress disorder in females (Lee et al., 2016) and low socioeconomic status (Camacho et al., 2012), .

The pathogenesis of RA is largely due to a dysregulated adaptive immunity and a breakdown in self-tolerance involving many different cell types (Humby et al., 2019). Immune cell activation and interaction with the synovium promotes the transition of a quiescent, acellular membranous lining into an active and invasive tissue. Innate and adaptive immune cells migrate into the normally immune cell-devoid synovial cavity and release inflammatory mediators. Neutrophils and monocytes play an important role in the effector stage of RA through the chronic production of cytokines such as TNF-α and IL-6 (McInnes & Schett, 2017). B cells contribute to the pathogenesis through the production of autoantibodies including rheumatoid factor and anti-citrullinated protein antibodies, alongside anti-collagen antibodies (Mewar & Wilson, 2006). Approximately 50 – 80 % of all RA patients present with

autoantibodies, whilst the remaining RA patients are deemed seronegative. Activation from immune complexes via Fc-γ receptors (McInnes & Schett, 2017), as well as by mast cells (tryptase) and neutrophils (trypsin), can induce cytokine production in macrophage-like synovial cells (McInnes & Schett, 2017). Chronic and sustained inflammation of the joint leads to increased vascularity, encouraging the recruitment of further immune cells. The resultant synovitis permanently alters the phenotype of synoviocytes triggering an invasive and aggressive phenotype in FLS which become proinflammatory and invasive, culminating in ECM degradation (D'Aura Swanson et al., 2009). FLS are key for the local production of cytokines, proteolytic enzymes and inflammatory mediators, which actively degrade the ECM and promote the activity of other cell types (Bartok & Firestein, 2010). FLS are the primary cell type responsible for cartilage destruction due to their production of copious degradative enzymes.

The intima lining layer of the synovial membrane increases significantly in cellularity in RA due to proliferation of both macrophage-like-synovial cells and FLS (Bartok & Firestein, 2010). Consequently, a structure known as a pannus forms at the interface of cartilage, eroding and degrading cartilage and bone, much like an invasive tumour. This structure is formed of macrophages, invasive FLS and osteoclasts. Osteoclasts can become activated by many cytokines present in the rheumatic milieu including macrophage colony stimulating factor, TNF-α and receptor activator of nuclear factor κ-B ligand (RANK-L) and are the primary effectors of bone erosion (Bartok & Firestein, 2010; Pap T & S, 2009).

Extracellular vesicles

EVs are a key mechanism of mechanism of paracellular communication within the body and have been attributed a multitude of roles in both homeostatic and disease states (Deatherage & Cookson, 2012; Hyenne et al., 2019; van der Pol et al., 2012). They have a multitude of reported cellular effects and can be classified into three main sub-groups based on their biogenesis: microvesicles (MVs), exosomes and apoptotic bodies (Beaudoin & Grondin, 1991; van der Pol et al., 2012). New EV subtypes are still being described, for example a class of EVs formed from mitochondria termed 'MitoEVs', highlights this diverse and everchanging field (D'Acunzo et al., 2021). Exosomes are the smallest of the EVs, which range in diameter from 20 – 100 nm. Exosomes are formed inside of the cell as part of the endosomal sorting network; they encompass cytosolic products and are released from the plasma membrane as intact vesicles (van der Pol et al., 2012). Microvesicles (MV) are larger than exosomes and can be between 100 – 1000 nm in diameter, they are generated by outward budding at the cell surface membrane (van der Pol et al., 2012). Apoptotic bodies are much larger than both exosomes and MVs and arise as a response to programmed cell death cues that prevent intracellular contents from releasing pro-inflammatory mediators into the extracellular space (Figure 1.) (Elmore, 2007).

EVs are now recognised for being largely heterogenous, mainly due to their differing origins of biogenesis, parent cell derivation and internal and surface contents, such as an

assortment of proteins, lipids and RNAs (Dalli et al., 2013; Mack et al., 2000; Ng et al., 2013). This diversity is due to the type of cell the EV is derived from, its activation state and microenvironment (Dalli et al., 2013; Peterson et al., 2008). Additionally, studies have shown single stranded and double stranded DNA can be found within EVs (Balaj et al., 2011; Guescini et al., 2010; Thakur et al., 2014). Classical markers of EVs include the tetraspanins, a family of proteins involved in microdomain formation, including CD63**,** CD81, CD82 and CD9 (Buschow et al., 2009; Chairoungdua et al., 2010; Charrin et al., 2014), as well as other components of endosomal sorting and transport; syntenin-1 and TSG101 (Kowal et al., 2016). EVs also present markers that indicate their cell of origin. EVs are a vehicle for the paracellular transport of cargoes, but also facilitate the transportation of membrane-bound cargoes such as antigen presentation complexes and receptors (Li et al., 2013; Rauschenberger et al., 2016). EVs have been shown to display protein, lipid and microRNA (miRNA/miR) profiles that are distinct from their parent cell, suggesting that EVs do not just merely represent their cell of origin, but are in fact actively packaged, distinct organelles (Collino et al., 2010; Diehl et al., 2012).

Figure 1. Extracellular Vesicle family biogenesis

The EV family is composed of 3 main members: exosomes, MVs and apoptotic bodies. Exosomes are generated intracellularly as part of the cells recycling network and are formed by inward budding of multi-vesicular bodies and are then released from the plasma membrane by exocytosis. MVs are generated at the plasma membrane and apoptotic bodies arise due to cues for programmed cell death and membrane budding that occurs in apoptosis to encapsulate potentially damaging intracellular material.

Immunomodulatory potential of EVs in Arthritis

Osteoarthritis

The use of naturally occurring EVs represents a promising therapeutic strategy for the treatment of arthropathies, including osteoarthritis (Duan et al., 2020). This potential is highlighted by the use of exosomes from healthy articular chondrocytes to modify the activity of OA chondrocytes. Histone deacetylase 2/8 (HDAC2/8) is expressed at higher levels within the OA joint and specifically in the exosomes of OA chondrocytes. Increased expression of HDAC2/8 can induce cartilage degradation and suppress ECM production (Culley et al., 2013). In this way, it is thought that pathogenicity can be spread via exosomes from diseased chondrocytes to healthy chondrocytes of the same joint. MiR-95-5p can inhibit the expression of HDAC2/8 and in chondrocytes under physiological conditions, miR-95-5p expression is higher than during OA, as well as in derived exosomes. To this end, Mao *et al*. investigated the therapeutic benefit of treating OA chondrocytes with EVs isolated from healthy chondrocytes and in doing so showed their efficacy as promoters of anabolic gene expression through the direct inhibition of HDAC2/8 activity (Guping Mao et al., 2018).

Autologous blood has been explored recently as a potential therapeutic for the treatment of OA, which includes the use of citrate anti-coagulated platelet rich plasma (CPRP) or hyper acute serum (hypACT). Researchers have shown that both CPRP and hypACT can induce chondrogenesis *in vitro* and this is specifically mediated by their EV contents (Otahal et al., 2020). Interestingly, EVs from CPRP and hypACT could efficiently induce the expression of anabolic proteins such as SOX-9, COL2A and aggrecan.

Currently, several groups are investigating various drug delivery systems utilising synthetic nanoparticles as a means of achieving sustained long-term drug release, increased joint specificity and protection from environmental destruction (Mehta 2021). Intra-articular administration of hydrogel containing micelle-encapsulated eicosapentaenoic acid suppress glycosaminoglycan loss and reduce inflammation within the OA joint (Tsubosaka et al., 2020). Similar reductions in inflammation were achieved by intra-articular administration of liposome-encapsulated gold nanoparticles conjugated with proteins extracted from fish oil glands (Sarkar et al., 2019). Intra-articularly administered liposomes containing drugs such as dexamethasone and triamcinolone acetonide are currently in clinical trials at various stages, however, a previously completed phase II clinical trial showed efficacy for nanoparticle delivered dexamethasone to the OA knee (Hunter et al., 2019).

More sophisticated particle-based vehicles have been developed that release their therapeutically beneficial cargo in response to changes in pH, temperature, or treatment with near infrared radiation (Mehta et al., 2021). Zerrillo *et al.,* demonstrated that pH sensitive ammonium bicarbonate encapsulated poly(lactic-co-glycolic acid) nanoparticles could efficiently and systematically release their pro-reparative cargo - hyaluronic acid in damaged regions of the OA joint, due to altered pH kinetics in the enzymatically-rich diseased joint. Interestingly, they were able to show that using a combination of pH responsive and non-responsive particles they could provide an initial burst of therapeutic (pH responsive particles) which was then followed by a sustained/slow drug release from unresponsive particles. The authors found that by taking advantage of the pH dynamics, they could attenuate cartilage destruction in a murine model of OA (Zerrillo et al., 2019). Alternatively, Chen *et al.,* generated a novel nanogenerator, which combines photothermal haemoglobin with an anti-OA therapeutic such as nitric oxide (NO) or a notch1-siRNA, encapsulated within a nanoparticle. Following intra-articular administration of these nanoparticles, treatment with near-infrared light allowed the controlled release of therapeutic agents into the joint and in doing so, the authors were able to reliably and sustainably prevent local inflammation and cartilage erosion (Chen et al., 2019). Unfortunately, such systems remain limited in their translatability as preparation processes are lengthy and the particle sizes remain too large for effective cartilage penetration, limiting their use to control inflammation and pain. Interestingly, it was found that artificial particles have a size requirement of less than 10 nm in order to effectively penetrate the joint (Mehta et al., 2021). Our group has previously shown that neutrophil EVs ~100 nm in diameter, are able to access the cartilage to a depth greater than 100 µm, where they deliver their anti-inflammatory payloads, as opposed to ~70 µm with artificial particles (Mehta et al., 2021; Louise M. Topping et al., 2020). The evidence thus far, suggests that a

greater understanding of naturally and physiologically occurring EVs and their integration with existing drug delivery modalities can offer greater therapeutic effects.

Inflammatory Arthritis

Within the context of inflammatory arthritis, several useful effects of different subsets of EVs have been described. For example, bovine milk derived EVs administered orally by gavage or in drinking water, were found to not only delay the onset of arthritis in the collagen-induced arthritis (CIA) model but also reduced cartilage damage and bone marrow inflammation. Serum levels of the cytokines MCP-1 and IL-6 were lower and reductions in anti-collagen IgG2a levels were observed (Arntz et al., 2015). These exciting discoveries are yet to be tested in RA patients but are promising due to the easily accessible nature of cow's milk.

The therapeutic potential of neutrophil-EVs was first shown in 2004 by Gasser and Schifferli, whereby incubation of macrophages with neutrophil-EVs did not induce proinflammatory cytokine release, but instead induced the production of TGF-β1 and dampened proinflammatory responses to zymosan and LPS (Gasser & Schifferli, 2004). Subsequently, our group found these EVs contained the pro-resolving mediator Annexin-1 (AnxA1) (Dalli et al., 2008). Neutrophil-EVs exhibit many beneficial properties including the production of specialised pro-resolving mediators (SPMs) in macrophages: RvD1, PGE2, MaR1 and $PGF_{2\alpha}$ which hampered the classical activation of macrophages. This process was found to require AnxA1 as well as PtdSer expression (Dalli & Serhan, 2012). These neutrophil-EVs were also found to alter FLS phenotype. FLS co-cultured with classically activated macrophages exhibited upregulated expression of TNF-α, CD55, IL-6, VCAM-1 and MCP-1, compared to FLS exposed to naïve macrophages. Macrophages activated with LPS and IFN-y in the presence of neutrophil-EVs were unable to increase the expression of these markers in FLS (Rhys et al., 2018).

Given the extensive proposed anti-inflammatory properties of neutrophil-EVs and that perturbations to EV production using the $Tmem16f^{-/-}$ mouse, impaired cartilage integrity in inflammatory arthritis, our group sought to explore the effects of neutrophil-EVs in the K/BxN serum transfer model of inflammatory arthritis. Here administration of neutrophil-EVs protected against cartilage degradation and neutrophil-EVs but not synthetic microcapsules or $CD14^+$ monocyte-EVs, were able to penetrate the cartilage and co-localise with the lacunae where they exerted their chondroprotective effects through the promotion of pro-anabolic genes. This protection again required AnxA1 and FPR2/ALX receptor mediated interactions leading to TGF-β activation (Headland et al., 2015). These findings were extremely novel, given that the cartilage is impenetrable to cells. Other immune cell-derived EVs such as primary antigen presenting cells - dendritic cells (DC)-EVs, have also been shown to have inherent capacity to modulate immune responses such as by altering T cell immunity through enhancing $CD4^+CD25^+$ Treg activity (Yang et al., 2011).

MSCs derived EVs

Alongside the more conventional cell types present in the joint niche are resident pools of mesenchymal stem/stromal cells (MSCs). This cell type is present in almost all organs and tissues and is endowed with multipotent and regenerative properties. As such, the beneficial properties of MSCs have been directed at clinically regenerating and protecting tissues during inflammation (Regmi 2019) and injury (Pittenger et al., 2002; Spees et al., 2016). Great efforts have been made to optimise the tissue specific homing of MSCs, albeit with limited success. In more recent years, MSC-EVs have garnered significant interest as they are reported to closely mimic the anti-inflammatory effects observed by MSCs and therefore represent an efficacious cell-free therapy (Mianehsaz et al., 2019).

Zhang *et al.,* have shown that EVs from bone marrow MSCs have pro-resolving properties as they aided osteochondral regeneration following surgical injury at the trochlear groove, in the distal femurs of rats. MSC-EVs supported ECM deposition and the restoration of normative joint architecture without inducing adverse effects (Zhang et al., 2016). Intra-articular administration of bone marrow MSC-EVs following the sodium iodoacetate model of OA, led to an improvement in clinical scores, elevated type II collagen deposition and a reduction of MMP-13 within the cartilage (He et al., 2020). Cosenza *et al.,* reported similar beneficial effects of bone marrow MSC-EVs in a collagenase-induced model of OA in mice. The authors found that MSC-EVs induced type II collagen and aggrecan expression in OA-like chondrocytes while simultaneously supressing catabolic genes such as, A disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS5) and MMP-13 (Cosenza et al., 2017).

MSC-EVs derived from joint specific niches, can also facilitate the delivery of anti-inflammatory cargos to the joint (Mianehsaz et al., 2019). Exosomes from synovial membrane MSCs have been observed to transfer Wnt3a and Wnt5a to articular chondrocytes and promote their migration and proliferation via the alternative Wnt signalling pathway (S. C. Tao et al., 2017). Exosomes from MSCs found in the infrapatellar fat pad have also been shown to aid cartilage homeostasis during OA through inhibition of mechanistic target of rapamycin (mTOR) mediated autophagy due to miR-100-5p in chondrocytes. These MSC-EVs augmented chondrocyte proliferation and matrix synthesis and in doing so, protected against cartilage degradation in pre-clinical models of OA (Wu et al., 2019). Alternative studies have also shown that EVs from autologous blood products can induce chondrogenic differentiation in adipose MSCs (Neubauer et al., 2019). However, the clinical evidence for the efficacy of autologous blood products in the treatment of OA remains low and as such, its application is currently still under debate (Gato-Calvo et al., 2019).

T cells have been demonstrated to play a role in the pathogenesis of RA (Mellado et al., 2015). $CD4^+$ T cells infiltrate the synovium where they differentiate into different subsets depending on the dominance of transcription factors present in the synovium. A homeostatic environment favours the upregulation of fork head or winged helix transcription 3 (Foxp3) which encourages cell differentiation into regulatory T cells (Tregs) which produce anti-inflammatory mediators such as TGF-β, whereas an inflammatory environment upregulates

retinoic acid receptor-related orphan nuclear receptor-γt (RORγt), subsequently generating T helper (Th)17 cells which produce IL-17 with downstream pro-inflammatory effects (Ma et al., 2019). It is known that MSCs can regulate the survival and effector functions of T cells, for example reduce the production of pro-inflammatory factors. In a mouse CIA model the administration of MSCs suppressed the skewing of activated $CD4^+$ T cells into Th17 effector cells and increased the production of Tregs that produce IL-10 (Zhou et al., 2011). Recently, Ma and colleagues described the immunomodulatory effect of human umbilical cord MSC (hUCMSCs)-EVs in a CIA model in rats, which were shown to replicate many of the therapeutic effects observed by hUCMSCs. Both EVs and parent cell were responsible for: the promotion of T cell apoptosis, reduction in RORγt levels, increased Foxp3, regulating the balance and polarisation of Treg/Th17 cells, delayed radiological changes associated with RA and synovial hyperplasia (Ma et al., 2019). Similarly, the lateral transfer of Let-7d from Tregs to Th1 cells by exosomes has been shown to suppress Th1 proliferation and pro-inflammatory cytokine secretion (Okoye et al., 2014). $CD8^+$ $CD25^+$ Treg exosomes have also been shown to supress $CD8^+$ T cell effector functions and anti-tumour immunity (Xie et al., 2013). Furthermore, MSC-EVs stimulated the switch of Th1 into Th2 cells (Chen et al., 2016) and enhanced the expression of the immune checkpoint regulator, cytotoxic T lymphocyte-associated protein 4 (Chen et al., 2016).

Notably, in some publications, only MSC-exosomes enhanced the production of Tregs and reduced the presence of IFN-γ producing T cells (Ji et al., 2019). Cosenza and colleagues showed that whilst bone marrow MSC-EVs inhibited inflammatory T cell proliferation and promoted Treg differentiation in a dose-dependent manner, this effect was not recapitulated by the parent MSCs in murine models of RA. Interestingly, only exosomes increased Treg populations while parental MSCs and MVs did not (Cosenza et al., 2018). This data suggests once again the heterogeneity of different subsets of EVs, as well as the enrichment of EVs with different cargo when compared to the parent cells (Oggero et al., 2019). Interestingly, MSC-EVs were shown to polarise cells and generate anti-inflammatory effects, only when the immune system was activated, thus preventing the increased risk of infection seen with the use of immunosuppressive drugs in RA (B. Zhang et al., 2018). Moreover, the proliferation of B cells and natural killer (NK) cells was decreased by MSC-EVs (Budoni et al., 2013; Di Trapani et al., 2016).

Besides their role in modulating T cell proliferation and differentiation, MSC-EVs have been shown to reduce DC maturation by altering antigen acquisition dynamics (Reis et al., 2018). The authors showed that MSC-EVs reduced the expression of maturation and activation markers, decreased IL-6 and IL-12p70 release and thus, represented a switch from pro-inflammatory to an immunoregulatory phenotype, typified by the enhanced production of TGF-β. Additionally, MSC-EVs also reduced CCR7 expression on DC following LPS stimulation which translated to a marked inhibition of their chemotactic capabilities in response to CCL21. This suggests that MSC-EVs may limit T cell homing to secondary lymphoid organs. LPS preconditioning of MSCs has been shown to enhance their protective properties; the stimulation of hUCMSCs with LPS improved the anti-inflammatory potential of the

resultant EVs due to let-7b enrichment. The exosomal transfer of let-7b to recipient macrophages polarised cells towards an anti-inflammatory M2 phenotype (Ti et al., 2015).

During OA, it has been shown that autologous or allogenic MSCs can repair cartilage in *in vitro* experiments utilising co-cultures of MSCs and chondrocytes to improve MSC chondrogenesis (Cooke et al., 2011; de Windt et al., 2017). Similarly in RA, the role of MSC-EVs in modulating the inflammatory milieu and in promoting a regenerative microenvironment has recently been investigated by several groups (Kim et al., 2019). For example, intra-articular administration of human induced pluripotent stem cells or synovial MSC-EVs following the collagenase induced model of OA protected against cartilage degradation. *In vitro*, both sources of MSC-EVs boosted human chondrocyte proliferation and migration (Zhu et al., 2017). EVs from human MSCs can be internalised by OA synoviocytes or chondrocytes via the binding of surface molecules such as CD44 and can subsequently downregulate the secretion of inflammatory cytokines such as IL-6, CCL-2, CCL-5 and IL-1α, IL-1β, IL-8 and IL-17 (Ragni et al., 2019; Vonk et al., 2018). Moreover, TNF-α stimulated chondrocytes treated with MSC-EVs, decreased COX-2 expression, collagenase activity and promoted chondrocyte proliferation with no impact on apoptosis (Vonk et al., 2018). These anti-inflammatory and anabolic outcomes were dependent on the inhibition of NF-κB activation (Vonk et al., 2018). Others have shown the protective potential of human adipose tissue MSC-EVs in OA chondrocytes. These MSC-EVs were able to reduce the release of catabolic mediators while simultaneously inducing the increased secretion of IL-10 and type II collagen expression (Tofiño-Vian et al., 2018).

Human embryonic stem cells (hESCs) are another appropriate source of exosomes for therapeutic use. EVs from immortalised E1-MYC 16.3 hESCs were tested in a rat osteochondral defect model, whereby 1.5 mm by 1 mm holes were made in the trochlear grooves of distal femurs. Intra-articular injection with exosomes following surgery, on a weekly basis, promoted stable hyaline cartilage formation. 12 weeks after surgery the EV-treated group displayed full repair and a smooth surface with adjacent cartilage, compared with the untreated group which displayed insufficient neotissue growth and an irregular surface. This treatment regime also increased the infiltration of pro-resolving M2 macrophages while decreasing the levels of pro-inflammatory mediators within the synovium. Consequently, the authors observed increased proliferation and migration of chondrocytes, an effect mediated by exosomal CD73 (S. Zhang et al., 2018). Similarly, intra-articular injection of human ESC-MSC-EVs, 4 weeks after DMM surgery significantly protected these animals from cartilage erosion by enhancing type II collagen expression and reducing ADAMTS5 levels (Wang et al., 2017). Another study also utilising E1-MYC 16.3 ESC-MSC-EVs demonstrated their ability to reduce inflammation, pain and fibrosis while enhancing cartilage and subchondral bone repair following the induction of OA in rats (Zhang et al., 2019). These EVs were able to mediate their anabolic effects by upregulating TIMP-2 expression and ameliorating gene expression associated with fibrosis. Specifically, MSC-EVs enhanced sulphated glycosaminoglycans (sGAG) synthesis and suppressed IL-1β-induced NO and MMP-13 production (Zhang et al., 2019). MSC-EVs from human exfoliated deciduous

teeth inhibited pro-inflammatory and MMP gene expression in human temporomandibular chondrocytes stimulated with IL-1β. These EVs were found to contain miR-100-5p, which may mediate the beneficial effects of these EVs through mTOR (Luo et al., 2019). MiRNAs are known to play roles in the process of tissue repair and regeneration. Their efficacy having been shown in restoring vascular integrity and inducing cell differentiation (Jia et al., 2013; Wang et al., 2008). The downregulation of miR-192-5p was recently described in a microarray analysis of human RA-FLS, this miR was found to directly bind and modulate ras-related C3 botulinum toxin substrate 2 signalling, which was consequently elevated in human RA-FLS (Zheng et al., 2020). In the same study using a rat CIA model, MSC-EVs overexpressing miR-192-5p was shown to reduce: PGE_2 release, pro-inflammatory cytokine levels in synovial tissue and serum NO release, as well as circulating levels of inducible NO synthase (iNOS) (Zheng et al., 2020). In a different set of experiments, enhanced proliferation of chondrocytes and downregulation of the production of specificity protein-1 was attributed to the delivery of miR-135b through MSC -EVs following stimulation with TGF-β1 (Wang et al., 2018). The current literature highlights the therapeutic potential of MSC-EVs in preventing and treating both RA and OA.

Bone biology

One of the hallmarks of arthropathies such as OA and RA, are changes in bone metabolism and during RA, but not OA, this is represented as erosion originating in the cortex extending into subchondral bone with disease progression. The disproportionate catabolic events observed in these conditions is thought to be dependent in part, on changes in the profile of EVs found within the diseased joint (Behera & Tyagi, 2018). Maeda *et al.,* have recently shown the expression of several miRNAs expressed in the joint are altered in RA. The authors observed that the top 22 miRNAs with the most significant up or down-regulation are thought to mediate processes related to bone resorption or compensatory bone formation via Wnt and bone morphogenetic protein (BMP). Interestingly, a bone resorption mediating RNA, miR-221-31p was identified as an exosomal cargo and demonstrated to be released by fibroblasts following inflammatory stimuli. Whilst the disease state promotes the expression of pro-osteoclastic cargo, this is accompanied by the downregulation of pro-bone forming cargo. However, under physiological conditions, exosomal communication can mediate bone formation (Maeda et al., 2017).

It is understood that EVs from a specific cellular source retain much of their parent cell's biological capabilities. This has been effectively demonstrated as osteoblast-EVs can effectively initiate osteogenic differentiation within MSC, whether that be in culture conditions favouring osteogenic or adipogenic differentiation and vice versa (Narayanan et al., 2018). Bone marrow stromal cells (BMSC) have been shown to contribute to the regeneration of a range of tissues, such as bone and cartilage. Osteoblast exosomes have been shown to promote osteogenic differentiation of BMSCs via growth factors found on exosomes, while simultaneously supressing their capability to self-renew or commit to an

adipogenic lineage (Zhao et al., 2010). Cui *et al.,* have similarly shown that exosomes from bone mineralising osteoblasts can promote osteogenic differentiation in BMSC through the lateral transfer of miRNA (Cui et al., 2016). Interestingly, even during the process of osteogenic differentiation, the release of a specific profile of EV associated miRNAs compared to undifferentiated BMSC, can support further lineage commitment in an autocrine and paracrine manner. For example, the miRNA *let-7,* was observed to be increased on osteogenic BMSCs and others have observed that *let-7* can induce ectopic bone formation within MSCs *in vitro* (Wei et al., 2014; Xu et al., 2014). Ekstrom *et al.,* demonstrated that LPS stimulated monocyte-exosomes induced osteogenic MSC differentiation, with enhanced expression of *RUNX2* and *BMP-2*; two genes crucial for bone regeneration (Ekström et al., 2013). There is evidence for mutual crosstalk and regulation between bone forming cells and osteoclasts as a specific subset of osteoblast exosomes have been observed to also promote osteoclastogenesis via the expression of RANK-L and TRAP (Deng et al., 2015; Solberg et al., 2015). The homeostatic maintenance of osteoclastogenesis also relies on negative regulation via osteoblast exosomes, for instance, exosomes expressing osteoprotegerin; the physiological antagonist of RANK-L (Solberg et al., 2015). At present, several osteoclast exosome cargos which promote osteoblast differentiation, migration and enhanced mineralisation have been reported, such as ephrin B2-B4, IGF and cardiotropin 1 (Behera & Tyagi, 2018; Rigoutsos & Furnari, 2010; Wang et al., 2013; Zhao et al., 2006).

During OA and RA, changes in bone metabolism are seen alongside the destruction of articular cartilage within the joint capsule. EVs have been shown to exist in both articular and growth plate cartilage (Miyaki & Lotz, 2018). Articular cartilage vesicle (ACV) cargo is highly heterogenous and includes cytoskeletal constituents, growth factors, inflammatory components and ECM proteins (Rosenthal et al., 2011). Interestingly, a number of cargoes present in healthy ACVs, such as lubricin and perlecan, are reduced in expression during OA, while others, like complement and collagen VI components are upregulated (Rosenthal et al., 2011). Certain ACV cargoes are only expressed in healthy or OA cartilage, for example, several apolipoproteins and immunoglobulin-γκ chains are expressed by OA ACVs. Indeed, immune complexes have been isolated previously from both the RA and OA synovium and EV bound immunoglobulins are demonstrated to contribute to their generation (Monach et al., 2009). EVs isolated from unstimulated chondrocytes have been observed to promote anabolic gene expression profiles in recipient primary human articular chondrocytes, while simultaneously supressing genes associated with catabolic processes; this included the promotion of genes associated with collagen II and aggrecan production (Liu et al., 2020).

Table 1. Immunomodulatory potential of endogenous extracellular vesicles within the joint
Summary of the key endogenous interactions mediated by extracellular vesicles between major cell types found within the joint during homeostasis, RA and OA.

Reactive oxygen species involvement in OA and RA

Reactive oxygen species (ROS) play a regulatory role in the normal functioning of chondrocyte activities, such as proliferation and matrix remodelling (Henrotin et al., 2005). ROS are carefully balanced by protective antioxidant mechanisms under homeostatic conditions, however, when this redox state is significantly imbalanced, structural, functional and molecular changes occur. Within the context of joint diseases, elevated production of ROS and depletion of antioxidants are implicated in disease progression via a multitude of different mechanisms (Henrotin et al., 2003; Matés et al., 1999). ROS and MMPs, the main culprits of ECM degradation, are both elevated in OA cartilage and synovium. Nitrated type II collagen peptides are found at elevated levels in OA patient serum as well as peroxynitrite in OA cartilage (Henrotin et al., 2004). Similarly, reduced antioxidant levels are reported in RA patient's plasma and depressed antioxidant levels are associated with increased risk of developing RA and disease activity (Heliövaara et al., 1994; Kamanli et al., 2004).

ROS have the ability to oxidise many cellular components including nucleic acids, phospholipids and transcriptional factors, which can result in ECM breakdown and cell death (Henrotin et al., 2003; Lo et al., 1998; Tiku et al., 1999). For example, superoxide and its derivates play a key role in chondrocyte cell death (Carlo & Loeser, 2003), which can be blocked using a superoxide dismutase (SOD)-mimetic (Kurz et al., 2004). In contrast, high levels of NO do not induce cell death in chondrocytes (Carlo & Loeser, 2003) and some authors have suggested that NO could have anti-apoptotic actions when antioxidant levels are low; NO was able to reduce hydrogen peroxide-induced apoptosis *in vitro* (Del Carlo & Loeser, 2002; Matsushita et al., 2004). However, NO can play a detrimental role in cartilage matrix synthesis. A key element of matrix loss is driven by insensitivity to growth factors such as insulin-like growth factor (IGF)-1 that promotes matrix formation (Henrotin et al., 2005). Using iNOS knock-out mice, NO was found to be responsible for resistance to IGF-1 in chondrocytes, since in the absence of iNOS, chondrocytes retained their sensitivity to IGF-1-mediated actions in a zymosan induced arthritis model (van de Loo et al., 1998). Similarly, NO dose-dependently inhibits IGF-1 mediated proteoglycan synthesis (Studer, 2004; Studer et al., 2000).

EVs and oxidative stress in OA and RA

Whilst the role of oxidative stress, mitochondrial dysfunction and ROS are well documented within the context of RA and OA pathophysiology, the role of EVs in this context is less well studied. Yang *et al.,* demonstrated that exosomes from vascular endothelial cells reduce autophagy and p21 expression in chondrocytes resulting in increased cellular ROS levels and promoted apoptosis (Yang et al., 2021). Similarly, exosomes from osteoarthritic chondrocytes inhibit autophagy in macrophages which induces mitochondrial ROS production leading to further inflammasome activity, IL-1β and ultimately aggravating synovitis in a murine model of OA (Ni et al., 2019). As well as having roles in cellular fate and synovial inflammation, it is thought that EVs in the context of oxidative stress are involved in the propagation of neuropathic pain (Ni et al., 2020). Macrophages recruited to the sites of injury can sensitise

nociceptive neurons by elevating ROS levels and in doing so contribute to evoking a pain response. In the dorsal root ganglion, neuronal-immune crosstalk can be mediated by neuronal exosomes which are taken up by macrophages and can initiate a feedback loop of elevations in ROS and exosomes, ultimately propagating long-term pain (Malcangio, 2019).

In a tissue microenvironment that is undergoing oxidative stress, biological molecules such as DNA, phospholipids and proteins can become modified to form DAMPS (Chakravarti & Chakravarti, 2007; Cooke et al., 2003; Poli et al., 2004). TLRs such as TLR4 and inflammasomes recognise endogenous molecules such as DAMPS, resulting from sterile cellular stress (Ha et al., 2020). DAMPS act as stress-signalling molecules and have been reported to be contained within EVs (Yarana & St Clair, 2017). Ha and colleagues described a subset of EVs termed 'stressEVs', generated from cells under oxidative stress which have divergent effects compared to EVs generated by LPS stimulation. StressEVs but not LPS generated EVs, activated gene expression of proinflammatory cytokines *Ccl24* and *Il23* as well as anti-inflammatory *Tnfaip6* and *Socs2*. StressEVs had the ability to activate TLR4/myeloid differentiation factor 2 complex on target cells due to the expression of eicosanoid-lysolipids, specifically lysophospholipids (LysoPLs) with oxidized arachidonoyl acyl chains (Ha et al., 2020; Manček-Keber et al., 2015). The formation of LysoPLs that are delivered to cells via stressEVs, required the enzyme activity of secretory phospholipase A2 (sPLA2s). This is significant as RA and gout patient synovial fluid demonstrated sPLA2 activity (Pruzanski et al., 1992). Further to this, injection of $sPLA_2$-IIA into the K/BxN acute arthritic mouse model, increased ankle swelling that was found to be, in part due to TLR4 activation (Ha et al., 2020).

Profiling synovial fluid EVs from 12 RA patients using size exclusion chromatography coupled with RNA sequencing, uncovered miRNAs derived from EVs that were found in joints with either low- or high-grade inflammation (Foers et al., 2021). The results revealed 78 EV miRNAs that were differentially expressed; 29 of the miRNAs enriched in EVs from low-inflamed joints were targets for ROS signalling, indicating that modulation of ROS signalling pathways are associated with reduced synovial inflammation (Foers et al., 2021).

Thiol groups have been implicated in the release of EVs since as early as 1993 and were shown to be a requirement for MV generation by platelets (Dachary-Prigent et al., 1993). Since then, a number of other studies have shown redox processes play a part in EV release from plasma membranes (Szabó-Taylor et al., 2015). Szabo-Taylor and colleagues revealed a homeostatic role of thiols on EVs. Monocyte thiol surface expression was shown to be increased following stimulation *in vitro* with LPS or TNF, whilst their newly released EVs had decreased thiol groups as compared with EVs from unstimulated cells (Szabó-Taylor et al., 2017). RA patients presented a similar trend, with increased thiols on $CD14^+$ monocytes and decreased expression of thiols on newly released EVs when compared with healthy controls. Similarly, peroxiredoxin 1 (Prdx), a group of thiol containing proteins were shown to be elevated on the surface EVs in RA plasma. The authors also detected overoxidised Prdx species which are enzymatically inactive. The authors suggest that a large proportion of EV-associated Prdx is therefore inactive and thus could be a protective mechanism to dispose of inactive Prdx and enhance cell plasma membrane thiol groups. Upregulation of cell surface

free thiols has been previously described as a protective mechanism during infection and ROS production to prevent the overoxidation of surface proteins (Pellom et al., 2013; Szabó-Taylor et al., 2015).

There is a growing effort to utilise highly oxidative microenvironments to bolster exosomal targeting and specifically to the pathophysiological joint. Wu and colleagues have used hydrogen peroxide sensitive lipid nanoparticles as vehicles for the transfer of therapeutic cargo in oxidative conditions. The authors showed modified nanoparticles carrying dexamethasone had <10% drug release rates in PBS which increased to as much as 60% in the presence of hydrogen peroxide (Wu et al., 2021). This strategy not only increases the half-life of the drug but also improves delivery. Importantly, the payloads of these hydrogen peroxide sensitive micelles, could efficiently drive chondrogenic differentiation and cartilage production in BMSC and decrease pro-inflammatory cytokine secretion *in vitro* (Wu et al., 2021). Others have observed similar success of ROS reactive exosomes *in vivo*. Tolerogenic DC exosomes (TolDex) have been shown to have therapeutic benefits in RA. However, their efficacy *in vivo* is reduced by poor systemic stability and ineffective accumulation within the joint (Kim et al., 2005). Conferring ROS reactivity by the addition of a thioketal linker embedded polyethylene glycosylation increased the systemic stability of modified TolDex and improved accumulation within the joint. ROS responsiveness enabled preferential cargo transfer to mature DCs owing to their increased ROS activity. Modified TolDex decreased co-stimulatory molecule expression, pro-inflammatory cytokine secretion and increased Treg differentiation in the CIA model (Lee et al., 2021). It could therefore be argued that to maximally address aberrant oxidative stress within the diseased joint it would be prudent to deliver anti-oxidating cargo, while also taking advantage of improved trafficking by ROS sensitive exosomes.

Recent high throughput screening efforts have yielded promising disease modifying OA drugs, of which, small molecule modulators of ROS activity have appeared to exhibit effectiveness in initial studies. Shi *et al.*, have treated osteoarthritic mice with N-[2-bromo-4-(phenylsulfonyl)-3-thienyl]-2-chlorobenzamide, a small molecule inducer of SOD 3. In doing so, the authors were able to: reduce intracellular superoxide anions, chondrocyte apoptosis, increased anabolic gene expression and protect the cartilage from degradation *in vivo* (Shi et al., 2019*)*. Similarly, 3-phenylcoumarin derivative 6,7-dihydroxy-3-[3',4'-methylenedioxyphenyl]-coumarin (3-PD-5) has been shown to reduce neutrophil oxidative stress and ROS production following activation with immune complexes (Andrade et al., 2018). It was recently observed that 3-PD-5 bearing liposomes could reduce neutrophil migration into the RA joint and decrease activation by reducing elastase expression and superoxide generation (Albiero et al., 2020).

Biologically occurring EVs may also hold promise when it comes to tackling the oxidising microenvironment of the OA and RA joint. While the exosomes isolated from IL-1β chondrocytes are conducive to increased matrix degradation and mitochondrial dysfunction, those obtained from healthy primary chondrocytes have been observed to yield a relatively increased number of mitochondrial proteins. These exosomes could prevent the

development of OA *in vivo* by restoring mitochondrial function and inducing macrophage polarisation to a pro-resolving phenotype (Zheng et al., 2019). The exact mechanism by which biologically occurring exosomes can prevent OA and whether exosomes from other cellular sources are similarly capable, requires further study.

Modulation of EVs for enhanced therapeutic payload

EVs have a number of useful properties that make them effective tools for the encapsulation of drugs or biological material. Vesicles may be derived from cells inoculated with the compound of interest or may be chemically or mechanically disrupted to encapsulate the required material. Vesicles can protect their internal contents from rapid degradation due to their phospholipid bilayer and surface expression indicating 'self' as well as having the unique ability to cross different biological membranes.

Loading and Overexpressing in EVs

Among the myriad of technical strategies employed to bolster the therapeutic efficacy of EVs, one technique is to manipulate them by the addition or overexpression of specific cargoes (Malda et al., 2016). Overexpression of miRNAs encapsulated in EVs has been a promising strategy towards cartilage protection. As a relevant example miR-320, which was found to be decreased in OA cartilage (Meng et al., 2016), overexpression in human BM-MSCs led to the release of EVs which downregulated MMP-13, enhanced SOX9 and type II collagen expression in human OA chondrocytes (Sun et al., 2019). As previously discussed, synovial MSC exosomes can transfer Wnt5a and support chondrocyte proliferation and migration. One consequence of this transfer is a subsequent decrease in ECM production; it is thought that this occurs due to upregulation of IL-6 following Wnt5a stimulation (S. C. Tao et al., 2017). One exosome-based strategy to combat the negative effects of Wnt5a in this setting, is to utilise exosomes from MSCs carrying miR-92a-3p. Mao *et al.*, transfected MSCs with a miR-92a-3p mimetic and observed the expression of this miRNA within the derived exosomes. The authors reported that miR-92a-3p-MSC-exosomes could efficiently induce increased upregulation of matrix genes and cartilage production from chondrocytes by inhibiting the function of Wnt5a (G. Mao et al., 2018). In an alternative approach to combat the catabolic effect of Wnt5a while keeping its beneficial properties, Tao *et al.,* have genetically engineered synovial MSCs using a lentiviral construct, in order to stably induce the overexpression of miR-140-5p alongside Wnt5a. In doing so, the authors kept the proliferative effects of Wnt5a on chondrocytes while returning ECM production in recipient chondrocytes to normal levels (S.-C. Tao et al., 2017).

EVs derived from MSCs adenovirally infected to over-express microRNA-124A were assessed for their ability to modulate synovial fibroblast activity. HMSC-124A-EVs inhibited proliferation of MH7A - an immortalised human synovial fibroblast line, to a greater degree than non-transfected EVs alone, although HMSC-EVs did display an inherent ability to dampen proliferation. The rate of wound closure, migration and invasion in MH7A was delayed and

apoptosis was increased in the presence of HMSC-EVs and to a greater extent with HMSC-124A-EVs (Meng et al., 2020). In RA, miR-150-5p was successfully transfected into bone marrow-MSCs and transferred via exosomes to RA-FLS. The delivery of this miRNA inhibited the expression of genes MMP-14 and VEGF by binding to their three prime untranslated region, thereby reversing RA-FLS migration, invasion and endothelial cell tube formation induced by IL-1β, TGF-β and TNF-α *in vitro*. Angiogenesis and joint inflammation were also inhibited *in vivo* with the injection of miR-150-5p-EVs (Chen et al., 2018). When comparing with OA, miR-150-5p expression levels in the serum and synovial tissue FLS of RA patients, were all greatly reduced whilst MMP-14 and VEGF were enhanced (Chen et al., 2018).

A group headed by Dr. Paul Robbins carried out a number of experiments looking at over-expression in DCs and their derived EVs in the treatment of arthritis. DC-EVs overexpressing IL-10, induced a 4-fold reduction in T cell proliferation *in vitro* and *in vivo* studies; these EVs protected against paw oedema in a delayed-type hypersensitivity (DTH) model, whereby mice are immunised using keyhole limpet hemocyanin (KLH) and 2-weeks post immunisation a Th1 inflammatory response ensues, following the administration of KLH into the foot pad. In this model, EVs were found to be more efficacious than their parental cell, although this difference could be concentration dependent. Virally transfected IL-10 (vIL-10) DC-EVs alone, were also able to delay the onset of CIA in mice and were *as* effective at suppressing arthritis progression when compared to cells – vIL-10 DCs (Kim et al., 2005). The following year the same group generated DC-exosomes overexpressing Fas ligand a pro-apoptotic member of the TNF family which has been described to suppress T cell responses *in vivo*. These EVs were able to inhibit inflammation in the DTH model using KLH, in both the treated and untreated contralateral paw and systemic injection partially rescued established CIA in mice (S. H. Kim et al., 2006). These effects required MHC Class II and the presence of Fas receptor in the host mice. They also went on to over-express IL-4 in DCs, IL-4 is a cytokine with anti-inflammatory actions, reported to supress IL-1 and TNF-α generation from macrophages (Essner et al., 1989; Hart et al., 1989). Adenoviral transfection of 10^6 DCs with both soluble and membrane bound IL-4, was sufficient to suppress the progression of arthritis using 1 mg of exosomes (the number of exosomes generated by DCs over a 24 hour period) (Kim et al., 2007). Exosomes derived from IL-4 transfected DCs, also acted with similar efficacy to their parent cells and suppressed established CIA in mice. Therapeutic effects were also seen in the DTH model using KLH, with a single-dose of either IL-4 transfected DCs or exosomes being sufficient to significantly suppress paw swelling, even in the untreated contralateral paw. These therapeutic exosomes exerted their effects through interactions with antigen presenting cells and were able to modify $CD3^+$ T cells, again requiring MHC II and in part Fas L (Kim et al., 2007).

Using a similar method, the same group assessed the overexpression of indoleamine 2,3-dioxygenase (IDO) which is a tryptophan-degrading enzyme and is also involved in host defence and tolerance maintenance. IDO has been demonstrated to be involved in maintaining tolerance in RA, where inhibiting IDO accelerates inflammation in CIA models (Szántó et al., 2007) and inhibition of CIA progression was found to work via an IDO-

dependent mechanism (Seo et al., 2004). Robbins and colleagues utilised these properties of IDO, coupled with DCs to generate IDO-expressing DC-exosomes that exerted anti-inflammatory and therapeutic effects in both DTH and CIA models (Bianco et al., 2009).

Given that endogenous neutrophil-EVs contain lipid mediator precursors, our group exploited the anti-inflammatory properties of neutrophil-EVs by using them as scaffolds, enhancing their therapeutic efficacy by enriching them with lipid mediators such as aspirin-triggered resolvin D1 or a stable analogue of lipoxin A_4 (Norling et al., 2011). These nanoparticles showed therapeutic and pro-resolutive effects: limited neutrophil influx in a murine model of peritonitis, reduced the resolution interval and enhanced wound healing. These observed benefits are in part due to inherent anti-inflammatory properties of neutrophil-EVs, with a single dose of 1 x 10^5 non-enriched EVs being sufficient to counteract neutrophil infiltration in a model of peritonitis by ~25%. This was enhanced to ~60% with LXA_4 analogue enrichment. These enriched EVs were also protective against inflammation of the temporo-mandibular joint as determined by neutrophil infiltration into the site (Norling et al., 2011) (Figure 2).

Figure 2. Loading and overexpressing Extracellular Vesicles for enhanced therapeutic payload
EVs derived from cells adenovirally transfected to over-express certain molecules with therapeutic efficacy in different cell types. A. Dendritic cell (DC)-EVs adenovirally infected to over-express IL-10, 1L-4, indoleamine 2,3-dioxygenase (IDO) and Fas Ligand (Fas L). B. Mesenchymal stem cells (MSCs)-EVs overexpressed with many different miRNAs. C. Polymorphonuclear (PMNs)-EVs (Neutrophil-EVs) were enriched with aspirin-triggered resolvin D1 (AT-RvD1) or a stable analogue of lipoxin A_4.

Targeting

A number of challenges remain with regards to the therapeutic use of EVs, including how to direct their specific effects to the site of injury (Liang et al., 2021). While the above strategies are effective for loading EVs with cargo, others have used methods for genetically engineering the expression of specific proteins on the surface of EVs (Yang et al., 2018). Appending proteins to the surface of exosomes relies on the presence of highly ubiquitous EV proteins. One of the most widely utilised targets is the membrane embedded lysosome-associated membrane protein 2B (LAMP-2B). DC-EVs have been observed to be highly enriched for LAMP-2B, particularly the N-terminus of LAMP-2B on the surface of DC-EVs. Through genetic engineering techniques, it is possible to generate LAMP-2B fusion proteins whereby the target protein is fused to the N-terminus of LAMP-2B and is consequently also expressed at high levels on the surface of these manipulated EVs (Alvarez-Erviti et al., 2011). With the use of high-throughput screening platforms, it is then possible to identify proteins which facilitate organ or cell specific recruitment, thus allowing the engineering of tissue-targeting EVs (Liu et al., 2019). To this end, Liang *et al.,* aimed to target EVs by engineering DCs with a

chondrocyte-affinity peptide (CAP) fused to LAMP-2B. The exosomes of CAP-DCs were loaded with miR-140 via electroporation. The authors observed that these exosomes were able to deliver miR-140 specifically to chondrocytes *in vitro* and reduce the progression of OA in a rat model of medial meniscus destabilisation by the inhibition of cartilage degradation (Liang et al., 2020). It was observed that exosomes lacking CAP were recruited diffusely across various organs throughout the rat, while CAP-miR-140 exosomes were recruited more specifically to the joint and chondrocytes. The authors also showed that CAP expressing EVs were able to penetrate the cartilage with far greater depth than those without, further emphasising their potency (Liang et al., 2020).

Alternatively, the surface modification of EVs allows the specific delivery of small molecules and drugs. This is a particularly attractive opportunity for delivering therapeutic agents which may be hydrophobic or exert negative off-target effects. Xu *et al.,* generated DC exosomes with a LAMP-2B-E7 peptide fusion protein, specifically trafficking these EVs to synovial MSCs. Kartogenin is a small molecule which can induce chondrogenic differentiation within synovial fluid MSCs, however, the low water solubility of kartogenin means that it precipitates within the cytosol reducing its effective concentration. By loading E7 peptide exosomes with kartogenin, the authors could induce more potent chondrogenesis within synovial fluid MSCs vs kartogenin alone. Concurrently, co-administration of E7-kartogenin EVs with synovial fluid MSCs into the joint, potently inhibited the progression of OA *in vivo*, more so than either MSCs or E7-kartogenin EVs in isolation (Xu et al., 2020). Despite these promising results, the efficacy of targeted EVs remains limited, particularly within environments rich in proteolytic activity due to the degradation of EV surface proteins. Avoiding degradation remains a challenge but there has been success with maintaining the expression of LAMP-2B fusion proteins on exosomes by introducing a series of glycosylation motifs (Hung & Leonard, 2015).

Topping *et al.,* coupled the ability to not only target EVs to the joint, but to enrich them with desired compounds. An antibody specific to damaged cartilage – disease modified collagen-II (anti-ROS-CII), was incorporated onto neutrophil-EVs by sonication with free phospholipid, resulting in the generation of neutrophil-EV based liposomes that encapsulated the antibodies of interest. These EVs improved pharmacodynamic efficacy by accumulating at the arthritic knee and binding specifically to the damaged cartilage following systemic administration. These EV/anti-ROS-CII preparations were enriched with functionally active anti-TNF-α or IL-10 and accelerated the attenuation of joint inflammation compared to enriched EVs conjugated to a non-specific isotype control antibody (L. M. Topping et al., 2020).

Synthetic moieties allow for the direct targeting of EVs to their desired cell or organ of interest, however, the endogenous properties of EVs also can also be exploited due to their differential inherent targeting. In cancer cells integrins $\alpha_6\beta_1$ and integrins $\alpha_6\beta_4$ dictates adhesion to fibroblasts and epithelial cells resident in the lung, whilst integrin $\alpha_v\beta_5$ expressing EVs specifically bind to Kupffer cells, mediating liver tropism. In this way cancer cell derived EVs follow the metastatic organotropism potential of their parent cell, determined by integrin

expression (Hoshino et al., 2015; Murphy et al., 2019). The tetraspanin family of proteins, abundant on certain subsets of EVs, can also determine EV targeting capabilities. Tetraspanin-8 complexed with integrin α_4 on the surface of EVs, interestingly influences target cell selection, preferentially binding to endothelial cells and cells of the pancreas; these discoveries will facilitate tailoring EVs as therapeutics and drug delivery systems (Rana et al., 2012). Similarly, glycans have been shown to also regulate cell-uptake and binding. Glycans present on MSC-EVs from human adipose tissue are demonstrated to interact more strongly with sialic acid-binding lectins (Figure 3) (Shimoda et al., 2017). Further work is needed to explore the endogenous targeting properties of EVs and to exploit these for therapeutic approaches.

Tissue engineering approaches to assist cartilage regeneration are currently exploring the use of photo-induced imine linked hydrogels in order to produce a minimally invasive and injectable scaffold. These hydrogels have been administered *in vivo* at the surface of articular cartilage and are observed to be safely tolerated by the host. These hydrogel tissue patches have previously been loaded with cartilage protective cells, such as chondrocytes or platelet rich plasma and have yielded greater reparative efficacy as opposed to chondrocytes alone. It is thought this was achieved by enhanced localisation at the site of injury, instead of relying on cellular homing and trafficking which can be less effective (Qi et al., 2018). In addition, it is thought that hydrogel scaffolding alters the release kinetics of cellular factors and in doing so, increases their pharmacodynamics and thus, their reparative effects (Liu, Yang, Niu, et al., 2017). More recently, Liu *et al.,* have used hydrogel scaffolds in order to create acellular tissue patches loaded with stem-cell derived exosomes. It was shown that the hydrogel scaffold efficiently retained exosomes and allowed specific targeting of articular cartilage with EVs. The authors showed that exosome loaded tissue patches promoted chondrocyte migration to the site of cartilage defects and resulted in greater deposition of ECM and ultimately, resulted in elevated cartilage repair (Liu, Yang, Li, et al., 2017).

Figure 3. Endogenous and synthetic targeting of Extracellular Vesicles.
The composition of endogenous moieties found on EVs aid in their natural targeting properties, these include integrins, glycans and tetraspanins. Synthetic enhancements can direct EVs to target location – fusion proteins to lysosome-associated membrane protein 2 fusion proteins (LAMP-2B) including chondrocyte-affinity peptide (CAP) and E7. Antibody-mediated targeting anti-ROS-CII directs EVs to damaged cartilage. Adapted from Murphy *et al.,* 2019.

Semi-synthetic

Neutrophil-derived cell-membrane coated nanoparticles or 'semi-synthetic nanoparticles' have been generated through the fusion of natural cell membranes onto synthetic polymeric cores, exhibiting and outer-surface that mimics 'self'. These semi-synthetic neutrophil-nanoparticles have been suggested to act therapeutically by 'mopping up' or neutralising free

pro-inflammatory cytokines which in the synovium would otherwise activate neutrophils and other cells (Q. Zhang et al., 2018). Semi-synthetic neutrophil-nanoparticles possess many receptors for pro-inflammatory cytokines and chemokines, whilst they are unable to elicit downstream effects. Here, Zhang and colleagues have demonstrated that neutrophil-nanoparticles reduced: the *in vitro* activation of both chondrocytes and human umbilical vein endothelial cells; activated with either cytokines or RA synovial fluid, induced a stark inhibition of chondrocyte apoptosis, decreased MMP-3 generation and increased aggrecan production. These protective effects were observed *ex vivo* as shown by improved cartilage retention in a femoral head-explant model as well as in *in vivo* CIA and human transgenic TNF-α mouse models. The authors demonstrated increased sGAGs, phenotypic switch of FLS and reductions in knee diameter, all highlighting the usefulness of neutrophil-nanoparticles as anti-inflammatory strategies for RA (Q. Zhang et al., 2018).

Concluding remarks

There are many advantages to injecting EVs as opposed to cells, including reduced likelihood of toxicity and immunogenicity and enhanced stability during storage. Cells are also more likely to undergo phenotypic changes following injection, as opposed to EVs which retain their phenotype after release. Here we have discussed the numerous efficacious results with endogenous and modified EVs that can not only be enriched in compounds already in the clinic, but miRNAs and fusions proteins or antibodies for targeting to diseased joints. The use of EVs in the clinic will not only aid improved delivery of specific compounds but reduce off-target effects, whilst circumventing the challenges of cell-based therapy. Here we have described the use of EVs as a potentially safer alternative to cell and drug therapy due to their nanoscale size and stealth encapsulation of drugs or regulatory materials to deliver to target cells.

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Table 1. Immunomodulatory potential of endogenous extracellular vesicles within the joint

Summary of the key endogenous interactions mediated by extracellular vesicles between major cell types found within the joint during homeostasis, RA and OA.

Figure 1. Extracellular Vesicle family biogenesis

The EV family is composed of 3 main members exosomes, microvesicles, and apoptotic bodies. Exosomes are formed through the inward budding of multi-vesicular bodies (MVBs) and routed to and released from the plasma membrane by exocytosis. Microvesicles (MVs) are generated through direct budding at the plasma membrane. Apoptotic bodies, arise as a consequence of extensive membrane budding that occurs during apoptosis. Figure created with BioRender.com

Figure 2. Loading and overexpressing Extracellular Vesicles for enhanced therapeutic payload

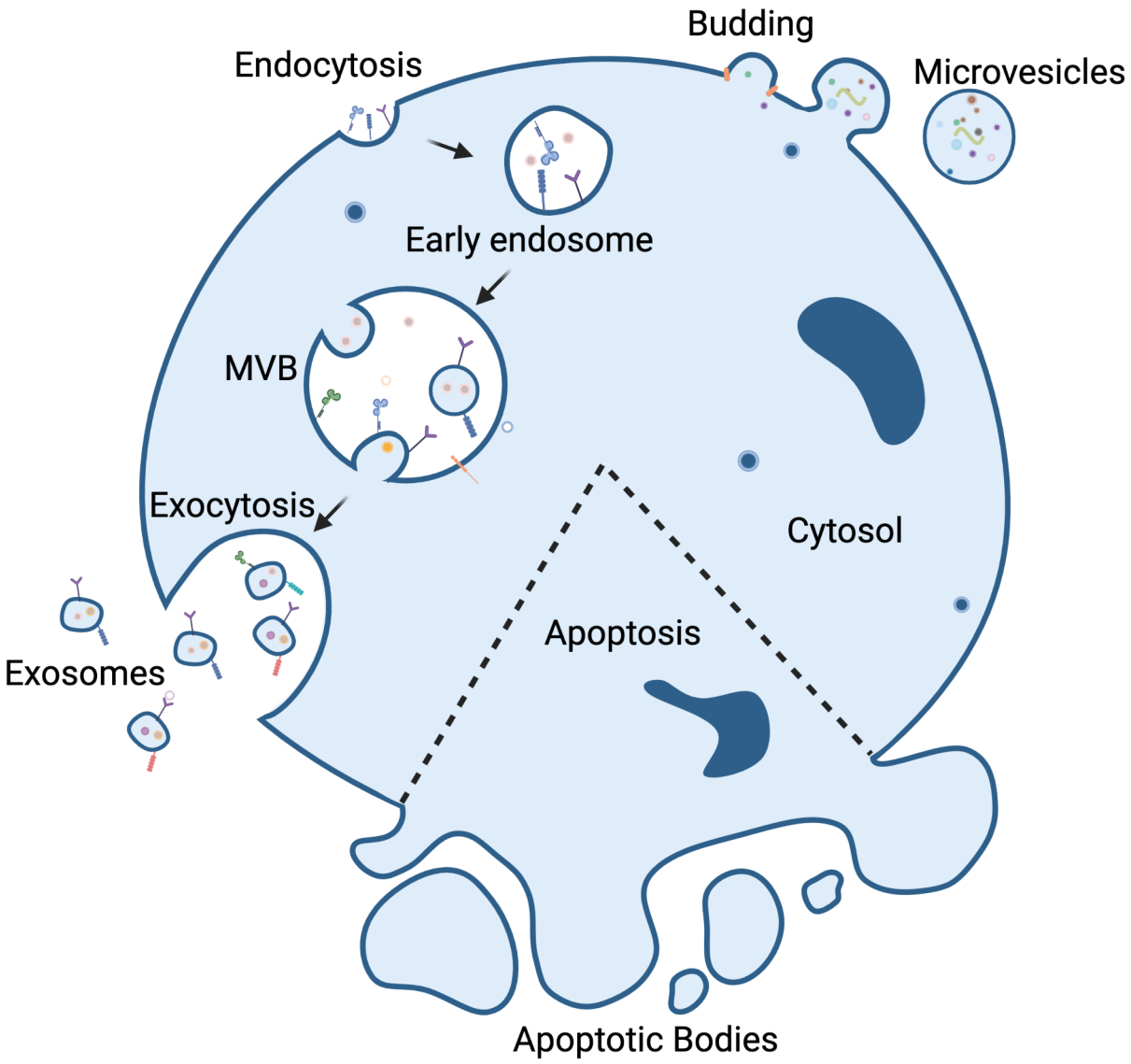
EVs derived from cells adenovirally transfected to over-express certain molecules showing therapeutic efficacy in different cell types. A. Dendritic cell (DC) derived EVs adenovirally infected to over-express IL-10, 1L-4, indoleamine 2,3-dioxygenase (IDO) and Fas Ligand (Fas L). B. Mesenchymal stem cells (MSCs) derived EVs overexpressed many different microRNAs (miRNAs). C. Polymorphonuclear (PMNs) derived EVs (Neutrophil-EVs) were enriched with aspirin-triggered resolvin D1 (AT-RvD1) or a stable analogue of lipoxin A_4. Figure created with BioRender.com

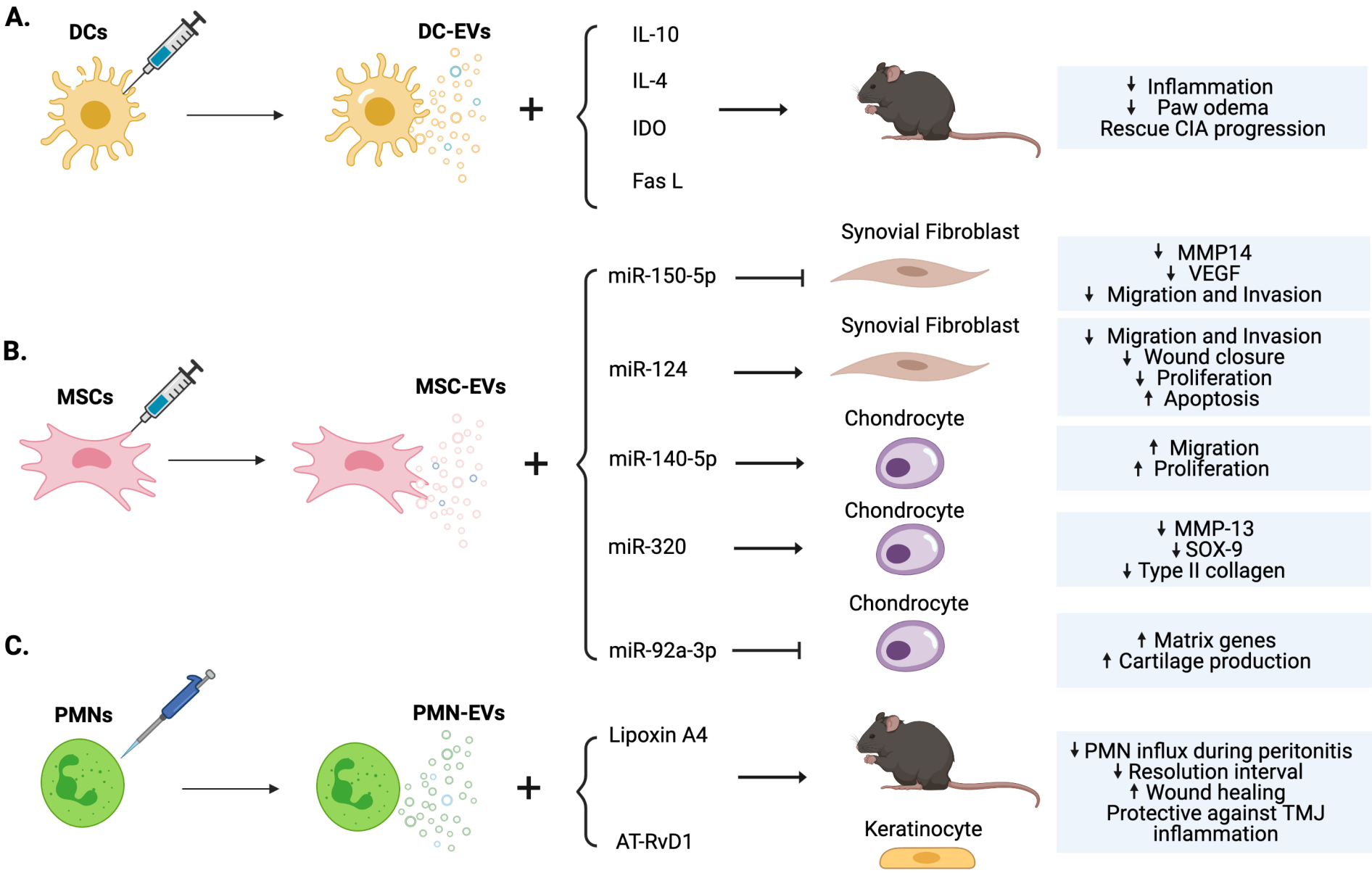
Figure 3. Endogenous and synthetic targeting of Extracellular Vesicles

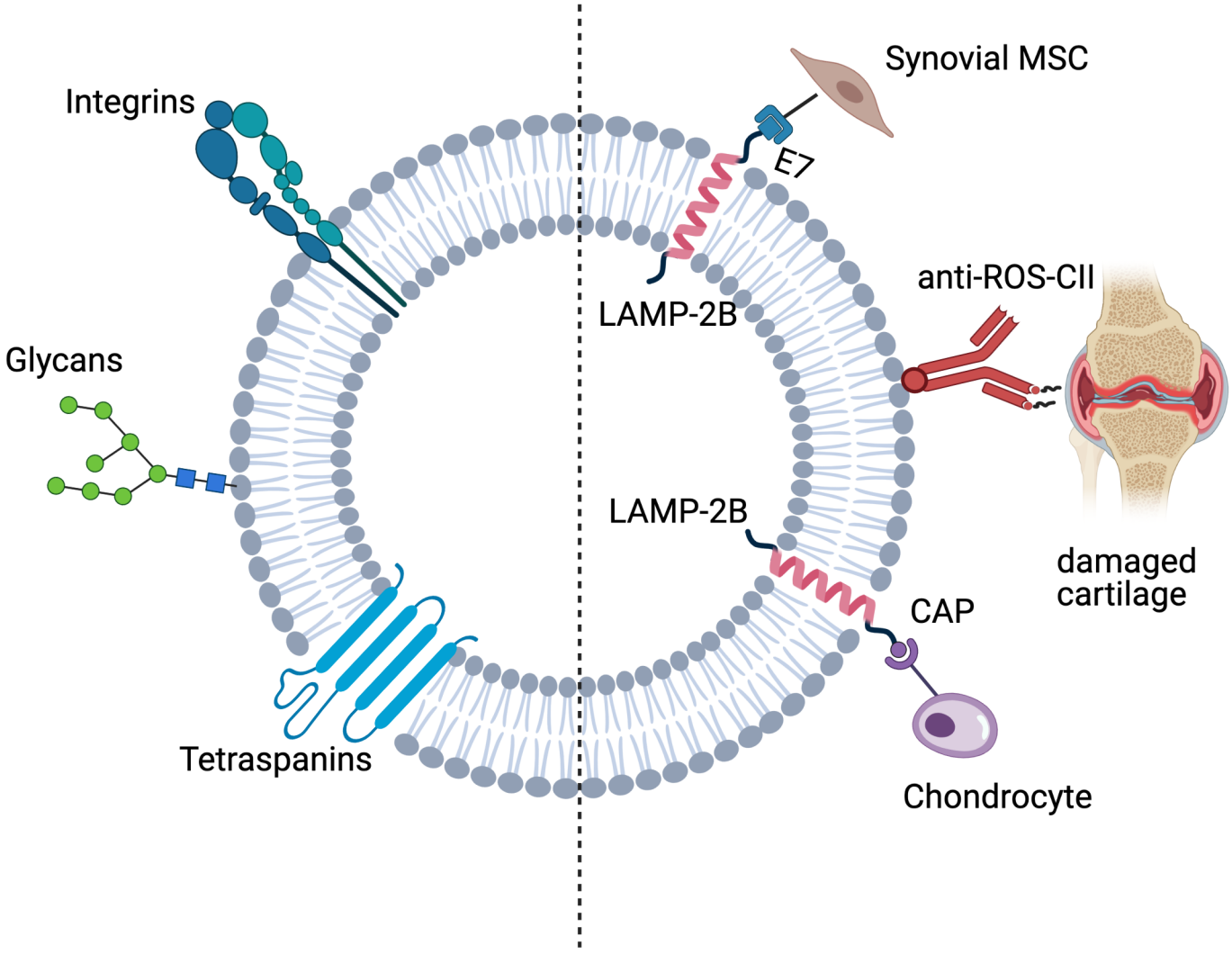
The composition of endogenous moieties found on EVs aid in their natural targeting properties, these include integrins, glycans and tetraspanins. Synthetic enhancements can direct EVs to target location; fusion proteins to lysosome-associated membrane protein 2 fusion proteins LAMP-2B including chondrocyte-affinity peptide (CAP) and E7. Antibody-mediated targeting anti-ROS-CII directs EVs to damaged cartilage. Figure created with BioRender.com. Adapted from Murphy et al., 2019

Table 1: *Immunomodulatory potential of endogenous extracellular vesicles within the joint*

Host	Source	Effects	Target	Reference
Homeostasis				
Human	Osteoclast	Inhibition of osteoclastogenesis osteoprotegrin	Osteoblast	Solberg et al., 2015
Human	Osteoblast	Promote osteoblast differentiation, migration, and enhanced mineralisation	Osteoclast	Behera & Tyagi, 2018
Human	Osteoblast	Induce osteoblast differentiation and inhibit adipogenesis	BMSC/ MSC	Zhao et al., 2010
Human	Osteogenic BMSC	Paracrine and autocrine induction of osteogenesis via the lateral transfer of miRNA	BMSC	Wei et al., 2014
Human	MSC	Inhibited DC maturation and pro-inflammatory cytokine secretion and increased TGF-β release	Dendritic cell	Reis et al., 2018
Human	MSC	Skew Th1 polarisation into Th2 and elevate CTLA-4 expression	Th1 cell	Chen et al., 2016
Mouse	Regulatory T-cell	Suppress proliferation and cytokine release	Th1 cell	Okoye et al., 2014
Osteoarthritis				
Human	Chondrocyte	Induction of chondrogenesis and collagen production	OA chondrocyte	Liu et al., 2020
Human	Chondrocyte	Reduction of HDAC2/8 via miR-95-5p	OA chondrocyte	Mao et al., 2018
Human	Hyper acute serum	Increase anabolic protein expression	Chondrocyte	Otahal et al., 2020
Human	Synovial MSC	Promote migration and proliferation by Wnt signalling	Chondrocyte	Tao et al., 2017
Human	Autologous blood product	Induction of chondrogenic differentiation	Adipose- MSC	Neubauer et al., 2019
Human	BMSC	Inhibit inflammatory cytokine and collagenase activity	OA chondrocyte	Vonk et al., 2018
Human	Adipose- MSC	Reduced catabolic mediator release and increased IL-10, and type II collagen expression	OA chondrocyte	Tofiño-Vian et al., 2018
Mouse	Infrapatellar fat pad MSC	Induced cell proliferation and cartilage production via miR-100-5p	OA chondrocyte	Wu et al., 2019
Mouse	Synovial MSC	Decreased cartilage loss and clinical scores during the collagenase induced OA	Joint capsule	Zhu et al., 2017
Mouse	BMSC	Improved clinical outcome in sodium iodoacetate OA	Joint capsule	He et al., 2020
Mouse	BMSC	Cartilage protection in collagenase induced OA	Joint capsule	Cosenza et al., 2017
Mouse	ESC-MSC	Reduced cartilage destruction, increased type II collagen deposition and ADAMTS5 inhibition	Joint capsule	Y. Wang et al., 2017
Rat	MSC	Enhanced chondrocyte proliferation via miR-135b	Chondrocyte	Wang et al., 2018
Rat	E1-MYC 16.3 ESC-MSC	Decreased cartilage loss, and iNOS and MMP-13 with increased TIMP-2 and GAG expression	OA chondrocyte	Zhang et al., 2019
Rheumatoid Arthritis				
Human	LPS stimulated Monocyte	Induced chondrogenic gene expression	MSC	Ekström et al., 2013
Human	Neutrophil	Decreased pro-inflammatory cytokine release in macrophage/FLS co-cultures	Macrophage	Rhys et al., 2018
Mouse	Neutrophil	Protected cartilage and induced chondrogenesis via annexin-1	Chondrocyte	Headland et al., 2015
Mouse	Neutrophil	Decreased vascular leakage and leukocyte extravasation	Vasculature	Lim et al., 2013
Mouse	LPS stimulated fibroblast	Increased osteoclastogenesis via miR-221-31p	Osteoblasts	Maeda et al., 2017
Mouse	BMSC	Inhibited adaptive immune cell proliferation and reduced infiltrating T-cell numbers	Immune cell	Di Trapani et al., 2016
Rat	BMSC	Delayed disease onset by systemic reduction in pro- inflammatory mediators	Joint capsule	Zheng et al., 2020
Rat	hUCMSC	Delay of radiographic changes and synovial hyperplasia and promoted Treg polarisation	Joint capsule	Ma et al., 2019


Budding
Endocytosis
Microvesicles
Early endosome
MVB
Exocytosis
Cytosol
Apoptosis
Exosomes
Apoptotic Bodies


A.
DCs
DC-EVs
IL-10
IL-4
IDO
Fas L
↓ Inflammation
↓ Paw odema
Rescue CIA progression
B.
MSCs
MSC-EVs
miR-150-5p
Synovial Fibroblast
↓ MMP14
↓ VEGF
↓ Migration and Invasion
miR-124
Synovial Fibroblast
↓ Migration and Invasion
↓ Wound closure
↓ Proliferation
↑ Apoptosis
miR-140-5p
Chondrocyte
↑ Migration
↑ Proliferation
miR-320
Chondrocyte
↓ MMP-13
↓ SOX-9
↓ Type II collagen
miR-92a-3p
Chondrocyte
↑ Matrix genes
↑ Cartilage production
C.
PMNs
PMN-EVs
Lipoxin A4
AT-RvD1
Keratinocyte
↓ PMN influx during peritonitis
↓ Resolution interval
↑ Wound healing
Protective against TMJ inflammation


Synovial MSC
Integrins
E7
LAMP-2B
anti-ROS-CII
Glycans
LAMP-2B
damaged
cartilage
CAP
Tetraspanins
Chondrocyte