The Impact of Inflammation on Extracellular Vesicles-Mediated Communication Between Dendritic Cells and Mesenchymal Stem/Stromal Cells

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Resumo

A reparação/regeneração do tecido ósseo é um processo que ocorre ao longo de três fases principais – inflamação, reparação e remodelação – e pressupõe a permuta de sinais biológicos entre diferentes tipos de células, nomeadamente células que se diferenciação noutras estruturais do tecido ósseo e células do sistema imunológico, para promover a transição de uma fase da regeneração para a seguinte. As vesículas extracelulares (EV) representam uma população de fragmentos com membrana lipídica libertados pelas células, que, dependendo da origem celular, podem ser exossomas ou microvesiculas. Estas vesículas têm sido intensamente estudadas desde o início do século, tendo já sido demonstrado que as mesmas transportam diferentes fatores biológicos, nos seus lúmen ou à superfície das suas membranas, capazes de promover o recrutamento, proliferação, ou diferenciação celular. As células dendríticas (DC) são as células apresentadoras de antigénios mais potentes do sistema imunológico, e fazem a ligação entre o sistema imunitário inato e o adaptativo, através da interação com linfócitos T. Diferentes subpopulações de DC estão presentes em quase todos os órgãos do corpo humano, estando suscetíveis à maturação mediada por citocinas pró-inflamatórias, nomeadamente o fator de necrose tumoral alfa. O nosso laboratório já demonstrou, anteriormente, que as DC naïve de dadores humanos promovem a migração de células estaminais/estromais mesenquimais (MSC) de dadores humanos, e que esta atividade é comprometida na presença de um microambiente inflamatório. Além disso, demonstrou-se também que as EV geradas por DC naïve também conseguem promover o recrutamento de MSC de uma maneira dependente da quantidade de EV fornecida. O presente trabalho visa descobrir o impacto do microambiente criado após lesão tecidual na capacidade das DC-EV em promover a migração das MSC em direção ao estímulo.

Monócitos primários de dadores humanos saudáveis foram isolados por um mecanismo de seleção negativa a partir de buffy coats provenientes do excedente de dádivas de sangue e as MSC de dadores humanos saudáveis foram isoladas a partir de amostras de medula óssea descartadas em cirurgia (gentilmente doadas pelo Centro Hospitalar Universitário de São João e CUF, sob protocolos eticamente aprovados). As DC foram diferenciadas a partir dos monócitos durante 4 dias usando IL-4 e GM-CSF, e divididas em dois conjuntos, um conjunto de DC permaneceu sem qualquer estímulo, enquanto o restante conjunto de células foi estimulado com TNF-α, para simular um ambiente pró-inflamatório que induz a maturação das DC. A ativação das DC foi avaliada através das mudanças morfológicas e dos níveis de citocinas pró-inflamatórios presentes nos sobrenadantes das células. Para ambos os subgrupos de DC formados, as EV foram produzidas em meio de cultura suplementado com soro de feto de bovino (FBS), sujeito a um processo de remoção das EV dessa espécie. As

EV produzidas pelas DC foram isoladas por um processo que inclui sucessivas centrifugações para eliminar células mortas e resíduos celulares, e uma centrifugação final de alta velocidade (ultracentrifugação) para recuperar as EV. As EV foram posteriormente caracterizadas através de microscopia eletrónica de transmissão, nanoparticle tracking analysis e Western blot. O impacto das DC e das respetivas EV no recrutamento de MSC foi investigado através de um ensaio de migração de transwell. Para a pesquisa e identificação de proteínas foi considerada a base de dados do Uniprot para as seleções taxonómicas *Homo sapiens* e *Bos taurus*. A quantificação de proteínas foi efetuada pela metodologia Label Free Quantification.

As DC derivadas de monócitos foram diferenciadas com sucesso através de um protocolo previamente otimizado, demonstrado pela morfologia, observada por microscopia ótica, compatível com a das DC. Além disso, os níveis de citocinas pró-inflamatórias, nomeadamente IL-1β, IL-6 e TNF-α, revelaram-se aumentados nos sobrenadantes das DC ativadas com TNF-α, sugerindo o estado de maturação das mesmas. As EV libertadas por DC naïve (nEV) e DC estimuladas com TNF-α (tEV) eram semelhantes em tamanho e morfologia, e exibiram a presença de CD63, que estava ausente nos sobrenadantes sujeitos à ultracentrifugação, que serviram como controlo nas experiências para avaliar a atividade das EV, e que as conclusões acerca da sua funcionalidade não são confundidas pela presença de contaminantes dos sobrenadantes que possam ter sedimentado com as EV aquando da ultracentrifugação. Notavelmente, as nEV e tEV estimularam a migração das MSC numa maior extensão do que o controlo negativo com apenas meio de cultura sem FBS, mas não demonstraram o mesmo potencial de recrutamento das DC que lhe deram origem. Apesar das DC naïve apresentarem uma maior capacidade do que aquelas ativadas com TNF-α para promover a passagem das MSC do compartimento de cima para o compartimento de baixo, a mesma diferença não é verificada entre as nEV e as tEV. Uma análise proteómica, ainda inconclusiva devido à análise de poucas amostras, revelou que a população de EV isolada dos sobrenadantes estavam enriquecidos em biomarcadores de exossomas, incluindo Alix, CD9, CD63, CD81, e Hsp70. Adicionalmente, a expressão de moléculas co-estimulatórias (CD40, CD80 e CD86), e de moléculas de MHC classe II estava aumentada na suspensão das tEV. Ademais, proteínas relacionadas com a migração das MSC foram encontradas em diferentes abundâncias nas suspensões de nEV e tEV. Enquanto que níveis elevados de SDF-1 foram encontrados na última, quantidades menores de OPN e TGF- β1 foram detetados na mesma, comparativamente com níveis detetados na suspensão de nEV. Isto pode possivelmente explicar a falta de diferenças observadas entre a capacidade de nEV e tEV de recrutar MSC.

Estes resultados procuram elucidar o impacto da estimulação dos DC nas respetivas EV, e o seu potencial como estratégia terapêutica, particularmente no recrutamento de células

progenitoras/estaminais para o local de lesão, de modo a promover a regeneração do tecido. No entanto, é preciso confirmar e clarificar estes resultados, e perceber melhor como o ambiente inflamatório influencia o conteúdo e papel biológico das EV libertadas por DC.

Palavras-chave: vesículas extracelulares; células dendríticas; células estaminais/estromais mesenquimais; regeneração; inflamação

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Abstract

Bone tissue repair/regeneration progresses along three main stages – inflammation, repair and remodeling – and requires the exchange of biological messages between different cell populations, including tissue and progenitor cells of the skeletal system and immune cells. Extracellular Vesicles (EV), including exosomes and microvesicles, have been shown to carry different molecular mediators, and to be capable of promoting cell recruitment, proliferation and differentiation. Dendritic Cells (DC) are the most potent antigen presenting cells (APC) of the immune system, bridging the innate and adaptive immune response, through T cells interaction. These cells are present in almost all human organs, and these are susceptible to cytokine-mediated maturation, namely by Tumour Necrosis Factor (TNF)-α. Our group previously showed that naïve DC could recruit Mesenchymal Stem/Stromal Cells (MSC) and that this ability was impaired by an inflammatory-like environment. Moreover, naïve DC-EV alone were able to promote MSC migration in a dose-dependent manner. Thus, the current work aims to evaluate the impact of inflammation on DC-EV, including their ability to recruit MSC.

Human peripheral blood primary monocytes were obtained by negative selection, differentiated to DC using IL-4 and GM-CSF, and either left naïve, without any further stimulation, or treated with TNF-α, for DC to undergo maturation. DC activation was evaluated by morphological changes and quantification of pro-inflammatory cytokines by ELISA. For EV production, the media was supplemented with EV-depleted foetal bovine serum. EV were isolated by differential (ultra)-centrifugation and characterised by transmission electron microscopy, nanoparticle tracking analysis and Western blot. The impact of DC and their EV on MSC recruitment was investigated by transwell migration assays. Proteomic analysis of the content of isolated EV, was performed using the Label-Free Quantification methodology. The database Uniprot was considered for the search and identification of proteins of *Homo sapiens* and *Bos taurus* species.

DC were successfully differentiated from monocytes using a previously optimized protocol, and DC maturation was confirmed by morphology evaluation and a significantly increased secretion of IL-1β, IL-6 and TNF-α. EV-derived from naïve (nEV) and TNF-α-stimulated (tEV) DC were similar in size and morphology, and showed the presence of CD63, which was absent from ultracentrifugation supernatant (UC) control. Also, a filtration step was tested in the EV isolation protocol, resulting in only slightly smaller EV and reduction of particle number, and so it was not pursued. In terms of MSC recruitment, results confirm that naïve DCare more effective than TNF-α-stimulated ones. Importantly, nEV and tEV were able to stimulate MSC recruitment, when compared with the serum free negative control, but did not mimic the

potential of their respective parent cells to promote MSC migration. Although naïve DC have a higher capacity than those activated with TNF-α to promote MSC migration, the same disparity was not observed between nEV and tEV. Preliminary proteomic analysis revealed that the recovered nEV and tEV populations were enriched in exosomes markers, including Alix, CD9, CD63, CD81, and Hsp70. Additionally, the expression of costimulatory molecules, such as CD40, CD80, and CD86, and MHC class II molecules was superior in the tEV suspension. Furthermore, factors described to be involved in MSC migration were differently found in nEV and tEV suspensions. While high levels of SDF-1 were discovered in the latter, smaller amounts of OPN and TGF-β1 were detected in comparison to the nEV suspension, which possibly explains the lack of differences observed between the capacity of nEV and tEV to recruit MSC. While EV of only one donor were examined, an analysis in the DAVID bioinformatics database was performed, and detected proteins were assigned to GO annotations for biological process and cellular compartment. Preliminary results indicate that detected proteins in nEV and tEV were mainly associated with exosomes, and, in tEV, proteins associated with the functions of matured DC were increased.

The results presented here point to an impact of DC pre-conditioning on DC-secreted EV, and their potential for MSC recruitment. However, further studies are essential to confirm and clarify these results, and understand the DC-EV-mediated message in inflammatory conditions.

Key words: extracellular vesicles; dendritic cells; mesenchymal stem cells; regeneration; inflammation

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Abbreviations

AB	Apoptotic bodies
AFM	Atomic Force Microscopy
ALP	Alkaline phosphatase
APC	Antigen presenting cells
BCL	B cell lymphoma
BMP	Bone morphogenetic protein
CCL	C-C motif chemokine ligand
CD	Cluster of differentiation
CXCR	CXC chemokine receptor
DC	Dendritic Cells
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
ELS	Ectopic lymphoid tissue
ESCRT	Endosomal sorting complex required for transport
EV	Extracellular Vesicles
FGF	Fibroblast growth factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HGF	Hepatocyte growth factor
HLA-DR	Human leucocyte antigen – DR isotype
ICAM-1	Intercellular adhesion molecule 1
IFN	Interferon
IL	Interleukin
ILV	Intraluminal vesicles
IP-10	Interferon gamma-induced protein
ISCT	International Society for Cell & Gene Therapy

ISEV	International Society of Extracellular Vesicles
LDH	Lactate dehydrogenase
LFA-1	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharide
MCP	Monocyte chemoattractant protein
MHC	Major histocompatibility complex
miR	micro RNA
MMP	Metalloproteinases
MSC	Mesenchymal stem/stromal sells
MV	Microvesicles
MVB	Multivesicular bodies
NF-κB	Nuclear factor kappa B
NTA	Nanoparticle tracking analysis
OB	Osteoblasts
OC	Osteoclasts
OCN	Osteocalcin
OPN	Osteopontin
PGE2	Prostaglandin E2
PTEN	Phosphatase and tensin homolog
RA	Rheumatoid arthritis
RANKL	Receptor-activator of nuclear factor kappa B (NF-κB) ligand
RNA	Ribonucleic acid
RT	Room temperature
SDF	Stromal derived factor
SEC	Size exclusion chromatography
TEM	Transmission electron microscopy
TGF	Transforming growth factor

Th	T helper
TIMP	Tissue inhibitor of metalloproteinase
TLR	Toll-like receptor
TNF	Tumour necrosis factor
UC	Ultracentrifugation
VEGF	Vascular endothelial growth factor

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1. CHAPTER I: INTRODUCTION

1.1. Inflammation in Bone Healing

Bone is a complex organ that encompasses bone marrow, endosteum, periosteum, cortical and trabecular bone, blood vessels, nerves, and whose homeostasis requires the orchestration between several populations of cells, including osteoblasts (OB), osteoclasts (OC), osteocytes, and immune cells. Bone architecture undergoes constant renewal due to a biological process commonly designated as “remodeling”. In healthy individuals, there is a balance between new bone formation and destruction, tightly mediated by OB and OC, respectively. OB arise from mesenchymal stem cells (MSC), and are responsible for the synthesis of new extracellular matrix, which, on the other hand, is degraded by multinucleated cells of myeloid origin, the OC. The coordination of these two types of cells is extremely important both locally, to preserve bone mass throughout individuals life, and systemically, to maintain mineral homeostasis [1]. The immune system is actively involved in bone homeostasis by releasing bioactive factors that directly impact osteogenic or osteoclastogenic mechanisms [2]. The importance of naïve T cells in bone physiology was demonstrated in T cell-deficient animals, whose bone density decreased comparing to healthy animals, suggesting the eradication of these cells results in an unbalanced osteoclastogenesis/osteogenesis. Furthermore, resident macrophages, also named osteomacs, that directly contact with mature osteoblasts represent 10-15% of the bone tissue. These are located in sites of active bone remodeling, and seem to play a key role in maintenance of mature osteoblasts, as depletion of macrophages *in vivo* leads to a complete loss of endosteal osteomacs along with their associated osteoblasts [3]. Moreover, macrophages, as professional phagocytic cells, remove apoptotic cells of the tissue, which is essential for bone homeostasis as failure in their clearance can be associated with inflammation and autoimmune diseases [4]. Dysfunction of the immune system in the bone context elicits conditions such as osteolysis, osteoporosis, osteoarthritis, or rheumatoid arthritis, that impair the quality of life of patients that bear these comorbidities [2].

Due to bone natural dynamic above mentioned, in case of tissue damage, in healthy conditions, bone benefits from timely controlled microenvironment changes that promote its healing. Within this time window, there are three fundamental stages: inflammation, repair and remodeling. These must be coordinated and tuned correctly in order to promote bone regeneration, i.e., recapitulation of the native tissue characteristics. Although bone benefits from its natural ability to regenerate itself, there are several risk factors, including inflammatory-associated conditions, namely rheumatoid arthritis (RA), which is associated

with bone loss across the axial skeleton primarily induced by the systemic inflammation characteristic of RA patients, that can contribute to an increased possibility of fracture and impairment of its restoration. In fact, 30-50% of patients with RA have osteoporosis, a chronic disease featured by the loss of bone mineral density, and therefore brittle bones [5]. Bone fractures still represent a public health issue, although rates of incidence, prevalence, years lived with the disability of fractures have slightly decreased from 1990 to 2019. An opposite trend is observed in the absolute count of new fractures, with an increase of 33.4% in the same period. Globally, in 2019, there were 178 million new fractures [6]. A study conducted within the same time window of the previous one revealed that, in the United States of America, compromised bone healing has been reported in approximately 600 000 fractures every year, of which 16.7% develop a non-union condition, i.e., a fractured bone that is not fully recovered from trauma 9 months post injury, and that lack signs of healing on consecutive radiographs along the following 3 months [7]. Along with high-cost treatments, failure in bone healing burdens critical problems associated with the patients quality of life and health, which impact, for instance, their work life. Therefore, new strategies to accelerate the recovery of difficult-to-heal bone fractures are necessary.

Acute inflammation is crucial in an early stage of bone healing in order to successfully achieve tissue regeneration without the formation of a fibrous scar. Perturbation of inflammation may impair the adequate revascularization, cell proliferation, or progenitor cells differentiation at the injury site. In humans, acute inflammation can persist for one week, and is essentially associated with immune cells infiltration. Upon fracture or other events related with bone damage, vascular disruption stimulates haematoma formation within the fracture gap that hosts neutrophil infiltration [8]. Neutrophils are rapidly recruited to the lesion site to do the clearance of dead cells, tissue debris and microorganisms, along with the release of soluble factors, including C-C motif chemokine ligand (CCL)2 and IL-6, that act as macrophages chemoattractant. Osteomacs are influenced by the inflammatory cues of the microenvironment to acquire a pro-inflammatory M1-like phenotype. These cells secrete a cocktail of pro-inflammatory cytokines, including TNF-α, IFN-γ, and IL-6, that stimulate the migration of T cells. Activation of these cells upregulates the expression of receptor-activator of nuclear factor kappa B (NF-κB) ligand (RANKL), a cytokine closely connected with OC activity. In physiological situations, there is a stable interaction between RANK (RANKL receptor) expressed by OC progenitor cells and OB that express RANKL as a membrane-associated molecule. In the presence of M-CSF, RANKL-OB promote osteoclast differentiation, favouring the physiological process of bone formation/destruction. However, in inflammatory environments, activated T cells produce RANKL, favouring OC differentiation, and resulting in an unbalanced osteoblast/osteoclast formation, which disturbs bone naturally

stable remodeling [9]. T helper (Th) 17 cells, characterized by an IL-17 secreting signature, are implicated in osteoclastogenesis. In humans, Th17 cells differentiate from T CD4+ cells exposed to transforming growth factor (TGF)-β and inflammatory cytokines [10]. An exacerbated and uncontrolled inflammatory response favours bone destruction, rather than its healing, that is why a tuned inflammatory response is key in the bone healing context, i. e., the shift of a pro-inflammatory microenvironment promoting bone destruction (resorption) to an anti-inflammatory one promoted by M2-like polarised macrophages. While M1-like macrophages trigger an inflammatory response and contribute to the removal of debris within the fracture site, M2-like macrophages promote MSC osteogenic commitment and bone formation in the advanced stage of the bone healing process [11]. Macrophage polarisation can be mediated by the secretome of other cells, namely MSC, which contains extracellular vesicles (EV), that enclose bioactive molecules and participate in cell crosstalk. Previous work has shown that BMSC-EV have the ability to inhibit the expression of M1-like macrophage marker CD86, which suggests the inhibition of the pro-inflammatory response, and this led to improved healing [12]. On the other hand, Gengival MSC-derived EV successfully promoted the polarization of M2-like macrophages, decreasing pro-inflammatory cytokines and downregulating osteoclastogenesis, and consequently preventing bone loss [13]. The EV-mediated communication between macrophages and MSC in the context of bone regeneration is bilateral and macrophages-derived EV also impact MSC behaviour, although differently depending on macrophages polarization [14].

1.1.1 Mesenchymal Stem/Stromal Cells

As part of the multipotent stromal population, MSC play an important role in tissue regeneration/repair [15]. MSC can be isolated from different tissues, including bone marrow, placenta, amnion, cord blood and adipose tissues [16]. Importantly, depending on the tissue origin, MSC display different behaviour that can impact their functional activity, therefore it is important to state their tissue of origin [17]. Regarding their descendance, MSC have been characterised using adherence to plastic and expression of specific markers such as CD73, CD90, CD105, and absence of markers from other cell populations, like those of the hematopoietic system CD11b, CD14, CD19, CD79a CD45, and HLA-DR, or the endothelial marker CD34 [18] and their ability to differentiate into osteoblasts, adipocytes, or chondroblasts [19]. The stemness features attributed to widely described MSC remain controversial. For instance, MSC characterization does not meet rigorous criteria of the stem cell definition, namely their ability to undergo asymmetric division and complete lineage renewal [20]. The International Society for Cell & Gene Therapy (ISCT) encourages the use of “Mesenchymal Stromal Cells” to describe a bulk population of cells with considerable secretory, immunomodulatory and homing properties [21]. However the aim to standardise

the nomenclature has not been achieved throughout the years as both “stromal” and “stem” designations have been used under the “MSC” umbrella. On the other hand, there are currently no specific markers that precisely segregate populations of mesenchymal stromal cells from those of mesenchymal stem cells [21]. Herein, the designation **Mesenchymal Stem/Stromal Cells** (MSC) was used, since it has become popular in recent publications [22-26], and acknowledge that even though these MSC populations may include fibroblasts and myofibroblasts, they are also enriched in stem/progenitor cells. The degree of heterogeneity of this broad population is strongly influenced by several factors, , namely their tissue origin and recovery technique, together with culture conditions and passage numbers.

MSC-based potential therapies have been widely studied in the tissue regeneration field due to the ability of these cells not only to give rise to tissue specific cells (osteoblasts, in the bone context), but also to modulate the behaviour of neighbouring cells and boost their pro-regenerative phenotypes (Figure 1.1). Bone is a dynamic tissue and the interplay of progenitor, bone, endothelial and immune cells is mandatory to achieve complete healing process. Therefore, more than MSC cell replacement capacity, new therapies are taking advantage of the impact of MSC on other cells through paracrine signalling [27]. MSC secrete pro-angiogenic factors, such as vascular endothelial growth factor (VEGF) which stimulates endothelial progenitor cells differentiation into endothelial cells [28] and consequently revascularization. Besides VEGF, MSC produce high levels of other mediators of angiogenesis, including hepatocyte growth factor (HGF) and fibroblast growth factor-2 (FGF-2), and antiapoptotic factors, such as Akt-1 [29]. When engrafted into the ischaemic hindlimbs of mice, MSC increase capillary density and blood perfusion [29]. Ischemic rats also benefit from the upregulation of other anti-apoptotic molecules levels, such as survivin and B cell lymphoma 2 (BCL-2), upon MSC administration [30].

Importantly, MSC can both enhance or inhibit the immune response, depending essentially on the inflammatory stimuli they are exposed to [31]. MSC immunomodulatory capacity is mainly attributed to their secretome, which varies depending on the cells microenvironment. For instance, inflammatory cues stimulate MSC secretion of IL-6, HGF, VEGF, and TGF-β [32]. The latter influences not only the shift of M1-like macrophages activated with lipopolysaccharide (LPS) towards a more anti-inflammatory-like phenotype through Akt/FoxO1 signalling pathway, but also the phagocytosis potential of macrophages [33] that in a precise period of tissue healing may favour its reorganization and regeneration. MSC can affect other antigen presenting cells (APC), namely dendritic cells (DC), either by direct contact or paracrine signalling. Using a transwell barrier method, which physically separates two populations of cells, Park, Kim [34] reported that indirect co-culture of MSC and DC resulted in impairment of DC maturation, which indicates that a fraction of MSC

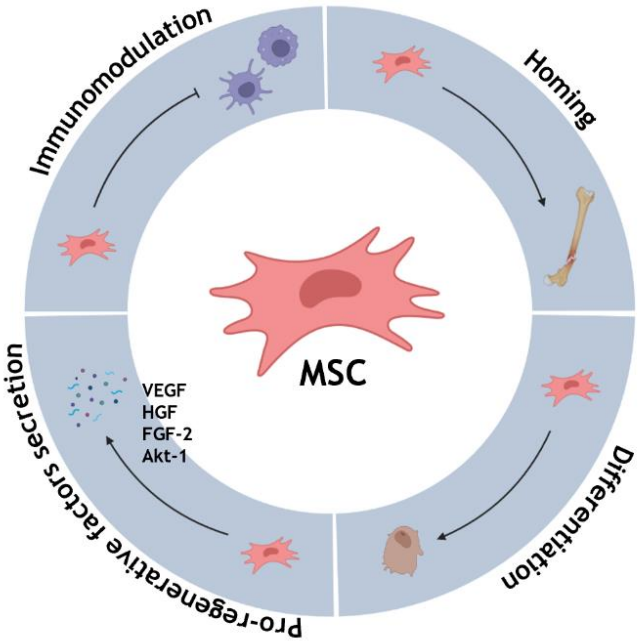

Figure 1.1. Mesenchymal Stem/Stromal Cells key features for cell-based bone therapies. Characteristics that make MSC attractive for regenerative medicine include differentiation into osteoblasts, pro-regenerative factors secretion, capacity to modulate immune response, and natural ability to migrate towards injured tissues.

immunoregulatory properties rest in their conditioned media. Along with soluble bioactive molecules, MSC secrete EV that pack other molecules that also impact DC fate. MSC-derived EV demonstrated to supress the expression of costimulatory markers, such as CD86, CD40, or CD83, on DC activated with LPS. Also, lymphocytes proliferation was negatively impacted by DC treated with MSC-derived EV, which suggests that DC acquire a tolerogenic phenotype upon EV internalization [35].

MSC natural ability to migrate towards injury sites is an attractive point for their use in therapeutic approaches. Recruitment of endogenous MSC to bone fracture occurs naturally in physiological conditions as an endogenous reaction, and has been demonstrated to be mainly mediated by the stromal derived factor (SDF)-1/CXC chemokine receptor (CXCR) 4 axis. A previous report elegantly showed that SDF-1 levels are increased during the acute phase of a mouse structural femoral bone graft, and that it enhances transplanted MSC homing to bone injury sites [36]. Among chemical regulators of MSC migration, osteopontin (OPN), which has elevated levels in inflammatory and injury conditions, acts as chemoattractant of several types of cells, including macrophages, DC and T cells [37], and ultimately has been demonstrated to recruit MSC in a dose-dependent manner *via* stimulation of integrin β1 expression [38], and activation of focal adhesion kinase (FAK) and extracellular signal-regulated kinase (ERK) signalling pathways [39]. Injured tissues bear increased levels

of pro-inflammatory cytokines, such as TNF-α, which was already shown to stimulate MSC migration [40]. The route of administration of exogenous MSC also conditions their ability to reach the place of injury. This is critical as it is described that the majority of administered MSC die within 2 days. Moreover, despite being the most popular route of delivery, intravascular administration causes the loss of a great fraction of injected cells due to triggering of instant blood-mediated inflammatory reactions [41].

1.2. Dendritic Cells: the most potent antigen presenting cells

Dendritic Cells are professional APC, and, as B cells and macrophages, they connect the innate and adaptive immune response, being considered the "*crème de la crème*" of cells on immature T cells priming [42]. DC were only officially discovered in 1973 by Ralph Sleiman and Zanvil Cohn that reported a population of cells with a dendriform-like shape [43], which is a result of cytoplasmic protrusions formation upon their maturation, and a remarkably ability to migrate to secondary lymphoid organs. However, only a few decades later, the study of DC expanded exponentially with the development of protocols that allow the differentiation of immature DC from peripheral blood monocytes, in the presence of GM-CSF and IL-4 [44]. The description of what we know today as DC was recognized of great importance by the Nobel Committee for Medicine and Physiology, awarding the above-mentioned researchers with the Nobel Prize, in 2011 [45]. Earlier, in the 19th century, Langerhans Cells (LC) were discovered as an epidermal cell population and more than 100 years later, LC were, for a long time, thought to be the first described type of DC, as LC have the ability to migrate and present antigen to T cells. Curiously, LC are now classified, based on the cells developmental relationship, as a tissue-specific subset of macrophages, whose potential to migrate to lymph nodes had not been previously described [46]. Remarkably, this highlights how the current scientific knowledge is not a forever truth and is continuously evolving.

A wide range of DC subsets is found entrapped in the interstice of human organs, including skin, liver, lung, gastrointestinal tract, spleen, and lymph nodes, and in the lumen of vessels in the bloodstream [47]. In humans, hematopoietic stem cells (HSC) of the bone marrow are the progenitors of DC, which are an heterogeneous population of cells as a consequence of multiple progenies, including myeloid and lymphoid. The latter are predominantly in the T cells zones of lymph nodes and spleen, while myeloid DC are primarily found in the spleen marginal zone. Furthermore, there is a so-called "non-conventional group" of DC that includes those derived from circulating monocytes that infiltrate tissues during inflammation or infection. Indeed, following an inflammatory injury, the number of monocytes in the blood rises, reaching a peak around 48h post-injury. The magnitude of mobilisation increases proportionally with the severity of inflammation, and, for this reason, monocyte-

derived DC are also termed “inflammatory DC”. In this line, monocytes contribute to a more elevated level of APC to effectively react to the danger signals and initiate an adaptive immune response [48]. Contrarily to conventional DC, human monocyte-derived DC express DC-SIGN, a lectin receptor, which was important to localize monocyte-DC in the paracortical T cell areas of lymph nodes, where they present and cross-present antigens that have been internalised and associated with MHC molecules to T cells, even with more efficiency than the lymph nodes resident DC [49].

Classically, immature or naïve DC have a fundamental role in maintaining T cell anergy. Tolerance to self-antigens is primarily established in the thymus through a negative selection process mediated by DC and medullary thymic epithelial cells that present self-antigen to T cells and eliminate those that show elevated autoreactivity. Autoreactive T cells that may escape thymic deletion are also susceptible to the activity of tolerogenic DC which are distributed throughout the human body, and not only regulate effector T cells activity but also expand the regulatory T cells population [50]. Indeed, immature DC behave as a surveillance system within nonlymphoid tissues with the potential to capture antigens, exhibiting high intracellular levels of MHC class II, low expression of costimulatory molecules, which include CD80, CD83, and CD86, inducing T cell tolerance, and high endocytic/phagocytic capacity. Upon encountering and processing a potential signal of danger, DC mature, which is phenotypically translated in MHC class II, and costimulatory molecules CD80, CD83 and CD86 overexpression, migrate to the local lymph node via afferent lymph, and then activate T cells [51]. Other than antigen uptake, DC are susceptible to cytokine-mediated maturation, namely by TNF-α, which is particularly described in autoimmune diseases [52].

DC, in physiological conditions, are generally assumed to not be involved in bone homeostasis, as DC deficient animals have a normal bone structure [3]. Nevertheless, and even though scarce, an investigation conducted to understand the role of toll-like receptor (TLR) 4 during calvarial bone healing has demonstrated that animals with DC (CD11c+) lacking TLR4 expression showed significantly larger bone volume 28 days after injury, a phenomenon not observed for TLR4 knockout lysozyme+ myeloid cells, proposing a possible role for DC [53]. A more recent study pinpoints a possible role of DC in maintaining oral mucosal and alveolar bone integrity, reporting that DC deficiency in mice could accelerate bone necrosis following dental extraction [54]. Although the involvement of DC in the crosstalk with bone populations in physiological conditions remains unclear, in some pathological conditions, such RA or periodontitis, DC participation in bone destruction, mainly through T cells interactions, is reported, as further exposed.

1.2.1. Dendritic Cells in Inflammation-Mediated Bone Destruction

In RA, high levels of pro-inflammatory cytokines in the rheumatoid synovium, including TNF-α, IL-1β, IL-6, IL-8 and others, contribute to persistent inflammation and disease progression, and contribute to DC migration towards the inflammation foci of the synovial tissue, where, in healthy conditions, DC are normally rare [55]. RA is a chronic systemic autoimmune disorder that primarily affects synovial joints, resulting in cartilage and subsequent bone destruction, which leads patients to experience massive pain, impaired mobility, and lower lifespan [56, 57]. DC have been found in synovial joint fluid and the overproduction of pro-inflammatory cytokines, including TNF-α, mediates their migration and, importantly, switch from an immature stage to a T cell activator stage. In RA, DC are usually surrounded by T CD4+ cells [58], and the formation of these clusters is reported to trigger the generation of autoimmune events in RA. Curiously, in inflamed tissues there is a trend of ectopic lymphoid tissue (ELS) assembly, and, in RA, synovial fibroblasts create a microenvironment enriched in chemokines and cytokines that support to ELS formation. In these clusters, DC, T cells and B cells are maintained in close contact which contributes to T cell activation and polarisation [59]. For instance, IL-1 and IL-12 production by DC leads to the differentiation in T helper type 1 cells (Th1), while the combination of IL-1β, IL-6, and IL-23 secretion induces T CD4+ cells to acquire a Th17-like phenotype. These subpopulations of T CD4+ cells exacerbate the inflammatory response by stimulating the migration and activation of other immune cells, such as macrophages, neutrophils and B cells. On the other hand, mature DC are also key drivers of class switch recombination, autoantibody generation, or new lymphatic vessel formation, and their depletion have been shown to affect ELS architecture [59].

Importantly, T cells found in RA synovium can participate in disease progression, as Th17 cells are associated with bone destruction by stimulating osteoclast differentiation by RANKL signalling and cytokine production [60]. Besides indirect activation of osteoclastogenesis *via* T cell activation, DC themselves can differentiate into osteoclasts (DC-OC). The presence of DC-OC has been already described in murine arthritis models and in RA-human patients. Importantly, DC-OC display stronger bone destruction capacity compared to monocyte-derived osteoclasts, while maintaining their APC characteristics, such as expression of MHC class II and CD80 and CD86, and ability to stimulate T cells [61]. DC and osteoclasts arise from the same progenitor cells which validates their potential of transdifferentiation, alongside the expression of the same receptors on the cell membrane and the overlap of several signalling pathways. For instance, disturbance in RANKL signalling impairs OC activity which results in both abnormal bone growth and may disturb DC survival [62]. These cells work synergistically as DC can mature upon uptake fragments of osteoclast-

mediated bone resorption and osteoclasts functions can be sustained by DC activity. Together, these evidences suggest DC are major drivers initially on the onset of the disease and then on RA progression, and subsequent bone destruction.

1.3. Extracellular Vesicles

1.3.1. EV biogenesis and interaction with target cell

Extracellular Vesicles (EV) are lipid vesicles naturally released by cells that have been gaining considerable attention in different fields of research. Peter Wolf, trying to clarify the manner in which platelets are involved in the mechanism of blood clotting, identified the presence of lipid-rich particles upon ultracentrifugation of serum and plasma, using electron microscopy. Also, the implication of these particles in the activation of platelets was suggested [63]. Early, electron microscopy examination demonstrated vesicular structures within the cartilage matrix of mice, indicating an association of these vesicles with cartilage calcification and naming them matrix vesicles [64]. In 1981, the term “exosome” was suggested to describe the plasma membrane derived vesicles [65]. Pioneering work showed that immune cells, such as B cells and DC release functional EV, containing antigen presenting molecules and able to activate T cells [66, 67]. In the recent years, it has become clear that all types of cells, both prokaryotic and eukaryotic, produce EV and they play an important role in the communication between cells.

The International Society for Extracellular Vesicles (ISEV) endorses the use of “EV" to describe the heterogeneous population vesicles delimited by a lipid bilayer and naturally released from cells, which includes apoptotic bodies, microvesicles and exosomes [68]. Each type of EV is primarily classified based on their size and biogenesis [69]. Apoptotic bodies (AB) may reach larger sizes (> 1 μm) [70] and, as the nomenclature suggests, they are released through the disintegration of the plasma membrane of apoptotic or dying cells [71]. AB are rapidly cleared by other cells to avoid inflammation but their role in cell proliferation and differentiation has been reported [72]. Microvesicles (MV) are vesicles with sizes ranging from 100-1000 nm formed directly from the surface membrane of the cells, while exosomes are derived from the intraluminal bud of multivesicular endosomes and have sizes smaller than 200 nm [70]. In the past, EV were thought to be cell debris, but, exosomes in particular have been reported to contain biologically important factors, such as proteins and genetic material [73].

Exosomes are the EV that have attracted the most attention from researchers. Their composition has been extensively studied and proteomics revealed a set of proteins that may

be used as markers, including the tetraspanins CD63, CD81 and CD9, Alix, TSG101 and Hsp70 [74]. Nonetheless, establishing the intracellular origin of EV is technically very challenging, so the recommendation of the ISEV community is to describe the characteristics of EV, like size, density and molecular markers [68]. These small EV are vesicles surrounded by a lipid layer, composed of different types of phospholipids, sphingomyelin, cholesterol and ceramide enclose membrane proteins (Alix, TSG 101), the aforementioned tetraspanins, heat-shock proteins (e.g., Hsp60/70/90, Hsc70), antigens (MHC class I and MHC class II), enzymes (phosphate isomerase, peroxiredoxin, aldehyde reductase, fatty acid synthase), cytoskeleton components (e.g., actin and tubulin), cytokines, DNA, different types of RNA (microRNAs and messenger RNA), and metabolites [75, 76]. Secreted alone, these molecules would be susceptible to degradation in the extracellular microenvironment, thus exosomes are of great significance in paracrine signal as they shield the bioactive molecules, protecting them from proteolytic attack [77].

Besides size, the fundamental biological distinction between MV and exosomes relies on their cellular origin. Whereas the first are derived from the surface membrane of the cells in a process dependent on actin rearrangements, exosomes are formed by the inward budding of endosomal membrane during the maturation of endosomes. These invaginations then form intraluminal vesicles (ILV) inside the endosomes, which are, at this point, named multivesicular bodies (MVB) [78]. The ubiquitylation of proteins that have been internalised or transported from the trans-Golgi network functions as an incorporation mechanism of molecules into ILV. Among assembly and incorporation of molecules in exosomes mechanisms, the route dependent on the Endosomal Sorting Complex Required for Transport (ESCRT), which comprises 4 major complexes (ESCRT-0, -I, -II, and -III) has been intensively studied,. Importantly, these complexes are responsible for the recognition and docking of ubiquitinated proteins in ILV upon further deformation of the endosomal membrane into a vesicle-like shape [79]. Subsequently, MVB bearing ILV/exosomes can then follow the secretory pathway and fuse with the plasma membrane of the cell, releasing their content to the extracellular milieu, or the lysosomal pathway for degradation (Figure 1.2) [80]. Conversely, there are also ubiquitylation-independent pathways, and, particularly in APC, the sorting of MHC-II to ILV is facilitated by membrane microdomains formed by tetraspanins, such as CD9, CD63, CD81, and CD82 [81]. In DC, the route followed by peptide-MHC-II complexes depends on cells maturation stage. MVB have been suggested to be the cellular compartment where MHC-II is maintained until DC activation. DC maturation triggers the fusion of ILV-bearing MHC-II in the MVB membrane and subsequent transport to the plasma membrane [82]. Moreover, ubiquitination and further lysosomal degradation, and transportation held by CD9 to exosomes have been routes proposed for peptide-loaded MHC-II fate in immature DC and mature DC,

respectively. Particularly in DC, AlP1/Alix/Vps31, TGS101/Vps23 and ubiquitinated proteins have been demonstrated to be mandatory for the release of exosomes by these APC. Additionally, the ESCRT-0 is also required for exosome assembly and secretion by DC, and their antigen presentation characteristics [81]. Importantly, CD9 seems to be a key molecule in exosomes release to the extracellular milieu as DC obtained from CD9 knockout mice produce reduced amounts of EV compared to those obtained from wild-type DC [83].

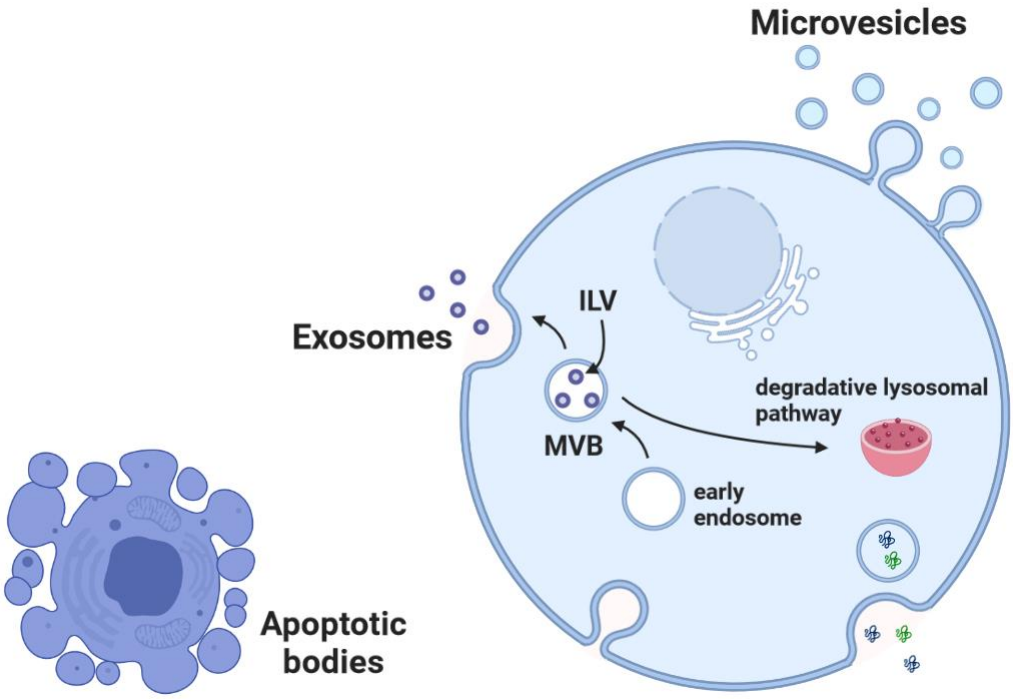

Figure 1.2. Biogenesis of extracellular vesicles. Exosomes are released when the multivesicular bodies (MVB), derived from early endosomes, fuse with the cell membrane. Microvesicles are derived from the plasma membrane of the cells. Apoptotic bodies are vesicles derived from apoptotic cells.

The interest behind EV and their biological role arises from their ability to induce phenotypic changes in target cells. EV have adhesion molecules on their surface that bind to the corresponding receptors on the recipient cell, which might be crucial to determine in what cells EV can act. For instance, DC-EV express intercellular adhesion molecule 1 (ICAM-1), and phosphatidylserine that binds to the LFA-1-bearing activated cytotoxic T cells and milk fat globule EGF/factor VIII, respectively, docking the EV on the cell surface [84, 85]. Other immune cells, namely T cells, also release EV carrying integrins, such as integrin α4β7, which promotes the interaction with its ligand MadCAM-1 expressed in the small intestine. Regarding non-immune cells-derived EV, equal anchorage of the vesicles on the cell membrane is observed, for instance, EV released by murine oviductal fluid carries αV subunit of αVβ3 which binds to the corresponding integrin on mouse sperm [86]. EV surface decoration influences

the targeting and might also impact the process of interaction with recipient cells as several have been described, namely receptor-ligand interaction, internalisation, or fusion with the cell membrane (Figure 1.3).

In **receptor-ligand interaction** molecules that decorate the membrane of EV can bind to surface molecules exposed on the cell membrane, activating intracellular signalling pathways, without the delivery of their content. This is particularly evident in EV-mediated communication between cells of the immune system. APC shed EV-bearing MHC and co-stimulatory molecules that induce activation of T lymphocytes [66]. Also, DC-derived EV with peptide-loaded MHC can anchor on DC surface to potentiate T cell responses, or directly affect T cells fate through CD40 and DC-SIGN signalling, which promotes IFN-γ secretion and Th1 polarization [87]. Moreover, the presence/absence of certain molecules generates different reactions, which underlines the great importance of this type of contact. For instance, APC-derived EV can interact with T CD8+ cells *via* MHC class I and ICAM-1. EV co-expressing both ICAM-1 and B7 are strongly immunogenic and induce T CD8+ cell to mount a peptide-specific proliferative responses, while EV lacking B7 can be nonimmunogenic [88].

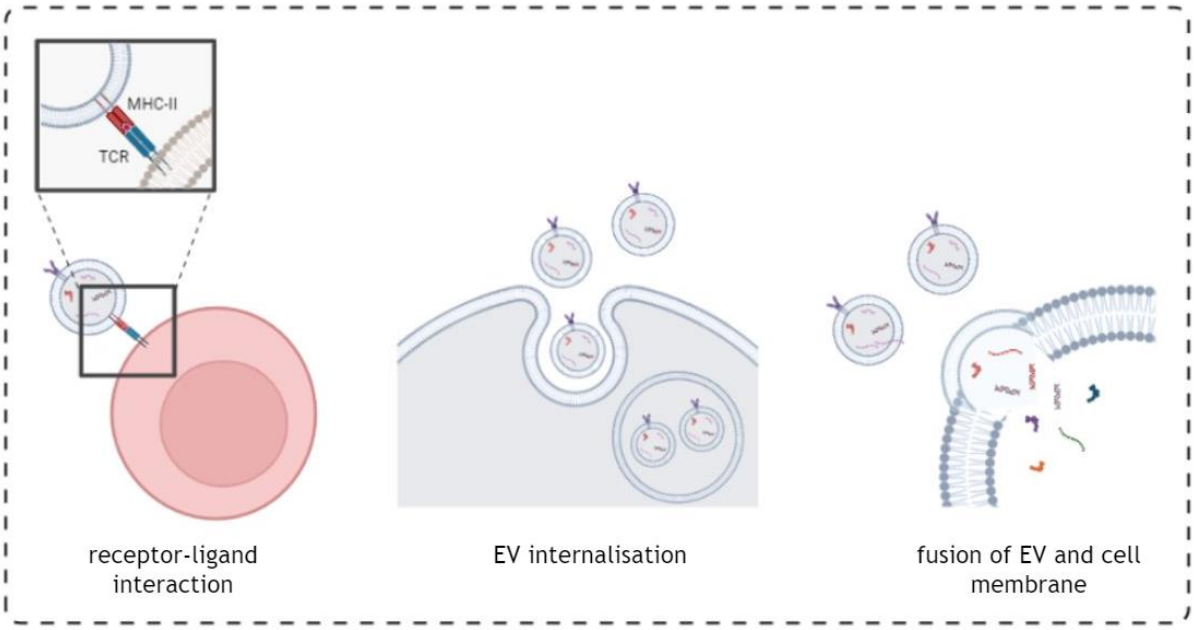

Figure 1.3. Routes of EV-mediated communication. The communication between EV and target cells is mainly described to be through receptor-ligand signalling, internalisation mainly by endocytosis, and fusion of both EV and target cell lipidic membranes with released of the content of EV within the cytosol.

Internalisation is a process mediated by EV membrane proteins, such as CD9 or CD81. These tetraspanins are key molecules in EV biogenesis, as previously described, and are additionally implicated on EV uptake by recipient cells, as knockdown, for instance, of CD9 in

EV derived from tumour cells impairs their internalisation [89]. Cellular internalisation of EV relies on endocytosis, phagocytosis, or macropinocytosis processes, although the first is the most described pathway of cellular uptake [90]. Interestingly, endocytosis experiments pinpoint to a low capacity of EV internalisation by cells, even at considered high doses (100 µg/mL) [87]. However, EV internalisation capacity may be highly correlated with cell type, as they behave differently, and distinct uptake rates may be taken into consideration. Concerning endocytosis, several mechanisms have been described for EV internalisation, including clathrin-depend, caveolae, and/or cholesterol-rich lipid rafts ones [91]. When inside the cell, the fate of EV remains unclear, however a great number of EV may follow the lysosome pathway for further degradation, as fluorescent reporters combined with EV markers have unraveled their colocalization [92]. Considering internalisation by endocytosis, the endosome is the cellular compartment that shelters EV inside the cell, however, at this point, the EV cargo remains inaccessible as it is still surrounded by a lipidic physical barrier. The fusion of EV and endosome membranes mediated by the acidic environment of the endosome and further release of EV content to the cytosol has been proposed. Alternatively, direct contact of endosomes enclosing EV with target cellular compartments, such as the nucleus and endoplasmic reticulum has been described [93]. Moreover, cells that uptake EV can simply function as hosts for a certain period of time as EV in early endosomes can move to late endosomes or MVB that fuse with the cell membrane releasing the EV again. This route might be important concerning cellular barrier crossing such as epithelium or blood brain barrier and reach the actual target cells [91].

Fusion with the cell membrane involves direct delivery of enclosed bioactive molecules into the cytosol that is less addressed due to difficulties that researchers face to study this process. In this regard, the mechanism proposed for the integration of EV membrane with the cell membrane, and consequent release of the molecules in their lumen, is similar to the above mentioned concerning the fusion of EV with endosomes. Briefly, an acidic microenvironment might promote modifications in the membranes that favour their fusion, and consequently release of their content within the cytosol [91].

Upon secretion, EV hold mechanisms to “fly under the radar” and remain longer in circulation. These mechanisms include the expression of CD47, commonly known as “don’t eat me” molecule, that protects from phagocytosis by macrophages and monocytes [94]. Remarkably, this leads to a more effective EV delivery to target cells, pinpointing a passive targeting mechanism that underlies a negative selection process. Importantly, studies investigating the possible mechanisms of EV interaction with cells are mainly operated *in vitro*, as *in vivo* studies require high-resolution image labelling techniques to EV detection. Future studies should discuss whether the preference for a type of interaction over another is related

to EV features, such as biogenesis, size, or content. To clarify the role and mode of action of EV, their recovery from biological fluids or cell culture supernatants is a key step to conduct robust investigations.

1.3.2. EV isolation methods

To study EV there is a need to isolate, purify or at least enrich them in the sample to be analysed. EV can be secreted in *in vitro* culture, and can also be found in biological fluids, such as blood plasma [95], urine [96], saliva [97], milk [98], or synovial fluid [99]. Suitable methods to isolate EV from cell culture conditioned media or bodily fluids are of great importance, as the EV samples purity and concentration depends on the effectiveness of the isolation method. There are several methods to recover EV with different efficiencies degrees: differential (ultra)centrifugation, size-exclusion chromatography (SEC), polymer precipitation, filtration, immunoaffinity isolation, or microfluidics techniques. A worldwide survey on isolation methods indicated that ultracentrifugation (UC), which includes differential centrifugations, remains the “gold standard” method of isolation, primarily for EV recovery from cell culture conditioned media [100]. The same survey also reports that the combination of different isolation methods is quite frequent in laboratories.

UC isolation method, with intermediate gradually increasing speed centrifugations, is dependent on particle density, and, consequently, sedimentation. The first low-speed centrifugations are conceived to eliminate cells, and their larger residues, while the final spinning step, whose speeds may range from 100,000-200,000x *g*, produces a pellet enriched in small EV, that can be further resuspended in phosphate-buffered solution (PBS), and then stored at –80 ºC [101]. Some disadvantages have been attributed to this method, as pelleting by UC provides low yields, induces EV aggregation and membrane can rupture during resuspension of EV pellets [102]. Conversely, recovery by SEC has been increasingly used as it may enhance EV membrane stability, because EV are not subjected to shear stress. Isolation of EV by SEC relies on the different sizes of the components of the fluid. In this separation technique, a stationary phase column with a porous gel filtration polymer is used, and particles smaller than the pore size get entrapped in the matrix, whereas larger molecules get eluted first [101]. These two conventional methods of isolation have been coupled together, particularly to recover EV from plasma, and have revealed great yield and purity [103, 104]. The UC step allows cells and debris segregation from EV, while SEC removes soluble proteins that pellet along EV [105]. Other strategies combined with UC, such as the use of a sucrose gradient or prior filtration steps, may help as well to separate EV from contaminants.

Other EV purification methods involve polymer precipitation, and low-speed centrifugations. The decrease of EV solubility, mediated using a polymer solution is the fundamental principle behind this technique. Polyethylene glycol (PEG) is commonly used to entrap EV, and its combination with protamine has shown to be more efficient on EV recovery from small volumes of fluids than UC [106]. Interestingly, novel approaches of EV purification arise, and the combination of immunoaffinity and microfluidic devices present considerable advantages as well as great EV recovery. These advantages are related with microfluidic devices small size, and minimized reagent volumes required, or less time-consuming protocols. For this approach, the microfluidic platforms are typically coated with antibodies that bind to signature EV receptors, namely CD9, CD63, and CD81, and further released for EV recovery [107].

Ranging from pelleting of non-vesicular particles along with EV to low recovery yield, all separation techniques display their own limitations, and for that reason, choosing the most adequate EV purification method remains a very challenging task. This becomes more complex depending on the starting fluid, as, in principle, cell culture conditioned media have less contaminants than, for instance, blood. Heterogeneity within the EV family and complexity of fluids are some aspects that hamper EV segregation, therefore, separation of different EV types and optimization of isolation protocols are still a challenge in the EV field.

1.3.3. EV characterization methods

After isolation, EV characterization is very important to evaluate the characteristics (e.g., morphology, size, concentration) and purity of the obtained population.

Electron microscopy is one of the tools that helps to identify the presence, heterogeneity and purity of EV samples. For example, transmission electron microscopy (TEM) enables the rapid recognition of EV since they display a typical morphology under this technique and determination of the size of individual EV [108, 109]. Despite having a natural round-shape, EV acquire a cup-shaped morphology as TEM is performed in a vacuum environment which demands fixation and dehydration of the samples, causing EV collapse and changes in their morphology. However, cryo electron microscopy reveals their typical round-shaped morphology [110]. Visualization of EV under TEM requires a relatively simple negative staining protocol, in which imaging results meet the necessary parameters for EV samples evaluation already stated. In this method, samples are allowed to adsorb onto a substrate, commonly a layer of amorphous carbon supported by a thin layer of a polymer, followed by removal of excess liquid, and staining [109]. Among negative staining reagents, uranyl acetate is widely applied as a contrast agent in EV preparations [111-114]. Briefly, an image is captured by electron perturbation when the electron beam hits the sample, and, as

EV have a nanoscale size, TEM is especially useful since images are recorded with 1 nm resolution [115].

Besides, other techniques, including nanoparticle tracking analysis (NTA), dynamic light scattering (DLS), atomic force microscopy (AFM), and resistive pulse sensing are commonly performed for physical and quantitative analysis [116]. Commonly detailed in EV-related publications to characterize isolated samples, NTA technology takes advantage of the Brownian motion and movement of particles in suspension, and analyses the vesicles on a single-particle level, allowing results such as EV concentration and size distribution. Despite providing information of great importance for EV characterization, NTA is associated with some limitations, namely failing to acknowledge particle sizes smaller than 50 nm and overestimating the sample concentration [117]. AFM is also a very powerful tool to take a closer look to EV, especially the surface topography of the vesicles. This technique relies on the interactions between a probing tip and EV surface, as when the tip gets closer to the vesicles, the interaction of forces leads to its deflection, and further recorded by a system based on a laser and detector. In order to be successful, the samples must adhere to a non- or functionalized substrate (e.g., antibody-coated surfaces), such as mica or glass [118].

The biochemical and compositional analysis is usually performed using flow cytometry and Western blotting to confirm the presence of the classically tetraspanins, namely CD9, CD63, CD81, or proteomic analysis to determine the EV content [116].

1.4. EV as mediators of intercellular communication

1.4.1. EV in the context of inflammation and regeneration

EV participate in cell communication during key biological processes of tissue repair and regeneration, including angiogenesis [119], cell proliferation, migration and invasion [120], immune cell regulation and differentiation [121], and modulation of the inflammatory response. Upon injury, acute inflammation is the first stage of repair and regeneration of all tissues, and during this stage several cues create an environment prone to cells recruitment and proliferation that contribute to tissue restoration. However, early inflammation must be controlled in order to avoid chronic inflammatory conditions that bear serious clinical complications [122]. In physiological conditions, EV seem to play an important role in mediating the transition from a pro- to an anti-inflammatory state. For instance, EV loaded with miR-126-3p and derived from synovial fibroblasts promoted rat chondrocyte migration and proliferation and downregulated the expression of pro-inflammatory factors (IL-1β, IL-6, and TNF-α). These EV displayed anti-apoptotic and anti-inflammatory effects, preventing cartilage degeneration [123]. Moreover, EV also reduce inflammation and apoptosis in the spinal cord

by inhibition of pro-inflammatory cytokines produced by microglia [124, 125]. Interestingly, naïve macrophages from bone-marrow produce EV containing anti-inflammatory microRNAs (miR-99a, miR-146b and miR-378a), that acts on recipient macrophages, influencing them to polarise into M2 macrophages and suppress inflammation, by targeting NF-κF and TNF-α signalling [126].

However, in severe inflammatory conditions, EV may contribute to tissue damage. EV from the serum of patients with coronary artery disease were shown to significantly impair angiogenesis and human umbilical vein endothelial cells (HUVECs) migration. Also, IL-1β and TNF-α were overexpressed in HUVECs stimulated with EV from those patients [127]. Furthermore, EV carrying miRNA-21 released by cells of hepatocellular carcinoma, an inflammation-related tumour, activate cancer-associated fibroblasts (CAF) to produce VEGF, matrix metalloproteinases (MMP)-2, MMP-9, basic fibroblast growth factor (bFGF) and TGF-β that enable tumour progression [128]. In other liver-related pathologies, particularly non-alcoholic fatty liver disease, EV released by hepatocytes promote endothelial damage through miR-1-induced inflammation marked by increased NF-κB activity and downregulation of Kruppel-like factor 4 (KLF4) [129]. Furthermore, the acute-phase protein C-reactive protein (CRP) can be transported by EV from the liver and dispersed throughout the bloodstream. In sepsis patients, circulating EV are associated with monomeric CRP, while undetectable in healthy individuals. Monomeric CRP pro-inflammatory characteristics, as opposed to the majority of CRP that circulates in the vasculature, which has a pentameric structure. The release of chemoattractant molecules, namely IL-8, by monocytes is induced by EV-associated monomeric CRP, which promotes neutrophil recruitment and the spread of inflammation [130]. These pieces of evidence suggest that EV activity in target cells strongly depends on the physiological and pathological conditions of the tissue and that inflammation plays a central role in the impairment of repair and regeneration, including through EV communication and the mediators that they transport.

EV biological role in repair and regeneration is a growing object of research, but several questions remain answered. Some studies pinpoint the positive effects of EV on tissue regeneration. For instance, EV produced by adipose-derived stem cells promote regeneration of the myelin sheath in rat peripheral nerve injury (PNI) models. These EV contained miRNA-26b that downregulated karyopherin subunit alpha 2 (Kpna2) expression in Schwann cells, inhibiting the enhanced autophagy upon PNI [131]. Moreover, EV from skin precursor-derived Schwann cells were shown to regulate neuronal outgrowth or regrowth on motoneurons both in vitro and in vivo. The recovery of injured neurons is attributed to the Akt/mTOR/p70S6K signalling pathway activation mediated by EV [132]. Particularly in bone repair/regeneration, the same duality aforementioned can be observed, as EV can both promote osteogenesis or

bone destruction. Importantly, EV impact on enhancement/impairment of regeneration seem to strongly depend on cell source and, among others, their microRNA (miR) content as briefly illustrated in Table 1.

Although EV from other cells have been implicated in tissue healing, MSC-derived EV are the most intensively studied in the context of regeneration. Lately, it has been suggested that MSC-EV alone have the same MSC functions and therapeuticl effects [133]. Recent studies have demonstrated how MSC-derived EV impact the regenerative process. For example, EV from MSC were injected in rats with osteochondral defects created on the trochlear grooves of both distal femurs, and, after 12 weeks, the animals exhibited complete restoration of cartilage and subchondral bone, which indicates that EV from MSC may be effective in cartilage repair [134]. Also, MSC-derived EV improve cellular migration, angiogenic and osteogenic gene expression, and bone formation, confirmed *in vivo* [135]. As their parent cells, MSC-derived EV have immunomodulatory properties. In fact, the immunomodulatory response is one of central functions of MSC-derived EV, and evidence shows that EV derived from MSC modulate both innate and adaptive immune responses, affecting, for example, the behaviour of DC [136], macrophages and B cells [137] and T cells [138]. MSC-derived EV administered to a mouse model of liver injury suppressed tissue inflammation and increased the levels of TGF-β and HGF, which are involved in liver regeneration [139]. EV produced by MSC stimulated with LPS have the ability to promote macrophage polarisation to the anti-inflammatory M2 phenotype. It was identified the distinctive presence of let-7b in the EV from cells treated with LPS, which may work as a mediator of inflammatory suppression [140]. Also, it was demonstrated that bone marrow-derived MSC can suppress inflammation triggered by Th1 cells expressing CD39 through the delivery of EV bearing CD73 capable of producing adenosine [4].

Table 1.1. MicroRNA associated with EV that influence osteoblast or osteoclast activity.

Content	Cell Source	Effect	Reference
miR-29a and miR-1260	BMSC	Promote angiogenesis and osteogenesis	[141, 142]
miR-690	M2-like macrophages	Upregulate IRS-1 and TAZ levels	[143]
miR-122-5p	BMSC	Promote proliferation of osteoblasts	[144]
miR-335	DC	Inhibit the Hippo signalling pathway and enhance bone regeneration *in vivo*	[19]

miR-181b-5p	Osteocyte	Promote human periodontal ligament stem cell osteogenic differentiation through PTEN downregulation	[145]
miR-378a	M2-like macrophages	Enhance osteogenesis through positive regulation of BMP2 signalling	[14]
miR-5106	M2-like macrophages	Promote MSC osteogenic differentiation by targeting Salt-inducible kinase 2 and 3 (SIK2 and SIK3)	[146]
miR-23a-5p	OC	Inhibit osteogenic differentiation by regulating RUNX2	[147]
miR-214-3p	OC	Inhibit osteoblastic bone formation through the PI3K/AKT pathway	[148]
miR-31	HUVECS (aging endothelial cells)	Inhibit osteogenic differentiation of MSC via Frizzled-3 restraint	[149]
miR-30a and miR-23a	Neoplastic mast cells	Inhibit osteoblast maturation by downregulating expression of RUNX2 and SMAD1/5	[150]
miR-143	OB	Inhibit osteoblastogenesis by targeting RUNX2 dimerization partner, core-binding factor β	[151]
miR-155	M1-like macrophages	Inhibit osteoblastic differentiation by targeting BMP signalling	[14]

1.4.2. Immune cells-derived EV in regeneration and communication with MSC

EV secreted by APC have been intensively studied in the connection of the innate and adaptive immune response, namely in infection and cancer conditions. Particularly DC, have been intensively studied as capable of stimulating the adaptive immune response, activating T cells. Demonstration of APC ability to release EV bearing antigen-MHC class I or MHC class II complexes and co-stimulatory molecules (e.g., CD86), and participate in antigen presentation was a key milestone in the EV field [66]. In 1998, mouse DC-derived EV (DC-EV) were shown to be able to induce anti-tumour T-cell activation, through their MHC class I and class II and co-stimulatory molecules [67]. Additionally, the efficiency of DC-EV in antigen presentation is magnified when anchored to the DC surface [152]. Moreover, *in vitro* DC-EV promoted the differentiation of monocytes into immature DC and the exchange of functional peptide-MHC complexes between DC and MHC class II negative mature DC for indirect antigen presentation. On the other hand, *in vivo*, DC-EV stimulated antigen-specific activation of naïve CD4+ T cells [153, 154]. Altogether, these evidences suggest that DC-EV are important mediators during the immune innate and adaptive crosstalk, and could contribute to perpetuate chronic inflammation through T cell activation. However, there is also evidence that EV produced by immature DC induce a tolerogenic response in transplanted rats by upregulating T regulatory cells (CD4+CD25+FoxP3+ T cells) (Treg) [155]. Importantly, EV

content is strongly influenced by the microenvironment that the parent cells are in. Lipopolysaccharide (LPS), tumour necrosis factor (TNF)-α, interferon (IFN)-γ, hypoxia, and even the environmental pH have been shown to affect the type of molecules that EV carry at the surface and in their lumen, and the quantity of EV that parent cells release [75, 156]. For example, stimulation of DC with LPS leads to the upregulation of MHC class II, CD86, and the adhesion molecule ICAM-1 on the EV they produce [157].

Therapies based on EV from DC have been gaining considerable attention throughout recent years, especially in the cancer field, and seem to have advantages over the classical cell-based therapies, regarding for instance the storage process that enables EV to keep their immunotherapeutic activity [158]. Evidence suggests that DC-derived EV, with TNF and Fas ligand (FasL) receptors on their surface can kill tumour cells through ligands-receptors interaction. Also, the killing of cancer cells was more evident when DC that produced the EV were exposed to danger signals [159]. The EV derived from mature DC ability to kill several types of tumour cells, including melanoma, squamous lung carcinoma, or colon carcinoma, were dependent on exposure time and dose provided. Several clinical trials are ongoing to test the efficiency of EV as an anti-cancer therapy. For example, a phase II clinical trial aiming to test the clinical effect of DC-derived EV in patients with inoperable non-small cell lung cancer after chemotherapy confirmed the capacity of DC-EV to enhance Natural Killer (NK) cells anti-tumour activity [160].

Identical to DC, macrophages release EV containing both MHC-I and MHC-II, and costimulatory molecules (e.g., CD86) and capable of stimulating T cells [161, 162]. EV produced by *Mycobacterium bovis* BCG-infected macrophages promote CD4+ and CD8+ T cells memory, and IFN-γ production, showing the importance of EV in regulating an effective immune response [162]. When infected with *Mycobacterium tuberculosis*, *Salmonella typhimurium*, or *Toxoplasma gondii*, macrophages release EV that carry pathogen-associated molecular patterns (PAMPs) that activate non-infected macrophages to produce a proinflammatory response and stimulate the secretion of TNF-α and IL-12 *in vivo* [161]. Importantly, EV may carry cytokines capable of mediating the inflammatory status of the microenvironment they are in. Macrophages stimulated with LPS produce EV with anti-inflammatory mediators (e.g., G-CSF and IL-1ra), TNF-α, and LPS-responsive RNAs, that activate the NF-kB pathway, supporting the EV importance and mainly the molecules they carry in recipient cells behaviour switch [163, 164]. Furthermore, and interestingly, macrophages cooperate with DC through the EV they release, and consequently participate in CD4+ T cell response, by transporting phagocytosed antigen to DC via EV [165]. The study of EV released by macrophages seems to be focused on their effect on target cells upon microbial infection of macrophages.

In the context of repair and regeneration, immune cells-derived EV potential therapeutic role and communication with MSC remain relatively unexplored. However, interest has been growing and several recent studies have explored the roles of particularly macrophage and DC secreted EV in tissue repair/regeneration. Angiogenesis is an important stage in tissue repair and regeneration, and EV produced by macrophages upon cobalt, copper, and zinc ion stimulation were shown to drive this process [166-168]. These studies support that EV may promote endothelial migration and upregulation of nitric oxide, VEGF, and integrin-β1 gene expression, that are correlated with the mechanisms that regulate angiogenesis. Moreover, EV secreted by macrophages treated with zinc also promote osteogenesis, as the uptake of EV by osteoblasts promoted elevated ALP activity. Additionally, EV released from LPS-stimulated monocytes were shown to induce increased osteogenic activity in MSC, associated with the upregulation of RUNX2 and BMP-2 compared to the control group [169]. Importantly, a recent study compared the impact of EV from different macrophage phenotypes on tissue repair/regeneration. The authors demonstrated that EV derived from M1 polarised macrophages (M1-EV) inhibit bone repair by negatively regulating bone morphogenetic protein (BMP) signalling, specially BMP2 and BMP9, while naïve M0-EV and anti-inflammatory M2-EV promote repair and regeneration. Interestingly, the miRNA profiles of the M0-EV and M2-EV are different from the M1-EV [14]. The migration of cells to damaged sites requires MMP activity for extracellular matrix remodeling. Exposing macrophages to harmful substances influences them to release EV carrying increased levels of MMP [170], that may enable target cells to migrate to the lesioned site. Also, monocytes transdifferentiated into “keratinocyte-like cells” stimulated skin fibroblasts to produce higher levels of MMP-1, through EV containing 14-3-3 proteins, which alleviates fibrogenic activity [171]. Besides macrophages, also DC have been reported to have the ability to recruit MSC via paracrine mediators, and that recruitment was significantly impaired by a TNF-α enriched environment [172]. Later EV were shown to be the secreted component responsible for MSC recruitment by DC [173]. Besides influencing MSC migration, DC-derived EV may also contribute to MSC differentiation of bone marrow-MSC, by delivering miR-335. EV miR-335 inhibited Hippo signalling pathway and improved bone regeneration in vivo [19]. Furthermore, DC-EV carrying TGF-β1 and IL-10 demonstrated to have anti-degenerative properties in the bone context, by inhibiting alveolar bone loss *in vivo*. Data suggested that these vesicles are internalised by DC and T cells, and affect their phenotype and function [77]. Experiments conducted with *in vivo* models also demonstrate the potential of EV derived from immunosuppressive DC treated with IL-10 to promote inflammation resolution in arthritis [174]. Thus, EV from immune cells participate in the tissue repair and regeneration processes, particularly the crosstalk between different immune cell populations and between immune and

tissue cells. Nevertheless, studies regarding this communication are still scarce and need to be pursued to support their use in new therapeutic applications.

1.5. EV-based Therapeutic Approaches

Recently, EV biological features, including 1) small size, 2) low immunogenicity, 3) ability to cross biological barriers, 4) targeted delivery, 5) easy uptake by cells, 6) delivery of therapeutic molecules, 7) cargo loading, and 8) stability in circulation [175], made them into attractive candidates for cell-based therapies, but cell-free therapies. Beyond their therapeutic potential, EV content analysis may also be helpful in the clinic for a diagnostic and prognostic purposes since they provide a source of biomarkers particularly overexpressed in some diseases such as cancer [176].

As they seem to recapitulate parent cell function, EV can possibly substitute therapies that include delivery of cells. Injection of free cells still carries some problems associated with cells extraordinary responsiveness to the microenvironment cues they are exposed to that can promote a non-therapeutic phenotype. Furthermore, stem cells-based therapies can induce tumour [177]. EV potential cargoes include nucleic acids, proteins, and drugs, and methods of EV loading can be separated into *ex vivo* and *in vitro* strategies [178]. EV can be isolated and then loaded with exogenous cargo. For this purpose, electroporation, a process that promotes the temporary formation of pores in membranes, is commonly used as a mechanism to introduce new cargo in the vesicles. However, a previous study points out that electroporation of EV with RNA may induce the aggregation of nucleic acids [179]. Alternatively, molecules can be introduced into EV during their biogenesis (endogenous loading), by incubating the parent cells with the therapeutic molecules, or transforming them to express particular proteins or nucleic acids. For the incorporation method during EV formation, the compatibility of the molecules is necessary, in order to avoid toxic effects on cells [180]. Manipulation of the isolated EV may potentially interfere with vesicle stability, thus incorporation of molecules during their biogenesis may have advantages in this aspect.

The delivery of selected molecules associated with EV has shown significant advantages in comparison to their delivery without any selected molecules loading [181, 182]. Delivery of anti-inflammatory agents, such as curcumin, associated with EV was demonstrated to improve solubility, stability and bioavailability of curcumin, with a significant increase of concentration in blood. Here, curcumin was loaded into the nanovesicles through entrapment of curcumin within their lipid bilayer mediated by hydrophobic interactions between drug and membrane, which protected curcumin from degradation. Results showed that macrophages produced lower levels of pro-inflammatory cytokines when treated with curcumin associated

with EV compared with curcumin alone [182]. Importantly, for drug loading based on active encapsulation, during EV biogenesis, it is crucial that cells survive to high concentrations of selected molecules. In this regard, MSC seem like good candidates to produce EV loaded with Paclitaxel, since they were shown to be resistant at concentrations higher than 10,000 ng/mL [183]. Paclitaxel is a drug used to suppress the proliferation of tumours and endothelial cells [183], and, in fact, their load in EV has already been explored by exogenously loading the drug into EV isolated from autologous cancer cells. In this study, the cytotoxic effect of Paclitaxel was shown to be enhanced when associated with EV [181]. More recently, and interestingly, there were also attempts to deliver Cas9 ribonucleoprotein for CRISPR-based genome editing *via* EV [184].

The strategies for EV administration are crucial for their ability to reach the target tissues and cells. Different routes of administration have been explored in *in vivo* and in clinical trials. These include strategies for EV systemic delivery, such as intravenous, oral, or intranasal administrations, and for local effects, which includes subcutaneous or intratumorally injections (Figure 1.4) [76]. Upon EV administration, it is important to monitor their biodistribution. The fate of EV can be followed both *in vivo* and *ex vivo*, and is mainly studied by labelling EV with fluorescent molecules, although other methods have been approached, including tracking of bioluminescent and radiolabelled EV [185]. The intravenous route of EV administration is the most reported in *in vivo* studies, therefore EV distribution is commonly examined following that administration approach [185]. For instance, luciferase-labelled EV administered via vein tail into mice were detected mainly in the liver and spleen, 30 minutes after injection [186]. Interestingly, the levels of EV in the blood decreased after 30 minutes, and a small amount increased in urine between 60 and 120 minutes, suggesting that some levels of EV are cleared through urine. The biodistribution of systemic delivered EV is not still clear, and injection of EV suspensions hampers the EV accumulation on target tissues and retention of the ideal concentrations for therapeutic impact in those sites [76]. Retention and timely controlled release of EV can be achieved following strategies like incorporation into biomaterials, which may enhance their bioavailability in target tissues. Many biomaterial-based approaches have been conducted for the delivery of EV, and their incorporation, for example, in sodium alginate hydrogels [187], collagen chitosan [188], and tricalcium phosphate scaffolds [189] is reported. The association of EV with scaffolds to promote bone regeneration has been intensively studied with *in vivo* models as illustrated in Table 1.2.

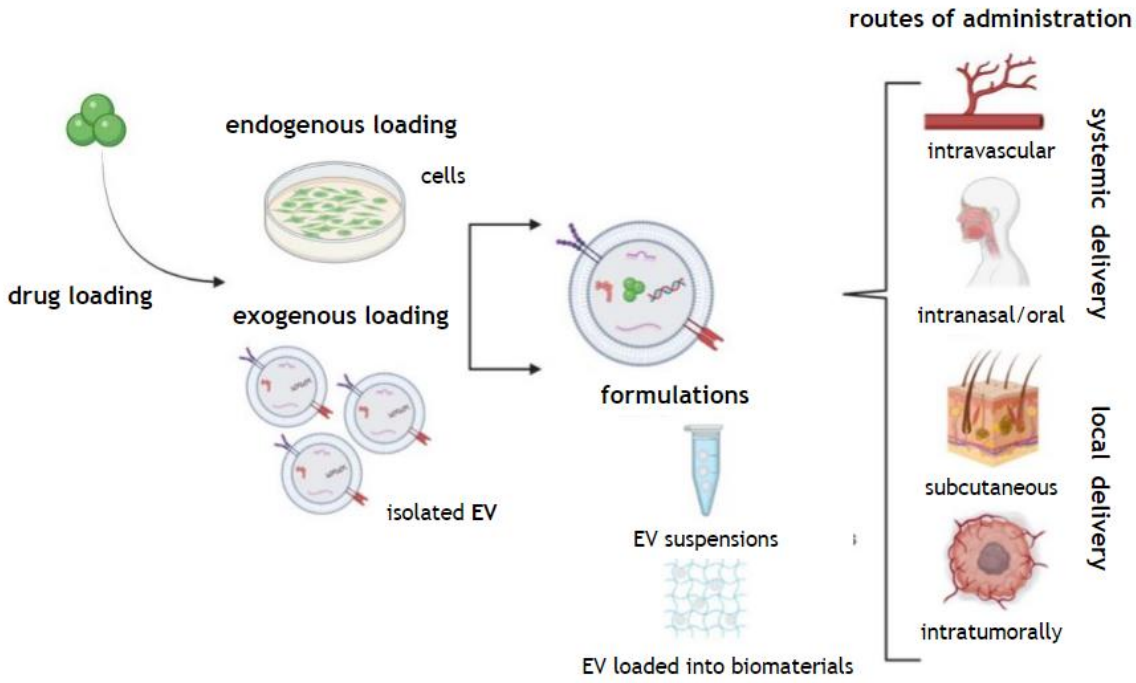

Figure 1.4. EV loading and administration. EV can carry nucleic acids, proteins or drugs of interest, which may be loaded into EV by endogenous or exogenous methods. Upon loading, EV can be administered in vivo in free suspensions or incorporated in biomaterials, following different routes.

The use of EV is recognised as a precious therapeutic tool, as EV can carry active molecules, such as nucleic acids, proteins, enzymes, or drugs, that modulate the behaviour of cells. When the word “exosomes” was submitted on clinicaltrials.gov, more than 200 studies were shown to be ongoing. These clinical trials address several pathological conditions (e.g., cancer, cerebrovascular disorders, or coronavirus disease, COVID-19, demonstrating the interest of the scientific community in these naturally produced nanoparticles for therapeutic applications.

Although considerable advances in EV characterization, isolation, and manipulation methods, and clarification of their participation in intercellular communication, several aspects need to be explored. Particularly, in the context of tissue repair/regeneration, understanding EV role in the transition between inflammation and repair upon injury remain underexplored and is crucial for their use in new therapeutic strategies.

Table 1.2. Bone cell-derived EV-integrated biomaterial scaffolds for bone regeneration. (Adapted from [190])

Source of Exosomes	Scaffold	*In vivo* Model	Function of Exosome-Integrated Scaffold
rBMSC	Alginate-PCL	Nude mouse subcutaneous bone formation model	Pro-angiogenic and pro-none regeneration activities in vitro and in vivo
rBMSC	Decalcified bone matrix (DBM)	Nude mouse subcutaneous bone formation model	Pro-angiogenic and pro-none regeneration activities in vitro and in vivo
hBMSC	Commercial hydroxyapatites scaffold	Rat calvarial defect model	Pro-angiogenic activities via Akt/mTOR pathway
hBMSC	Injectable chitosan hydrogel	Mouse calvarial defect model	Elevated osteogenesis via inhibition of miR-29a in BMP/Smad signalling
rBMSC	Decalcified bone matrix (DBM)	Rat subcutaneous implantation and Rabbit femoral condyle bone defect model	Human BMP2 plasmids were coated onto the vesicles Promoted bone formation and angiogenesis in animal model
hBMSC	Calcium sulphate/ nanohydroxyapatite based nanocement	Femur neck canal defect model in osteoporotic rats	Enhanced bone formation in the absence of BMP
hBMSC	Titanium allow	Osteoporotic bone defect model	miR-20a in hBMSC-EV was shown to play a key role in promoting osteogenesis
rBMSC	Mesoporous bioactive glass	Rat calvarial defect model	Osteoinductivity attributed to exosomal miRNAs (let-7a-5p, let-7c-5p, miR-328a, and miR-31a-5p)
hBMSC	Alginate-RGD hydrogel	Rat calvarial defect model	EV were tethered to biomaterial scaffolds with ECM proteins, which promote bone repair and prolonged delivery in vivo
Osteoclasts from mice	DBM	Rat calvarial defect model	Elevated osteogenesis via miR-324 in ARHGAP1/RhoA/ROCK signalling

1.6. Objectives

In the context of what was discussed, this thesis focuses on the evaluation of the impact of inflammation on Dendritic Cells (DC) and the characteristics of their secreted EV, including the capacity to recruit Mesenchymal Stem/Stromal Cells (MSC). To achieve that goal three specific objectives were delineated:

A. Optimize DC culture conditions to obtain EV of naïve and pro-inflammatory cells

Primary human peripheral blood monocytes were used to differentiate DC, that were then cultured in EV-free conditions. Different concentrations of FBS were tested, and DC maturation and viability assessed.

B. Characterize DC-EV secreted in naïve and pro-inflammatory conditions

EV morphology, size distribution and protein markers were evaluated to understand the impact of inflammatory conditions.

C. Determine the impact of DC-EV from inflammatory/non-inflammatory conditions on MSC recruitment

The effect of DC-EV produced in naïve or pro-inflammatory conditions on MSC migration was assessed by transwell migration assays.

2. MATERIALS AND METHODS

2.1. Ethics statement

Human primary monocytes were isolated from surplus buffy coats (BC) from healthy blood donors, kindly donated by Serviço de Imunohemoterapia, Centro Hospitalar Universitário de São João (CHUSJ), Porto. Human mesenchymal stem/stromal cells (MSCs) were obtained from healthy human bone marrow from patients undergoing knee ligament surgery, kindly donated by Serviço de Ortopedia, CHUSJ, Porto. All obtained human samples and procedures were performed in agreement with the principles of the Declaration of Helsinki and all experimental protocols were conducted following the approval and recommendations of the CHUSJ Ethics Committee for Health (references 90/19 and 260/11). A written informed consent was obtained from all subjects before sample collection. All samples were provided anonymized and patient identification was not provided to researchers.

2.2. Monocyte isolation, DC differentiation and EV production

Monocytes were isolated by a negative selection protocol as previously described (Figure 2.1) [173]. Briefly, blood was centrifuged (1,200 × g, for 20 min, room temperature (RT), no brake), and the peripheral blood mononuclear cells (PBMC) layer was collected. RosetteSep™ Monocyte Enrichment antibody cocktail (STEMCELL Technologies) was added to PBMC suspension, and then incubated for 20 min, under slow agitation. This mixture was diluted in 1:1 in 2% immune cells compatible FBS in sterile PBS, overlayed in Histopaque-1077 (Sigma-Aldrich Co), and centrifuged (1,200 × g, for 20 min, RT, no brake) (Figure 2.1). Finally, the monocyte layer was collected and washed in sterile PBS (300 × g, for 7 min, RT), at least 3 times.

Human peripheral blood monocytes were plated 2 × 10^6 cells/mL, plated at 10-15 × 10^6 cells in 100 mm diameter petri dishes, and allowed to differentiate to DC usingin RPMI-1640 + Glutamax (Invitrogen) cell culture media, with 10% heat inactivated FBS (Lonza) and 1% penicillin/streptomycin (v/v) (P/S; Invitrogen), further supplemented with 50 ng/mL recombinant human (rh)-IL-4 and rh-GM-CSF (Immunotools). After 4 days of differentiation, cells were re-cultured in RPMI supplemented with 0.5-1% FBS (previously ultracentrifuged at 100,000 × g, for 2 h at 4 °C),1% P/S (EV producing media), with or without inflammatory stimulation with rh-TNF-α (100 ng/mL) for 3 additional days. Cells were maintained in a humidified incubator, at 37 °C and 5% CO_2.

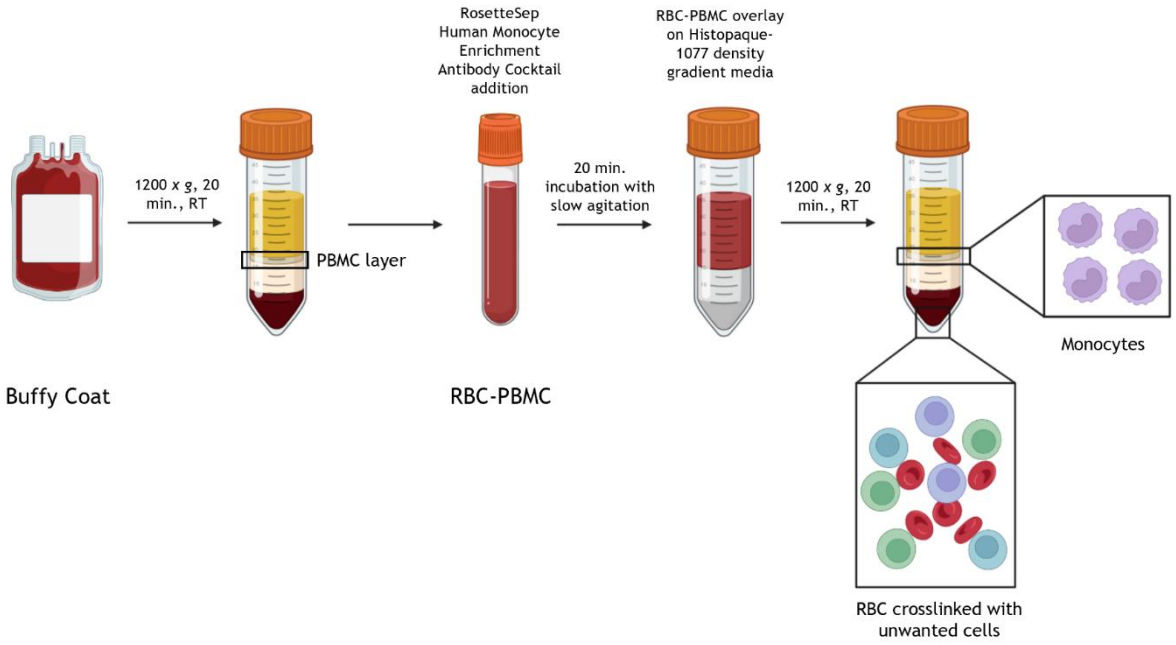

Figure 2.1. Monocyte isolation protocol schematics. The procedure is optimized under the recommendations of the manufacturer (STEMCELL Technologies). RBC – red blood cells; PBMC – peripheral blood mononuclear cells; RT – room temperature.

2.3. Viability assay

2.3.1. LDH activity

Viability of DC deprived from FBS was assessed using LDH assay (Promega), which quantitively measures lactate dehydrogenase (LDH). LDH released into culture media was quantified after a coupled enzymatic assay in which tetrazolium salt yields a formazan product. Absorbance was recorded at 490 nm.

2.4. Cytokines production

Conditioned media of DC cultures, stimulated as described, were centrifuged (300 × g, for 20 min., RT) at day 7, to remove cells, and stored at -20 ºC. A set of cytokines was assessed by enzyme-linked immunosorbent assay (ELISA, Legend Max Human ELISA kits, BioLegend, CA, USA): IL-1β, IL-6, IL-10, IL-12 (p70 subunit), and TNF-α. The assay was performed according to the manufacturer's protocol. Briefly, the Capture Antibody solution was added to the wells of a 96-well plate, and incubated overnight at 4 ºC. The plate was washed 4 times with Wash Buffer (PBS + 0.05% Tween-20), and non-specific binding blocked with Assay Diluent A solution, for 1h, RT, with slow agitation. Wells were again washed 4 times with Wash Buffer, diluted standards and samples were added, and incubated for 2h, RT, protected from light, under slow agitation. Wells were again washed with Wash Buffer, and Detection Antibody solution was added to each well, and incubated

for 1h, RT, with agitation. Wells were washed with Avidin-HRP solution to each well and incubated for 30 min., RT, with shaking. Wells were washed again 5 times with Wash Buffer, TMB substrate solution was added to each well, and incubated in the dark for 15 min. Finally, Stop Solution (2N H_2SO_4) was added to each well, and absorbances were read at 450 nm. Sample concentrations (pg/mL) were determined from absorbance values for each set of samples, and compared to a standard calibration curve.

2.5. EV isolation

DC-derived EV were isolated from cell culture supernatants, by the differential (ultra)-centrifugation protocol (Figure 2.2) [173]: cell culture media was collected and centrifuged at 300 × g, for 10 min., RT; 2,000 × g, for 20 min, at 4 °C; the supernatant was recovered and centrifuged at 10,000 × g, for 20 min, at 4 °C; the supernatant was again recovered and ultracentrifuged, at 100,000 × g, for 2 h, at 4 °C, using a 70Ti rotor in a Beckman Optima XE 100 ultracentrifuge. An additional purification of supernatants was tested by gravity filtration, to avoid the rupture of the vesicles, in some samples before the ultracentrifugation using 0.22 µm filters as described in [191]. EV pellets were resuspended in PBS and ultracentrifugation supernatants (UC control) were collected to be used as control. All samples were stored at −80 °C. For each EV or control sample, protein was quantified directly using DC™ Protein Assay (Bio-Rad) [173], and the calculated protein concentration was used as a measure for functional assays.

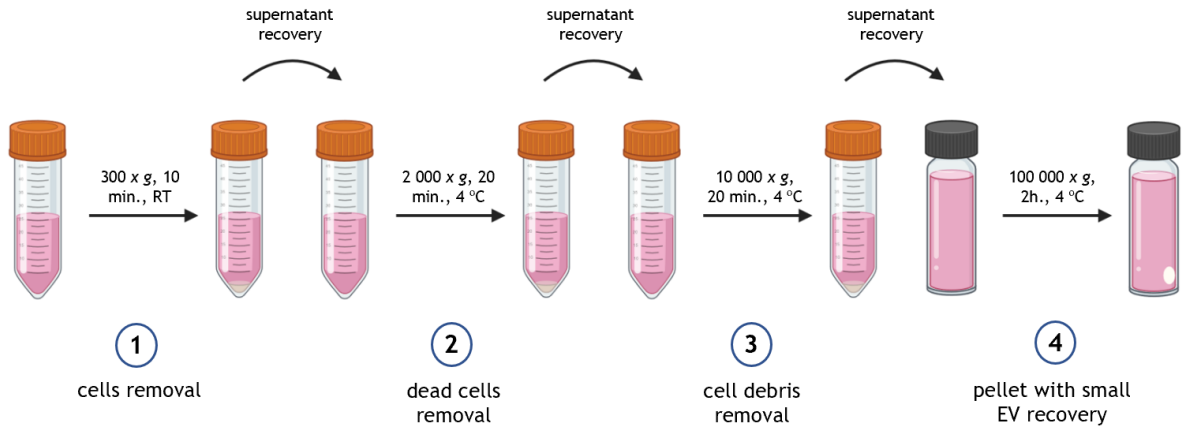

Figure 2.2. Sequence of centrifugations for EV isolation from cell culture media.

2.6. EV morphology and size characterization

EV morphology, size and concentration were characterised by transmission electron microscopy (TEM) and Nanoparticle Tracking Analysis (NTA).

2.6.1. Transmission Electron Microscopy

For TEM, DC-EV suspensions were diluted 1:1 in PBS, and 6 µL adsorbed onto 300-mesh formvar nickel grids and then negatively stained with uranyl acetate 5% for 10 seconds. Samples were imaged in a Jeol JEM 1400 electron microscope (Figure 2.3).

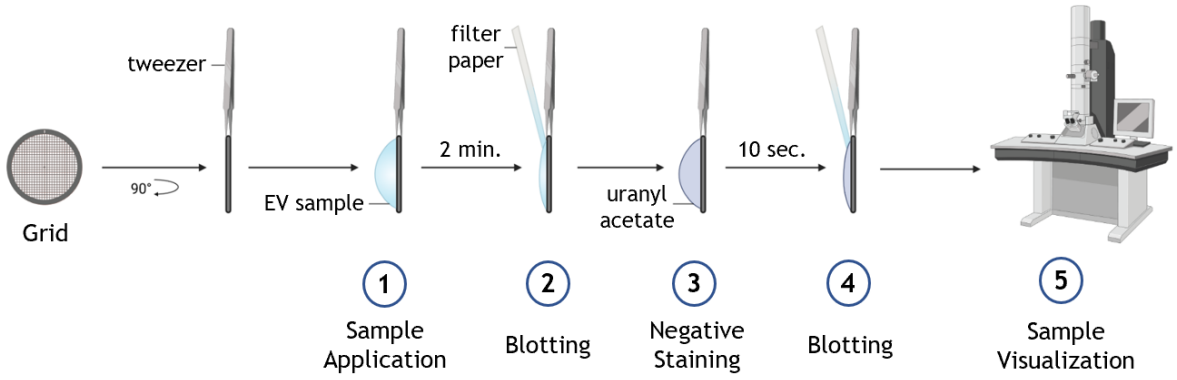

Figure 2.3. Sequence of steps for EV visualization under transmission electron microscopy.

2.6.2. Nanoparticle Tracking Analysis

For NTA, DC-EV and corresponding UC controls were diluted 1:1000 and 1:100, respectively, in PBS, to a final volume of 1 mL and analysed in a NanoSight NS300 with NTA3.0 software.

2.7. Western blotting

Protein amount of EV pellets and UC controls was quantified using DC™ Protein Assay (Bio-Rad), and the same amount of protein (7 µg) was used. Laemmli buffer was added to the samples, that were then denatured at 95 °C for 5 min, before being loaded on SDS-PAGE 10% polyacrylamide gels (Bio-Rad), and then transferred to nitrocellulose membranes. Membranes were blocked with 5% low fat milk (w/v) in TBS-T, and incubated overnight with mouse anti-human CD63 antibody (BD Pharmingen™), and rabbit anti-human calnexin at 4 ºC, followed by incubation with HRP-conjugated anti-mouse antibody and anti-rabbit antibody (GE Healthcare), respectively, for 1h, RT. Membranes were then washed with TBS-T and incubated with ECL substrate (GE Healthcare) for 2 min., and chemiluminescent signal detected with autoradiographic films (Amersham).

2.8. MSC isolation and culture

MSC had been previously isolated from human bone marrow tissue, and characterised to follow the criteria of the international society for cellular therapy. MSC were seeded in 75 cm2 culture flasks and cultured in MSC growth medium - low glucose Dulbecco's modified Eagle's medium (DMEM) + Glutamax (Invitrogen), supplemented with 10% heat inactivated

MSC-compatible Foetal Bovine Serum (FBS, Gibco™) and 1% P/S. Cells were maintained in a humidified incubator, at 37 °C and 5% CO_2. MSC from passages 7 and 8 were used in the current work.

2.9. MSC recruitment assays

Transwell assays were set up in 24-well plates using inserts with membranes of 8 µm pore size (BD Falcon™), as previously described [173]. Insert membranes coated with 100 µL of 1% gelatin (w/v) (1 h, at 37 °C) were seeded with 0.04×10^6 MSC (TC, top compartment) and stimuli were placed on the bottom wells for experiments with gradient (BC, bottom compartment). Serum-free media (SFM), without any further supplementation, was used as negative control, to assess basal levels of cell migration, and DMEM supplemented with 30% MSC-compatible FBS was used as positive control. For test condition, 50 µg of EV and respective UC controls were diluted in serum-free DMEM and media distributed in the bottom compartments. Also, 1×10^6 of differentiated DC were plated on the BC in MSC serum-free culture media. A subset of DC was stimulated with rh-TNF-α (100 ng/mL), 3 days before the recruitment assay, while the other subset remained naïve. After 8 h, the assays were stopped, culture media collected and stored at −20 °C, and insert membranes washed with PBS and fixed with paraformaldehyde (PFA) 4% (w/v) for 15 min., and washed again twice with PBS. Non-migrated cells on TC were removed with cotton swabs, membranes cut from the inserts, mounted on slides, and stained using fluoroshield with DAPI. Migrated cells were imaged in the entire insert membranes using an inverted fluorescence microscope (Axiovert 200M), counted with IN Cell Analyzer 2000 and IN Cell Investigator software (GE Healthcare) and migration index calculated for normalization across experiments as the ratio of migrated MSC in the test condition to the MSC migrated in the respective negative control condition, SFM.

2.10. Label-free quantitative EV proteomic analysis

Two samples of each experimental condition (EV-derived from non-stimulated DC and TNF-α stimulated DC, nEV and tEV, respectively) were analysed for their proteomic content. Protein quantification was performed using Label-Free Quantification as previously described in [192]. 500 ng of peptides from each EV suspension were analysed by LC-MS Liquid Chromatography-Mass Spectrometry, and the data obtained was explored with the Proteome Discover software. The Uniprot database was used for identification of peptides from both human and bovine species.

The lists of proteins identified in nEV and tEV suspensions were imported into the DAVID Bioinformatics database (https://david.ncifcrf.gov/) and assigned to their GO annotations for biological process and cellular compartment.

2.11. Statistical analysis

Data were analysed using GraphPad Prism 8.3.0 software was used. For ELISA statistical analysis data were tested for normal distribution by D'Agostino Pearson normality test and shown to be parametric, and statistical significance between the two groups was calculated using t-test. For migration assay statistical analysis data was tested for normal distribution by Shapiro-Wilk test and shown to be parametric. For multiple comparisons One-Way ANOVA was used. Statistical significance was considered for $p\text{-value}<0.05$.

3. CHAPTER III: RESULTS

3.1. DC differentiation: optimizing culture conditions for EV production

The first step was to optimize DC culture conditions and activation protocol to obtain EV from DC in naïve and stimulated conditions. DC are differentiated from monocytes using a combination of IL-4 and GM-CSF (Figure 3.1A and B). To induce DC maturation, cells were stimulated with TNF-α, mimicking a pro-inflammatory environment. Results obtained show that TNF-α stimulated DC change their morphology, becoming more spindle-like (Figure 3.1C), and secrete abundant pro-inflammatory cytokines, like IL-1β, IL-6 and TNF-α (Figure 3.1D), but not IL-12p70, whose levels remained below the limits of detection of the ELISA kit used (data not shown).

Importantly, DC cultures are performed in presence of FBS, a commonly used supplement for mammalian cell culture. FBS contains bovine EV, lipoproteins, protein complexes and even RNA components, which, in the context of EV research, represent important contaminants. Thus, it was important to use EV-depleted FBS and reduce FBS in cultures, at least in EV-producing stage. Our previous work showed that culture media with 1% of EV-depleted FBS could be used during DC-EV production [173]. However, to decrease further sample contamination, we tested whether the decreasing percentage of FBS to 0.5% would significantly impact DC viability. Preliminary results indicate that reduction of FBS affects DC viability, as LDH activity shows a tendency for increase in the supernatant of DC cultured with 0.5% of EV-depleted FBS media, particularly for some donors and in TNF-α stimulated conditions (Figure 3.1E).

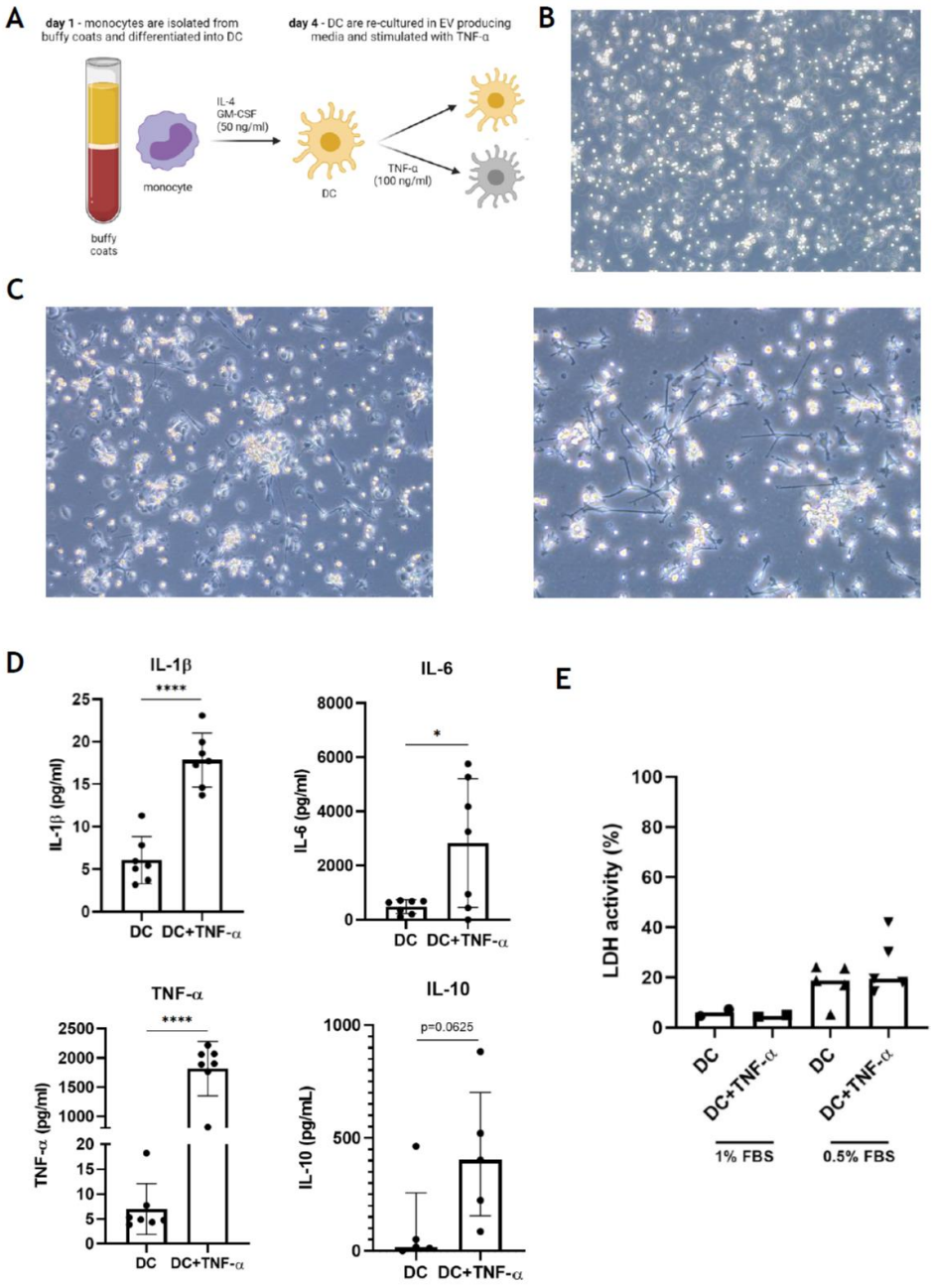

Figure 3.1. TNF-α promotes DC maturation. (A) Schematic representation of DC differentiation and activation: DC are differentiated from peripheral blood monocytes using GM-CSF and IL-4, for 4 days. After, DC are left unstimulated (naïve) or stimulated with TNF-α. **(B)** Morphology of monocytes just after isolation from buffy coats **(C)** Morphology of primary human DC unstimulated (left) and stimulated with TNF-α (right). **(D)** DC culture conditioned media was collected and IL1β, IL-6, IL-10 and TNF-α content was analysed by ELISA (n=7). Statistical analysis data was tested for normal distribution by D'Agostino Pearson normality test and shown to be parametric. Statistical significance between the two groups was calculated using t-test **(E)** Percentage of LDH activity calculated across 7 different donors for DC cultured with 1% and 0.5% of EV-depleted media. In the plots each donor is represented by a symbol, and the median is represented by bars (*p<0.05; ****p<0.001)

3.2. TNF-α does not significantly impact DC-EV production and characteristics

Next, DC were cultured in EV-free media, containing the reduced percentage of previously ultracentrifuged FBS, left unstimulated or stimulated with TNF-α and allowed to produce EV for 72h. EV were isolated from culture supernatants by performing the differential (ultra)centrifugation protocol, and characterised using a combination of methodologies, TEM, NTA and Western blot, to evaluate their morphology, size distribution and protein markers. TEM results show that isolated EV have the typical cup-shape morphology, while no EV were visible in the respective UC controls (Figure 3.2A). Also, no EV were visible when media that had not been in contact with cells was ultracentrifuged (Figure S1). EV concentration and size distribution were further analysed using NTA (Figure 3.2B). To further separate the EV population a filtration step, with a 0.22 µm filter, was introduced before the UC. The NTA data indicated that EV in unfiltered samples had a predominant population around 100 to 150 nm for both EV derived from naïve DC and TNF-α-activated DC, a size compatible with exosomes (Figure 3.2C), while in filtered samples particles were slightly smaller, around 100 nm, and concentrations of particles were lower (Figure 3.2C and Figure S3). Thus, the filtration step was not used for further experiments.

Then, Western blotting was used to probe the presence of EV markers. Results are illustrated in Figure 3.2D and show that the clssic EV marker, CD63, was present in ÊV samples but not in UC controls, while the endoplasmic reticulum marker, calnexin, was present in cell lysates. A non-specific band slightly smaller than calnexin could be observed in cell lysates and EV (Figure 3.2D).

Taken together these results show that naïve and TNF-α stimulated DC produce EV with similar sizes and the presence of the exosome marker CD63.

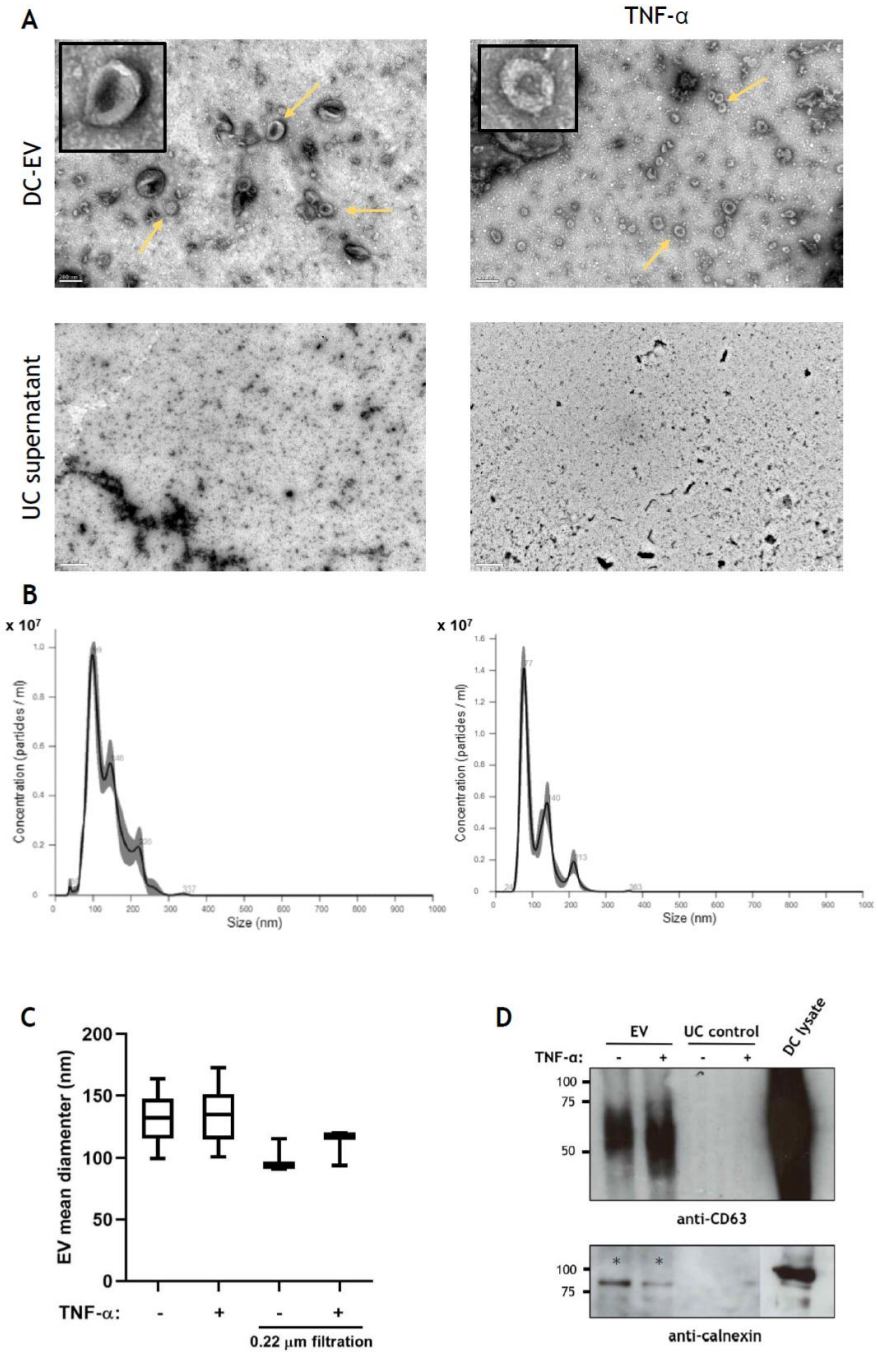

Figure 3.2. DC-EV characterization. (A) Morphology of the isolated DC-nEV (top left) and DC-tEV (top right) and respective UC supernatant controls (bottom left and right, respectively) analysed using TEM. Yellow arrows point to EV of different sizes (Scale bar: 200 nm). Inserts show single EV for detailed morphology. **(B)** Concentration versus size distribution of isolated DC-nEV (left) and DC-tEV (right), by NTA. Plot is representative of EV size and concentration analysis with NanoSight NS300**. (C)** Box plot of mean diameter of EV isolated by differential (ultra)centrifugation (n=10) and by (ultra)centrifugation preceded by 0.22 µm filtration (n=3). **(D)** Representative western blots for detection of the exosomal marker, CD63 (50-60 kDa), and the endoplasmic reticulum marker, calnexin (96 kDa), in isolated EV, respective UC controls, and DC lysates. EV produced by naïve DC (nEV); DC treated with TNF-α (tEV). *non-specific band.

3.3. DC-EV in MSC recruitment

Previous work from our team revealed that DC are able to induce significant MSC migration, which is impaired in an inflammatory-like environment [172]. In this work, we used a transwell system (Figure 3.3B), where DC-EV were placed in the bottom compartment and MSC (Figure 3.3A) seeded on the top compartment. DC themselves were also used as stimuli, and 30% FBS was used as positive control for cell migration. Migrated cells were stained with DAPI (Figure 3.3C) and counted.

When the number of migrated MSC were counted across different donors (Figure 3.3D), results obtained confirmed that preconditioning DC with the pro-inflammatory cytokine TNF-α supressed their ability to promote MSC migration. However, while EV from both conditions could promote MSC migration, above the negative control (serum-free media), no significant differences in MSC migration were observed between nEV and tEV groups (Figure 3.3D), or between EV and their respective supernatant, thus indicating that the low migration observed was not specifically induced by EV (Figure 3.3D).

3.4. The impact of DC stimulation with TNF-α on EV content

To uncover the protein content in EV suspensions and identify potential differences between the content of EV shed by naïve and mature DC, a proteomic analysis was performed on one sample of each condition (nEV and tEV). Results obtained show proteins involved in exosomes biogenesis, namely tetraspanins CD9, CD63, and CD81, and Alix. Albeit this was a preliminary study with nEV and tEV from only one donor, we wanted to take this analysis further and considered three groups of proteins: EV markers, inflammatory proteins and those potentially playing a role in cell migration (Figure 3.4A-C). First, the endoplasmic reticulum protein calnexin, which should be absent in exosomes, was used to calculate the relative proportion of exosome markers in both EV samples. Results show increased proportion of exosome markers CD9, CD63, CD81 and Alix, relative to calnexin in both samples, thus supporting that these suspensions are enriched in exosome (Figure 3.4A). Analysing the abundance ratio between tEV/nEV confirms that the amount of inflammatory molecules, including CD40, CD80, and CD86, and MHC class II was increased in tEV (Figure 3.4B).

Furthermore, analysing the differences of expression of different molecules reported to stimulate MSC migration, (e.g. OPN, SDF-1/CXCL12, and TGF-β1) in nEV and tEV, showed a marked decrease of OPN and TGF-β1 in the tEV sample compared to nEV. Conversely, SDF-1 was detected in much higher amounts in the tEV suspension than in the nEV sample.

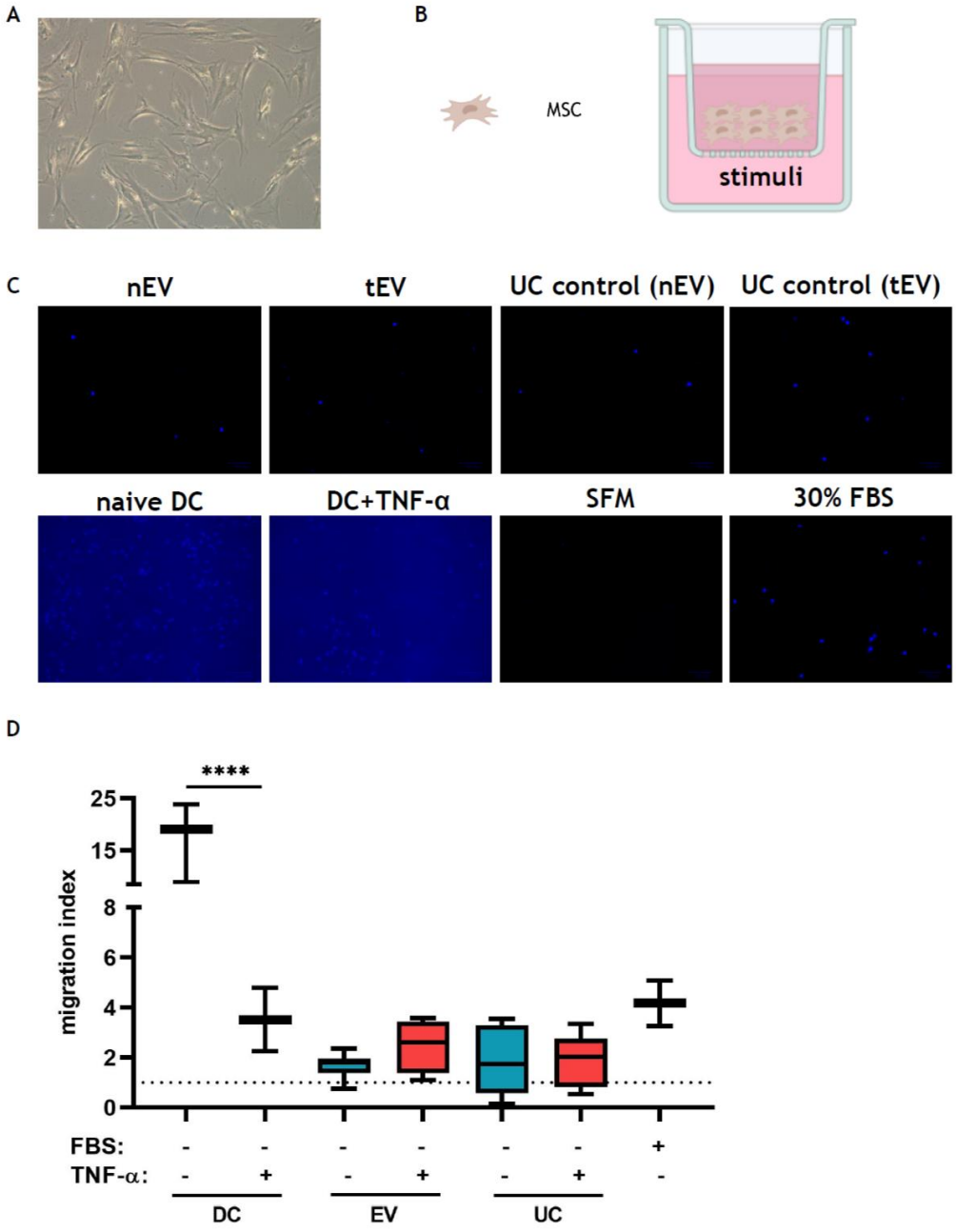

Figure 3.3. MSC recruitment. (A) Primary human MSC **(B)** Transwell assay experiment setting: naïve DC and TNF-α-treated DC or their EV (tEV and nEV) were placed in the bottom compartments, in serum-free media (SFM). SFM, without any supplementation, was used as negative control, and DMEM supplemented with 30% FBS (DMEM+30%FBS) was used as positive control. **(C)** Migrated MSC were stained with DAPI, and imaged using inverted fluorescence microscope. Representative images are shown (D) Migration index represents the ratio between each condition and negative control. The dashed line represents the migration index of negative control. Representative results of MSC migration towards DC and EV stimuli are shown for 3 and 6 DC donors, respectively (2 and 4 independent experiments). Statistical analysis data was tested for normal distribution by Shapiro-Wilk test and shown to be parametric. For multiple comparisons One-Way ANOVA was used (****<0.001)

MMP9 and MMP2 displayed similar abundances in both EV suspensions. Moreover, IP-10 was abundantly represented in tEV suspension in comparison with nEV, whereas MCP-1/CCL2 was noticeably lower in the tEV suspension (Figure 3.4C).

Lastly, the proteins identified in each suspension were introduced into the DAVID Bioinformatics database, to perform gene ontology (GO) cellular component (CC) and biological process (BP) analyses. The obtained data demonstrated the identified proteins in both suspensions are mostly associated with EV, and particularly exosomes (50% and 53% of proteins in tEV and nEV suspensions, respectively) (Figure 3.3D). Other detected proteins are mainly associated with other cellular compartments, namely the Golgi apparatus (9-10%), and endoplasmic reticulum (8-9%). Additionally, the analysis of GO biological processes related to DC antigen-presenting functions, regeneration and differentiation of bone-related cells were executed. Globally, proteins associated with T cell activation and antigen processing and presentation were detected in marginally higher amounts in the tEV suspension. Moreover, tEV suspension was enriched in proteins associated with angiogenesis and positive regulation of cell migration, which are important during tissue regeneration. On the other hand, proteins involved in osteoblast differentiation were detected in higher quantities in the nEV suspension, whereas those associated with osteoclast differentiation were found in higher numbers in tEV (Figure 3.3E).

These very preliminary results are encouraging in the differences observed between nEV and tEV, but an analysis with more EV donors is required to support any conclusion.

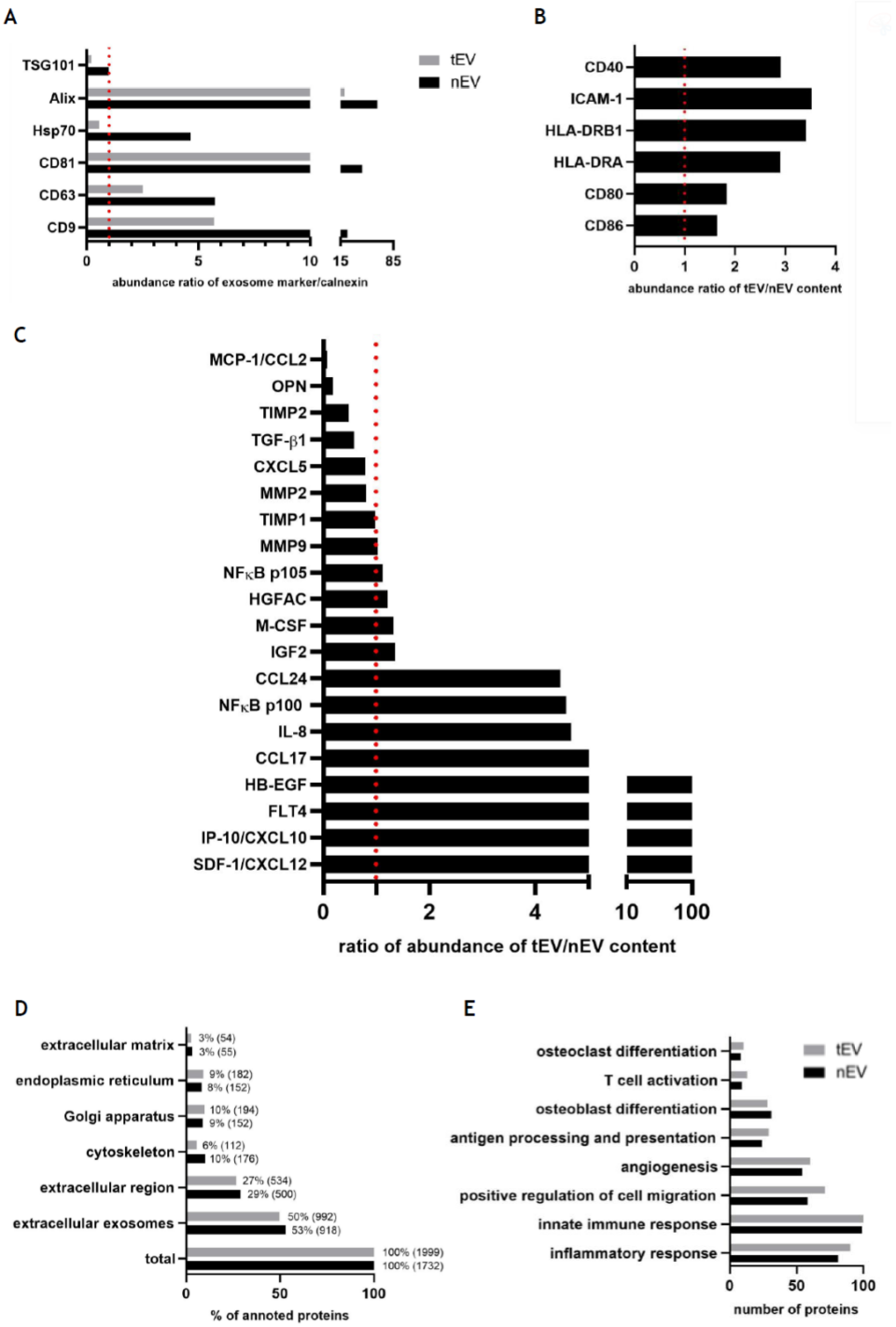

Figure 3.4. EV proteomic analysis. EV from naïve and TNF-α stimulated DC were analysed by LC-MS. **(A)** Ratio between abundance of exosome markers and calnexin, an endoplasmic reticulum-associated protein, in nEV and tEV suspensions. The relative abundance between tEV and nEV for **(B)** costimulatory molecules (CD40, CD80, CD86), MHC class II and ICAM-1 and **(C)** several chemoattractant (e.g. IL-8, SDF-1, OPN) and extracellular matrix modifying (e.g. MMP and TIMP) molecules were calculated. The number of human proteins found in nEV and tEV suspensions were categorized according to their gene ontology **(D)** cellular compartment and **(E)** biological processes.

4. CHAPTER IV: DISCUSSION AND FUTURE PERSPECTIVES

DC maturation using inflammatory cytokine, TNF-α or bacterial LPS [193] remain the most popular, despite other factors, such as CD40L, IFN-γ, IFN- α alone or combined, being also used to achieve an efficient DC activation. Previous work from our lab showed that stimulation of DC with TNF-α efficiently promotes a significant increase of CD83 and CD86 costimulatory molecules and HLA-DR, compared to immature DC [172]. Additionally, DC treated with TNF-α positively influence T cells proliferation, which is in line with their APC functions [172]. Herein, DC maturation could be confirmed by cell morphology and secretion of pro-inflammatory cytokine IL-1β, IL-6 and TNF-α, but not IL-12p70. In agreement with our results, previous reports have shown that DC matured with TNF-α produce do not secrete IL-12 or IFN-γ, which may impair the ability of DC to promote T CD8+ cells responses *in vitro* [194]. Additionally, TNF-α combined with IL-1β, IL-6 and PGE2 also failed to promote increased levels of IL12p70, but induced a considerable higher secretion of IL-12p40 [195], leading to Th2-like responses. On the other hand, secretion of large amounts of IL-12 is detected upon stimulation of DC with IFN-γ. Interestingly, an analysis of the impact of different serum-free media on the production of clinical grade DC for cancer immunotherapy revealed these have a massive impact on DC cytokine profile. Particularly, it has been demonstrated that mature DC cultured in RPMI release residual amounts of IL-12p70, while those maintained in X-VIVO 15 or AIM-V, both culture medias used in clinical DC-based immunotherapy, produce significant higher levels of IL-12p70 upon maturation [196]. Also, the release of pro-inflammatory cytokines, including IL-1β, IL-6, and TNF-α, was significantly more elevated in DC activated with TNF-α than in immature DC, which suggests that when exposed to an pro-inflammatory environment, DC may contribute to perpetuate inflammation, which is relevant for instance in chronic disorders, like RA. The anti-inflammatory and immunosuppressive cytokine, IL-10, presence in cells supernatants was also evaluated, and no significant differences were found between immature and mature DC were detected, which is not in line with previous studies [173, 195]. Finally, although cytokines are produced and consumed by cells in culture, it would be important to unveil whether TNF-α levels found within mature DC supernatants are influenced by the stimulation of cell with this cytokine. To test this, TNF-α gene expression could be assessed.

The *in vitro* analysis of EV-production, biology and function, are key to understand various mechanisms associated with their biogenesis, molecular composition, and impact on recipient or target cells. However, this faces several methodological challenges, starting with EV isolation and characterization, which are widely debated in the international EV research

community [68]. Reducing EV contamination by FBS components, including bovine EV and protein complexes within the vesicles size range remains a hot discussion topic. To reduce FBS-derived contamination, Thery, Witwer [68] previously proposed the use of either 1) serum-free media; 2) 1% bovine serum albumin instead of whole FBS; or 3) FBS EV-'depletion' protocols, termed EV-depleted FBS, if the cultured cells require serum supplementation for their growth. The most applied method for FBS EV-depletion includes diluting FBS media and performing high-speed UC, removing the contained bovine EV within the pellet, and using the supernatant as the media supplement [68]. In this work, the successful EV removal from ultracentrifuged FBS-containing media was confirmed by TEM analysis (Figure S1). We further tested the reduction of EV-depleted FBS in DC cultures for EV production. This led to an increased in LDH activity in cell supernatants, denoting cell stress and death. This is in line with previous studies have demonstrated that EV-depleted FBS media impacts cells behaviour. For instance, primary mouse astrocytes cultured in media lacking EV from the bovine serum demonstrated lower levels of growth and viability compared to culture with FBS-supplemented media [197], suggesting either the EV from FBS, the co-precipitating particles, or combination of both are important for cell proliferation in *in vitro* cultures. Several studies illustrate how different concentrations of FBS affect differently cell proliferation and biological properties, with lower concentrations having a negative impact [198, 199]. The potential increase of dead cells in EV producing cultures may increase EV contamination, for instance with apoptotic bodies. This would need to be confirmed and if required FBS concentration may need to be adjusted.

DC-EV were recovered by differential UC, the most reported EV recovery method from cell culture supernatants [100]. The isolated EV were characterised using a combination of methodologies, TEM, NTA and Western blot, to evaluate their morphology, size distribution and protein markers. The ISEV community recomends a combination of methodologies for EV isolation and characterization [68]. In our or work, cup-shaped vesicles were observed in TEM images, nervetheless this in not their native morphology but an artefact of the technique. Previous work confirms using using cryo electron microscopy that the EV original shape is round [200]. Additionally, TEM allows the observation of the size heterogenity within the recovered EV population and presence of possible contaminants that may hinder the interpretation of experimental results in EV research. TEM images also expose that DC-EV samples contain some protein aggregates that pellet along with them during ultracentrifugation. Interestingly, a recent study considered the formation of a protein corona as an underlying biological characteristic of EV in blood circulation, rather than a "contaminant" as it is generally suggested to be in EV samples. This external molecular components primarily attach to EV surface through electrostatic interaction and protein aggregation phenomenon,

and may affect EV movement and size [201]. Many attemps have been performed to collect only the fraction of exosomes from fluids, including SEC or gradient density ultracentrifugation. This separates EV according to their density (1.10-1.19 g/mL), and remove contaminating proteins non-specifically associated with them or large protein aggregates [202]. However, despite resulting in more pure EV populations, density gradients reduce their yield [203]. In this study, to reduce the pelleting of non-vesicular components during UC, and inspired by Saludas, Garbayo [191] work, a 0.22 µm filtration prior to ultracentrifugation was tested. Though, we decided to abandon the filtration step as lower concetration of particles were obtained, and EV sizes were only slightly smaller. NTA data revealed that EV samples recovered by ultracentrifugation were enriched in particules with mean sizes ranging from 100-200 nm in both approaches. Tkach, Kowal [87] also reported a similar range of sizes for EV recovered by ultracentrifugation from the supernatants of monocyte-derived DC, revealing an elevated concentration of particles smaller than 200 nm. Additionally, the presence of the exosome marker CD63 was detected by Western blotting, while no calnexin signal in EV samples was detected. Together, these results suggest that the methology used here allows the isolation of EV, possibly enriched in those of endosomal origin.

Many biological processes, including embryological development, tissue formation and organization, immune defence, inflammation, and tumour progression, depend on the ability of cells to migrate and perform their function. Cells locomotion can be guided by different stimuli, including chemical. Cell migration toward soluble signals is driven by activation of receptors, such as G protein-coupled chemokine receptors [204]. EV secretion has been reported to promote chemotaxis of a varity of cells. Small EV shed by rat bone marrow-derived MSC were shown to enhance the migration of human peridontal stem cells (hPLSC) across a transwell system. Furthermore, the combination of those small EV with an hydrogel exerted a therapeutical effect on periodontis in rats, by promoting migration, proliferation, and osteogenic differentiation of hPLSC, and reducing inflammatory cell infiltration [205]. MSC-derived EV were also reported to drive meniscus regeneration, by facilitating the migration and proliferation of endogenous chondrocytes and synovial MSC. The communication between immune cells also relies on secreted EV. Polymorphonuclear leuckocytes respond to EV released by APC, containing functional enzymes for leukotrienes, and migrate towards them [206]. Remarkable progress has been made in uncovering the role of EV in cell migration processes, nevertheless the interaction of blood-derived cells-derived EV and MSC remains unexplored.

The movement of BMSC from their niche to target tissues is a complex process, which is mediated by chemokines, cytokines, and growth factors, and mechanical factors, such as

extracellular matrix stiffness [207]. The trafficking of MSC is important for bone formation and bone fracture healing, and dysregulation of that process significantly impacts bone health. Additionally, for bone treatment, the migration of endogenous or exogenous MSC to injury sites is necessary to replenish bone cells, therefore the development of strategies that enhance MSC recruitment and migration may improve the bone regeneration process [208]. Our previous work showed the capacity of immune cells, particularly DC, to enhance MSC recruitment *in vitro*, while their stimulation with TNF-α negatively affected recruitment [172]. Later, EV from naïve DC were shown to be the secreted component responsible for paracrine MSC recruitment [173]. Our results showed that nEV do not have the same ability of their parent cells to recruit MSC, which is not in agreement with previously reported data. In this work, results seemed to be hampered by the low number of migrated MSC observed in most experiments and across all testing conditions, including the positive control. Herein, nEV and tEV seem to display a similar capacity to recruit MSC. Interestingly, Hosseinkhani, Kuypers [209] also did not find any substantial differences in the chemotactic effect of EV derived from unstimulated endothelial cells and EV derived from endothelial cells treated with TNF-α. Despite containing different molecules, the two groups of EV had the same ability to promote the transmembrane migration of monocytes. Nevertheless, EV released from TNF-α-stimulated endothelial cells were shown to upregulate the expression several inflammatory markers, such as ICAM-1, IL-6, IL-8 IL-6R, CCL-2, CCL-4, and CCL-5, on targeted cells [209].

Additionally, it is worth mentioning that observation of the top of the transwell membranes immediately after the 8h incubation, before removal of non-migrated cells, under optical microscopy, revealed that MSC seeded in serum free media were aggregatin in regions near the walls of the inserts, while those where 30% diluted FBS was used as a stiuli appeared to spread more evenly across the membrane (Figure S4). It is reported that switching cells from FBS-containing media to serum free media can induce cellular stress and autophagic flux, and that serum deprivation in MSC cultures significantly induces early apoptosis within 6 to 24 hours. The withdrawal of survival growth factors is even more harmful for MSC than incubation of MSC under hypoxic conditions [210]. This should be addressed in future work, as they may influence the smaller numbers of migrated MSC observed. Moreover, culture conditions and cells passage number affect the migration performance of MSC. Herein, cells in passages 7-8 were used for migration assays. It is reported that freshly isolated MSC had a better efficiency of homing than cultured cells, and that the migration efficiency of MSC decreases as time in culture increases [211]. Additionaly, the CXCR4 chemokine receptor, which is highly expresed in bone marrow-derived MSC, and plays an important role in MSC migration along with the binding molecule SDF-1, is downregulated upon culturing. Preconditioning MSC with cytokines, such as HGF and IL-6, may revert this trend [211].

Proteomic analysis revealed that the nEV an tEV suspension display elevated amounts of CD9, CD63, CD81, Alix, and Hsp70 exosomes markers. The proportion between those and calnexin was lower in the tEV suspension, and despite being preliminary results, as only one donor was analysed, these can suggest that the amounts of exosome recovered in parallel (at the same time) by UC changes between samples. The expression of costimulatory molecules (CD40, CD80, and CD86) and MHC class II molecules was found elevated in the tEV suspension. Similarly, preconditioning DC with IFN-γ increased the expression of MHC class II molecules and the costimulatory molecules CD40, CD80, CD86, and ICAM-1 on EV released by those cells in comparison to immature DC-derived EV [212].

EV released by naïve DC were previously shown to contain important biologically active mediators implicated in MSC migration, such as OPN and MMP9 [173]. OPN increases upon injury and in response to inflammation and its capacity to promote MSC migration is described [39]. In this work, OPN was relatively detected in lower amounts in the tEV suspension. However, SDF-1 (also known as CXCL12), described as critical for BMSC homing through the SDF-1/CXCR4 axis [207], was highly represented in the tEV suspension, and not expressed in the nEV sample. A recent study elegantly demonstrated that EV released by Schwann cells enclose SDF-1, and that these vesicles were able to promote the migration of HUVEC, dental pulp stem cells and, importantly, MSC. Furthermore, it was proved that SDF-1 in EV is crucial to promote the recruitment of MSC, as inhibiting its receptor (CXCR4) decreases the their migration [213]. MMP9 and MMP2, have gelatinase activity, so may participate in the degradation of the gelatin coating of the transwell membranes used in the migration assays. However no apparent differences were observed between the two EV preparations. Interestingly, IP-10, that was previously detected in small amounts in nEV [173], followed a different trend in tEV suspension. The overconcentration of IP-10, in inflammatory conditions, positively regulates MSC migration towards the damaged site [214]. The MSC migration in the direction of IP-10 stimuli was also shown to be enhanced by preconditioning the cells with the pro-inflammatory cytokines TNF-α and IFN-γ [214]. On the other hand, MCP-1/CCL2, that was increased in nEV in comparison to the ultracentrifugation supernatant control [173], was found in smaller amounts in the tEV suspension. MCP-1 is implicated in chemotaxis of several cells, including monocytes, microglia and memory T lymphocytes [215], and, importantly, it was already reported that this inflammatory signal promotes MSC recruitment [216]. Moreover, patterns of differently expressed TGF-β1 were detected between nEV and tEV samples. Pioneering work dissected the differences on the EV cargo levels of different subtypes of mouse DC, and revealed that TGF- β1 was exclusively present in EV released by regulatory DC [217]. The content of EV released by cells strongly depends on the stimulus the cells get from the microenvironment.

Previous analyses of all human EV proteins already reported in EVpedia revealed similar results regarding the cellular origin of proteins identified in EV. This investigation showed that, of total human vesicular proteins, 10.7, 6.4, and 6.1% derived from the cytoskeleton, Golgi apparatus, and endoplasmic reticulum, respectively [218], which reflects what was observed in nEV and tEV suspensions in our preliminary proteomics analysis. However, while it is described that 11.8% of human EV proteins are derived from the extracellular region [218], GO CC analysis of proteins found in nEV and tEV suspensions revealed that 27-29% of the proteins identified in DC-derived EV suspensions are from the extracellular region. Together, these observations suggest that the proteins found in EV, and particularly DC-EV, in our study, enclose proteins derived from multiple cellular compartments, namely Golgi apparatus and endoplasmic reticulum, which suggest the presence of contaminating non-vesicular proteins in EV suspension. So, it would be interesting to determine whether the content found in EV suspensions is inside of the vesicles or may be a protein contaminant. This could be achieved by assessing if the proteins are resistant to protein K digestion. EV will protect the molecules that they bear from proteolytic attack, therefore only proteins inside the EV will be detected upon proteinase K digestion. Nonetheless, as discussed above, EV have proteins on their surface and may have a biologically relevant protein corona, which will likely be destroyed by proteinase K digestion, so this analysis would have to be carefully controlled.

Furthermore, proteins associated with activated APC functions were detected in relatively higher amounts in the tEV suspension, which is compatible to their parent cells functions [219]. Maturation of DC is reported to support the osteoclast activity in some inflammatory-related diseases, namely in RA, mainly through T cell activation [62]. Interestingly, EV shed by inflammatory-activated DC expressed slightly elevated numbers of proteins associated with osteoclast activity, which suggest that these vesicles may represent an additional mechanism of bone destruction perpetuation, which could be further dissected with more robust EV proteomic analysis from more DC donors. Preliminary results of nEV content indicate that these vesicles bear some proteins associated with osteoblast differentiation, nevertheless the relevance of those proteins may not be sufficient to drive MSC differentiation into bone cells. However, previous work from our group demonstrated that EV secreted by naïve DC do not impact MSC proliferation or differentiation. The results showed that the stimulation of MSC with DC-EV did not impact the expression of osteogenic (ALP and RUNX2) and chondrogenic (SOX9) lineage markers [173].

Thus, while our preliminary data suggests that nEV and tEV bear different mediators that may impact MSC recruitment, further studies are necessary to support this conclusion and validate the results presented here.

In this work, the potential role of DC secreted EV in mediating MSC migration was evaluated along with the impact of exposing the EV producing cells to inflammatory conditions. Herein, we observed DC in naïve and pro-inflammatory conditions produced EV with similar morphology and size. Furthermore, nEV and tEV displayed an identical capacity to recruit MSC. Nevertheless, a robust analysis was hindered by the low number of migrated cells, therefore further improvements on the recruitment assays should be accomplished. Strategies to reliably understand the role of EV can include seeding increased numbers of MSC on the top compartment of the transwell system and allowing them to migrate for an extended period, and eventually testing different times of incubation, or even perform other types of migration assays. Additionally, this study would also benefit from the use of MSC from lower passages, since the “ageing” of cells in culture affects their ability to migrate. Finally, after the refinement of the migration protocol, the effect of EV produced in naïve and inflammatory conditions should be evaluated on another group of control cells (e.g. fibroblasts) to dissect the specificity of DC-EV to recruit MSC.

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REFERENCES

1. Kangari, P., et al., *Mesenchymal stem cells: amazing remedies for bone and cartilage defects.* Stem Cell Res Ther, 2020. **11**(1): p. 492.
2. Chen, Z., et al., *Osteoimmunomodulation for the development of advanced bone biomaterials.* Materials Today, 2016. **19**(6): p. 304-321.
3. Dar, H.Y., et al., *Osteoimmunology: The Nexus between bone and immune system.* Front Biosci (Landmark Ed), 2018. **23**(3): p. 464-492.
4. Amarnath, S., et al., *Bone marrow-derived mesenchymal stromal cells harness purinergenic signaling to tolerize human Th1 cells in vivo.* Stem Cells, 2015. **33**(4): p. 1200-12.
5. Ashai, S. and N.C. Harvey, *Rheumatoid arthritis and bone health.* Clin Med (Lond), 2020. **20**(6): p. 565-567.
6. Collaborators, G.B.D.F., *Global, regional, and national burden of bone fractures in 204 countries and territories, 1990-2019: a systematic analysis from the Global Burden of Disease Study 2019.* Lancet Healthy Longev, 2021. **2**(9): p. e580-e592.
7. Bishop, J.A., et al., *Assessment of compromised fracture healing.* J Am Acad Orthop Surg, 2012. **20**(5): p. 273-82.
8. Claes, L., S. Recknagel, and A. Ignatius, *Fracture healing under healthy and inflammatory conditions.* Nat Rev Rheumatol, 2012. **8**(3): p. 133-43.
9. Udagawa, N., et al., *Osteoclast differentiation by RANKL and OPG signaling pathways.* J Bone Miner Metab, 2021. **39**(1): p. 19-26.
10. Pacifici, R., *T cells: critical bone regulators in health and disease.* Bone, 2010. **47**(3): p. 461-71.
11. Pajarinen, J., et al., *Mesenchymal stem cell-macrophage crosstalk and bone healing.* Biomaterials, 2019. **196**: p. 80-89.
12. Huang, Y., et al., *Bone marrow mesenchymal stem cell-derived exosomes promote rotator cuff tendon-bone healing by promoting angiogenesis and regulating M1 macrophages in rats.* Stem Cell Res Ther, 2020. **11**(1): p. 496.
13. Nakao, Y., et al., *Exosomes from TNF-alpha-treated human gingiva-derived MSCs enhance M2 macrophage polarization and inhibit periodontal bone loss.* Acta Biomater, 2021. **122**: p. 306-324.
14. Kang, M., et al., *Bone regeneration is mediated by macrophage extracellular vesicles.* Bone, 2020. **141**: p. 115627.
15. Andreeva, E., et al., *Interaction of multipotent mesenchymal stromal and immune cells: Bidirectional effects.* Cytotherapy, 2017: p. 1152-1166.
16. Mohammadpour, H., et al., *TNF-alpha modulates the immunosuppressive effects of MSCs on dendritic cells and T cells.* Int Immunopharmacol, 2015. **28**(2): p. 1009-17.
17. Sacchetti, B., et al., *No Identical "Mesenchymal Stem Cells" at Different Times and Sites: Human Committed Progenitors of Distinct Origin and Differentiation Potential Are Incorporated as Adventitial Cells in Microvessels.* Stem Cell Reports, 2016. **6**(6): p. 897-913.
18. Fabre, H., et al., *Characterization of Different Sources of Human MSCs Expanded in Serum-Free Conditions with Quantification of Chondrogenic Induction in 3D.* Stem Cells Int, 2019. **2019**: p. 2186728.
19. Cao, Z., et al., *Exosomal miR-335 derived from mature dendritic cells enhanced mesenchymal stem cell-mediated bone regeneration of bone defects in athymic rats.* Mol Med, 2021. **27**(1): p. 20.
20. Sipp, D., P.G. Robey, and L. Turner, *Clear up this stem-cell mess.* Nature, 2018. **561**(7724): p. 455-457.
21. Viswanathan, S., et al., *Mesenchymal stem versus stromal cells: International Society for Cell & Gene Therapy (ISCT(R)) Mesenchymal Stromal Cell committee position statement on nomenclature.* Cytotherapy, 2019. **21**(10): p. 1019-1024.

22. Dai, Y., et al., *Development of a novel feeding regime for large scale production of human umbilical cord mesenchymal stem/stromal cells.* Cytotechnology, 2022. **74**(3): p. 351-369.
23. Deo, D., M. Marchioni, and P. Rao, *Mesenchymal Stem/Stromal Cells in Organ Transplantation.* Pharmaceutics, 2022. **14**(4).
24. Frisbie, L., R.J. Buckanovich, and L. Coffman, *Carcinoma-Associated Mesenchymal Stem/Stromal Cells: Architects of the Pro-tumorigenic Tumor Microenvironment.* Stem Cells, 2022. **40**(8): p. 705-715.
25. Li, C. and B. Wang, *Mesenchymal Stem/Stromal Cells in Progressive Fibrogenic Involvement and Anti-Fibrosis Therapeutic Properties.* Front Cell Dev Biol, 2022. **10**: p. 902677.
26. Song, W., et al., *Craniomaxillofacial derived bone marrow mesenchymal stem/stromal cells (BMSCs) for craniomaxillofacial bone tissue engineering: A literature review.* J Stomatol Oral Maxillofac Surg, 2022.
27. Madrigal, M., K.S. Rao, and N.H. Riordan, *A review of therapeutic effects of mesenchymal stem cell secretions and induction of secretory modification by different culture methods.* J Transl Med, 2014. **12**: p. 260.
28. Ge, Q., et al., *VEGF secreted by mesenchymal stem cells mediates the differentiation of endothelial progenitor cells into endothelial cells via paracrine mechanisms.* Mol Med Rep, 2018. **17**(1): p. 1667-1675.
29. Kim, S.W., et al., *Amniotic mesenchymal stem cells have robust angiogenic properties and are effective in treating hindlimb ischaemia.* Cardiovasc Res, 2012. **93**(3): p. 525-34.
30. Okazaki, T., et al., *Intravenous administration of bone marrow stromal cells increases survivin and Bcl-2 protein expression and improves sensorimotor function following ischemia in rats.* Neurosci Lett, 2008. **430**(2): p. 109-14.
31. Wang, Y., et al., *Plasticity of mesenchymal stem cells in immunomodulation: pathological and therapeutic implications.* Nat Immunol, 2014. **15**(11): p. 1009-16.
32. Li, C., et al., *Paracrine effect of inflammatory cytokine-activated bone marrow mesenchymal stem cells and its role in osteoblast function.* J Biosci Bioeng, 2016. **121**(2): p. 213-9.
33. Liu, F., et al., *MSC-secreted TGF-beta regulates lipopolysaccharide-stimulated macrophage M2-like polarization via the Akt/FoxO1 pathway.* Stem Cell Res Ther, 2019. **10**(1): p. 345.
34. Park, M., et al., *Immune suppressive effects of tonsil-derived mesenchymal stem cells on mouse bone-marrow-derived dendritic cells.* Stem Cells Int, 2015. **2015**: p. 106540.
35. Shahir, M., et al., *Effect of mesenchymal stem cell-derived exosomes on the induction of mouse tolerogenic dendritic cells.* J Cell Physiol, 2020. **235**(10): p. 7043-7055.
36. Kitaori, T., et al., *Stromal cell-derived factor 1/CXCR4 signaling is critical for the recruitment of mesenchymal stem cells to the fracture site during skeletal repair in a mouse model.* Arthritis Rheum, 2009. **60**(3): p. 813-23.
37. Wang, K.X. and D.T. Denhardt, *Osteopontin: role in immune regulation and stress responses.* Cytokine Growth Factor Rev, 2008. **19**(5-6): p. 333-45.
38. Zou, C., et al., *Mesenchymal stem cells require integrin beta1 for directed migration induced by osteopontin in vitro.* In Vitro Cell Dev Biol Anim, 2011. **47**(3): p. 241-50.
39. Zou, C., et al., *Osteopontin promotes mesenchymal stem cell migration and lessens cell stiffness via integrin beta1, FAK, and ERK pathways.* Cell Biochem Biophys, 2013. **65**(3): p. 455-62.
40. Hemeda, H., et al., *Interferon-gamma and tumor necrosis factor-alpha differentially affect cytokine expression and migration properties of mesenchymal stem cells.* Stem Cells Dev, 2010. **19**(5): p. 693-706.
41. Lin, W., et al., *Mesenchymal stem cells homing to improve bone healing.* J Orthop Translat, 2017. **9**: p. 19-27.
42. Santamaria, J., et al., *Antigen-presenting cells and T-lymphocytes homing to the thymus shape T cell development.* Immunol Lett, 2018. **204**: p. 9-15.

43. Steinman, R.M. and Z.A. Cohn, *Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution.* J Exp Med, 1973. **137**(5): p. 1142-62.
44. Sallusto, F. and A. Lanzavecchia, *Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha.* J Exp Med, 1994. **179**(4): p. 1109-18.
45. Randolph, G.J., *Dendritic cells: The first step.* J Exp Med, 2021. **218**(3).
46. Doebel, T., B. Voisin, and K. Nagao, *Langerhans Cells - The Macrophage in Dendritic Cell Clothing.* Trends Immunol, 2017. **38**(11): p. 817-828.
47. Chen, K., et al., *Tissue-resident dendritic cells and diseases involving dendritic cell malfunction.* Int Immunopharmacol, 2016. **34**: p. 1-15.
48. Zanna, M.Y., et al., *Review of Dendritic Cells, Their Role in Clinical Immunology, and Distribution in Various Animal Species.* Int J Mol Sci, 2021. **22**(15).
49. Sallusto, F. and A. Lanzavecchia, *Monocytes join the dendritic cell family.* Cell, 2010. **143**(3): p. 339-40.
50. Hasegawa, H. and T. Matsumoto, *Mechanisms of Tolerance Induction by Dendritic Cells In Vivo.* Front Immunol, 2018. **9**: p. 350.
51. Cechim, G. and J.A.B. Chies, *In vitro generation of human monocyte-derived dendritic cells methodological aspects in a comprehensive review.* Anais da Academia Brasileira de Ciências, 2019. **91**(4).
52. Pettit, A.R. and R. Thomas, *Dendritic cells: the driving force behind autoimmunity in rheumatoid arthritis?* Immunol Cell Biol, 1999. **77**(5): p. 420-7.
53. Wang, D., et al., *Enhanced Calvarial Bone Healing in CD11c-TLR4-/- and MyD88-/- Mice.* Plast Reconstr Surg, 2017. **139**(4): p. 933e-940e.
54. Elsayed, R., et al., *Role of dendritic cell-mediated immune response in oral homeostasis: A new mechanism of osteonecrosis of the jaw.* FASEB J, 2020. **34**(2): p. 2595-2608.
55. Yu, M.B. and W.H.R. Langridge, *The function of myeloid dendritic cells in rheumatoid arthritis.* Rheumatol Int, 2017. **37**(7): p. 1043-1051.
56. Burmester, G.R. and J.E. Pope, *Novel treatment strategies in rheumatoid arthritis.* Lancet, 2017. **389**(10086): p. 2338-2348.
57. Littlejohn, E.A. and S.U. Monrad, *Early Diagnosis and Treatment of Rheumatoid Arthritis.* Prim Care, 2018. **45**(2): p. 237-255.
58. Page, G. and P. Miossec, *RANK and RANKL expression as markers of dendritic cell-T cell interactions in paired samples of rheumatoid synovium and lymph nodes.* Arthritis Rheum, 2005. **52**(8): p. 2307-12.
59. Pitzalis, C., et al., *Ectopic lymphoid-like structures in infection, cancer and autoimmunity.* Nat Rev Immunol, 2014. **14**(7): p. 447-62.
60. Tu, J., et al., *A Tale of Two Immune Cells in Rheumatoid Arthritis: The Crosstalk Between Macrophages and T Cells in the Synovium.* Front Immunol, 2021. **12**: p. 655477.
61. Narisawa, M., et al., *Human dendritic cell-derived osteoclasts with high bone resorption capacity and T cell stimulation ability.* Bone, 2021. **142**: p. 115616.
62. Alnaeeli, M., et al., *Dendritic cells at the osteo-immune interface: implications for inflammation-induced bone loss.* J Bone Miner Res, 2007. **22**(6): p. 775-80.
63. Wolf, P., *The nature and significance of platelet products in human plasma.* Br J Haematol, 1967. **13**(3): p. 269-88.
64. Anderson, H.C., *Vesicles associated with calcification in the matrix of epiphyseal cartilage.* J Cell Biol, 1969. **41**(1): p. 59-72.
65. Trams, E.G., et al., *Exfoliation of membrane ecto-enzymes in the form of micro-vesicles.* Biochim Biophys Acta, 1981. **645**(1): p. 63-70.
66. Raposo, G., et al., *B lymphocytes secrete antigen-presenting vesicles.* J Exp Med, 1996. **183**(3): p. 1161-72.

67. Zitvogel, L., et al., *Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell derived exosomes.* Nat. Med. 4, 1998: p. 594-600.
68. Thery, C., et al., *Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines.* J Extracell Vesicles, 2018. **7**(1): p. 1535750.
69. van Niel, G., G. D'Angelo, and G. Raposo, *Shedding light on the cell biology of extracellular vesicles.* Nat Rev Mol Cell Biol, 2018. **19**(4): p. 213-228.
70. Teixeira, J.H., et al., *Circulating extracellular vesicles: Their role in tissue repair and regeneration.* Transfus Apher Sci, 2016. **55**(1): p. 53-61.
71. Kalra, H., G.P. Drummen, and S. Mathivanan, *Focus on Extracellular Vesicles: Introducing the Next Small Big Thing.* Int J Mol Sci, 2016. **17**(2): p. 170.
72. Hristov, M., et al., *Apoptotic bodies from endothelial cells enhance the number and initiate the differentiation of human endothelial progenitor cells in vitro.* Blood, 2004. **104**(9): p. 2761-6.
73. Camussi, G., et al., *Exosomes/microvesicles as a mechanism of cell-to-cell communication.* Kidney Int, 2010. **78**(9): p. 838-48.
74. Mathivanan, S., H. Ji, and R.J. Simpson, *Exosomes: extracellular organelles important in intercellular communication.* J Proteomics, 2010. **73**(10): p. 1907-20.
75. Nakase, I., et al., *Environmental pH stress influences cellular secretion and uptake of extracellular vesicles.* FEBS Open Bio, 2021.
76. Pinheiro, A., et al., *Extracellular vesicles: intelligent delivery strategies for therapeutic applications.* J Control Release, 2018. **289**: p. 56-69.
77. Elashiry, M., et al., *Dendritic cell derived exosomes loaded with immunoregulatory cargo reprogram local immune responses and inhibit degenerative bone disease in vivo.* J Extracell Vesicles, 2020. **9**(1): p. 1795362.
78. Russell, A.E., et al., *Biological membranes in EV biogenesis, stability, uptake, and cargo transfer: an ISEV position paper arising from the ISEV membranes and EVs workshop.* J Extracell Vesicles, 2019. **8**(1): p. 1684862.
79. Robbins, P.D. and A.E. Morelli, *Regulation of immune responses by extracellular vesicles.* Nat Rev Immunol, 2014. **14**(3): p. 195-208.
80. Chen, C., et al., *Imaging and Intracellular Tracking of Cancer-Derived Exosomes Using Single-Molecule Localization-Based Super-Resolution Microscope.* ACS Appl Mater Interfaces, 2016. **8**(39): p. 25825-25833.
81. Andreu, Z. and M. Yanez-Mo, *Tetraspanins in extracellular vesicle formation and function.* Front Immunol, 2014. **5**: p. 442.
82. Buschow, S.I., et al., *Exosomes contain ubiquitinated proteins.* Blood Cells Mol Dis, 2005. **35**(3): p. 398-403.
83. Chairoungdua, A., et al., *Exosome release of beta-catenin: a novel mechanism that antagonizes Wnt signaling.* J Cell Biol, 2010. **190**(6): p. 1079-91.
84. Nolte-'t Hoen, E.N., et al., *Activated T cells recruit exosomes secreted by dendritic cells via LFA-1.* Blood, 2009. **113**(9): p. 1977-81.
85. Kowal, J. and M. Tkach, *Dendritic cell extracellular vesicles.* Int Rev Cell Mol Biol, 2019. **349**: p. 213-249.
86. Kooijmans, S.A.A., O.G. de Jong, and R.M. Schiffelers, *Exploring interactions between extracellular vesicles and cells for innovative drug delivery system design.* Adv Drug Deliv Rev, 2021. **173**: p. 252-278.
87. Tkach, M., et al., *Qualitative differences in T-cell activation by dendritic cell-derived extracellular vesicle subtypes.* EMBO J, 2017. **36**(20): p. 3012-3028.
88. Hwang, I., X. Shen, and J. Sprent, *Direct stimulation of naive T cells by membrane vesicles from antigen-presenting cells: distinct roles for CD54 and B7 molecules.* Proc Natl Acad Sci U S A, 2003. **100**(11): p. 6670-5.
89. Rappa, G., et al., *Nuclear transport of cancer extracellular vesicle-derived biomaterials through nuclear envelope invagination-associated late endosomes.* Oncotarget, 2017. **8**(9): p. 14443-14461.

90. Pedrioli, G. and P. Paganetti, *Hijacking Endocytosis and Autophagy in Extracellular Vesicle Communication: Where the Inside Meets the Outside.* Front Cell Dev Biol, 2020. **8**: p. 595515.
91. Corbeil, D., et al., *Uptake and Fate of Extracellular Membrane Vesicles: Nucleoplasmic Reticulum-Associated Late Endosomes as a New Gate to Intercellular Communication.* Cells, 2020. **9**(9).
92. Bonsergent, E., et al., *Quantitative characterization of extracellular vesicle uptake and content delivery within mammalian cells.* Nat Commun, 2021. **12**(1): p. 1864.
93. Mathieu, M., et al., *Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication.* Nat Cell Biol, 2019. **21**(1): p. 9-17.
94. Kamerkar, S., et al., *Exosomes facilitate therapeutic targeting of oncogenic KRAS in pancreatic cancer.* Nature, 2017. **546**(7659): p. 498-503.
95. Madeira, R.P., et al., *Isolation and molecular characterization of circulating extracellular vesicles from blood of chronic Chagas disease patients.* Cell Biol Int, 2022. **46**(6): p. 883-894.
96. Li, H., et al., *Extracellular Vesicles from Human Urine-Derived Stem Cells Ameliorate Particulate Polyethylene-Induced Osteolysis.* Int J Nanomedicine, 2021. **16**: p. 7479-7494.
97. Comfort, N., et al., *Isolation and characterization of extracellular vesicles in saliva of children with asthma.* Extracell Vesicles Circ Nucl Acids, 2021. **2**: p. 29-48.
98. Lin, Y., et al., *Milk-Derived Small Extracellular Vesicles Promote Recovery of Intestinal Damage by Accelerating Intestinal Stem Cell-Mediated Epithelial Regeneration.* Mol Nutr Food Res, 2022. **66**(11): p. e2100551.
99. Boere, J., et al., *Synovial fluid pretreatment with hyaluronidase facilitates isolation of CD44+ extracellular vesicles.* J Extracell Vesicles, 2016. **5**: p. 31751.
100. Gardiner, C., et al., *Techniques used for the isolation and characterization of extracellular vesicles: results of a worldwide survey.* J Extracell Vesicles, 2016. **5**: p. 32945.
101. Sidhom, K., P.O. Obi, and A. Saleem, *A review of exosomal isolation methods: is size exclusion chromatography the best option?* 2020.
102. Zhang, X., et al., *A novel three step protocol to isolate extracellular vesicles from plasma or cell culture medium with both high yield and purity.* J Extracell Vesicles, 2020. **9**(1): p. 1791450.
103. Koh, Y.Q., et al., *Exosome enrichment by ultracentrifugation and size exclusion chromatography.* Front Biosci (Landmark Ed), 2018. **23**(5): p. 865-874.
104. Wei, R., et al., *Combination of Size-Exclusion Chromatography and Ultracentrifugation Improves the Proteomic Profiling of Plasma-Derived Small Extracellular Vesicles.* Biol Proced Online, 2020. **22**: p. 12.
105. Pegtel, D.M., L. Peferoen, and S. Amor, *Extracellular vesicles as modulators of cell-to-cell communication in the healthy and diseased brain.* Philos Trans R Soc Lond B Biol Sci, 2014. **369**(1652).
106. Deregibus, M.C., et al., *Charge-based precipitation of extracellular vesicles.* Int J Mol Med, 2016. **38**(5): p. 1359-1366.
107. Lo, T.W., et al., *Microfluidic device for high-throughput affinity-based isolation of extracellular vesicles.* Lab Chip, 2020. **20**(10): p. 1762-1770.
108. Rikkert, L.G., et al., *Quality of extracellular vesicle images by transmission electron microscopy is operator and protocol dependent.* J Extracell Vesicles, 2019. **8**(1): p. 1555419.
109. Scarff, C.A., et al., *Variations on Negative Stain Electron Microscopy Methods: Tools for Tackling Challenging Systems.* J Vis Exp, 2018(132).
110. Cizmar, P. and Y. Yuana, *Detection and Characterization of Extracellular Vesicles by Transmission and Cryo-Transmission Electron Microscopy.* Methods Mol Biol, 2017. **1660**: p. 221-232.
111. Keerthikumar, S., et al., *Proteogenomic analysis reveals exosomes are more oncogenic than ectosomes.* Oncotarget, 2015. **6**(17): p. 15375-96.

112. Lunavat, T.R., et al., *Small RNA deep sequencing discriminates subsets of extracellular vesicles released by melanoma cells--Evidence of unique microRNA cargos.* RNA Biol, 2015. **12**(8): p. 810-23.
113. Polanco, J.C., et al., *Extracellular Vesicles Isolated from the Brains of rTg4510 Mice Seed Tau Protein Aggregation in a Threshold-dependent Manner.* J Biol Chem, 2016. **291**(24): p. 12445-12466.
114. van der Pol, E., et al., *Particle size distribution of exosomes and microvesicles determined by transmission electron microscopy, flow cytometry, nanoparticle tracking analysis, and resistive pulse sensing.* J Thromb Haemost, 2014. **12**(7): p. 1182-92.
115. Szatanek, R., et al., *The Methods of Choice for Extracellular Vesicles (EVs) Characterization.* Int J Mol Sci, 2017. **18**(6).
116. Doyle, L.M. and M.Z. Wang, *Overview of Extracellular Vesicles, Their Origin, Composition, Purpose, and Methods for Exosome Isolation and Analysis.* Cells, 2019. **8**(7).
117. Bachurski, D., et al., *Extracellular vesicle measurements with nanoparticle tracking analysis - An accuracy and repeatability comparison between NanoSight NS300 and ZetaView.* J Extracell Vesicles, 2019. **8**(1): p. 1596016.
118. Parisse, P., et al., *Atomic force microscopy analysis of extracellular vesicles.* Eur Biophys J, 2017. **46**(8): p. 813-820.
119. Beez, C.M., et al., *Cardiac Extracellular Vesicles (EVs) Released in the Presence or Absence of Inflammatory Cues Support Angiogenesis in Different Manners.* Int J Mol Sci, 2019. **20**(24).
120. Zhou, X., et al., *Mesenchymal stem cellderived extracellular vesicles promote the in vitro proliferation and migration of breast cancer cells through the activation of the ERK pathway.* Int J Oncol, 2019. **54**(5): p. 1843-1852.
121. Chen, Q., et al., *BMSC-EVs regulate Th17 cell differentiation in UC via H3K27me3.* Mol Immunol, 2020. **118**: p. 191-200.
122. Silva, A.M., et al., *Extracellular Vesicles: Immunomodulatory messengers in the context of tissue repair/regeneration.* Eur J Pharm Sci, 2017. **98**: p. 86-95.
123. Zhou, Y., et al., *Exosomes derived from miR-126-3p-overexpressing synovial fibroblasts suppress chondrocyte inflammation and cartilage degradation in a rat model of osteoarthritis.* Cell Death Discov, 2021. **7**(1): p. 37.
124. Jiang, Z. and J. Zhang, *Mesenchymal stem cell-derived exosomes containing miR-145-5p reduce inflammation in spinal cord injury by regulating the TLR4/NF-kappaB signaling pathway.* Cell Cycle, 2021. **20**(10): p. 993-1009.
125. Zhang, M., et al., *Exosomes with high level of miR-181c from bone marrow-derived mesenchymal stem cells inhibit inflammation and apoptosis to alleviate spinal cord injury.* J Mol Histol, 2021. **52**(2): p. 301-311.
126. Bouchareychas, L., et al., *Macrophage Exosomes Resolve Atherosclerosis by Regulating Hematopoiesis and Inflammation via MicroRNA Cargo.* Cell Rep, 2020. **32**(2): p. 107881.
127. Zhang, P., et al., *Serum-Derived Exosomes from Patients with Coronary Artery Disease Induce Endothelial Injury and Inflammation in Human Umbilical Vein Endothelial Cells.* Int Heart J, 2021. **62**(2): p. 396-406.
128. Zhou, Y., et al., *Hepatocellular carcinoma-derived exosomal miRNA-21 contributes to tumor progression by converting hepatocyte stellate cells to cancer-associated fibroblasts.* J Exp Clin Cancer Res, 2018. **37**(1): p. 324.
129. Jiang, F., et al., *Hepatocyte-derived extracellular vesicles promote endothelial inflammation and atherogenesis via microRNA-1.* J Hepatol, 2020. **72**(1): p. 156-166.
130. Fendl, B., et al., *Extracellular vesicles are associated with C-reactive protein in sepsis.* Sci Rep, 2021. **11**(1): p. 6996.
131. Yin, G., et al., *Exosomes produced by adipose-derived stem cells inhibit schwann cells autophagy and promote the regeneration of the myelin sheath.* Int J Biochem Cell Biol, 2021. **132**: p. 105921.

132. Wu, X., et al., *Extracellular vesicles from skin precursor-derived Schwann cells promote axonal outgrowth and regeneration of motoneurons via Akt/mTOR/p70S6K pathway.* Ann Transl Med, 2020. **8**(24): p. 1640.
133. Keshtkar, S., N. Azarpira, and M.H. Ghahremani, *Mesenchymal stem cell-derived extracellular vesicles: novel frontiers in regenerative medicine.* Stem Cell Res Ther, 2018. **9**(1): p. 63.
134. Zhang, S., et al., *Exosomes derived from human embryonic mesenchymal stem cells promote osteochondral regeneration.* Osteoarthritis Cartilage, 2016. **24**(12): p. 2135-2140.
135. Takeuchi, R., et al., *Exosomes from conditioned media of bone marrow-derived mesenchymal stem cells promote bone regeneration by enhancing angiogenesis.* PLoS One, 2019. **14**(11): p. e0225472.
136. Favaro, E., et al., *Human mesenchymal stem cells and derived extracellular vesicles induce regulatory dendritic cells in type 1 diabetic patients.* Diabetologia, 2016. **59**(2): p. 325-33.
137. Guo, L., et al., *Extracellular vesicles derived from mesenchymal stem cells prevent skin fibrosis in the cGVHD mouse model by suppressing the activation of macrophages and B cells immune response.* Int Immunopharmacol, 2020. **84**: p. 106541.
138. Li, J., et al., *Correction: Extracellular vesicles derived from miR-199a-5p-modified adipose-derived mesenchymal stem cells alleviate immune thrombocytopenia by inhibiting T helper 17 differentiation.* Lab Invest, 2021.
139. Tamura, R., S. Uemoto, and Y. Tabata, *Immunosuppressive effect of mesenchymal stem cell-derived exosomes on a concanavalin A-induced liver injury model.* Inflamm Regen, 2016. **36**: p. 26.
140. Ti, D., et al., *LPS-preconditioned mesenchymal stromal cells modify macrophage polarization for resolution of chronic inflammation via exosome-shuttled let-7b.* J Transl Med, 2015. **13**: p. 308.
141. Lu, G.D., et al., *BMSC-Derived Exosomal miR-29a Promotes Angiogenesis and Osteogenesis.* Front Cell Dev Biol, 2020. **8**: p. 608521.
142. Wu, D., et al., *Bone mesenchymal stem cells stimulation by magnetic nanoparticles and a static magnetic field: release of exosomal miR-1260a improves osteogenesis and angiogenesis.* J Nanobiotechnology, 2021. **19**(1): p. 209.
143. Li, Z., et al., *Exosomes Derived From M2 Macrophages Facilitate Osteogenesis and Reduce Adipogenesis of BMSCs.* Front Endocrinol (Lausanne), 2021. **12**: p. 680328.
144. Liao, W., et al., *BMSC-derived exosomes carrying microRNA-122-5p promote proliferation of osteoblasts in osteonecrosis of the femoral head.* Clin Sci (Lond), 2019. **133**(18): p. 1955-1975.
145. Lv, P.Y., et al., *Osteocyte-derived exosomes induced by mechanical strain promote human periodontal ligament stem cell proliferation and osteogenic differentiation via the miR-181b-5p/PTEN/AKT signaling pathway.* Stem Cell Res Ther, 2020. **11**(1): p. 295.
146. Xiong, Y., et al., *M2 Macrophagy-derived exosomal miRNA-5106 induces bone mesenchymal stem cells towards osteoblastic fate by targeting salt-inducible kinase 2 and 3.* J Nanobiotechnology, 2020. **18**(1): p. 66.
147. Yang, J.X., et al., *Osteoclast-derived miR-23a-5p-containing exosomes inhibit osteogenic differentiation by regulating Runx2.* Cell Signal, 2020. **70**: p. 109504.
148. Li, D., et al., *Osteoclast-derived exosomal miR-214-3p inhibits osteoblastic bone formation.* Nat Commun, 2016. **7**: p. 10872.
149. Weilner, S., et al., *Secreted microvesicular miR-31 inhibits osteogenic differentiation of mesenchymal stem cells.* Aging Cell, 2016. **15**(4): p. 744-54.
150. Kim, D.K., et al., *Mastocytosis-derived extracellular vesicles deliver miR-23a and miR-30a into pre-osteoblasts and prevent osteoblastogenesis and bone formation.* Nat Commun, 2021. **12**(1): p. 2527.
151. Uenaka, M., et al., *Osteoblast-derived vesicles induce a switch from bone-formation to bone-resorption in vivo.* Nat Commun, 2022. **13**(1): p. 1066.

152. Vincent-Schneider, H., et al., *Exosomes bearing HLA-DR1 molecules need dendritic cells to efficiently stimulate specific T cells.* Int Immunol, 2002. **14**(7): p. 713-22.
153. Schierer, S., et al., *Extracellular vesicles from mature dendritic cells (DC) differentiate monocytes into immature DC.* Life Sci Alliance, 2018. **1**(6): p. e201800093.
154. Thery, C., et al., *Indirect activation of naive CD4+ T cells by dendritic cell-derived exosomes.* Nat Immunol, 2002. **3**(12): p. 1156-62.
155. Yang, X., et al., *Exosomes derived from immature bone marrow dendritic cells induce tolerogenicity of intestinal transplantation in rats.* J Surg Res, 2011. **171**(2): p. 826-32.
156. Jeske, R., et al., *Human Pluripotent Stem Cell-Derived Extracellular Vesicles: Characteristics and Applications.* Tissue Eng Part B Rev, 2020. **26**(2): p. 129-144.
157. Segura, E., S. Amigorena, and C. Thery, *Mature dendritic cells secrete exosomes with strong ability to induce antigen-specific effector immune responses.* Blood Cells Mol Dis, 2005. **35**(2): p. 89-93.
158. Markov, O., A. Oshchepkova, and N. Mironova, *Immunotherapy Based on Dendritic Cell-Targeted/-Derived Extracellular Vesicles-A Novel Strategy for Enhancement of the Anti-tumor Immune Response.* Front Pharmacol, 2019. **10**: p. 1152.
159. Munich, S., et al., *Dendritic cell exosomes directly kill tumor cells and activate natural killer cells via TNF superfamily ligands.* Oncoimmunology, 2012. **1**(7): p. 1074-1083.
160. Besse, B., et al., *Dendritic cell-derived exosomes as maintenance immunotherapy after first line chemotherapy in NSCLC.* Oncoimmunology, 2016. **5**(4): p. e1071008.
161. Bhatnagar, S., et al., *Exosomes released from macrophages infected with intracellular pathogens stimulate a proinflammatory response in vitro and in vivo.* Blood, 2007. **110**(9): p. 3234-44.
162. Giri, P.K. and J.S. Schorey, *Exosomes derived from M. Bovis BCG infected macrophages activate antigen-specific CD4+ and CD8+ T cells in vitro and in vivo.* PLoS One, 2008. **3**(6): p. e2461.
163. Bacher, P., et al., *Low-Avidity CD4(+) T Cell Responses to SARS-CoV-2 in Unexposed Individuals and Humans with Severe COVID-19.* Immunity, 2020. **53**(6): p. 1258-1271 e5.
164. McDonald, M.K., et al., *Functional significance of macrophage-derived exosomes in inflammation and pain.* Pain, 2014. **155**(8): p. 1527-1539.
165. Xu, Y., et al., *Macrophages transfer antigens to dendritic cells by releasing exosomes containing dead-cell-associated antigens partially through a ceramide-dependent pathway to enhance CD4(+) T-cell responses.* Immunology, 2016. **149**(2): p. 157-71.
166. Liu, J., et al., *Exosomes derived from macrophages upon Zn ion stimulation promote osteoblast and endothelial cell functions.* J Mater Chem B, 2021. **9**(18): p. 3800-3807.
167. Wang, Z., et al., *Exosomes secreted by macrophages upon copper ion stimulation can promote angiogenesis.* Mater Sci Eng C Mater Biol Appl, 2021. **123**: p. 111981.
168. Zhang, H., et al., *Exosomes derived from macrophages upon cobalt ion stimulation promote angiogenesis.* Colloids Surf B Biointerfaces, 2021. **203**: p. 111742.
169. Ekstrom, K., et al., *Monocyte exosomes stimulate the osteogenic gene expression of mesenchymal stem cells.* PLoS One, 2013. **8**(9): p. e75227.
170. Li, C.J., et al., *Novel proteolytic microvesicles released from human macrophages after exposure to tobacco smoke.* Am J Pathol, 2013. **182**(5): p. 1552-62.
171. Medina, A. and A. Ghahary, *Transdifferentiated circulating monocytes release exosomes containing 14-3-3 proteins with matrix metalloproteinase-1 stimulating effect for dermal fibroblasts.* Wound Repair Regen, 2010. **18**(2): p. 245-53.
172. Silva, A.M., et al., *Resveratrol as a natural anti-tumor necrosis factor-alpha molecule: implications to dendritic cells and their crosstalk with mesenchymal stromal cells.* PLoS One, 2014. **9**(3): p. e91406.
173. Silva, A.M., et al., *Dendritic Cell-derived Extracellular Vesicles mediate Mesenchymal Stem/Stromal Cell recruitment.* Sci Rep, 2017. **7**(1): p. 1667.
174. Kim, S.H., et al., *Exosomes derived from IL-10-treated dendritic cells can suppress inflammation and collagen-induced arthritis.* J Immunol, 2005. **174**(10): p. 6440-8.

175. Jarrige, M., et al., *The Future of Regenerative Medicine: Cell Therapy Using Pluripotent Stem Cells and Acellular Therapies Based on Extracellular Vesicles.* Cells, 2021. **10**(2).
176. de Jong, B., et al., *Recent Advances in Extracellular Vesicles as Drug Delivery Systems and Their Potential in Precision Medicine.* Pharmaceutics, 2020. **12**(11).
177. Amariglio, N., et al., *Donor-derived brain tumor following neural stem cell transplantation in an ataxia telangiectasia patient.* PLoS Med, 2009. **6**(2): p. e1000029.
178. Gyorgy, B., et al., *Therapeutic applications of extracellular vesicles: clinical promise and open questions.* Annu Rev Pharmacol Toxicol, 2015. **55**: p. 439-464.
179. Kooijmans, S.A.A., et al., *Electroporation-induced siRNA precipitation obscures the efficiency of siRNA loading into extracellular vesicles.* J Control Release, 2013. **172**(1): p. 229-238.
180. de Jong, O.G., et al., *Drug Delivery with Extracellular Vesicles: From Imagination to Innovation.* Acc Chem Res, 2019. **52**(7): p. 1761-1770.
181. Saari, H., et al., *Microvesicle- and exosome-mediated drug delivery enhances the cytotoxicity of Paclitaxel in autologous prostate cancer cells.* J Control Release, 2015. **220**(Pt B): p. 727-37.
182. Sun, D., et al., *A novel nanoparticle drug delivery system: the anti-inflammatory activity of curcumin is enhanced when encapsulated in exosomes.* Mol Ther, 2010. **18**(9): p. 1606-14.
183. Pessina, A., et al., *Mesenchymal stromal cells primed with paclitaxel provide a new approach for cancer therapy.* PLoS One, 2011. **6**(12): p. e28321.
184. Yao, X., et al., *Engineered extracellular vesicles as versatile ribonucleoprotein delivery vehicles for efficient and safe CRISPR genome editing.* J Extracell Vesicles, 2021. **10**(5): p. e12076.
185. Kang, M., et al., *Biodistribution of extracellular vesicles following administration into animals: A systematic review.* J Extracell Vesicles, 2021. **10**(8): p. e12085.
186. Lai, C.P., et al., *Dynamic biodistribution of extracellular vesicles in vivo using a multimodal imaging reporter.* ACS Nano, 2014. **8**(1): p. 483-494.
187. Lv, K., et al., *Incorporation of small extracellular vesicles in sodium alginate hydrogel as a novel therapeutic strategy for myocardial infarction.* Theranostics, 2019. **9**(24): p. 7403-7416.
188. Abolgheit, S., et al., *Bone marrow-derived mesenchymal stem cells and extracellular vesicles enriched collagen chitosan scaffold in skin wound healing (a rat model).* J Biomater Appl, 2021. **36**(1): p. 128-139.
189. Zhang, J., et al., *Exosomes/tricalcium phosphate combination scaffolds can enhance bone regeneration by activating the PI3K/Akt signaling pathway.* Stem Cell Res Ther, 2016. **7**(1): p. 136.
190. Kang, M., C.S. Lee, and M. Lee, *Bioactive Scaffolds Integrated with Liposomal or Extracellular Vesicles for Bone Regeneration.* Bioengineering (Basel), 2021. **8**(10).
191. Saludas, L., et al., *Isolation methods of large and small extracellular vesicles derived from cardiovascular progenitors: A comparative study.* Eur J Pharm Biopharm, 2022. **170**: p. 187-196.
192. Osorio, H., et al., *Proteomics Analysis of Gastric Cancer Patients with Diabetes Mellitus.* J Clin Med, 2021. **10**(3).
193. Landi, A., L.A. Babiuk, and S. van Drunen Littel-van den Hurk, *Dendritic cells matured by a prostaglandin E2-containing cocktail can produce high levels of IL-12p70 and are more mature and Th1-biased than dendritic cells treated with TNF-alpha or LPS.* Immunobiology, 2011. **216**(6): p. 649-62.
194. Decker, W.K., et al., *Deficient T(H)-1 responses from TNF-alpha-matured and alpha-CD40-matured dendritic cells.* J Immunother, 2008. **31**(2): p. 157-65.
195. Wu, X., et al., *Comparative study of dendritic cells matured by using IL-1beta, IL-6, TNF-alpha and prostaglandins E2 for different time span.* Exp Ther Med, 2017. **14**(2): p. 1389-1394.

196. Calmeiro, J., et al., *In-Depth Analysis of the Impact of Different Serum-Free Media on the Production of Clinical Grade Dendritic Cells for Cancer Immunotherapy.* Front Immunol, 2020. **11**: p. 593363.
197. Lehrich, B.M., et al., *Fetal Bovine Serum-Derived Extracellular Vesicles Persist within Vesicle-Depleted Culture Media.* Int J Mol Sci, 2018. **19**(11).
198. Khasawneh, R.R., et al., *Addressing the impact of different fetal bovine serum percentages on mesenchymal stem cells biological performance.* Mol Biol Rep, 2019. **46**(4): p. 4437-4441.
199. Kwon, D., et al., *The Effect of Fetal Bovine Serum (FBS) on Efficacy of Cellular Reprogramming for Induced Pluripotent Stem Cell (iPSC) Generation.* Cell Transplant, 2016. **25**(6): p. 1025-42.
200. Mardpour, S., et al., *Interaction between mesenchymal stromal cell-derived extracellular vesicles and immune cells by distinct protein content.* J Cell Physiol, 2019. **234**(6): p. 8249-8258.
201. Toth, E.A., et al., *Formation of a protein corona on the surface of extracellular vesicles in blood plasma.* J Extracell Vesicles, 2021. **10**(11): p. e12140.
202. Thery, C., et al., *Isolation and characterization of exosomes from cell culture supernatants and biological fluids.* Curr Protoc Cell Biol, 2006. **Chapter 3**: p. Unit 3 22.
203. Onodi, Z., et al., *Isolation of High-Purity Extracellular Vesicles by the Combination of Iodixanol Density Gradient Ultracentrifugation and Bind-Elute Chromatography From Blood Plasma.* Front Physiol, 2018. **9**: p. 1479.
204. Sung, B.H. and A.M. Weaver, *Exosome secretion promotes chemotaxis of cancer cells.* Cell Adh Migr, 2017. **11**(2): p. 187-195.
205. Liu, L., et al., *Bone Marrow Mesenchymal Stem Cell-Derived Small Extracellular Vesicles Promote Periodontal Regeneration.* Tissue Eng Part A, 2021. **27**(13-14): p. 962-976.
206. Esser, J., et al., *Exosomes from human macrophages and dendritic cells contain enzymes for leukotriene biosynthesis and promote granulocyte migration.* J Allergy Clin Immunol, 2010. **126**(5): p. 1032-40, 1040 e1-4.
207. Fu, X., et al., *Mesenchymal Stem Cell Migration and Tissue Repair.* Cells, 2019. **8**(8).
208. Su, P., et al., *Mesenchymal Stem Cell Migration during Bone Formation and Bone Diseases Therapy.* Int J Mol Sci, 2018. **19**(8).
209. Hosseinkhani, B., et al., *Extracellular Vesicles Work as a Functional Inflammatory Mediator Between Vascular Endothelial Cells and Immune Cells.* Front Immunol, 2018. **9**: p. 1789.
210. Zhu, W., et al., *Hypoxia and serum deprivation-induced apoptosis in mesenchymal stem cells.* Stem Cells, 2006. **24**(2): p. 416-25.
211. Sohni, A. and C.M. Verfaillie, *Mesenchymal stem cells migration homing and tracking.* Stem Cells Int, 2013. **2013**: p. 130763.
212. Viaud, S., et al., *Updated technology to produce highly immunogenic dendritic cell-derived exosomes of clinical grade: a critical role of interferon-gamma.* J Immunother, 2011. **34**(1): p. 65-75.
213. Wang, D., et al., *Schwann cell-derived EVs facilitate dental pulp regeneration through endogenous stem cell recruitment via SDF-1/CXCR4 axis.* Acta Biomater, 2022. **140**: p. 610-624.
214. Croitoru-Lamoury, J., et al., *Human mesenchymal stem cells constitutively express chemokines and chemokine receptors that can be upregulated by cytokines, IFN-beta, and Copaxone.* J Interferon Cytokine Res, 2007. **27**(1): p. 53-64.
215. Singh, S., D. Anshita, and V. Ravichandiran, *MCP-1: Function, regulation, and involvement in disease.* Int Immunopharmacol, 2021. **101**(Pt B): p. 107598.
216. Boomsma, R.A. and D.L. Geenen, *Mesenchymal stem cells secrete multiple cytokines that promote angiogenesis and have contrasting effects on chemotaxis and apoptosis.* PLoS One, 2012. **7**(4): p. e35685.

217. Elashiry, M., et al., *Proteomic Characterization, Biodistribution, and Functional Studies of Immune-Therapeutic Exosomes: Implications for Inflammatory Lung Diseases.* Front Immunol, 2021. **12**: p. 636222.
218. Choi, D., et al., *Quantitative proteomic analysis of trypsin-treated extracellular vesicles to identify the real-vesicular proteins.* J Extracell Vesicles, 2020. **9**(1): p. 1757209.
219. Mbongue, J.C., et al., *The Role of Dendritic Cell Maturation in the Induction of Insulin-Dependent Diabetes Mellitus.* Front Immunol, 2017. **8**: p. 327.

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Supplementary Information

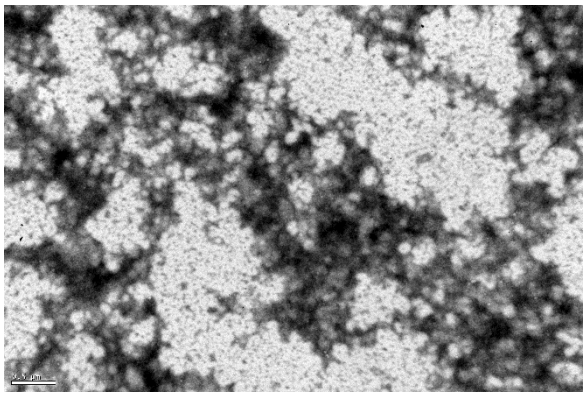

Figure S 1. Ultracentrifuged RPMI-1640 + 10% FBS observation under TEM.

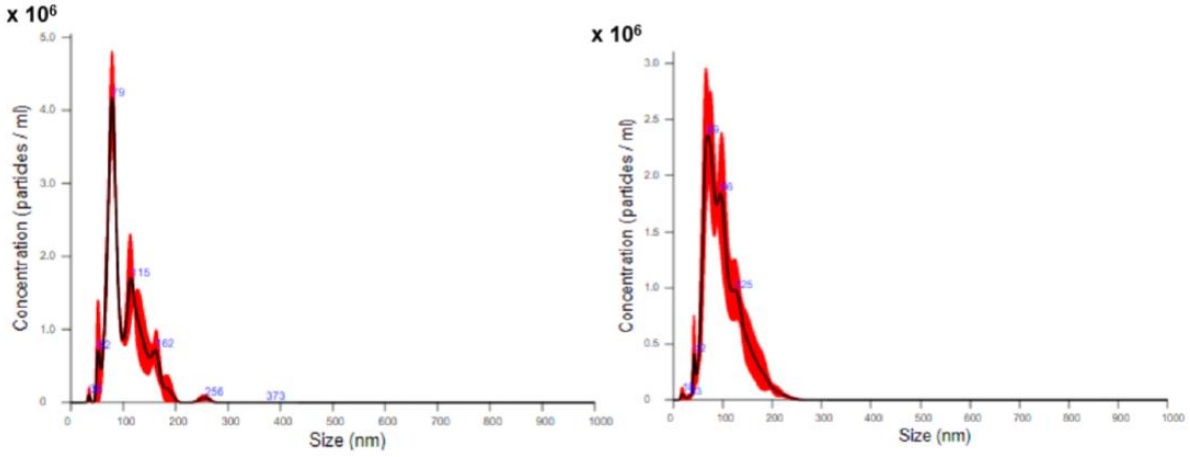

Figure S 2. Concentration versus size distribution of ultracentrifugation controls. NTA analysis was performed across supernatants of DC and DC activated with TNF-α cultures (left and right, respctively).

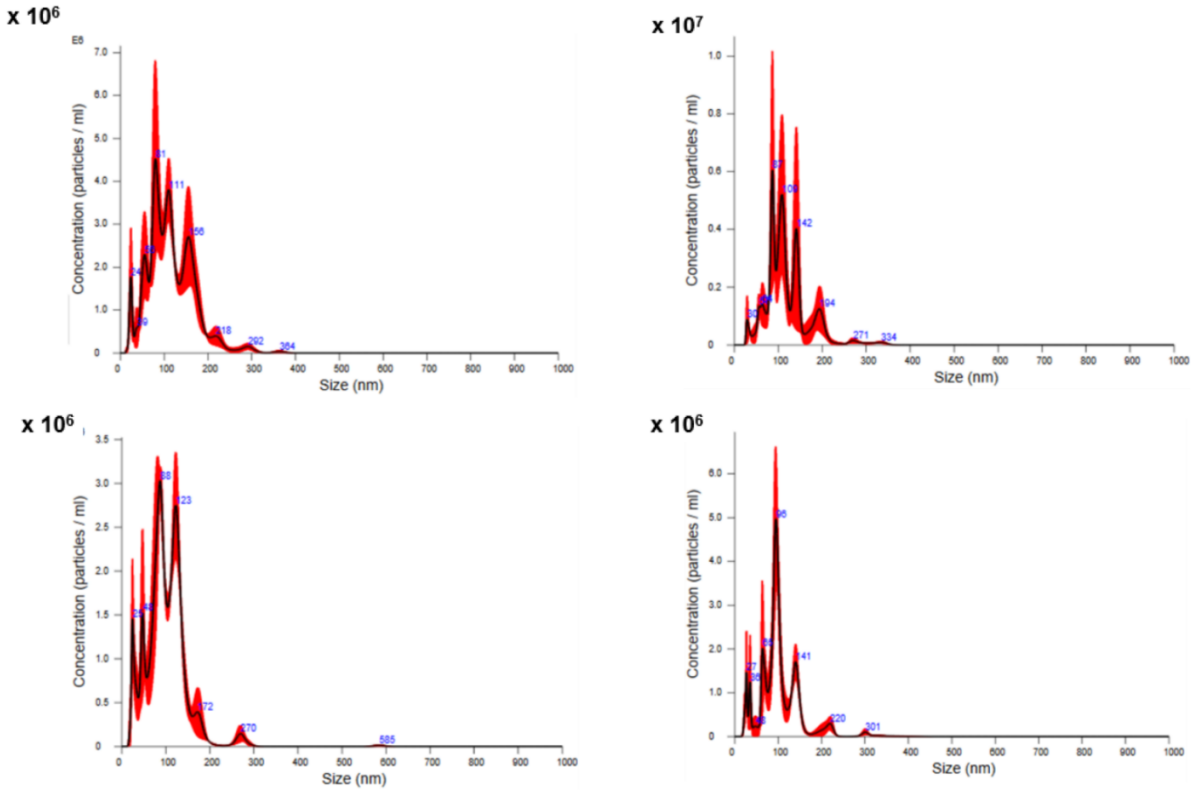

Figure S 3. Concentration versus size distribution of 0.22 µm filtered samples. Upper graphs correspond to nEV (left) and tEV (right) and bottom graphs correspond to respective UC supernatant controls.

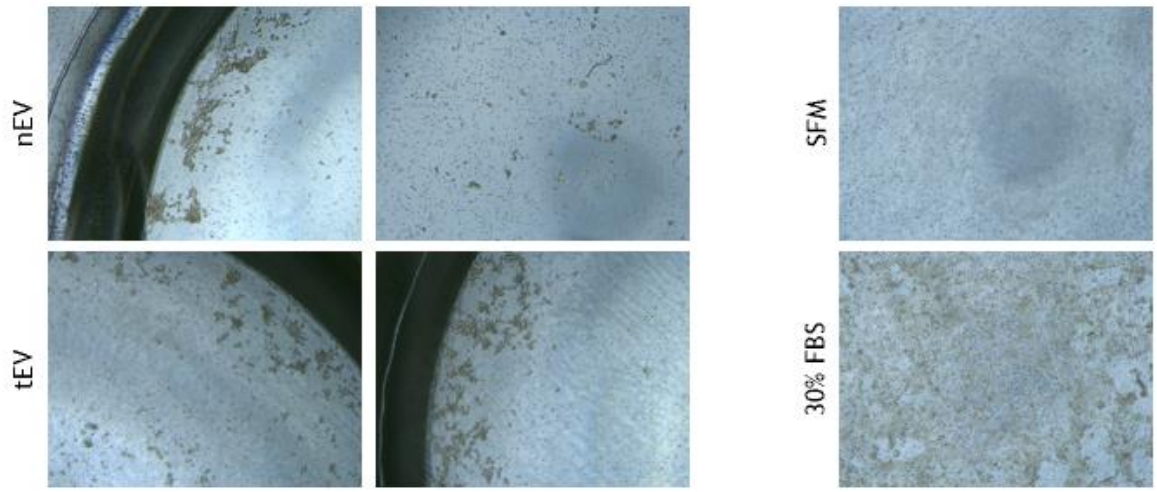

Figure S 4. Visualization of membranes of the transwell inserts immeadiately after fixation of MSC with PFA 4%.