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EXTRACELLULAR VESICLES AS MESSENGERS FOR TISSUE REGENERATION: IMPACT ON MESENCHYMAL STEM/STROMAL CELLS

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Table of Contents

Abstract

The homeostatic mechanism of bone remodelling may be disturbed by a variety of traumatic and pathophysiological conditions affecting the skeletal system. Musculoskeletal disorders constitute a major economical and health care burden, particularly in aging populations. In otherwise healthy individuals, bone repair follows a regulated sequence of events usually divided into three phases: inflammation, repair and remodelling. Upon bone injury, an inflammatory response is elicited, both at the local and systemic levels, which needs to be resolved along time in order to create a microenvironment conducive to tissue repair/regeneration. Immune cells, which are recruited to the injury site, are known to modulate the activity of osteoprogenitor cells, including multipotent mesenchymal stem/stromal cells (MSC), which are involved in the formation of new bone tissue. This crosstalk between immune and skeletal cell populations may occur via direct cell-to-cell communication and through secreted factors, which may be conveyed inside extracellular vesicles (EV).

Previous work in the lab revealed that naive human dendritic cells (DC) promote human MSC recruitment, particularly through the EV they secrete. Following these results, it was hypothesized that the microenvironment created upon bone injury could precondition DC and the EV they secrete, impacting their capacity to regulate MSC biology. Thus, a critical size femur defect *in vivo* model was performed, and animals were sacrificed at 3 and 14 days after bone injury. Non-operated animals were used as controls. EV derived from DC differentiated *ex vivo* from bone marrow (BM) at 14 days post-bone injury were shown to significantly promote MSC migration whereas EV from day 3 post-injury DC significantly impaired it. A membrane array screening indicated that EV from 14 days post-injury DC were depleted of TIMP-1, TNF-α and Fas ligand, when compared to EV from day 3. In addition, it was also demonstrated that the response to bone injury was characterized by timely-controlled alterations in the profile of microRNAs (miRNA) circulating systemically in plasma, which were predicted to regulate several cellular mechanisms, including cell proliferation. In line with this, the work developed during this dissertation was intended to complement these previous findings and aimed at further dissect the systemic and local responses triggered upon bone injury. In particular, three specific objectives were established: A) evaluate the impact of bone injury in the systemic inflammatory response, namely, the influence of plasma from different timepoints after injury on MSC proliferation, and the inflammatory cytokine expression in splenocytes; B) explore the impact of DC-EV on MSC biology, in particular, confirm the specificity of DC-EV from different times after bone injury in recruiting MSC; C) validate the temporal changes in EV content, observed in the membrane array and their impact on MSC. Rat

biological samples collected from the *in vivo* critical size bone defect model and sham-operated animals, previously obtained in the lab, were used for this work.

Results obtained showed that plasma from rats at 3 and 14 days after bone injury significantly promoted MSC proliferation, compared to the non-stimulated control condition. These results confirmed the initial bioinformatics predictions, and were included in the scientific article *Silva et al. (2018) Theranostics 8(14),* published during the development of this thesis. The systemic response to injury was further evaluated by gene expression analysis of cytokines levels in splenocytes from injured and control animals, with the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 being elevated in rats at 3 days post-injury. In line with these results, the inflammatory response to bone injury appeared to impact also cytokine expression by BM-derived DC, with TNF-α and IL-6 expression levels showing a tendency to decrease from day 3 to day 14 post-injury.

After bone injury, DC-EV concentration was lower at day 3, but recovered by day 14, while it remained low along time after surgery in sham-operated animals. At the functional level, EV secreted by DC from injured animals promoted MSC proliferation relative to control conditions, albeit not significantly and to levels similar to those of ultracentrifugation supernatant controls. Also, DC-EV affected the expression levels of IL-6 by MSC, particularly those from 3 days post-injury cells. Importantly, our previous results on the impact of bone injury on the ability of DC-EV to modulate MSC migration were confirmed herein to be specific, as DC-EV from sham-operated controls were not able to promote MSC recruitment in a transwell migration system.

In order to further explore our previous findings on the effect of time post-injury on DC-EV content, conditioned media from DC was first analysed by western blotting for the levels of TIMP-1, confirming that this protein was enriched in conditioned media of DC from 3 days after injury rats. In addition, transwell migration assays were conducted to test the ability of TNF-α, also found timely-regulated in DC-EV, to control MSC recruitment in a dose-dependent manner. According to our results, TNF-α alone did not impact MSC migration, and thus might not be responsible for the effect observed for DC-EV.

Overall, this work contributed to further characterize the systemic and local responses to bone injury, suggesting the specificity of such response in comparison with soft tissue injury, as performed for sham-operated animals. It also provided valuable insights into the paracrine crosstalk between immune and skeletal cell populations in the context of bone repair and regeneration, which may contribute to the development of novel therapies for bone-associated pathologies, using natural or engineered EV.

Sumário

O mecanismo homeostático da remodelação óssea pode ser alterado por uma diversidade de condições traumáticas e patofisiológicas que afetam o sistema esquelético. As doenças musculoesqueléticas constituem um enorme peso, a nível económico e dos sistemas de saúde, particularmente em populações envelhecidas. Em indivíduos saudáveis, a reparação óssea segue uma sequência regulada de acontecimentos, usualmente divididos em três fases: inflamação, reparação e remodelação. Quando ocorre uma lesão óssea, uma resposta inflamatória é iniciada, tanto a nível local como sistémico, que deve ser resolvida no tempo por forma a criar um microambiente que conduza à reparação/regeneração do tecido. As células imunes que são recrutadas para o local da lesão são capazes de modular a atividade das células osteoprogenitoras, incluindo células mesenquimais estaminais ou do estroma (MSC, do inglês *mesenchymal stem/stromal cells*), que estão envolvidas na formação de novo tecido ósseo. Esta inter-comunicação entre populações celulares dos sistemas imune e esquelético pode ocorrer por comunição direta entre células e através de fatores secretados, que podem ser veiculados no interior de vesículas extracelulares (EV, do inglês, *extracellular vesicles*).

O trabalho previamente desenvolvido no laboratório revelou que as células dendríticas (DC, do inglês *dendritic cells*) humanas imaturas promovem o recrutamento das MSC humanas, particularmente através das EV que secretam. No seguimento destes resultados, foi colocada a hipótese de que o microambiente criado em contexto de lesão óssea poderia pré-condicionar as DC e as EV por elas secretadas, influenciando a capacidade de estas células regularem a biologia das MSC. Assim, um modelo *in vivo* de lesão óssea de tamanho crítico foi desenvolvido e os animais foram sacrificados aos dias 3 e 14 após a cirurgia. Animais não operados foram usados como controlos. As EV derivadas de DC diferenciadas *ex vivo* a partir da medula óssea (BM, do inglês *bone marrow*) aos 14 dias após-lesão promoveram significativamente a migração das MSC, enquanto as EV de DC do dia 3 apresentaram um efeito inibidor. Foi realizado um array de membrana cujos resultados indicavam que as EV das DC de 14 dias após-lesão tinham, em relação aos 3 dias, quantidades reduzidas do Inibidor Tecidual de Metaloproteases-1 (TIMP-1, do inglês *Tissue Inhibitor of Metalloproteinase-1*), Fator de Necrose Tumoral-α (TNF-α, do inglês *Tumor Necrosis Factor-α*) e Fas Ligando (FasL, do inglês *Fas Ligand*). Para além disso, foi também demonstrado que em resposta à lesão óssea ocorriam alterações no perfil de microRNAs (miRNA) em circulação sistémica no plasma, dependentes do tempo pós-lesão, que se previu estarem associadas à regulação de vários mecanismos celulares, incluindo da proliferação celular. Assim, o trabalho desenvolvido no âmbito desta dissertação veio complementar estes resultados, com o objetivo de explorar as respostas local e sistémica desencadeadas em resposta à lesão no osso. Em particular foram delineados três objetivos:

A) avaliar o impacto da lesão óssea na resposta inflamatória sistémica, nomeadamente a influência do plasma proveniente de animais em diferentes tempos pós-lesão na proliferação das MSC, e o perfil de expressão de citocinas inflamatórias pelas células do baço; B) explorar o impacto das EV de DC na biologia das MSC, em particular confirmar a especificidade das EV das DC de tempos diferentes pós-lesão no recrutamento de MSC; C) validar as mudanças temporais no conteúdo das EV, que tinham sido observadas no array de membrana e o seu impacto nas MSC. As amostras biológicas de rato do modelo *in vivo* de lesão óssea de tamanho crítico e de animais sham, obtidas previamente no laboratório, foram utilizadas para a realização deste trabalho.

Os resultados obtidos mostraram que o plasma dos dias 3 e 14 após a lesão óssea promoveram significativamente a proliferação das MSC, comparativamente com a condição controlo não-estimulada. Estes resultados confirmaram as previsões bioinformáticas iniciais e foram incluídos no artigo científico *Silva et al. (2018) Theranostics 8(14)*, publicado durante o período de desenvolvimento desta tese. A resposta sistémica à lesão foi avaliada por análise de expressão génica dos níveis de citocinas nas células do baço de animais lesados e controlo, tendo-se verificado que os níveis das citocinas pro-inflamatórias TNF-α, IL-1ß e IL-6 se encontravam elevados nas células ao dia 3 após a lesão. A resposta inflamatória local à lesão também teve impacto no perfil de expressão de citoquinas pelas DC derivadas da BM, com uma tendência de diminuição dos níveis de expressão de TNF-α e IL-6 do dia 3 para o dia 14 pós-lesão.

Após a lesão óssea, a concentração de EV secretadas pelas DC mostrou uma diminuição ao dia 3, recuperando valores semelhantes aos das células de animais não operados ao dia 14. No entanto, em animais sham as concentrações de EV secretadas permaneceram baixas aos 3 e 14 dias após a cirurgia. A nível funcional, as EV produzidas pelas DC de animais com lesão óssea promoveram a proliferação das MSC, comparativamente com as condições controlo, embora não significativamente e a níveis semelhantes aos dos controlos (sobrenadantes da ultracentrifugação). As EV das DC afetaram a expressão de IL-6 pelas MSC, particularmente as de DC do dia 3 pós-cirurgia. Os resultados obtidos demonstraram ainda que a capacidade de as EV das DC modularem a migração das MSC é específica do contexto de lesão óssea, uma vez que as EV secretadas pelas DC de controlos sham não foram capazes de promover o recrutamento das MSC num sistema de migração em *transwell*.

O efeito do tempo pós-lesão óssea no conteúdo das EV foi aqui explorado, numa primeira fase através da avaliação dos níveis da proteína TIMP-1 no meio condicionado das DC por *western blotting*, tendo-se confirmado que esta proteína se encontrava enriquecida no meio condicionado destas células ao dia 3 após lesão. Em seguida, foram realizados ensaios de migração em sistema *transwell* para testar se a citoquina TNF-α teria a capacidade de controlar o recrutamento das MSC de um modo dependente da dose. De

acordo com os nossos resultados, o TNF-α não teve impacto na migração das MSC e, portanto, poderá não ser o fator responsável pelo efeito observado com as EV derivadas de DC.

Globalmente, o trabalho desenvolvido no âmbito desta dissertação contribuiu para melhor caracterizar as respostas local e sistémica desencadeadas em resposta à lesão óssea. Os resultados obtidos sugerem a especificidade destas respostas, por comparação com as condições de lesão dos tecidos moles, nos controlos sham. Além disso, este trabalho fornece também novos conhecimentos acerca da inter-comunicação parácrina entre as populações de células dos sistemas imunitário e esquelético, no contexto da reparação/regeneração óssea, que poderão vir a contribuir para o desenvolvimento de novas terapias para o tratamento de doenças que afetam o osso, usando EV naturais ou modificadas.

List of Figures

List of Tables

List of Abbreviations

ALP	Alkaline phosphatase
BC	Bottom compartment
b-FGF	Basic fibroblast growth factor
BM	Bone marrow
BMP-2	Bone morphogenetic protein-2
BMUs	Basic multicellular units
BSA	Bovine serum albumin
BTE	Bone tissue engineering
CCL-2	Chemokine (C-C motif) ligand-2
CXCR-4	C-X-C chemokine receptor type 4
CD	Cluster of differentiation
cDNA	Complementary DNA
CM	Conditioned media
DAMP	Damage associated molecular patterns
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic Cell
DGAV	Direção-Geral de Alimentação e Veterinária
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
EV	Extracellular Vesicles
FBS	Fetal bovine serum
FGFR3	Fibroblast growth factor receptor 3
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GBD	Global Burden of Disease
GM-CSF	Granulocyte-macrophage colony-stimulating factor
hFOB	Human fetal osteoblastic cells
hiPS	Human-induced pluripotent stem cell
HRP	Horseradish peroxidase
IL	Interleukin
Ly6G	Lymphocyte antigen 6 complex locus G6D
Mac	Macrophage
MAFIA	Macrophage Fas-induced apoptosis

MAPK	Mitogen-activated protein-kinase
M-CSF	Macrophage Colony-Stimulating Factor
mRNA	Messenger RNA
miRNA	microRNA
MMP	Matrix Metalloproteinase
mRNA	Messenger RNA
MSC	Mesenchymal stem/stromal cell
MV	Matrix Vesicles
MVB	Multivesicular bodies
NO	Non-operated
NTA	Nanoparticle tracking analysis
OB	Osteoblasts
OC	Osteoclasts
OCN	Osteocalcin
OI	Osteogenesis imperfecta
OPG	Osteoprotegerin
OPN	Osteopontin
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PFA	Paraformaldehyde
PI3k-Akt	Phosphatidylinositol 3-kinase-Protein kinase B
PL	Platelet lysate
P/S	Penicillin/G-streptomycin
RA	Rheumatoid arthritis
RANK	Receptor activator of nuclear factor kappa-B
RANKL	Receptor activator of nuclear factor kappa-B ligand
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute media
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
RUNX-2	Runt-related transcription factor 2
SDF-1	Stromal cell-derived factor-1
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SFM	Serum-free media
TBS	Tris-buffered saline
TC	Top compartment
TEM	Transmission electron microscopy
TGF-β1	Transforming growth factor-beta 1

TIMP	Tissue inhibitor of matrix metalloproteinases
TLR	Tool-like receptor
TNF-α	Tumour necrosis factor-alpha
VEGF	Vascular endothelial growth factor
α-MEM	Minimum essential medium eagle alpha modification
β-TCP	Beta-tricalcium phosphate

1. INTRODUCTION

1.1. Bone in normal and pathophysiological conditions

Bone is a unique type of connective tissue, composed of cells, a mineralized matrix of crystalline hydroxyapatite, also enriched in type I collagen, and water. It is highly vascularized and metabolically active and constitutes the skeletal framework characteristic of all vertebrate organisms, serving several physiological functions. Besides from providing support, mobility and protection for vital organs, bone hosts the hematopoietic compartment, and constitutes a reservoir for calcium and other inorganic ions [1]. In terms of structure, bone tissue can be organized in the form of compact bone, which constitutes the dense outer layer, and trabecular bone, a porous lattice enclosing several spaces in the most interior regions. Two specialized membranes, the periosteum and the endosteum, line the outer and inner surfaces of bone, respectively, constituting important niches of osteoprogenitor cell populations [1].

In humans, the process of bone development starts during the embryonic period, but is not yet fully completed at birth, as bone mass continues to increase during childhood and more markedly during puberty, by the end of which more than 95% of the skeleton is fully formed [2]. This process of modeling leads to alterations in bone shape and size, since new tissue is formed in places where resorption, a physiological process of bone destruction, did not occur yet. During adulthood, gains in bone mass are reduced to a minimum and bone homeostasis is maintained by a dynamic and continuous process of remodelling, which differs from modeling, since the formation of new bone is intended to replace bone at a site where it was previously removed by resorption [3, 4]. Bone remodelling is carried out asynchronously at distinct anatomical places termed basic multicellular units (BMUs) [5], where the formation of new bone by osteoblasts (OB), which are cells differentiated from mesenchymal precursors, is coupled with the resorption activity of osteoclasts (OC), multinucleated cells with hematopoietic origin [6]. This remodelling process, which lasts about 4 months per site, allows the replacement of bone that has outlived its functionality and the continuous adaptation to environmental cues and mechanical stressors, and also repair in the case of injury, maintaining tissue function throughout life [6]. Nevertheless, with advancing age, this highly sophisticated mechanism becomes dysregulated, with a decrease in the quantity of bone formed in each cycle of remodelling, which leads to bone mass reduction and altered mechanical properties [7]. Moreover, several pathological conditions can accelerate this natural process, to different extents, leaving the skeleton susceptible to damage at an earlier age.

1.1.1. Bone disorders

Musculoskeletal disorders are extremely common, affecting approximately one in three people worldwide [8]. They constitute the most common cause of severe long-term pain and are highly debilitating, posing a serious economic burden to society, both through direct costs of treating the condition and indirect ones related with decreased productivity. In fact, according to the 2016 Global Burden of Disease (GBD) data for noncommunicable diseases, musculoskeletal diseases were the second leading cause of global disability, which was estimated to have increased by 61.6% between 1990 and 2016 [8]. Also, since these conditions present an augmented prevalence with age [8], the number of people suffering from them and the burden associated are expected to markedly increase over the next decades. Among musculoskeletal disorders, bone diseases constitute a heterogeneous group with different underlying pathophysiologies, which can also have manifestations in the joints and muscles.

Although relatively rare, bone diseases can be caused by genetic mutations, as in the case of skeletal dysplasias [9]. These conditions are characterized by generalized skeletal abnormalities, which may display different degrees of clinical severity, ranging from mild and asymptomatic to highly severe and incompatible with life. This category includes about 372 conditions, being achondroplasia, osteogenesis imperfecta (OI) and osteopetrosis the most common [10]. Achondroplasia is caused by a mutation in the fibroblast growth factor receptor 3 (FGFR 3) gene and results in retarded cartilage formation, which is translated into short and abnormally shaped bones [11]. Osteogenesis imperfecta and osteopetrosis are both marked by altered bone density and increased fracture risk. However, while in osteopetrosis, abnormalities in osteoclast formation and function result in high bone density, in OI, defects in bone formation, particularly in collagen synthesis, lead to osteopenia and low bone mass [12, 13].

Most commonly, bone pathologies are caused by mineral or chemical imbalances and dysregulations in the process of bone turnover, being collectively known as metabolic bone diseases [14]. Among them, osteoporosis is by far the most prevalent, affecting about 10 million people over the age of 50 years in the United States, a number expected to reach more than 14 million by 2020 [15]. This condition is characterized by decreased bone mass and defective microarchitecture which results in increased susceptibility to fracture, the main manifestation of this condition [16]. More than 8.9 million osteoporotic fractures occur every year worldwide [17], being a major cause of disability and reduced quality of life, and representing a huge economic burden. Data from 2010 pointed to an annual cost of osteoporosis, including pharmacological intervention and without accounting for intangible costs, of €37 billion, only in Europe, a number expected to increase by 25% until 2025 [18]. Impairments in bone remodelling may also occur due to chronic systemic

inflammation, as in the case of rheumatoid arthritis (RA). The high levels of pro-inflammatory cytokines characteristic of this autoimmune condition can promote osteoclast activation, which in turn accentuates bone resorption, leading to bone loss and increased propensity to fracture [19].

Bone can host different types of tumors, including those originated in the bone tissue itself (benign or malignant primary bone tumors) and bone metastases. Osteosarcoma, the most common primary bone tumor, although rare among adults, is the most common primary cancer in adolescents aside from leukemia and lymphoma [20], presenting a 5-year survival rate around 65% [21]. Bone metastases are formed in 65 to 80% of patients with metastatic breast and prostate cancer, and they are indicative of an advanced stage cancer, being normally incurable [22, 23]. Once established in the bone microenvironment, these metastases interfere with bone remodelling, by inducing the resorption of normal bone (osteolytic metastasis) and/or promoting the deposition of new tissue, with the formation of osteoblastic lesions [24]. These remodelling imbalances are in turn associated with the secretion of different molecules which support the survival of tumor cells, leading to a vicious cycle which hinders treatment [25]. Patients with bone metastasis normally suffer from pathological fractures, decreased mobility and severe pain, which is highly difficult to manage and generally intensifies over time [26].

One common manifestation of most bone conditions described so far is the increased susceptibility to fracture. Fractures are discontinuities in the normal bone anatomy and represent one of the most common injuries of the musculoskeletal system. Every time a fracture occurs, the patient stands at an increased risk of developing new fractures and rehabilitation is needed in up to 75% of individuals [17]. In the case of hip fractures, 24-33% may even die in the first-year post-injury [27, 28]. Moreover, in 5-10% of all fracture cases, bone fails to heal completely, originating non-unions, or complications such as bone delayed unions may occur, leading to pain and disability, and increasing costs in fracture management [29].

1.1.2. Bone repair and regeneration: the role of Mesenchymal Stem/Stromal Cells

When bone is injured, either by a traumatic event or in the context of a pathological condition, the process of bone healing is initiated, proceeding though a series of three main overlapping and well-coordinated phases: inflammation, repair and remodelling (Figure 1) [30]. Of note, these stages are highly dependent on a tight communication between different intervenient cells, which can occur either by direct cell-to-cell contact [31], or via secreted paracrine factors, including cytokines, chemokines, growth factors, among others [32], also likely including extracellular vesicles (EV).

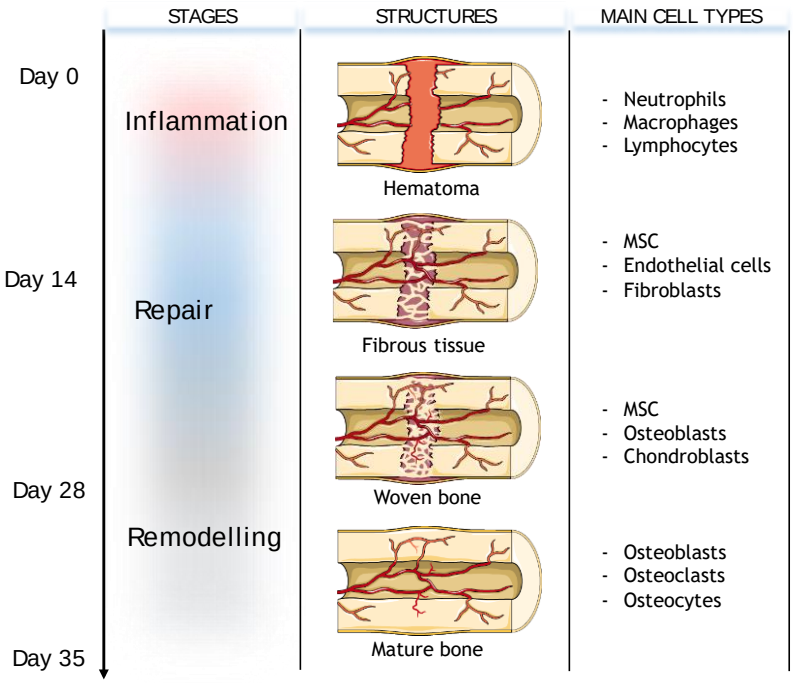

Figure 1. The process of bone repair follows three main overlapping stages. Upon bone injury, a local inflammatory reaction is elicited which leads to the recruitment of circulating immune cells, of fibroblasts, endothelial cells and osteoprogenitor cells, including MSC. After two weeks, this inflammatory reaction resolves allowing the transition to the events of tissue repair. The process of osteogenesis is initiated, and woven bone is formed either by endochondral or intramembranous ossification during the following two weeks. The stage of bone remodelling, which occurs through the concerted action of osteoblasts and osteoclasts, starts around day 28 after injury and allows the conversion of the immature woven bone into mature bone, with recovery of tissue function. The timepoints indicated correspond to the ones reported for the process of bone repair in rats. Based on [33, 34].

Damage in the blood vessels adjacent to the injury site activates the coagulation cascade and induces the formation of a hematoma, providing a short-term scaffold for the sequential arrival of immune cells, which clear the wound, interact with the tissue resident cells and act in a synergistic and time-dependent manner to prime the microenvironment for the following events of the repair and regeneration process [35]. This constitutes the initial acute inflammatory phase, which occurs during the first 7 days post-injury in rats, and is characterized by an elevation in the levels of pro-inflammatory cytokines (such as IL-6, TNF-α, IL-1ß) and angiogenic factors (e.g. angiopoietin-1, VEGF), contributing to the process of revascularization, which re-establishes the blood supply to the damaged tissue [30]. Importantly, this pro-inflammatory microenvironment provides the signals for the recruitment of osteoprogenitor cells, including mesenchymal stromal/stem cells (MSC), which are thought to proliferate and differentiate towards the chondrogenic and osteogenic lineages in the following stages of repair, which are initiated around day 14 after injury, when the inflammatory response enters the resolution phase [30].

In optimal conditions, as in the case of fractures with extremely low interfragmentary movement, the bone repair phase can proceed directly through a process resembling normal bone remodelling (primary bone healing), skipping the formation of a healing callus [30]. However, in the vast majority of cases, bone heals indirectly with the formation of a soft callus (secondary bone healing) which is progressively converted into a bony one [36]. This route of bone repair follows both intramembranous and endochondral ossification at different locations of the injury site, and allows a stepwise increase in mechanical stability, eventually reaching levels suitable for revascularization and mineralization to occur [30]. In the periosteum, in regions at some distance from the injury site where vascularization is not disturbed, MSC differentiate into osteoblasts and woven bone is directly deposited through a process of intramembranous ossification, which starts as early as 3 to 7 days post-injury in rats and progresses towards the injury site [30]. In contrast, at close proximity to the damaged area, where blood supply is compromised, repair occurs through differentiation of MSC into chondrocytes which produce an extracellular matrix enriched in type II collagen and proteoglycans (soft callus) that serves as a template for endochondral ossification, events which are initiated at day 7 to 10 after injury in rats [30]. These cells eventually undergo hypertrophic differentiation and start the mineralization of their surrounding matrix, events accompanied by invasion of new blood vessels and recruitment of monocytes and MSC [37]. Monocytes differentiate into osteoclast-like cells which degrade the calcified cartilage and MSC differentiate into osteoblasts which start the deposition of immature woven bone [38]. As this process proceeds, the damaged area starts regaining enough mechanical stability to sustain physiological loads, a point normally reached around 28-35 days post-injury in rats and within 8-16 weeks after injury in fracture patients [30]. Finally, during the remodelling phase, immature woven bone is remodelled to lamellar bone, restoring normal bone anatomy and function, a process which takes 5 to 8 weeks in rats, but normally lasts for several years in humans [30].

Even though bone presents an innate ability to fully regenerate upon damage, without the formation of a fibrous scar and with fully recovery of function, numerous factors may interfere with this process opening the need for therapeutic intervention which intends to enhance and accelerate the bone healing process [34]. Autologous bone grafting is still considered the gold standard for severe cases of bone injury, but it is associated with numerous drawbacks, such as limited supply of donor tissue, donor-site morbidity and risks associated with the surgical procedures (e.g. bleeding, infection, disease transmission) [39]. The field of bone tissue engineering (BTE), which entails the use of cells, bioactive carriers and molecular factors, in different combinations, to promote bone tissue repair, appeared over three decades ago as a potential alternative to traditional therapeutic options [40, 41].

Among cell-based therapies for bone regeneration, those using MSC are the most widely described in the literature (reviewed in [42]). In fact, several works have shown the benefits of MSC transplantation for the healing of bone fractures and defects in different animal models [42], and 26 clinical trials are currently registered for the use of MSC in fracture treatment [43]. This is not surprising since MSC constitute a source of progenitor cells essential for bone repair, due to their ability to differentiate into the chondrogenic and osteogenic lineages [44]. Moreover, MSC have been successfully isolated from different tissues [45], and suggested to be immunoprivileged, constituting a promising source of cells off-the-shelf for heterologous transplantation [46]. Additionally, they were shown to migrate to places of injury upon transplantation [47], including in the case of bone injury [48], through what appears to be a multistep process primarily mediated by homing/chemokine receptors and endothelial co-receptors, among which the SDF-1/CXCR4 signalling axis seems to assume particular relevance [49, 50]. Interestingly, MSC were also shown to modulate the progression of inflammation, by secreting immunoregulatory factors, and empower tissue resident cells (such as endothelial cells, fibroblasts, progenitor cells) towards a regenerative phenotype, through the production of considerable amounts of growth factors [51]. Nevertheless, the exact mechanisms underlying the positive benefits of MSC for bone repair remain elusive.

Although tremendous achievements have been registered over the years, several hurdles need to be surpassed until BTE becomes a standard option in clinical settings. A better fundamental understanding of the molecular mechanisms and cellular crosstalk that govern the different phases of bone regeneration is therefore warranted to uncover novel potential therapies and facilitate the translation from bench to bedside.

1.2. Immune cells in bone regeneration

Since the pioneer works in the 1970s [52, 53], when the intricate relationship between the skeletal and immune systems was first unveiled, considerable amount of evidence has been gathered pointing to how immune cells impact bone biology, both in physiological and pathophysiological conditions (reviewed in [54]). In the context of bone regeneration, the initial inflammatory phase is now recognized as essential for the progression and success of the overall process, as immune cells provide important instructions for the recruitment, proliferation and differentiation of osteoprogenitor cells, including MSC, and for the anabolic and catabolic activities of osteoblasts and osteoclasts, respectively [33]. For instance, impaired fracture healing has been reported both in studies with IL-6 and TNF-α knockout animal models [55, 56] and in patients treated with anti-inflammatory drugs [57].

Neutrophils are the first innate immune cells to reach the injury site, during the first 24 h, attracted by cell debris and danger signals (e.g. damage associated molecular patterns (DAMP)) [33]. Even though early reports suggested that neutrophils could negatively impact bone regeneration [58], recent works have demonstrated that neutrophil activation may be essential for the transition from the initial inflammatory stages to the downstream events conducive to bone repair. In fact, neutrophils have been pinpointed as the immune cell population most abundantly present in the early fracture hematoma [59], where they seem to be involved in the production of a fibronectin-rich extracellular matrix which can serve as a scaffold for stromal cell recruitment, as recently suggested by Bastian *et al*. [60]. Moreover, work by Kovtun *et al*. using a femoral osteotomy mouse model showed decreased bone tissue formation and a fracture callus with impaired mechanical properties at 21 days post-injury when neutrophils were depleted by administration of an anti-Ly6G antibody prior to injury [61]. These results were in line with previous work from Chan *et al.*, which showed impaired fracture healing, with delayed mineralization and remodelling of the healing callus, when a similar approach to specifically deplete neutrophils in a murine tibial fracture model was applied [62]. Importantly, neutrophils are known to secrete certain chemical mediators, such as IL-6 and CCL-2, essential for the recruitment of inflammatory macrophages (derived from circulating monocytes), which together with osteomacs (bone tissue resident macrophages) constitute the two major macrophage populations in the bone microenvironment [63, 64]. Macrophages are in fact the immune cell type most extensively studied for their crosstalk with skeletal populations, which seems to occur not only during the early inflammatory events but also in the later stages of bone repair and remodelling [65].

A study using a murine tibial fracture model showed a differential spatial distribution of macrophages throughout the process of endochondral fracture healing, with these cells being preferentially present near the injury site in early timepoints, and in areas of active bone deposition in the later stages [66]. Moreover, when macrophages where depleted systemically, in the drug-inducible macrophage Fas-Induced apoptosis (MAFIA) mouse model, smaller fracture calluses and less deposition of woven bone were observed, which translated into impaired bone union at 21 days post injury. The authors also assessed the contribution of macrophages for the osteoblastic differentiation of mesenchymal progenitor cells. When macrophages were depleted in non-injured animals, the number of mesenchymal progenitor cells was reduced, together with the ability of these cells to differentiate into osteoblasts, suggesting a role for macrophages in the promotion of osteoblastic differentiation *in vivo* [66]. Several other works have also reported the ability of macrophages to induce MSC osteogenic differentiation *in vitro* [67, 68], which seems to be dependent on their inflammatory activation [69].

Alexander *et al.* used a murine tibial bone injury model that heals primarily through intramembranous ossification to uncover the contribution of osteomacs for the outcome of the bone healing process [70]. Both inflammatory macrophages ($F4/80^{+}Mac\text{-}2^{-/high}$) and osteomacs ($F4/80^{+}Mac\text{-}2^{-/low}$) were shown to be present at the injury site in all healing times. However, osteomacs were present in significantly higher numbers in areas of intramembranous bone formation, where inflammatory macrophages only appeared in small clusters. Moreover, osteomacs were shown to be positioned in close proximity with matrix-producing and mineralizing osteoblasts. When these cells were depleted *in vivo* in a timepoint coincident with the time of surgery, the anabolic processes of bone deposition and mineralization were significantly repressed, suggesting that this particular macrophage subset plays a major role in the anabolic phases of intramembranous bone healing [70].

The ability of macrophages to shift their inflammatory phenotype according to their surrounding environment is well known, with growing evidence pointing to how this plasticity may impact tissue regeneration [71]. Schlundt and colleges explored how macrophage phenotype shifts during the course of normal bone healing using a murine model of standard closed femoral fracture [72]. During the first 3 days post-injury, a shift from M1 (pro-inflammatory) to M2 (pro-regenerative) macrophages was observed around the osteotomy gap, and at 7 days post-injury, M2 macrophages were highly enriched whereas M1 macrophages were completely absent. Interestingly, at 21 days after surgery, during the later bone anabolic stages, the macrophages were still present but did not express the markers characteristic of neither M1 or M2 phenotypes, data suggesting the existence of another set of macrophages during remodelling. A proof of concept study was also performed by implanting a collagen scaffold containing IL-4 and IL-13 in the osteotomy gap, with the purpose of inducing the M2 phenotype *in vivo*. As expected, the artificial induction of a more anti-inflammatory environment in the injured area promoted tissue regeneration, with callus and bone volumes being significantly higher when compared with non-treated animals [72].

On the other hand, the involvement of dendritic cells (DC) in the crosstalk with bone populations during bone healing remains vastly unexplored. A recent investigation conducted by Wang *et al.* intended to evaluate the role of toll-like receptor 4 (TLR4) during the process of calvarial bone healing, ended up unveiling a possible role for dendritic cells [73]. Animals lacking TLR4 expression in their dendritic cells ($CD11c^{+}$) showed significantly larger bone volume at 28 days post-injury, a response that was not observed for TLR4 knockout in $lysozyme^{+}$ myeloid cells [73]. However, dendritic cells have been mainly reported in the literature on bone biology for their possible contribution for osteoclastogenesis and involvement in pathological conditions associated with bone destruction [74]. Osteoclasts and DC share a common myeloid precursor and are regulated by common molecular factors, such as RANKL, which is determinant for osteoclast

formation [75] and dendritic cell activation and survival [76]. These overlapping features prompted investigations into the possible connection between these two cell types. In 2004, a study by Rivollier *et al.* suggested for the first time the ability of human immature DC obtained in culture from circulating blood monocytes, to transdifferentiate into mature osteoclasts in the presence of M-CSF and RANKL, a process occurring through a cell fusion event [77]. Over the years, several other groups have corroborated these results, both in *in vitro* and *in vivo* settings [78-80]. Wakkach *et al.* showed that the injection of purified conventional DC into osteopetrotic oc/oc mice, which present differentiated OC unable to resorb bone, could partially revert the osteopetrotic phenotype, as these cells were able to differentiate into functional osteoclasts *in vivo* [79]. Additionally, this differentiation process was shown to be dependent on the presence of considerable amounts of osteogenic factors and controlled by inflammatory $CD4^+$ T cells, which can produce high amounts of RANKL and regulate its expression by bone marrow stromal cells [79]. In 2010, a study performed by Galois *et al.* used a genome-wide transcriptomic analysis to compare the novel OC differentiation pathway from DC to the classical pathway in which OC are differentiated from monocytes, in the presence of the same osteoclastogenic factors (M-CSF and RANKL) [81]. In both situations, a decreased expression of monocyte or DC characteristic markers was observed coupled with the upregulation of specific osteoclastic genes. Moreover, the results showed that DC and OC present a more similar molecular profile than DC and monocytes, suggesting that DC are better equipped to more efficiently generate OC when compared with monocytes [81]. Due to the lack of specific markers for DC-OC, it is currently not possible to differentiate between this type of OC and those classically differentiated from monocytes [74]. Moreover, the complete purity of DC populations tested for their osteoclastogenic potential, both in culture and in cell suspensions transplanted into animal models, is still debatable, due to the possibility of cross-contamination with OC precursors and monocytes/macrophages [82]. More studies are therefore warranted to uncover the real contribution of DC for osteoclastogenesis.

Both T and B lymphocytes are known to participate in bone remodelling, with their involvement in diseases marked by bone loss being increasingly recognized [83, 84]. T cells express RANKL, both in the soluble and membrane forms, being able to induce osteoclastogenesis [85], while B cells present the opposite role, being capable of blocking osteoclastogenesis through the secretion of osteoprotegerin (OPG), a decoy receptor for RANKL [86]. Moreover, the participation of these adaptive immune cells in the progression of bone healing has also been reported. Several studies suggested a negative effect of T cells during bone healing [87-89] while others have revealed a positive influence [90, 91]. In 2014, Könnecke *et al.* explored the spatiotemporal distribution of T and B cells during the course of bone healing in a murine femoral fracture model [92]. These cells were shown to infiltrate the injury site at two defined timepoints: at 3 days post-injury, when they

seemed to be equally distributed throughout the injury site, and at 14 days, at the time of hard callus formation, when these cells appeared in close proximity to osteoblast precursors and osteoclasts. In between these two waves of lymphocyte infiltration, at 7 days post-injury, a complete absence of lymphocytes within the cartilaginous callus was observed. Moreover, these cells seem to have a prolonged contribution to fracture healing, as they could still be detected during the remodelling phase at surprisingly high numbers [92].

Even though still in its infancy, the study of the interactions between immune and bone cells has provided important insights into the dynamics of bone regeneration, suggesting the crosstalk between immune cells and bone cells as a potential target for the development of novel therapies for bone repair and regeneration. Importantly, common to most of findings reported so far, there is the notion that, although essential for the onset and progression of tissue repair, inflammation needs to be tightly regulated and progressively resolved along time. In fact, pro-inflammatory environments have been proven to be highly deleterious for bone regeneration [93], as in the case of patients affected by chronic inflammatory conditions, which suffer from bone loss and impaired bone healing [94]. Bone tissue regeneration is thus dependent on proper cell-to-cell communication, in order to control immune cell activation and function, which can occur either through direct cell-cell contact or paracrine action, with extracellular vesicles being recently pinpointed as important players in this process [95].

1.3. Extracellular vesicles as new players in tissue repair

Since the early observations reporting their existence [96-98], EV were viewed for many years as "membrane debris" or as a way for cells to eliminate any unwanted material [99]. Only many years later, with the pioneer work from Raposo *et al.*, showing the ability of EV to promote adaptive immune responses [100], EV were placed in the spotlight as important regulators of cell communication both in homeostatic and pathological conditions [101]. EV have been classified in different populations mainly based on their size and biogenesis pathway, namely exosomes (50-200 nm), microvesicles (200 nm – 1 μm) and apoptotic bodies (> 1 μm) (reviewed in [102]) (Figure 2). Among these, exosomes are nanometric vesicles enriched in endosomal components which are formed from multivesicular bodies (MVB) when these structures fuse with the plasma membrane and release their intraluminal vesicles by exocytosis [103]. The lack of specific markers for the different EV subtypes and of standardized EV purification and characterization protocols, make the task of obtaining EV populations with high degree of purity still challenging [104, 105]. These difficulties persist in spite of the efforts of the EV research community [102, 106], and led the International Society for Extracellular Vesicles to recommend the use of

the broad designation “extracellular vesicles” whenever secreted vesicles are isolated by the most commonly used methods, namely, ultracentrifugation and phase separation [102, 105], and thus this nomenclature was adopted throughout this thesis.

EV are produced by virtually all cell types and they can be found in different biological fluids, including plasma/serum [107] and urine [108]. They are a reflection of their parental cells since they are loaded with selected molecules from the cell, such as proteins, RNA (miRNA, mRNA, long non-coding RNA) and DNA [109]. Thus, their content varies according to the different physiological contexts of the cell, an evidence which prompted the study of EV as biomarkers for pathological conditions [110]. Recent studies suggest that EV can be targeted to specific cells [111], and once they reach their target they may activate receptors on the cell surface, may be internalized, fuse with the cell membrane to directly deliver their content into the cytosol [112] or communicate with their recipient cell via connexin-containing channels, through a process similar to that of gap junction-mediated cell-to-cell communication [113]. Regardless of the mechanism involved, EV are able of modifying the physiological state of the recipient cell and can therefore be exploited as natural signalling mediators and delivery vehicles [114].

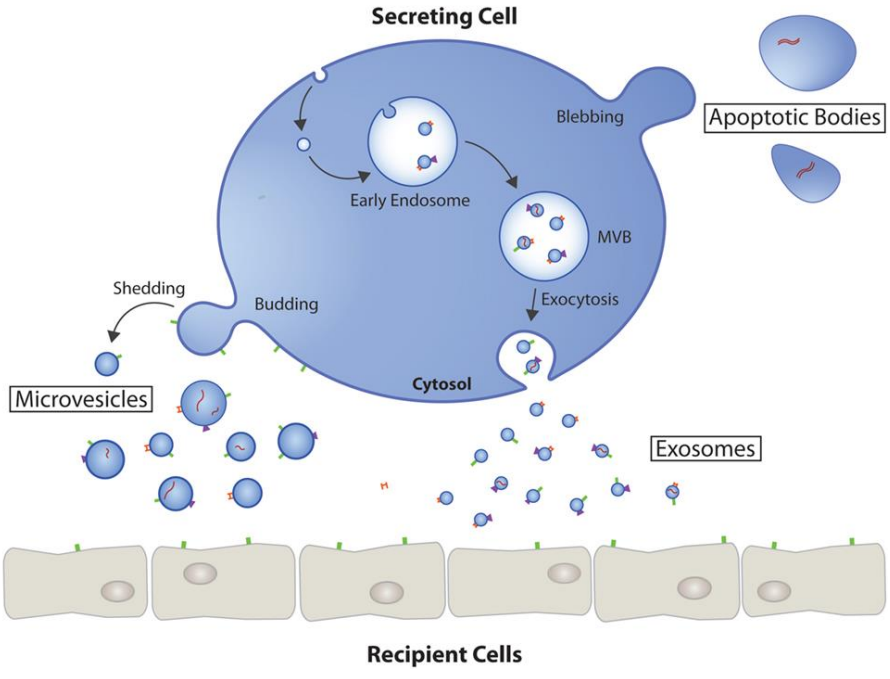

Figure 2. Extracellular vesicles (EV) include different populations of vesicles with specific biogenesis and secretion pathways. Exosomes are small-sized vesicles (50-200 nm) originated within the lumen of multivesicular bodies (MVB), which result from maturation of early endosomes, releasing their content through exocytosis upon fusion with the plasma membrane. Microvesicles are larger in size (200 nm – 1 μm) and are formed through the outward budding of the plasma membrane. Apoptotic bodies constitute portions of cells (>1 μm) resultant from membrane blebbing during the disintegration of cells undergoing programmed cell death (reproduced from [115]).

Most of the studies with EV conducted so far have been focused on their possible roles as mediators of immune responses and consequently on their promising application for the treatment of immunological disorders and cancer [116-118]. The immunomodulatory role of EV in the context of tissue repair and regeneration has attracted less attention, but a few works with different injury models have shown the benefits of EV administration in tissue recovery [109, 119]. In fact, EV have been implicated in different events associated with the tissue repair cascade, including immunomodulation, angiogenesis, extracellular matrix (ECM) deposition, cell differentiation and proliferation. Also, EV have the potential to be modified to deliver molecules of interest to target cells. These aspects were recently reviewed by our group [120] and are summarized in Figure 3.

For the purpose of this thesis, the role of EV in specifically controlling bone repair and regeneration, including bone remodelling, as well as the activity of the different cell types implicated in these processes, will be further described in the following section.

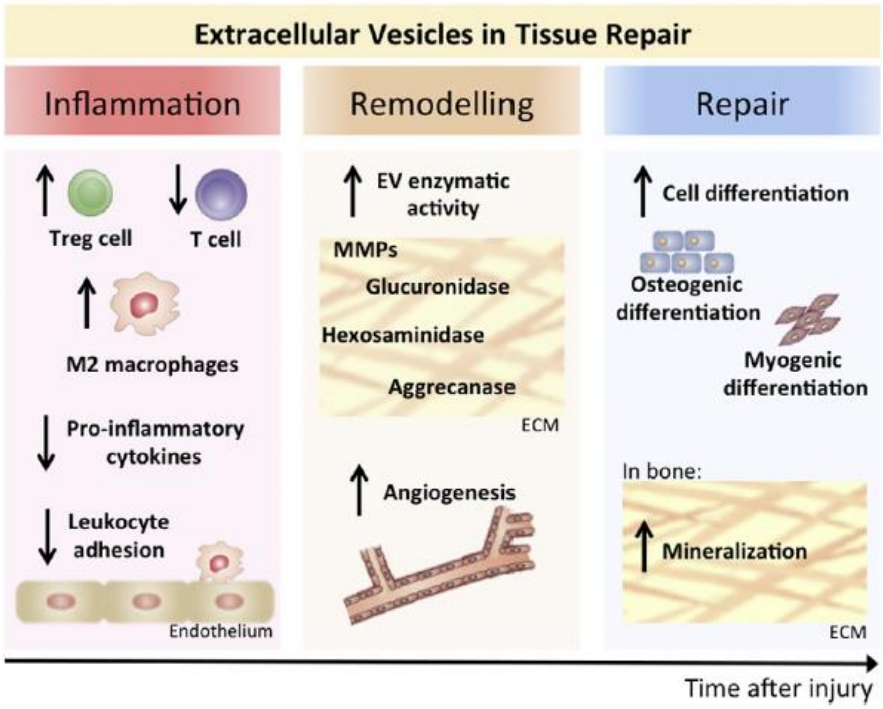

Figure 3. Extracellular vesicles with multiple cell origins play a role in the different stages of tissue repair and regeneration. EV may be associated with the resolution of the inflammatory response which is triggered after injury, by controlling the recruitment of immune cells, inhibiting the secretion of pro-inflammatory cytokines and activating immune cell subsets with an immunoregulatory phenotype. EV may enclose different enzymes which participate in the turnover of the damaged ECM and may also contribute to the process of angiogenesis, which is essential for the infiltration of progenitor cells responsible for the repopulation of the newly formed tissue. During the later stages of tissue repair, EV may also promote cell differentiation and proliferation and contribute to the generation of a fully mature and functional tissue. In bone repair, EV have been recognized as starting sites of bone tissue mineralization. (Reproduced from [120]).

1.3.1. EV contribution for bone repair and regeneration

Several studies have highlighted a role for EV in bone repair and regeneration. A very recent study from Xie and colleagues implicated EV in the systemic control of bone homeostasis, reporting that EV isolated from serum of elderly patients with bone loss presented altered proteomic profiles, when compared with those of healthy individuals, which were correlated with the ability of these EV to negatively interfere with the process of bone remodelling [121]. For instance, EV isolated from osteoporotic patients' serum were shown to promote osteoclast differentiation and inhibit osteoblast mineralization activity *in vitro*. Interestingly, serum-circulating EV isolated from aged healthy individuals were predicted to play a role in the maintenance of bone health, due to their enrichment in regulatory proteins involved in the facilitation of cell adhesion and inhibition of aging-induced oxidative stress [121].

Most works on the role of EV in bone repair and regeneration have been focusing on EV derived from MSC. A recent study from Zhao *et al*. showed that EV isolated from bone marrow-derived MSC could significantly promote the proliferation of human fetal osteoblastic 1.19 (hFOB) cells, when compared to the negative control, an effect associated with the activation of the MAPK signalling pathway [122]. Importantly, MSC-derived EV have been particularly described in the process of osteogenesis both *in vitro* [123-127] and *in vivo* [126, 128-132]. Martins *et al*. isolated EV from osteogenically induced hBMSC, at the early stages of differentiation, and showed that these EV present osteoinductive potential in the absence of other osteogenic stimuli, as they were able to induce activation of important osteogenic markers (BMP-2, ALP, SP7) [123]. In a different work, EV were obtained from hMSC at different stages over the entire time course of osteogenic differentiation [127]. Interestingly, even though EV from all timepoints could promote osteogenic differentiation of uncommitted MSC, only the EV derived from later stages were able to induce extracellular matrix mineralization, a stage-dependent behavior which was associated with differential miRNA expression [127]. These results suggest that cells secrete EV with different properties depending on their differentiation state. In a different study, Qin *et al*. showed that upon treatment with BMSC-derived EV, osteoblasts presented increased osteoblastic activity, with upregulation of important osteogenic genes (RUNX-2, ALP, OCN and OPN), compared to the control group, an effect at least partially mediated by miR-196a [124]. The authors went further and evaluated if the isolated EV were able to promote bone regeneration *in vivo*. For that, a calvarial defect was inflicted in Sprague Dawley rats where a hydrogel scaffold containing EV was implanted. The results showed a significantly higher area and density of newly formed bone in treated animals, compared to the hydrogel alone control group, with an evident

acceleration of bone regeneration [124]. Importantly, none of these articles included the EV-depleted ultracentrifugation control.

An interesting study by Furuta *et al*. provided strong evidence for the participation of MSC-EV in endochondral bone regeneration using a femoral fracture model in CD9 knockout ($CD9^{-/-}$) mice, a strain which secretes low levels of exosomes [132]. $CD9^{-/-}$ mice presented an impaired fracture healing, mainly due to a delay in callus formation, a scenario which was completely reversed when these mice were injected with MSC-derived EV. Importantly, this effect could not be observed with the EV-depleted ultracentrifugation control [132]. Another study showed enhanced bone regeneration in a model of critical-sized calvarial bone defect when EV isolated from human-induced pluripotent stem cell-derived MSC (hiPS-MSC) were implanted together with a tricalcium phosphate (ß-TCP) scaffold, an effect which was at least partially associated with the activation of the PI3K/Akt signalling pathway [130]. Additionally, hiPS-MSC-EV/ß-TCP scaffolds were also reported to promote bone regeneration in osteoporotic rats with calvarial critical-size bone defects, in a dose-dependent manner, through the induction of osteogenesis and angiogenesis [128]. Once again, EV-depleted ultracentrifugation controls were not included in neither one of these two studies.

EV may also contribute to bone repair and regeneration through their participation in the direct mineralization of the ECM. In fact, matrix vesicles (MV) are recognized as a specific type of EV, secreted by osteoblasts and chondrocytes, which contain membrane transporters for the influx of calcium and phosphate ions, which seem to create a particularly adequate microenvironment for the formation of hydroxyapatite crystals inside their lumen [133, 134]. These crystals are believed to eventually grow out of the MV and originate globular assemblies, known as calcifying nodules, which continue to grow and make contact with the surrounding ECM collagen fibrils, finally leading to their mineralization [133].

Other studies have been implicating osteoclast-derived EV in controlling bone remodelling. Huynh *et al*. reported that mature murine osteoclasts release a specific subset of RANK-containing EV with the ability to inhibit the process of osteoclastogenesis in 1,25-dihydroxyvitamin D3 (1,25(OH)2D3)-treated bone marrow cultures, probably through a competitive inhibition of RANK-RANKL interaction in osteoblasts [135]. Interestingly, when EV were isolated from osteoclast precursors, an opposite effect could be observed, with a significant promotion of osteoclast formation [135]. This study complements a previous work from Deng *et al*. showing that osteoblasts produce EV enriched in RANKL capable of stimulating osteoclastogenesis *in vitro* [136]. An interesting study by Sun and colleagues proposed a mechanism by which osteoclast-derived EV enriched in miR-214, could inhibit osteoblast function, through an interaction mediated by ephrinA2/EphA2 [137]. Interestingly, the levels of miR-214 in EV isolated from serum of

osteoporotic patients and ovariectomized mice were considerably increased when compared to those of the respective controls, suggesting a potential use for these EV as biomarkers for bone-loss [137] .

Very few works have implicated EV derived from immune cells in the processes of tissue repair and regeneration, particularly in the case of bone. Since the success of tissue repair is highly dependent on an initial inflammatory response, which needs to be resolved along time, it is reasonable to assume that immune cell-derived EV may play a role in the transition to reparative events. In 2013, Ekström and colleagues reported that MSC exposed to EV derived from LPS-activated monocytes presented increased expression of osteogenic genes (RUNX2, BMP2) when compared to the control media, but those values were still lower than the ones obtained with conditioned media (CM) from activated monocytes [138]. On the contrary, osteocalcin was also upregulated in the EV condition, but this time also to a larger extent than for monocyte-CM. These results suggest that the osteogenic stimulus conveyed to MSC may be, at least in part, carried by EV [138]. More recently, in the work of Gebraad and colleagues it was shown that EV derived from LPS-stimulated primary human monocytes could induce cytokine secretion by MSC, whereby potentially interfering with the immunomodulatory activity of these cells [139]. The expression levels of matrix metalloproteinases, particularly of MMP-3 and MMP-1, were also upregulated in MSC stimulated with these EV, suggesting that EV-mediated intercellular communication may also play a role in the regulation of tissue remodelling [139]. In a study by Torreggiani *et al.*, platelet lysate-derived EV (PL-EV) appeared to exert a similar influence on MSC biology *in vitro*. The levels of proliferation and migration were significantly increased when MSC were treated with PL-EV, when compared with the control medium, an effect which was dependent on the EV concentration used. Moreover, PL-EV promoted MSC osteogenic differentiation, also in a dose-specific manner, with EV in lower doses (5 µg) significantly increasing matrix mineralization when compared with platelet lysate controls, whereas higher EV doses (50 µg) showed the opposite effect. Interestingly, an enrichment in basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF-BB) and transforming growth factor beta 1 (TGF-ß1) was detected in PL-EV when compared to PL, suggesting that the positive effects of PL on bone regeneration is at least partially mediated by EV [140]. Despite the advances on the impact that immune cell-derived EV might have in controlling bone repair and regeneration via MSC biology modulation, the crosstalk between these two major cell populations, as mediated by EV, remains largely unknown. Thus, it is being currently explored in the lab where this thesis was developed, as detailed in the following section.

1.4. Previous work

The previous work developed in the lab provides important insights into the crosstalk between immune cells and MSC. In particular, human primary dendritic cells were shown to promote MSC recruitment *in vitro*, an effect largely impaired when DC were preconditioned with the pro-inflammatory cytokine TNF-α [141]. Moreover, the chemoattractant effect of immature DC upon MSC was shown to be mainly conveyed in EV secreted by DC [142]. In line with these results, it was hypothesized that, upon bone injury, the pro-inflammatory microenvironment in the injured bone could impact the phenotype of immune cells present in the damaged area and the EV they secrete, modulating their crosstalk with MSC. In order to assess this hypothesis, an *in vivo* model of critical size femur defect was performed, using as controls non-operated and sham-operated animals. Analysis of injured and sham-operated animals was performed at 3 and 14 days after surgery, timepoints coincident with the acute inflammatory phase that occurs after injury, and the resolution of that inflammation phase, respectively [34]. Results obtained showed that, upon injury, there is a systemic response characterized by temporally regulated changes in the profile of the regulatory molecules miRNAs circulating in plasma, which were predicted to be involved in the control of cell proliferation [143]. On the other hand, EV secreted by bone marrow (BM)-derived DC, differentiated from BM collected from control and bone injury animals, at 3 and 14 days after surgery, were evaluated for their impact on MSC biology *ex vivo* (Silva and Teixeira *et al.*, in preparation).

1.5. Objectives

A | Evaluate the impact of bone injury in the systemic inflammatory response, namely:

A.1 | the influence of plasma from different timepoints after injury on MSC proliferation.

Preliminary findings in the lab indicated that plasma from animals at different timepoints post-bone injury expressed different miRNAs, which were predicted to be involved in the regulation of cell proliferation. These predictions were validated by exposing MSC *in vitro* to total plasma collected from non-operated or at 3 and 14 days post-injury animals, followed by immunostaining for Ki67 to evaluate the levels of cell proliferation.

These results were included in a scientific article that is already published:

Silva AM, Almeida MI, Teixeira JH, Ivan C, **Oliveira J**, Vasconcelos D, Neves N, Ribeiro-Machado C, Cunha C, Barbosa MA, Calin GA, Santos SG. Profiling the circulating miRnome reveals a temporal regulation of the bone injury response. *Theranostics* 2018; 8(14):3902-3917. doi:10.7150/thno.24444.

A.2 | the effect of time post-injury on the inflammatory cytokine profile of splenocytes.

The systemic inflammatory response to bone injury was further dissected through the assessment of gene expression levels of pro- and anti-inflammatory cytokines in splenocytes isolated from non-operated, 3 and 14 days post-bone injury rats and sham-operated controls by RT-qPCR.

B | Explore the impact of DC-EV on MSC biology:

B.1| evaluate the effect of DC-EV on MSC proliferation and immunomodulatory activity *in vitro*.

MSC were exposed *in vitro* to DC-EV derived from bone marrow cells of non-operated, 3 and 14 days post-surgery animals, and immunostaining for Ki67 was performed to evaluate the levels of cell proliferation. The levels of pro- and anti-inflammatory cytokines expressed by MSC after stimulation with DC-EV from bone-injured and control animals were assessed by RT-qPCR.

B.2| investigate the specificity of the impact of bone injury in the capacity of DC-EV to promote MSC migration.

Previous results obtained in the lab indicated that DC-EV isolated from DC differentiated from bone marrow at different timepoints post-injury, had a different capacity to promote MSC migration. The specificity of this effect in response to bone injury, comparing with an acute injury, was evaluated here in MSC and fibroblast transwell migration assays using DC-EV from sham-operated animals.

C| Validate the temporal changes found in the content of EV derived from DC at different timepoints post-injury, and evaluate the contribution of target proteins in the promotion of MSC migration.

EV from DC at different timepoints post-injury and from non-operated control animals were previously characterized for the presence of chemokines and cytokines and found to contain different protein mediators in a time post-injury dependent manner. In this thesis, candidate proteins, such as TIMP-1, were further tested by western blotting for their presence in conditioned media from BM-derived DC. Furthermore, the effect of other candidate proteins, such as TNF-α, was tested for their impact on MSC migration, by transwell migration assays.

2. MATERIALS AND METHODS

2.1. Bone injury *in vivo* model and sample collection

Biological samples used to conduct this study were obtained from a critical size bone defect *in vivo* model that had been previously performed in the lab [143]. Briefly, 3 months-old Wistar-Han rats (N=6) were subjected, under general anaesthesia, to a lateral arthrotomy of the right knee, after the retraction of both skin and muscle. A cylindrical defect (with ~3 mm diameter and ~4 mm depth) was then inflicted in the anterolateral wall of the lateral condyle of the femur using a surgical drill [144]. Sham-operated animals (N=3) underwent the same surgical procedure without the infliction of a cylindrical defect and were used as the surgical control group. Animals were then sacrificed at days 3 and 14 after injury. Non-operated (NO) animals were used as control. Animals were dissected and femurs were collected for bone marrow recovery by flushing. Spleens were also recovered and were then digested with collagenase I and crushed on a cell strainer for single cell isolation. All the procedures were performed in accordance with the ethical requirements on animal welfare and experimentation (following protocols approved by DGAV).

2.2. Dendritic cell (DC) differentiation and extracellular vesicles (EV) isolation

Bone marrow cells were cultured for 10 days in DC differentiating media - RPMI-1640 + Glutamax (Invitrogen), with 10 %(v/v) heat inactivated Fetal Bovine Serum (FBS; Biowest) and 1 %(v/v) penicillin G-streptomycin (P/S; Invitrogen) - containing 25 ng/mL IL-4 and GM-CSF (Immunotools) for differentiation into DC. The aspect of the cell cultures obtained in the lab is presented in Supplementary Figure 1. At day 7, the media was changed for EV-producing media – ultracentrifuged (100,000 ×g, for 2 h, at 4 °C) cell culture media for depletion of bovine EV, diluted to 1 %(v/v) FBS, containing DC differentiating cytokines; 3 days later, EV were isolated from cell culture media by differential centrifugation, with a last step of ultracentrifugation (at 100, 000 x g, for 2 h, at 4 °C) [142]. EV pellets and supernatants (UC control) were recovered and stored at -80°C until use. Their protein concentration was determined by *DC*™ Protein Assay (Bio-Rad) and used as a quantitative measure for functional assays. These samples were provided to perform the tasks contemplated in this dissertation, which are described in the next sections. A previous characterization of the DC cell populations at day 10 had been performed in the lab by immunophenotyping, followed by analysis by flow cytometry and is presented in Supplementary Figure 2.

2.3. RNA isolation and RT-qPCR analysis

Splenocytes, DC and MSC were lysed with TRIzol® (Invitrogen), followed by total RNA extraction according to the manufacturer's protocol. RNA concentration was spectrophotometrically determined in a NanoDrop ND-1000 device (ThermoFisher Scientific), and its integrity confirmed by agarose gel electrophoresis. For samples of the same type, an equal quantity of RNA was then treated with TURBO DNAse following manufacturer's instructions, and used for cDNA synthesis with random hexamers and SuperScript® III Reverse Transcriptase (all from Life Technologies). RT-qPCR was then performed in a CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad) using cDNA, gene specific primers and iQ SYBR Green Supermix (Bio-Rad). Primer sequences are available in Table 1. GAPDH was used as reference gene and relative gene expression was calculated for Cq<35, by the ΔCq method (according to MIQE guidelines).

Table 1. Sequences of primers used for the analysis of gene expression by RT-qPCR

Gene	Primer Sequence
TNF-α	Forward: 5'- CCCAGACCCTCACACTCAGAT -3' Reverse: 5'- TTGTCCCTTGAAGAGAACCTG -3'
IL-1β	Forward: 5'- CACCTTCTTTTCCTTCATCTTTG -3' Reverse: 5'- GTCGTTGCTTGTCTCTCCTTGTA -3'
IL-6	Forward: 5'- CATATGTTCTCAGGGAGATCTTGGA -3' Reverse: 5'- CAGTGCATCATCGCTGTTCA -3'
IL-10	Forward: 5'- TGCCTTCAGTCAAGTGAAGAC -3' Reverse: 5'- AAACTCATTCATGGCCTTGTA -3'
GAPDH	Forward: 5'- CCTCAAGATCATCAGCAAT –3' Reverse: 5'- CCATCCACAGTCTTCTGGGT -3'

2.4. EV characterization

EV populations isolated from DC from sham-operated animals (3d and 14d) were characterized for morphology and size using a set of complementary techniques. Correspondent UC supernatants were also analysed as controls. The same analysis was already performed in the group at the time of this dissertation for the samples derived from NO and bone-injured animals (3d and 14d).

Size distribution and concentration of DC-EV suspensions and DC-SN control were assessed by Nanoparticle Tracking Analysis (NTA) in a NanoSight NS300 instrument (Malvern), equipped with a 488 nm laser and a sCMOS camera. 10 µg of each EV/SN suspension were pre-diluted in 1 mL of particle-free PBS (previously filtered with a 0.22 µm filter) prior to NTA analysis. Data analysis was performed using NTA software v3.2.

EV morphology was assessed by Transmission Electron Microscopy (TEM). Briefly, DC-EV and DC-SN samples, previously diluted in filtered PBS (in a 1:5 ratio), were adsorbed onto 400-mesh formvar carbon-coated nickel grids and negatively stained with 5% uranyl acetate for 10 sec. Imaging was performed in a Jeol JEM 1400 electron microscope.

2.5. Mesenchymal stem/stromal cell (MSC) and fibroblast cell culture

Primary rat BM-derived MSC and dermal fibroblasts used to perform this study were kindly provided by Carla Cunha (i3S, INEB), who previously isolated these cells from healthy Wistar-Han rats following protocols routinely applied in the lab [145]. MSC isolated were shown to follow the criteria of the International Society of Cell & Gene Therapy for human MSC definition [44, 145].

For each experiment, MSC or fibroblasts aliquots in passage 4/5 were thawed and expanded in culture in 175 cm^2 cell culture flasks (BD Falcon™) using the appropriate growth medium - α-minimal essential medium (α-MEM, Gibco™) supplemented with 10 %(v/v) MSC-qualified FBS (Gibco™) and 1 %(v/v) P/S (Gibco™), or low glucose Dulbecco's modified Eagle's medium (DMEM, Corning™) supplemented with 10 %(v/v) FBS (Biowest™) and 1 %(v/v) P/S (Gibco™), respectively. Cells were maintained in a humidified incubator (at 37˚C and 5% CO_2) and medium was changed every 3 days until 70-80% confluence was reached. Cells were detached from cell culture flasks with 0.05 % trypsin-EDTA (Gibco™) for 5 min at 37°C, counted, and seeded in new 175 cm^2 cell culture flasks (BD Falcon™) at a density of 3000 cells/cm^2. Cells in passages 6 to 8 were used to set up the assays.

2.6. MSC proliferation assays

Immunofluorescence for the Ki67 proliferation marker was performed to assess the effect of plasma and DC-derived EV from NO, 3 and 14 days post-injury rats on MSC proliferation. For assays with plasma, MSC (3000 cells/cm^2) were cultured in 96-well plates and grown until ~60% confluence. Media was changed to serum-free media with 3U heparin (to prevent coagulation), and the next day cells were stimulated for 48h with rat total plasma, diluted in media in a 1:3 ratio. For assays with EV, MSC (3000 cells/cm^2) were cultured in 24-well plates on top of glass coverslips and grown until ~60% confluence. Media was changed to growth media with only 1 %(v/v) FBS, and the next day cells were

stimulated for 48h with 10 µg of DC-EV, or EV-depleted ultracentrifugation supernatant (SN).

Cells were then fixed with paraformaldehyde (PFA 4 %(w/v) in PBS) and permeabilized with Triton X-100 (0.1 %(v/v) in PBS). After blocking with Bovine Serum Albumin (BSA 5 %(w/v) in PBS), cells were incubated with anti-Ki67 primary antibody solution (1:250 in BSA 1 %(w/v), ThermoFisher Scientific) followed by AlexaFluor-647 conjugated secondary antibody. Secondary antibody alone was used as the staining negative control. Cell nuclei were stained with DAPI. Coverslips were mounted on slides and cells were imaged on an IN Cell Analyzer at a magnification of 20x. IN Cell Developer Toolbox 1.9.2 software was used to automatically determine the ratio of Ki67-positive cells relative to the total number of cells.

2.7. MSC cytokines production

MSC were seeded at a density of 3000 cells/cm^2 and grown until 60-70% confluence. Media was changed to α-MEM supplemented with 1 %(v/v) FBS and MSC were stimulated in the following day with 10 µg of EV secreted by DC from NO, 3 days and 14 days post-injury animals. The same amount of UC supernatant and IL-4 and GM-CSF in the same concentration as for DC differentiation were used as control, and unstimulated cells in basal media were used as negative control. After 48 h, cells were thoroughly washed with PBS 1x and lysed with TRIzol. RNA was isolated, retrotranscribed to cDNA and used in RT-qPCR reactions with cytokines gene specific primers, as described in section 2.3.

2.8. Transwell migration assays

The capacity of DC-EV from 3d and 14 days sham-operated animals to promote the recruitment of MSC and fibroblasts was evaluated using transwell migration assays. Migration assays using as stimuli DC-EV derived from bone-injured and non-operated animals were performed previously to this thesis. The same set-up was applied to study the effect of different concentrations of TNF-α on MSC migration. In detail, 24-well plates with transwell inserts (BD Falcon™) with 8 µm pore-size membranes were set-up to conduct the experiments.

The transwell inserts were first coated with gelatine (1 %(w/v) in PBS) for 1 h at 37°C, rinsed with PBS, and then seeded with 80 000 cells (MSC/fibroblasts) resuspended in 500 µL of serum-free media (α-MEM, Gibco™ or DMEM, Corning™) (top compartment, TC). Inserts were then placed on the wells (bottom compartment, BC), containing 750µL of serum-free media with different stimuli, as indicated. For assays with EV, 10 µg of DC-EV or DC-SN samples were used, and for assays with TNF-α, different concentrations of rat recombinant TNF-α (0.01; 0.1; 0.25; 0.5; 1 and 10 ng/mL, Immunotools) were tested.

Cells were incubated at 37˚C and 5% CO_2 in a humidified incubator for 12 h for cell migration. Conditioned media were then collected separately from top and bottom compartments for each condition, centrifuged at 14 000 rpm (at 4 ºC, for 5 min), to remove cells and cell debris, and supernatants were stored at -20 ºC until use. Membrane inserts were fixed with PFA (4 %(w/v) in PBS) and cleaned from non-migrated cells with cotton swabs. Membranes were removed from the inserts, mounted on a microscope slide using fluoroshield with DAPI and imaged on IN Cell Analyzer 2000 at a magnification of 20x. The number of migrated cells were automatically counted on IN Cell Developer Toolbox 1.9.2 software. Migration index was determined as the ratio of migrated cells in each condition to the number of cells migrated in the absence of any stimulus (serum-free media, SFM).

2.9. Gelatin zymography

Conditioned media collected from top and bottom compartments (TC and BC) of the transwell assays were tested for the presence of MMP-2 and MMP-9 in gelatine-containing zymography gels. For each conditioned tested, conditioned media protein was first precipitated by mixing one volume of sample with 6 volumes of ice-cold acetone. Tubes were kept at -20 ˚C overnight, followed by centrifugation at 14 000 rpm (at 4 ºC, for 5 min). Supernatants were discarded, and tubes were left uncapped at room temperature for evaporation of acetone remnants. Protein pellets were then dissolved in 30 µL of ultrapure water on ice. The amount of protein in each sample was quantified using DC™ Protein Assay (Bio-Rad), following manufacturer's instructions, and absorbance measurements at 750 nm were performed in a Synergy™ microplate reader.

The same amount of protein from each sample (4-8 µg) was mixed with loading buffer (SDS 0.1 %, 0.04 % sucrose in Tris buffer 0.25 M, pH 6.8) in non-reducing conditions, loaded and resolved in a SDS-gel, consisting of a 5% polyacrylamide (Bio-Rad) stacking gel followed by a 10% polyacrylamide resolving gel containing 0.1% gelatine (from porcine skin, Sigma-Aldrich). Precision Plus Protein™ Dual Color Standard (Bio-Rad) was used as molecular weight protein standard. Electrophoresis was conducted at 80 V until the dye front entered the separating gel and then continued at 100 V until the front exited the gel. Afterwards, zymograms were incubated with Triton X-100 2 %(v/v) (for 3 periods of 10 min), rinsed with distilled water, and were then incubated in MMP substrate buffer ($CaCl_2$ 10 mM in Tris buffer 50 mM, pH 7.5) for 16-18 h, at 37 ºC. After incubation, gels were washed with distilled water and stained with Coomassie Brilliant Blue G-250 (Sigma Aldrich), for 20 minutes, at room temperature, with gentle agitation. The zymograms were then rinsed with distilled water until clear bands resultant from MMP gelatinolytic activity became visible in the blue background. Gels were then imaged in a calibrated GS-800 densitometer

(Bio-Rad) and the intensity of each band was quantified using the "Gel Analyzer" plugin on ImageJ software.

2.10. Western blotting

Samples of DC conditioned media from bone-injured animals and sham-operated and NO controls were precipitated and quantified as described in section 2.5. The same amount of protein from each condition (40 µg) was mixed with Laemmli loading buffer in non-reducing conditions, denaturated at 65 ºC for 15 min, and then resolved on 10% SDS-PAGE polyacrylamide gels (Bio-Rad), as described in section 2.5. After electrophoretic separation, resolved proteins were then transferred to nitrocellulose membranes, which were subsequently blocked with BSA (5 %(w/v), in TBS-Tween 0.1 %(v/v)) for 1h, at room temperature. The membranes were incubated with anti-TIMP-1 (Millipore) or anti-TNF-α (Abcam) primary antibodies, overnight, at 4 ºC, followed by washing with TBS-Tween 0.1 %(v/v), and then incubated with HRP-conjugated secondary antibody (1:1000, GE Healthcare). Membranes were washed again, incubated with ECL substrate and chemiluminescent signal detected by exposure to autoradiographic films (GE Healthcare Life Sciences). Films were imaged in a calibrated GS-800 densitometer (Bio-Rad) and the intensity of each band was quantified using the "Gel Analyzer" plugin on ImageJ software. Ponceau-stained membranes were also imaged, the band corresponding to the albumin quantified on ImageJ as described before and used as reference for western blot TIMP-1 band normalization.

2.11. Statistical analysis

Data was analysed using GraphPad Prism software. Data was tested for normal distribution by D'Agostino and Pearson normality test and shown to be non-parametric. For comparisons between multiple groups, the non-parametric test Kruskal-Wallis was computed, followed by Dunn's multiple comparison testing. Statistical significance was considered for $p\text{-value} < 0.05$.

3. RESULTS

3.1. Plasma from bone-injured animals affects MSC proliferation

To study the impact of bone injury in the molecular cues circulating systemically at different timepoints after injury, a critical size bone defect *in vivo* model performed in the femur of adult rats was used. Previous findings in the lab indicated that this bone injury leads to changes in the profile of miRNA circulating in animals' plasma along time after injury, and compared to NO and sham-operated animals. The miRNAs differentially regulated along time after bone injury were predicted to be involved in the regulation of cell proliferation. In order to functionally validate these predictions, the effect of plasma from NO, 3 and 14 days post-injury animals on the proliferation of bone marrow-derived MSC was evaluated *in vitro*. Results obtained for the expression of the proliferation marker Ki67 in MSC stimulated with plasma for 48 h are illustrated in Figure 4(A) and the quantification across different experiments, expressed as the ratio of Ki67 positive cells relative to the total number of cells, is depicted in Figure 4(B). Levels of MSC proliferation in the presence of plasma from animals at days 3 and 14 post-injury were significantly higher when compared to the non-stimulated negative control (SFM) and identical to those obtained with the FBS positive control. Plasma from NO animals also induced MSC proliferation, but at lower levels than that of 3 and 14 days post-injury animals, and did not reach statistical significance, when compared to the negative control. These results were included in a scientific article published during the development of this thesis [143].

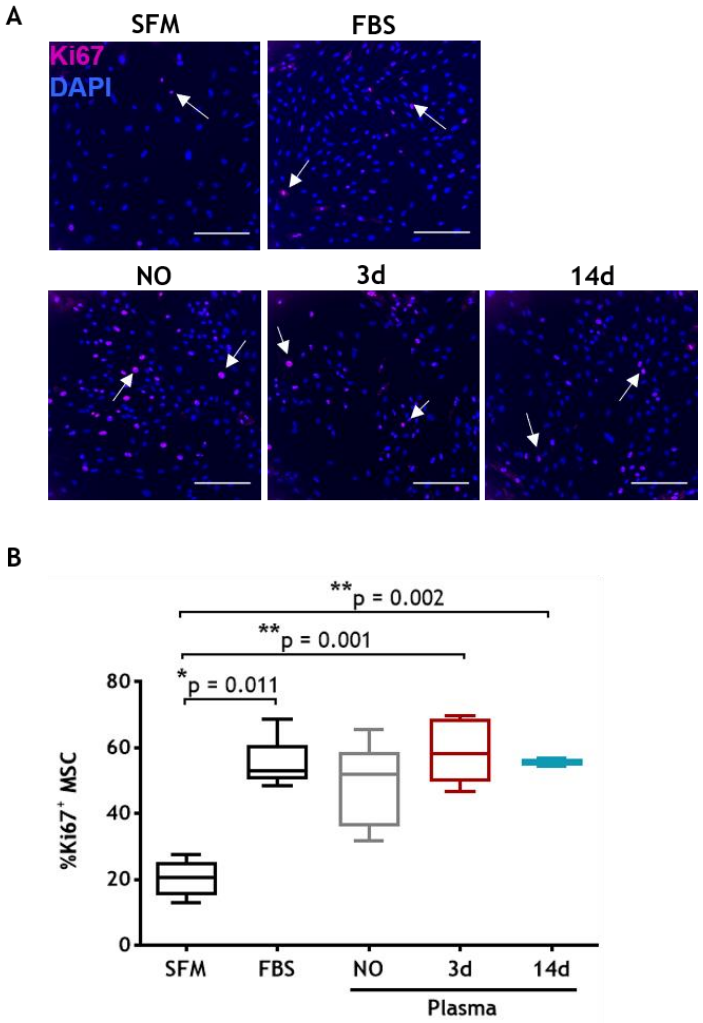

Figure 4. MSC proliferation is differently affected by rat total plasma from different timepoints after bone injury. (A) Representative fluorescence images of Ki67 immunostaining of rat MSC after 48h of stimulation with rat total plasma (diluted 1:3) from non-operated, 3 and 14 days post-injury animals (NO, 3d, 14d, respectively). SFM is the serum-free media negative control and FBS 10% (v/v) is the positive control. Ki67 (AlexaFluor647) is depicted in magenta and nuclei (DAPI) in blue; white arrows indicate Ki67-positive cells. Scale bar: 200µm. **(B)** Percentage of Ki67 positive MSC relative to the total number of cells, upon stimulation with plasma, quantified across multiple experiments. Box plots represent min-to-max distribution (NO: 5 animals; 3d and 14d: 6 animals per group). P-values relative to SFM control are indicated, as calculated with Kruskal-Wallis test followed by uncorrected Dunn's multiple comparison test. The comparisons between the other conditions tested were not statistically significant.

3.2. Bone injury affects the levels of cytokine expression at the systemic level

Considering the results obtained for MSC proliferation upon stimulation with plasma from bone-injured animals, and our previous findings on the circulating miRnome profile at different times after bone injury [143], we intended to further characterize the systemic response to injury that could condition MSC and bone repair/regeneration. Previous work performed in the lab evaluated the presence of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 in the plasma of bone-injured animals, by ELISA (Supplementary Figure 3). In

this thesis, the impact of the bone defect in the systemic inflammatory response to injury was evaluated in the spleen, since this is a peripheral secondary lymphoid organ crucial in mediating an inflammatory response to injury in rats [146]. The expression levels of the cytokines TNF-α, IL-1β, IL-6 and IL-10 were evaluated by RT-qPCR in whole splenocytes recovered from NO animals, and bone-injured and sham-operated animals, at 3 and 14 days after surgery, and are depicted in Figure 5. Results revealed a tendency for a decrease in the levels of all cytokines tested at 14 days post-bone injury, compared to 3 days post-bone injury animals, although no statistical differences could be found. Importantly, this temporal regulation was not observed in sham-operated animals and expression levels of TNF-α, IL-1β and IL-10 were significantly lower in 14 days post-bone injury animals than in sham-operated animals at the same timepoint.

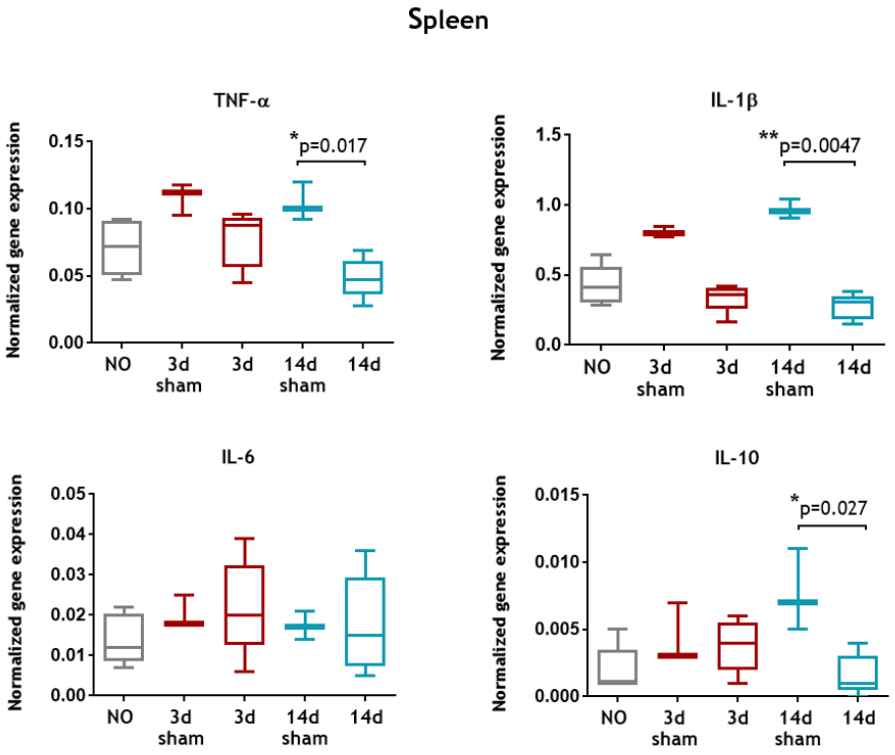

Figure 5. Bone injury regulates the systemic inflammatory response. mRNA expression levels of TNF-α, IL-1β, IL-6 and IL-10, normalized by GAPDH expression, were determined by RT-qPCR in whole splenocytes isolated from non-operated animals (NO), injured rats at day 3 (3d) and 14 (14d) after bone injury and respective sham-operated controls (3d sham, 14d sham). Box plots represent min-to-max distribution (NO, 3d, 14d: 5 animals per group; 3d sham, 14d sham: 3 animals per group). P-values were determined by Kruskal-Wallis test, followed by Dunn's multiple comparisons testing, and are indicated.

3.3. Bone injury influence on EV secretion by bone marrow-derived DC

Before analysing the EV produced by bone marrow-derived DC, we evaluated the impact of bone injury on the inflammatory profile of these immune cells. For that, femurs were collected from the bone-injured and sham-operated animals at 3 and 14 days after surgery, and from NO animals. Bone marrow was extracted, and DC were differentiated *ex vivo*. After 10 days of differentiation, levels of the activation marker CD86 at cell surface were previously assessed in the lab by flow cytometry (Supplementary Figure 2). In the scope of this thesis, the gene expression levels of the cytokines TNF-α, IL-1β, IL-6 and IL-10 in DC were evaluated, by RT-qPCR. Results are depicted in Figure 6, and revealed no significant differences between the groups tested. Nonetheless, TNF-α and IL-6 expression was lower at 14 days, than NO and 3 days post-bone injury, while in sham-operated animals, TNF-α levels were low in cells of both timepoints, and IL-6 had levels similar to the 3 days post-bone injury, not reducing at 14 days. IL-1β and IL-10 were expressed at very low levels by DC of NO and bone-injured animals, and were slightly higher in cells from sham-operated animals. However, these results should be interpreted carefully, due to the high variability observed.

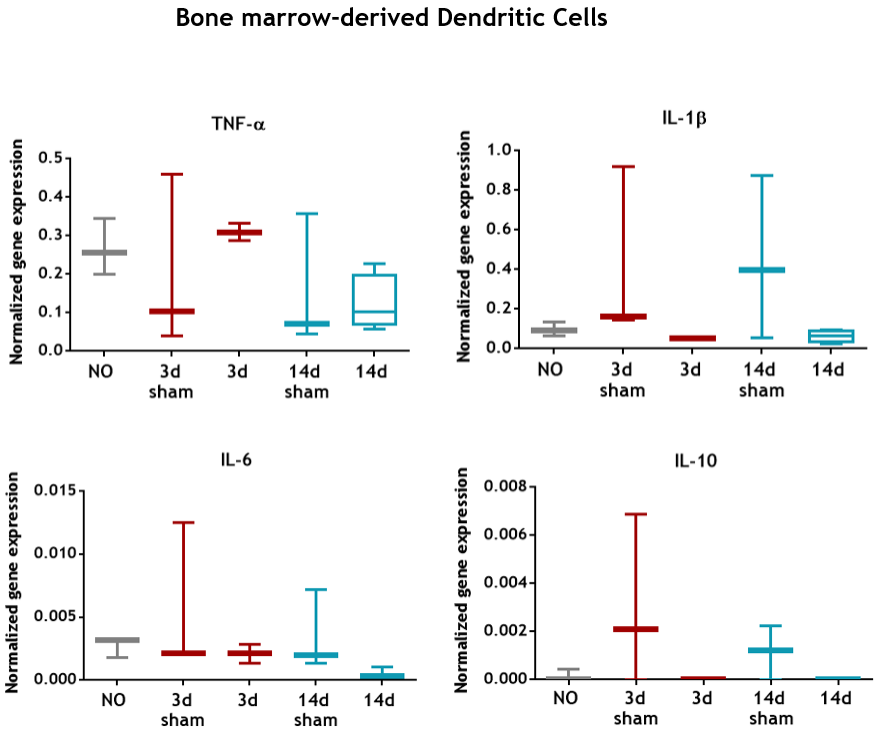

Figure 6. The microenvironment created upon bone injury seems to affect bone marrow-derived DC phenotype. Expression levels of TNF-α, IL-1β, IL-6 and IL-10 were assessed by RT-qPCR in dendritic cells isolated from bone marrow from non-operated (NO) and 3 days and 14 days animals after sham (3d sham, 14d sham) or bone injury (3d, 14d) surgeries. Box plots represent min-to-max distribution of n=3 to 4 animals per group. Statistical significance was assessed by Kruskal-Wallis test, followed by Dunn's multiple comparisons testing and no significant differences between groups could be found.

Of note, it was also our aim to analyse the expression of these cytokines in whole bone marrow isolates, however the RNA isolated from the bone marrow cells recovered was degraded, as supported by the absence of two bands corresponding to ribosomal RNA subunits 28S and 18S in the agarose gel performed (Supplementary Figure 4), preventing further analysis of coding genes expression.

The impact of bone injury in the secretion of EV by the bone marrow-derived DC was then evaluated. EV were isolated by ultracentrifugation from the supernatant of bone marrow-derived DC *ex vivo* cultures. The characterization of the isolated DC-EV morphology was performed by transmission electron microscopy (TEM) (Figure 7 (A)), and size and concentration were assessed by nanoparticle tracking analysis (NTA) (Figure 7 (B), (C), respectively). The data for DC-EV from NO and bone injured animals were previously obtained in the lab and the characterization of DC-EV from sham-operated controls was performed in the scope of this dissertation. TEM results for EV suspensions from NO, bone-injured and sham-operated animals, in all the conditions tested, revealed the presence of vesicles with a cup-shape morphology, characteristic of exosomes [147], which could not be detected in the UC control samples (Supplementary Figure 5). In terms of size distribution, NTA analysis showed EV populations isolated from DC of NO, bone injured animals and sham-operated controls, presented mean diameter values between 100-200 nm, which are comprised in the size range expected for exosomes [102]. No significant differences were observed when comparing the size of neither EV from DC of NO or injured animals nor EV from DC of sham-operated controls. Interestingly, bone injury seemed to affect the ability of DC to secrete EV, as evidenced in the characterization of EV suspension concentration performed by NTA. Indeed, the concentration of EV collected from DC of 3 days post-injury animals was reduced when compared to EV from cells of NO animals, although not statistically significant. Moreover, DC isolated from animals at 14 days post-injury seemed to recover their secretion capacity, showing EV concentration levels slightly higher than those of NO controls. This differential secretion pattern seems to specifically occur in response to bone injury, as EV secretion by DC from sham-operated animals is lower both at 3 and 14 days post-surgery. Of note, the concentration of particles in ultracentrifugation supernatants (UC control) was much lower than in EV samples, both for NO, bone-injured and sham-operated animals, data supporting the efficiency of the protocol followed for EV isolation.

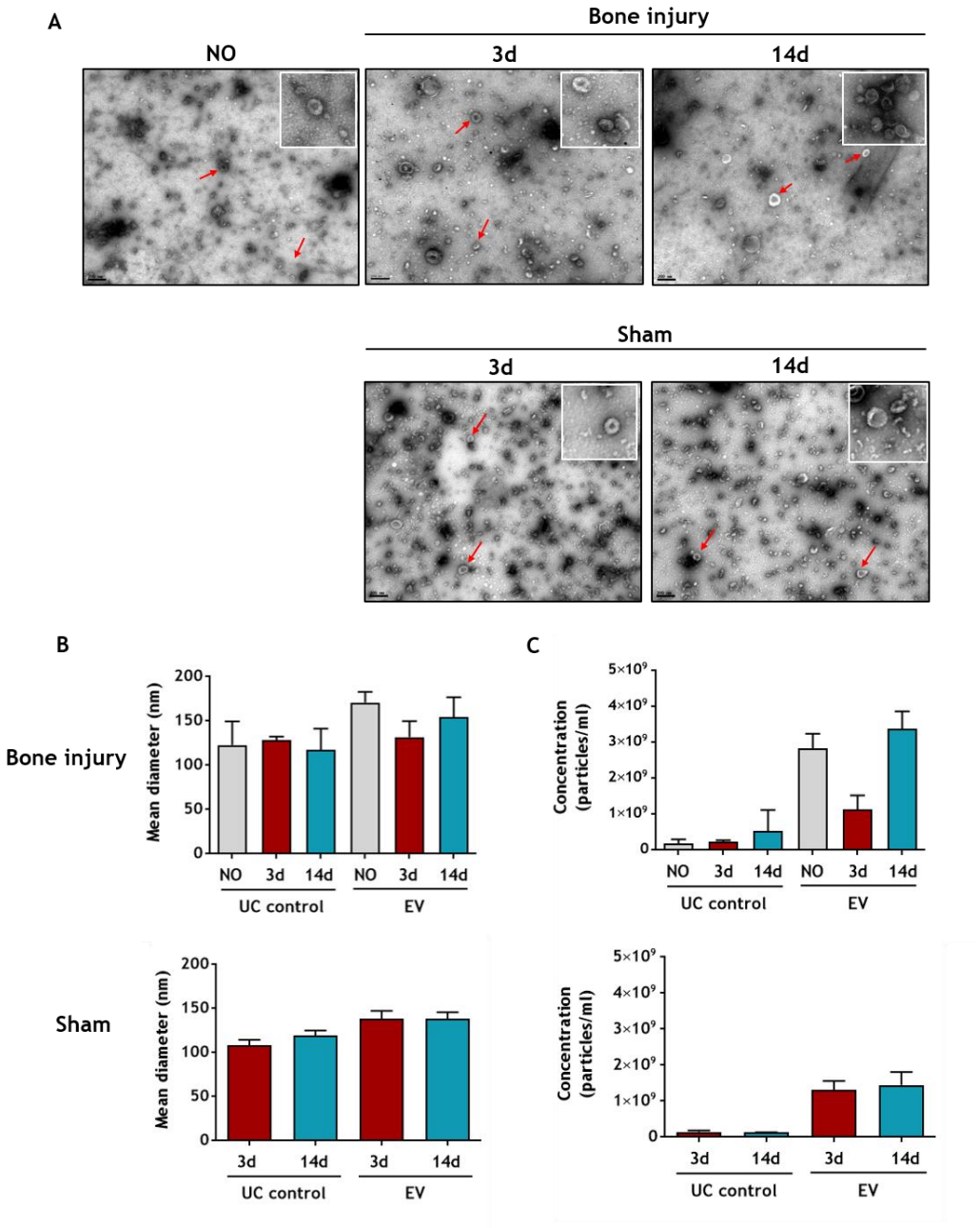

Figure 7. EV secretion by bone marrow-derived DC is affected by bone injury. (A) Representative TEM images of EV derived from dendritic cells differentiated *ex vivo* from bone-marrow of non-operated (NO), bone-injured animals at 3 (3d) and 14 days (14d) post-injury (top panel) and sham-operated animals at the same timepoints (3d sham, 14d sham) (bottom panel). Magnification: 50 000x. Scale bar: 200 nm. EV are indicated by red arrows and detailed in picture insets (magnification: 100 000x). NTA analysis was performed to characterize DC-EV populations and respective supernatants from EV ultracentrifugation (UC control) isolated from NO, bone-injured and sham-operated animals (n=3 per group), in terms of size distribution **(B)** and concentration **(C).** Bars represent the mean value±standard error for each condition. Statistical significance was tested by Kruskal-Wallis test, followed by Dunn's multiple comparisons testing and no significant differences were found.

3.4. Bone injury has a specific impact on the ability of DC-EV to modulate MSC biology

Taking into account the influence of bone injury in the secretion of EV by bone marrow-derived DC, we then aimed to explore the impact of the injury in the biological properties of these EV, namely upon MSC. The ability of EV from DC at different timepoints post-injury to impact MSC differentiation was previously evaluated in the lab, revealing no major effect compared to non-stimulated control MSC (data not shown). In this thesis, the impact of DC-EV on MSC proliferation, cytokine expression profile and migration was evaluated, as described in the following sections.

3.4.1. DC-EV effect on MSC proliferation

Having tested the action of plasma from injured rats on MSC proliferation, we next wanted to ascertain if DC-EV from the same animals could also have an impact on this process, particularly when these immune cells were derived from bone marrow at different timepoints after bone injury. Thus, EV secreted by bone marrow-derived DC from NO, 3 and 14 days post-bone injury animals were tested for their capacity to promote MSC proliferation, following a similar approach as for stimulation with total plasma. Results obtained were quantified across independent experiments and are represented in Figure 8.

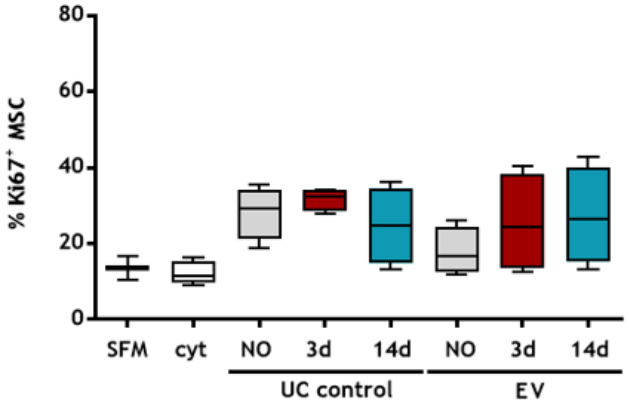

Figure 8. MSC proliferation upon DC-EV stimulation. Proliferative activity of MSC after stimulation for 48 h with EV from bone marrow-derived dendritic cells from non-operated (NO), 3 days (3d) and 14 days (14d) post-injury animals was assessed by immunostaining for Ki67. MSC proliferation in the absence of any stimuli (SFM) and in the presence of DC-differentiating cytokines - IL-4 and GM-CSF- (cyt) were also evaluated as controls. The ratio of Ki67 positive cells relative to the total number of cells was quantified across independent experiments (n=3 to 4 animals per group). Box plots represent min-to-max distribution. Statistical significance was assessed by Kruskal-Wallis test, followed by Dunn's multiple comparisons testing and no significant differences between groups were found.

The percentage of proliferating $Ki67^+$ MSC in the presence of DC-EV was slightly higher than that of the unstimulated control (SFM control), albeit no statistical differences were observed, and the ultracentrifugation controls were also able to promote MSC proliferation, to similar levels of DC-EV.

3.4.2. DC-EV effect on MSC immunomodulatory properties

The beneficial role of MSC for bone repair is possibly associated not only with their ability to differentiate into bone-forming cells but also with their immunomodulatory activity, which is known to be dependent on the molecular cues promoting their activation [148]. In line with this, we hypothesized that the MSC immunomodulatory action could be differentially regulated upon stimulation with DC-EV from different timepoints after bone injury. To test this hypothesis, the expression levels of TNF-α, IL-1ß, IL-6 and IL-10 were assessed by RT-qPCR in MSC stimulated with DC-EV from bone-injured animals or the respective UC supernatant controls. Both IL-1ß and IL-10 were not detected and TNF-α was also absent in almost all the conditions tested (data not shown). Results obtained for IL-6, depicted in Figure 9, showed that the expression of this cytokine could be induced in the presence of DC-EV, in a time-post-injury dependent fashion. In fact, IL-6 expression was increased upon stimulation with DC-EV from 3 days-post-injury animals, when compared to DC-EV from day 14 and NO control, which were still able to promote IL-6 expression to levels higher than those observed in the cytokine control condition. Interestingly, an inversed tendency could be detected in the UC supernatants controls, with an accentuated increase in IL-6 expression in the UC control from 14 days post-injury. However, the differences that could be found between the conditions tested were not statistically significant, likely due to the limited number of independent experiments that could be performed.

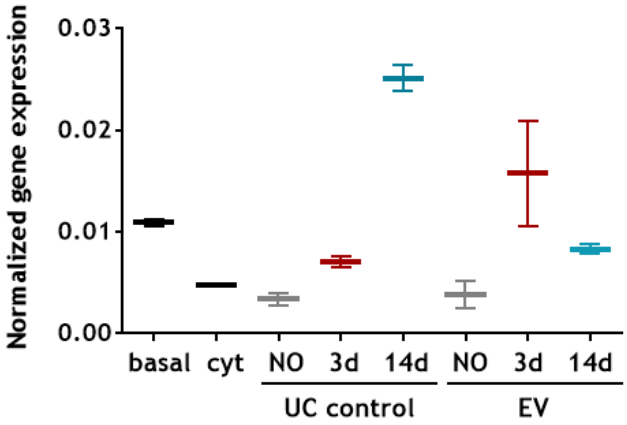

Figure 9. MSC IL-6 expression in response to DC-EV from different timepoints after bone injury. IL-6 expression levels were evaluated by RT-qPCR in MSC stimulated for 48 h with EV isolated from dendritic cells of non-operated (NO), 3 days (3d) and 14 days (14d) post-injury animals. Supernatants from EV ultracentrifugation (UC control) were used as control. Unstimulated MSC maintained in basal media (basal) or in the presence of DC-differentiating cytokines - IL-4 and GM-CSF- (cyt) were the negative controls of the experiment. Box plots represent min-to-max distribution (n=2). Statistical significance was assessed by Kruskal-Wallis test, followed by Dunn's multiple comparisons testing and no significant differences between groups were found.

3.4.3. DC-EV effect on MSC migration

Previous work performed in the lab have demonstrated the ability of human DC-EV to promote MSC migration [142]. Additionally, MSC are known to preferentially migrate towards sites of inflammation, when injected in different animal models of injury [148]. In line with this, EV isolated from DC of NO and bone-injured animals at different time points post-injury were previously evaluated in the lab for their ability to modulate MSC migration *in vitro*, using a transwell migration system. Briefly, rat MSC were seeded on the top compartment (TC) of the transwell and allowed to migrate for 12 h towards the bottom compartment (BC), where the same amount of different stimuli (DC-EV or the respective UC control) was added (Figure 10(A)). The number of migrated cells for each condition relative to the number of cells migrated in the absence of any stimuli (SFM condition) were quantified across independent experiments and are represented in Figure 10(B). Most interestingly, EV derived from DC of 3 days post-injury animals showed a significantly impaired ability to promote MSC migration, when compared to EV from DC of non-operated and 14 days post-injury animals. Considering these results, it was an objective of this dissertation to ascertain if the observed effects were a specific consequence of bone injury by testing if DC-EV from an acute injury scenario of a different organ could lead to similar outcomes. For this, DC-EV from sham-operated animals at day 3 and 14 after surgery bearing soft tissue trauma, were tested for their capacity to affect rat MSC migration, following the same protocol described above. Results indicated that DC-EV from sham-

operated animals were not able to promote MSC migration in any of the conditions tested, leading to levels of MSC migration similar to those observed with the unstimulated (SFM condition) and non-operated controls, contrarily to the effect observed for EV secreted by DC derived from the bone injury microenvironment (Figure 10(C)).

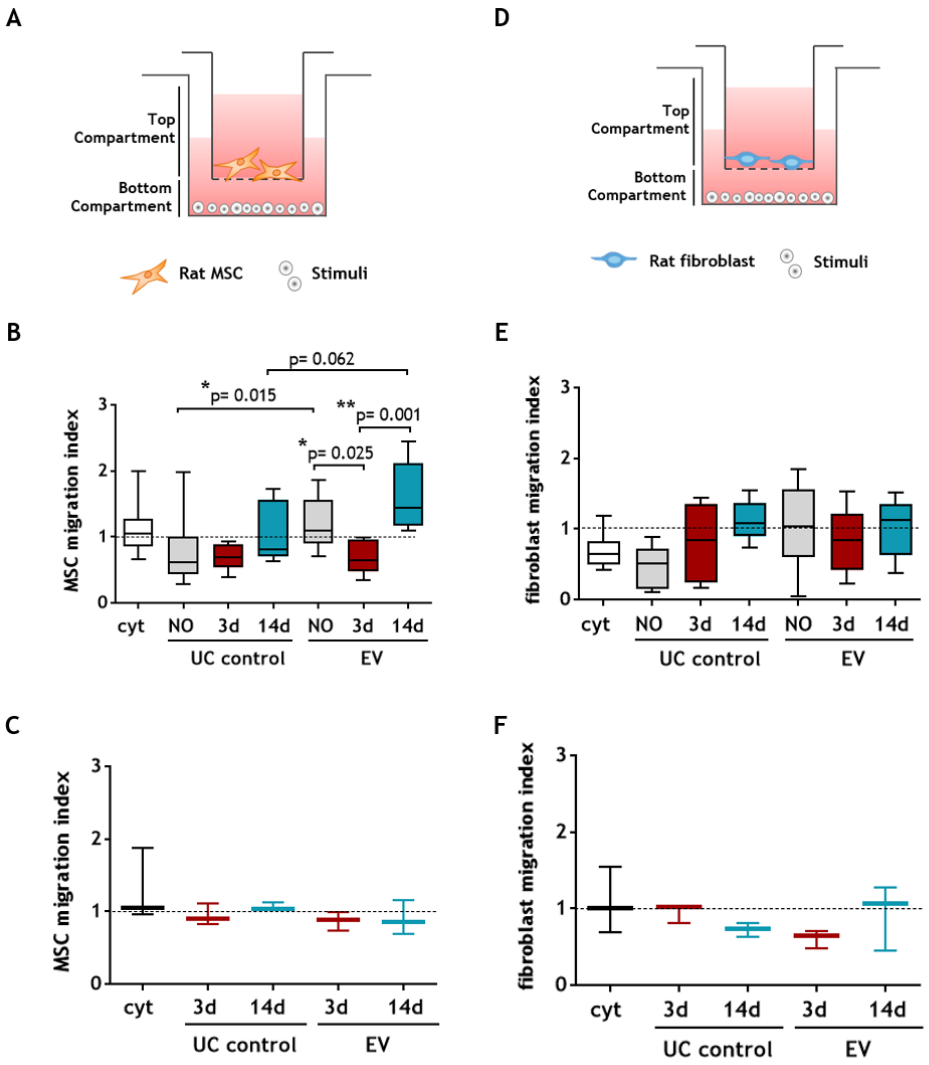

Figure 10. Bone injury affects the ability of EV from bone-marrow-derived dendritic cells to promote MSC migration. (A) Schematic representation of the transwell migration system used to assess the effect of dendritic cell (DC)-derived EV from non-operated (NO), 3 days (3d) and 14 days (14d) post-injury or sham-operated animals on MSC recruitment. MSC were seeded at the top compartment (TC) and allowed to migrate for 12 h towards stimuli in the bottom compartment (BC). The number of MSC migrated in the presence of the indicated stimuli from non-operated and bone-injured animals **(B)** or sham-operated controls **(C)**, relative to the number of migrated cells in the absence of any stimuli (dashed line) was quantified across independent experiments. The same transwell system was set-up to assess fibroblast migration **(D)** and migration indexes in the presence of the indicated stimuli from bone-injured animals **(E)** and sham-operated controls **(F)** were determined. EV ultracentrifugation supernatants (UC control) were used as controls. Cell migration in the presence of DC-differentiating cytokines was also evaluated as control. Box plots represent min-to-max distribution (n= 5 to 6 NO and bone-injured animals; n=3 sham-operated rats). P-values are indicated, as determined by Kruskal-Wallis test, followed by Dunn's multiple comparisons testing.

In order to evaluate the specificity of the effect of DC-EV on MSC migration, the same stimuli were previously tested for their ability to promote the recruitment of rat fibroblasts, using a similar experimental set-up (Figure 10(D)). Results confirmed that DC-EV from different timepoints post-injury specifically modulate MSC migration, as DC-EV were not able to significantly affect fibroblast migration, in any of the conditions tested (Figure 10(E)). Similar results were observed in the experiments performed in the scope of this thesis, testing the effect of DC-EV from sham-operated animals on fibroblast migration, as none of the tested conditions were capable of inducing significant fibroblast migration (Figure 10(F)). These results further support the specificity of the impact of DC-EV on MSC migration. Interestingly, EV from DC isolated from sham-operated animals at 3 days post-surgery seemed to inhibit fibroblast migration, but this effect was not significant.

Considering previous findings in the lab regarding MMP activity on EV-mediated MSC migration [142], the gelatinase activity in supernatants from top (TC) and bottom compartments (BC) of transwell assays performed to study the effect of EV derived from DC from non-operated and bone-injured animals on rat MSC migration was tested by zymography (Figure 11(A)). Results showed one band corresponding to MMP-9 (92 kDa) and two bands for MMP-2, likely pro-MMP2 (72 kDa) and active MMP-2 (67 kDa), which were quantified across independent experiments (Figure 11 (B, C)). Overall, high variability was evident for both MMP-9 and MMP-2 quantification and no statistical differences between conditions could be found. For most of the conditions tested, the activity of MMP-9 was found to be higher than that of MMP-2. Nonetheless, results showed that MMP-2 activity was found essentially in the top compartment, as it will likely be produced by MSC. Moreover, MMP-9 activity was slightly increased both in TC and BC in the presence of EV from 14 days post-injury, compared to day 3, being similar to that detected for EV from NO animals.

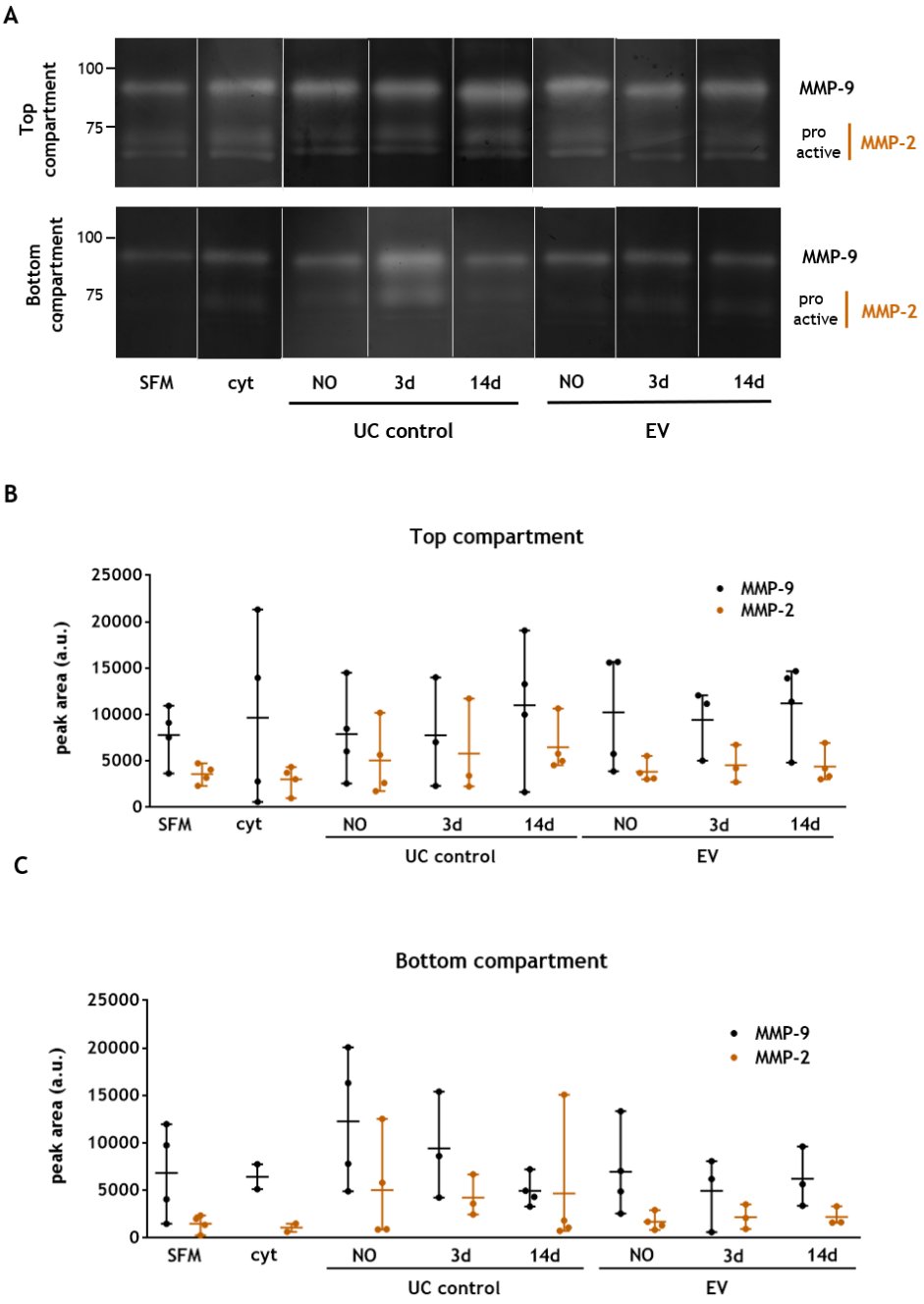

Figure 11. MMP-2 and MMP-9 activity in the supernatants of migration assays. (A) Representative zymograms obtained by gelatin zymography for the detection of MMP-2 and MMP-9 proteolytic activities in conditioned media collected from the MSC transwell migration systems, used to assess the effect of dendritic cell (DC)-derived EV, and respective EV ultracentrifugation controls (UC controls), from non-operated (NO), 3 days (3d) and 14 days (14 days) post-injury animals on MSC migration. Cell culture media was collected separately from top and bottom compartments (TC, BC, respectively) of the transwell system, protein was precipitated from these media and resolved in gelatin-zymography gels which were then stained with Coomassie blue for the detection of MMP-2 and MMP-9 gelatinolytic activities, visible as clear bands in a blue background. As indicated, one band corresponding to MMP-9 (92 kDa) and two bands for MMP-2, likely pro-MMP-2 (72 kDa) and active MMP-2 (67 kDa) could be detected. A densitometric analysis was performed to determine band intensities which were quantified in zymograms from TC **(B)** and BC **(C)**, across independent experiments (n=2 to n=4, respectively). Box plots represent min-to-max distribution and are presented as peak areas in arbitrary units (a.u.). The intensities of pro-MMP-2 and active MMP-2 bands were summed and are indicated as MMP-2. SFM and cyt are the negative controls of the transwell migration system experiments.

3.5. Assessment of DC-EV content

Considering the interesting results obtained regarding the impact of time post-bone injury on the ability of DC-EV to promote MSC recruitment, the next step was to analyse the content of EV from DC differentiated at different timepoints post-injury in order to uncover cytokines, chemokines or other molecules potentially implicated in cell migration. This task was accomplished in the lab prior to the time of this thesis using a rat cytokine membrane array (Supplementary Figure 6). The content of EV from DC of 14 days post-injury animals were shown to be more similar to that of DC-EV from NO animals and clearly divergent from the content of DC-EV from 3 days post-injury rats, as evidenced in the hierarchical clustering of cytokines/chemokines content depicted in Supplementary Figure 6(A). Moreover, a closer comparison between the content of DC-EV from 3 and 14 days post-injury, revealed that DC-EV at 14 days were highly depleted in TNF-α, Tissue Inhibitor of Metalloproteinase (TIMP)-1 and Fas ligand (FasL) (Supplementary Figure 6(B)), which were shown to be enriched in DC-EV from 3 days, together with IL-13 and IL-10, when compared to those from non-operated controls (Supplementary Figure 6(C)). Among the cytokines/chemokines tested, TNF-α was the molecule more highly enriched in EV from BM-derived DC at 3 days post-injury, relative to EV from BM-derived DC of NO rats, with a fold-change magnitude higher than 5 (Supplementary Figure 6(C)). Interestingly, when TNF-α levels were quantified by ELISA in total conditioned media from DC differentiated from bone marrow cells at different timepoints post-injury, a similar pattern of TNF-α depletion at 14 days compared to 3 days post-injury could be detected (Supplementary Figure 7). Indeed, the levels of TNF-α were nearly significantly lower in the supernatants of DC from 14 days post-injury, when compared to 3 days post-injury DC conditioned media, and significantly lower compared to conditioned media from DC of NO animals.

An objective of this thesis was to validate the time post-injury dependent variation in candidate molecules, namely TIMP-1 and TNF-α, in DC-EV of NO and bone-injured animals by western blotting. In line with this, we started by optimizing the western blot conditions to test the presence of those molecules using total conditioned media of DC differentiated from bone marrow cells of NO, bone-injured and sham-operated animals at 3 and 14 days after surgery. Despite several attempts to optimize the protocol applied, it was not possible to detect the band correspondent to TNF-α in any of the tested samples. On the other hand, immunoblots for TIMP-1 showed the presence of one band at 28kDa, correspondent to the monomeric form of TIMP-1 in all the conditions tested. These results are illustrated in Figure 12 (A) and quantified across independent animals in Figure 12 (B). TIMP-1 levels appeared to be increased in conditioned media of DC from 3 days post-bone injury animals, compared to those detected in DC-conditioned media from NO and 14 days post-bone injury rats, even though no statistical differences could be found between any

of the conditions tested. This temporal variation was not observed in conditioned media of DC differentiated from bone-marrow of sham-operated animals. Importantly, these results should be interpreted with caution due to the high variability observed. Unfortunately, TIMP-1 detection by western blotting on DC-EV could not be tested within the scope of this thesis.

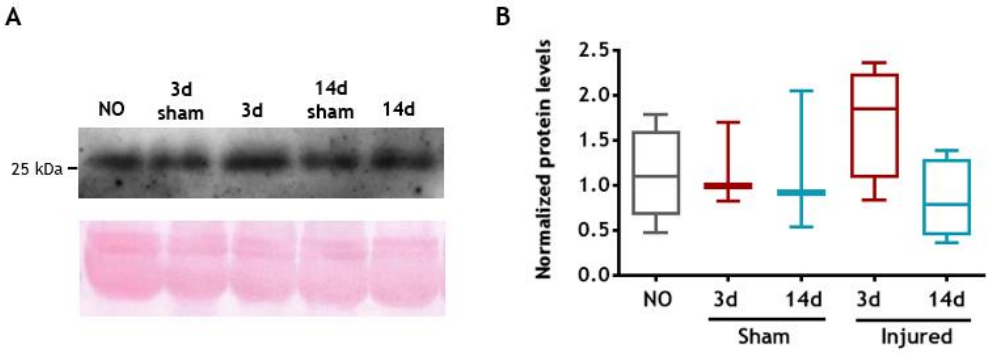

Figure 12. TIMP-1 was detected in DC conditioned media from different timepoints after bone injury. **(A)** Representative western blots showing one band at 28 kDa, correspondent to TIMP-1, in conditioned media of dendritic cells (DC) differentiated *ex vivo* from bone-marrow (BM) of non-operated (NO), bone-injured animals at 3 (3d) and 14 days (14d) post-injury and sham-operated animals at the same timepoints (3d sham, 14d sham) (top membrane). Ponceau-stained membranes were also imaged and the bands correspondent to albumin (bottom membrane) were quantified and used as normalizer. **(B)** Relative quantification of TIMP-1 bands across independent animals (n= 4 to 7 NO and bone injured animals; n=3 sham-operated rats). Box plots represent min-to-max distribution. Statistical significance was assessed by Kruskal-Wallis test, followed by Dunn's multiple comparisons testing and no significant differences between groups were found.

3.6. TNF-α concentration does not impact MSC migration

TNF-α is reported to be a mediator of MSC migration [149], an effect suggested to be dependent on its concentration [150]. Considering the results showing that time post-injury affects the amount of TNF-α present in DC-secreted EV and since these EV proved to have a differential impact on MSC migration, we then hypothesized that TNF-α could be a major mediator of DC-EV action on MSC migration, by modulating this process in a dose-dependent fashion. To test this hypothesis in our model, a transwell migration assay was conducted, whereby different concentrations of TNF-α (0.01; 0.1; 0.25; 0.5; 1; 10 ng/mL) were added to the bottom compartment (BC) acting as stimuli for rat MSC migration (Figure 13(A)). These concentrations were selected according to the levels of TNF-α detected by ELISA in total conditioned media from DC of NO, 3 and 14 days post-injury animals (Supplementary Figure 7). The number of MSC migrated in the presence of the indicated stimuli relative to the number of MSC migrated in the non-stimulated negative control (SFM) was quantified across independent experiments, and results are represented in Figure 13(B). Overall, the levels of MSC migration observed in the presence of TNF-α in

different concentrations were similar to those detected in the negative unstimulated control (SFM). Furthermore, no differences in MSC migration were found between the different TNF-α doses tested, with only 1 ng/mL and 10 ng/mL TNF-α showing a trend for increased cell migration.

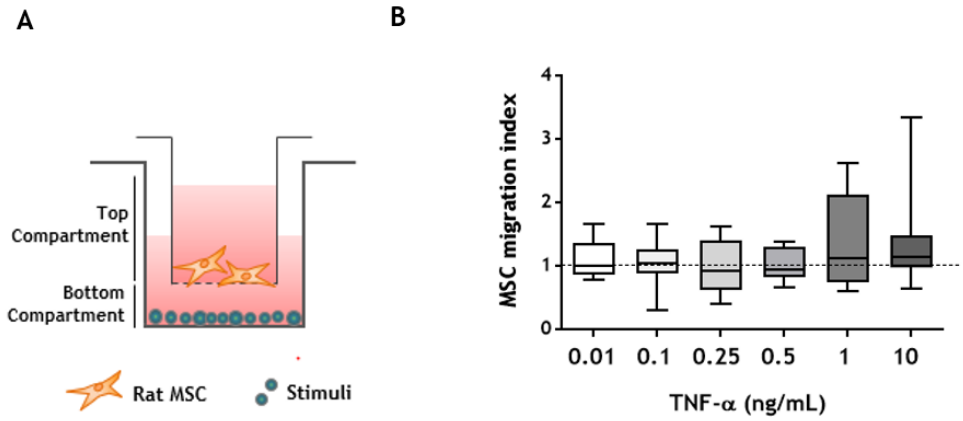

Figure 13. TNF-α *per se* does not replicate the effects observed for DC-EV on MSC migration. (A) Representation of the transwell set-up used to evaluate the effect of different doses of TNF-α on MSC recruitment. MSC were seeded inside the transwell insert (top compartment, TC) and allowed to migrate for 12 h towards the bottom compartment (BC), where rat recombinant TNF-α was present in different concentrations. **(B)** Migration index of MSC in the presence of the indicated stimuli relative to the negative control condition (dashed line); (n= 5 to 8 independent experiments, with MSC from 3 different rats). Box plots represent min-to-max distribution. Kruskal-Wallis test was performed, followed by Dunn's multiple comparisons test and no statistical differences could be found between conditions.

The results presented above will be integrated in a manuscript, currently being prepared for publication, on the impact of bone injury on DC-derived EV and their interaction with MSC.

4. DISCUSSION

Bone injury triggers a local inflammatory response that needs to be resolved over time to allow the initiation of reparative events [33]. This response is reflected at the systemic level changing the levels of immune cell populations and circulating mediators, such as cytokines [144, 151]. These systemic changes may potentially affect cells distant from the injury site, including endogenous MSC populations, which have been ascribed with immunomodulatory roles, and are able to differentiate into bone-forming cells [152], likely influencing their capacity to proliferate, migrate and differentiate. Previous work in the lab has further contributed to unravel the systemic response to bone injury in terms of secreted circulating mediators, by profiling the alterations in the plasma miRnome, particularly in the transition from the acute inflammatory phase after injury (day 3) to the inflammation resolution phase (day 14) [143]. Results showed an overall down-regulation of microRNAs (miRNA) at 3 days post-injury, which was then partially recovered at day 14, a temporal change that was predicted to mainly regulate processes of cell differentiation and proliferation [143]. During this thesis, these predictions were partially validated, with plasma from 3 and 14 days bone-injured animals promoting significantly increased levels of MSC proliferation, an effect that was not significant for plasma from NO animals. Several other investigations have been reported demonstrating an effect of plasma/sera from injury models on MSC proliferation. A study conducted by Kim *et al*. reported that serum obtained from a rat stroke model was able to increase MSC proliferation, relative to the positive control, and most interestingly through the upregulation of miRNA-20a expression. However, the timepoint post-injury when sera were collected was not specified and no comparison was made with plasma from healthy donors, even though miRNA expression profiles with both sera seem slightly different [153]. Importantly, another work described that sera collected from bone trauma patients enhanced the proliferation of healthy bone marrow-derived MSC, when compared to the positive control, but once again the sera collection timeline and the comparison with healthy donors were left undisclosed [154]. Nonetheless, the results obtained herein did not confirm the initial predictions of differential regulation of MSC proliferation in a time post-injury dependent manner. This leads to the hypothesis that the identified cocktail of miRNAs may not be the only circulating factors in plasma responsible for the effects observed on MSC proliferation. Additional investigations, using techniques such as mass spectrometry, could be employed to further dissect time-dependent changes in plasma content (for instance, for proteins and metabolites) after bone injury.

The systemic inflammatory response occurring upon injury is reflected not only in the plasma, but also in peripheral lymphoid organs, such as the lymph nodes and spleen. To further dissect the systemic response to bone injury, which remains vastly uncharacterized, gene expression of the cytokines TNF-α, IL-1ß, IL-6 and IL-10 in spleen isolated from NO, bone-injured, and sham-operated animals was assessed by RT-qPCR. The

levels of the pro-inflammatory cytokines TNF-α and IL-1β were decreased at 14 days post-bone injury, during the inflammation resolution stage, when compared to the acute inflammatory phase at 3 days post-injury, and particularly to sham-operated animals at the same timepoint. These results are similar to those observed for the levels of these cytokines in plasma, indicating a concerted systemic inflammatory response to bone injury. This time post-injury-dependent cytokine expression resembles also the one reported to occur at the local injury site [155], further supporting the notion that the local inflammatory response which occurs upon bone injury is reflected at the systemic level. Moreover, these results seem to implicate the spleen in the process of bone repair, in accordance with the work by Xiao *et al.*, which showed that the delayed bone healing in splenectomised rats was accompanied by reduced levels of TNF-α, IL-1β and IL-6, compared to animals with spleen [156]. The splenic mRNA levels of these pro-inflammatory cytokines were also reported to be upregulated in early timepoints in other models of injury, such as brain [157, 158] and ischemic acute kidney injury [159]. The expression of the anti-inflammatory cytokine IL-10 was also increased slightly in the spleen from day 3 post-injury animals and reduced at day 14, particularly in comparison with sham-operated controls. This suggests the existence of a compensatory mechanism, which may help counteract the inflammatory response to bone injury, facilitating the transition to the later reparative events. This type of regulatory mechanism has been suggested to occur early after intestinal injury in a mouse model, with the production of IL-10 by splenic regulatory B cells [160]. Moreover, Andres-Hernando *et al.* showed that circulating IL-6 could upregulate the production of IL-10 in the spleen, thus limiting lung inflammation in a mouse model of acute kidney injury [161]. Importantly, the temporal regulation observed in bone-injured animals could not be detected in sham-operated controls, which showed expression levels of TNF-α, IL-1β and IL-10 at 14 days significantly different than those observed in bone-injured animals at the same timepoints. These findings indicate that injury of different organs/tissues can lead to different systemic responses.

At the local level, the inflammatory response initiated upon injury leads to the recruitment of immune cell populations that help to create an adequate pro-regenerative microenvironment, providing signals for the recruitment, proliferation and differentiation of osteoprogenitor cells, including MSC, which will then be major players in the reparative events conducive to bone healing [33]. In fact, the crosstalk between immune and skeletal cell populations has been increasingly recognized over the years, both in physiological and pathophysiological scenarios [54]. Among cells of the innate immune system, DC are perhaps the less studied for their involvement in bone repair. Nevertheless, several studies have suggested the ability of DC to differentiate into osteoclasts, particularly when primed by a pro-inflammatory environment (reviewed in [74]). Moreover, DC constitute a versatile member of the innate immune system, essential for the translation to adaptive responses,

due to their role in the activation of T cells, whose function in bone repair has been increasingly explored [33]. The work developed during this thesis aimed at further unravelling the involvement of DC in the process of bone repair, particularly by dissecting the potential ability of DC-derived EV to modulate MSC biology.

To achieve this goal, DC were differentiated *ex vivo* from bone marrow collected from NO, bone-injured and sham-operated animals at days 3 and 14 after surgery. This approach was based on the hypothesis that DC differentiated *ex vivo* would have a phenotypic fingerprint remnant from the bone marrow environment where their progenitors were pre-conditioned. To confirm this hypothesis, DC obtained were characterized for their inflammatory activation status. Overall, DC differentiated from bone marrow expressed higher levels of TNF-α and IL-6 at day 3 than at day 14, albeit not statistically significantly different. This suggests that the microenvironment created upon bone injury may lead to alterations on the phenotype of these cells. This temporal regulation is in accordance to what was previously observed for the cytokine levels in DC conditioned media. However, as there were no significant differences among groups, these results need to be further validated. Moreover, we were not able to completely ascertain if the effects observed were mostly influenced by bone injury, due to the high variability of results observed for sham-operated animals. An assessment of the expression/secretion of a wider panel of cytokines and growth factors, in DC from a higher number of animals, should therefore be performed to fully assess cell phenotype. Future analysis of the expression levels of different cytokines, using quality RNA from whole bone marrow isolates from different time points post-injury could also help understand the variation of the inflammatory conditions created in the bone microenvironment at different time points post-injury, and how they may differentially precondition DC to produce EV with divergent biological properties.

Overall, our results show a concordant inflammatory response to bone injury both at local and systemic levels, which might be mediated by signals secreted by the local immune cells, including EV. For this reason, we were interested in dissecting the biological properties of the EV secreted by DC from bone marrow of bone-injured animals. The EV populations isolated from the culture media of DC from non-operated, bone-injured, and sham-operated animals had a morphology and size distribution compatible with exosomes. The morphological characterization conducted revealed the presence of vesicles with a cup-like morphology typical of exosomes, which were absent from the UC controls in all the conditions tested (Supplementary Figure 5), indicating the efficiency of the isolation protocol applied [147]. Moreover, these vesicles presented mean diameters between 100-200 nm which are included in the size range considered for exosomes [102]. Pusic *et al.* reported sizes of 50 nm for EV obtained from IFN-γ stimulated rat bone marrow-derived dendritic cells [162]. A different group isolated EV from immature rat bone marrow-derived

DC with sizes between 30-120 nm [163]. Of note, none of these groups performed NTA analysis or other complementary characterization techniques for a more accurate determination of size distribution, having only relied on TEM analysis. Importantly, the type of injury inflicted in the animals, namely bone injury or soft tissue trauma alone, did not impact the morphological characteristics of EV secreted by DC. Of note, the lack of EV specific-markers and standardized characterization techniques makes it imperative to perform a set of complementary techniques when conducting EV characterization [147]. In line with this, future work is needed to complete the characterization of EV populations used in the context of this dissertation, to fully comply with the criteria defined by ISEV and confirm the exosomal enrichment [102]. Western blotting for the detection of CD63, CD81, CD9, membrane proteins known to be present in exosomes [112], and calnexin, an integral protein of the endoplasmic reticulum absent from exosomes [112], will be performed in the near future.

Interestingly, the capacity of DC from bone injury animals to secrete EV appeared to vary in a time post-injury-dependent manner, as DC seemed to release less EV at 3 days post-injury, during the acute inflammatory phase. By the time of inflammation resolution, at 14 days post-injury, EV levels returned to those of NO animals. Interestingly, DC from sham-operated animals showed reduced EV secretion at both timepoints. Our results are not in line with previous *in vitro* studies, like Hu *et al.*, that observed increased secretion of EV by cardiomyocytes exposed to an *in vitro* model of oxidative stress injury [164], which is thought to occur during the acute inflammatory response to bone injury [34], an effect also reported by Saha *et al.* in primary human monocytes treated with ethanol *in vitro* [165]. Additionally, a different work reported enhancement in EV secretion by cardiomyocytes under hypoxic conditions [166], which are known to exist in the hematoma at early stages after bone injury [33]. Although these results are not in line with ours, it is possible that they reflect a specific response to different tissue injuries, or that the differences observed in those reports are due to the assays being conducted *in vitro*, while in our experiments DC precursors may have been pre-conditioned to respond in different ways *in vivo*.

Considering the importance of MSC for bone repair, the impact of DC-EV on MSC biology was further studied. DC-EV were tested for their ability to promote MSC proliferation. DC-EV from all the timepoints tested did not significantly impact MSC proliferation. Although there was a slight increase in MSC proliferation, it was not statistically significant, and it was also observed for the ultracentrifugation supernatant controls. This is in agreement with a recent publication from our team showing that EV isolated from immature human DC stimulate migration, but not proliferation of MSC [142]. A study reported by Paggetti *et al.* showed that exosomes derived from a chronic B cell leukemia cell line were able to promote the proliferation of bone marrow MSC, in a dose-

dependent manner [167], an effect also reported for exosomes derived from multiple myeloma cells [168] and human platelet lysate [140]. Importantly, we observed that MSC stimulated with plasma from the same injury model responded with increased proliferation. Thus, future studies with plasma-derived EV, aiming to characterize their effect and also their cellular origin, could clarify if they are responsible for the increased MSC proliferation and which cells are responsible for that effect.

The evaluation of the effect of DC-EV on MSC biology, revealed that these EV are able to impact MSC IL-6 expression and the capacity of these cells to migrate. MSC stimulation with EV from DC at 3 days post-injury increased their expression of IL-6, comparing with stimulation by EV from DC at day 14 post-injury and, in particular, from NO animals, revealing a modulation of the profile of cytokines expressed by MSC dependent on time post-injury. This is in accordance with the expected initial inflammatory response to bone injury, which should activate local cell populations, including MSC [33]. In fact, previous reports described that MSC pre-conditioning in different inflammatory environments modulates their secretome [169] . These changes of MSC IL-6 expression, mediated by DC-EV, might impact the immunomodulatory activity described for MSC [170], which could condition the inflammation resolution after bone injury and thus proper bone healing. Nonetheless, the differences between conditions reported herein were not statistically significant, probably due to the limited number of experiments that could be performed, and thus these results require further confirmation. Importantly, we did not ascertain if the effects observed for DC-EV on MSC proliferation and immunomodulatory activity were specifically mediated by the microenvironment created upon bone injury, and so the experiments should be repeated in the near future to determine if DC-EV from sham-operated controls induce similar results.

DC-EV demonstrated a differential ability to modulate MSC migration dependent on the time after bone injury when DC precursors were isolated. DC-EV from animals at 3 days post-injury had an inhibitory action upon MSC migration while DC-EV from day 14 post-injury, regain their ability to promote MSC migration, to levels slightly higher than those of control conditions. These findings are in line with early work developed in the lab showing that DC preconditioning with the pro-inflammatory cytokine TNF-α impaired the capacity of these cells to promote MSC migration [141], and the ability of EV from human immature DC to promote MSC migration *in vitro* [142]. Importantly, DC-EV from sham-operated animals were unable to promote MSC migration, suggesting that the inflammatory microenvironment created upon bone injury may play a role in the preconditioning of DC precursors, thus influencing the specificity of biological properties of EV secreted by these cells. Interestingly, the modulation of cell migration by DC-EV seems to be targeted for particular cell populations, since DC-EV also did not significantly impact the recruitment of fibroblasts, cells of mesenchymal origin with a recognized role in tissue repair, including

in bone injury, but also associated with tissue fibrosis [34, 171]. Fibroblast migration was also not promoted in the presence of DC-EV from sham-operated controls. In fact, EV isolated from DC from sham-operated animals at 3 days post-injury seemed to inhibit fibroblast migration, even though no statistical differences could be detected. However, EV derived from other cell types have been implicated in the recruitment of fibroblasts. For instance, Dörsam *et al*. showed that EV isolated from a Hodgkin lymphoma cell line could induce the migration of fibroblasts *in vitro* [172] and EV from platelet-rich plasma were also shown to induce the migration of primary human dermal fibroblasts [173]. Similarly, Shabbir and colleagues reported the ability of MSC-derived EV to promote the migration of fibroblasts from both healthy donors and chronic wound patients, in a dose-dependent fashion [174].

In order to unravel the molecular mechanisms underlying the effect of DC-EV on MSC migration, we started by testing the activity of gelatinases MMP-9 and MMP-2 in the supernatants collected separately from the top and bottom compartments of the transwell migration systems set-up. MMP are secreted proteolytic enzymes involved in the degradation of extracellular matrix components and direct activation of signalling molecules, being also known as facilitators of cell migration [175], including of MSC [176]. MMP have been implicated in bone development and disease [177] and different studies with MMP-null animal models have highlighted their role in the success of bone repair [178-180]. In the media from the top compartment, it is likely that the gelatinase activity detected is mainly due to MMP secreted by MSC, whereas gelatinase activity in the bottom compartment may be greatly impacted by the presence of EV. In fact, it was previously reported that EV derived from human DC contain MMP that may be exposed at their surface, likely contributing for the effect of these EV on MSC migration [142]. Contrarily, our results suggest that MMP-2 and MMP-9 activities do not play a major role in the ability of DC-EV from different timepoints post-injury to differentially modulate MSC migration, even though a slightly increase in MMP-9 activity could be detected in the presence of DC-EV from 14 days post-injury when compared to DC-EV from 3 days. Overall, the results obtained were highly variable for each condition tested, with persisting difficulties in performing the quantification of zymography band intensities, mainly due to the faint bands obtained across the different experiments performed. In fact, several attempts of optimization of the technique for the analysis of our samples were performed, namely by changing the concentration and the origin of the gelatine used. Therefore, gelatine zymography does not seem to be the most adequate technique for the detection of activity of rat gelatinases, and thus was not attempted for the detection of MMP-9 and MMP-2 in DC-EV. Alternative techniques could be applied in the future, such as western blotting for the evaluation of the levels of active MMP-9 and MMP-2, in order to confirm our results and validate the presence of these MMP in DC-EV. Interestingly, the results obtained with the

antibody membrane array, showed that MMP-8 was depleted in EV from DC at 3 days post-injury compared to DC from NO animals, suggesting that MMP other than MMP-2 and MMP-9 may be implicated in the time-post-injury-dependent effect of DC-EV on MSC migration, a hypothesis which will be further explored in the future.

Since EV from DC derived from different timepoints post-injury had an impact on MSC migration, and not on MSC proliferation or differentiation, an antibody membrane array was performed in the lab to detect the presence of chemokines and other molecules potentially implicated in cell migration. DC-EV from 14 days post-injury, which promoted MSC migration, were highly depleted in TIMP-1, an endogenous inhibitor of MMP with important roles in the regulation of cell migration [181]. In order to validate the presence of this protein in DC-EV, we started by optimizing the conditions of TIMP-1 detection by western blotting in total conditioned from DC cultures. We could successfully detect the presence TIMP-1 in all conditions tested, including culture media from DC from sham-operated animals, being increased at day 3 after bone injury. The detection of TIMP-1 specifically in DC-EV is currently under analysis. Importantly, in line with our results, Ries *et al*. reported increased MSC migration upon silencing of TIMP-1 expression [182]. Furthermore, Wang *et al*. showed an induction of TIMP-1 expression in the fracture callus early after tibial fracture in mice, which remained highly expressed until day 21 post-injury [183]. This is in accordance with TIMP-1 enrichment in EV from DC from 3 days post-injury animals, being possible that its levels at later timepoints after bone injury in mice are mainly due to their production by other cell populations, similarly to the cytokine secretion profile after bone injury [155]. Importantly, TIMP-1 levels were shown to be diminished in serum from non-union patients [184], pinpointing this molecule as a potential target for the development of new therapies for bone repair, namely mediated by DC-EV.

Along with TIMP-1, DC-EV from 14 days post-injury animals were also highly depleted in TNF-α. Importantly, previous work from the group have shown that DC stimulation with this pro-inflammatory cytokine lead to an impairment of their ability to promote MSC migration [141]. On the other hand, Naaldijk *et al*. reported increased MSC migration in the presence of TNF-α, using a transwell migration system [185], an effect also observed in a different study when MSC under hypoxic conditions were pre-conditioned with TNF-α [186]. Interestingly, TNF-α was reported to promote MSC migration in a dose-dependent fashion, with significantly higher migration at a concentration of 50 ng/mL, when compared to the SFM control [150]. However, at lower concentrations (<25 ng/mL) migration was only slightly increased, and no significant differences could be found [150]. Thus, we further tested the ability of different doses of rat recombinant TNF-α to influence MSC migration in our experimental setup. The doses tested were based on the levels of this cytokine found in total DC conditioned media by ELISA. TNF-α *per se*, in different concentrations, did not seem to majorly impact MSC migration. Slightly increased MSC

recruitment levels were only observed for high concentrations of TNF-α. These results seem to suggest that TNF-α is not a major player in the time-post-injury-dependent effect of DC-EV on MSC migration. Future studies are therefore needed to further explore the role of this cytokine and other potential candidates, among the chemokines differentially present in DC-EV from different timepoints after injury, alone or in combination, as mediators of DC-EV effects on MSC migration.

Considering the ability of DC-EV to regulate MSC migration, which was shown to be specifically impacted by the microenvironment created upon bone injury, and the potential of these EV to also modulate MSC proliferation and immunomodulatory activity, one can infer that DC-derived EV may constitute promising therapeutic agents for the improvement of bone repair and regeneration. Of note, our findings relied on *in vitro* and *ex vivo* models which are not sufficient to fully understand the contribution of DC and their secreted EV for the process of bone regeneration. The following task would be an *in vivo* proof-of-concept, for the evaluation of MSC recruitment to the injured area and overall improvement of bone healing, upon local or systemic administration of DC-EV in an animal model of bone injury. Moreover, the *in vitro* modification of these EV, for instance with miRNAs known to modulate MSC osteogenic differentiation [187] or with other mediators associated with improved bone repair, could also be envisioned as a future approach which would contribute to further accentuate the effects potentially mediated by DC-EV on MSC recruitment and bone tissue repair. Overall, the work developed during this dissertation contributed to further unravel the communication between immune and skeletal cell populations, particularly through secreted EV, in the context of bone repair. This work provides important knowledge which may contribute to the development of novel strategies for the treatment of traumatic bone injuries and other bone-associated pathologies.

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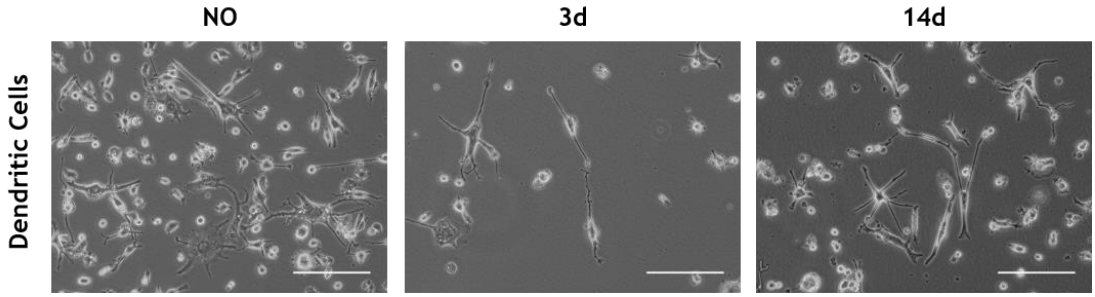
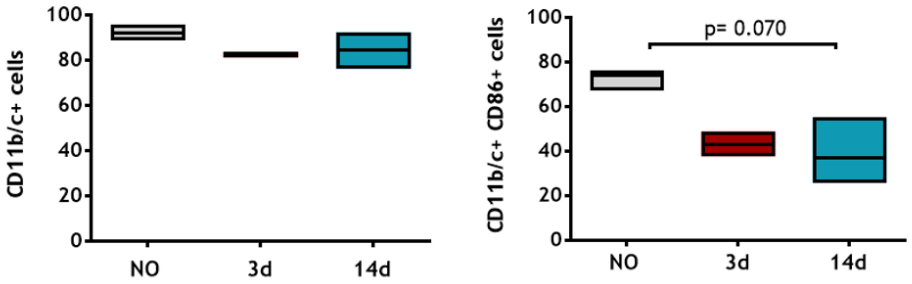
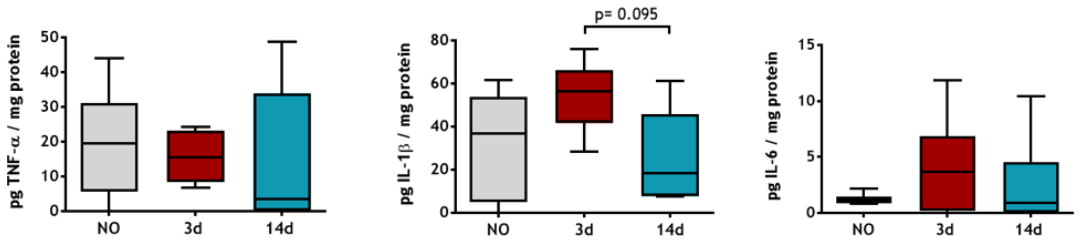
6. SUPPLEMENTARY FIGURES



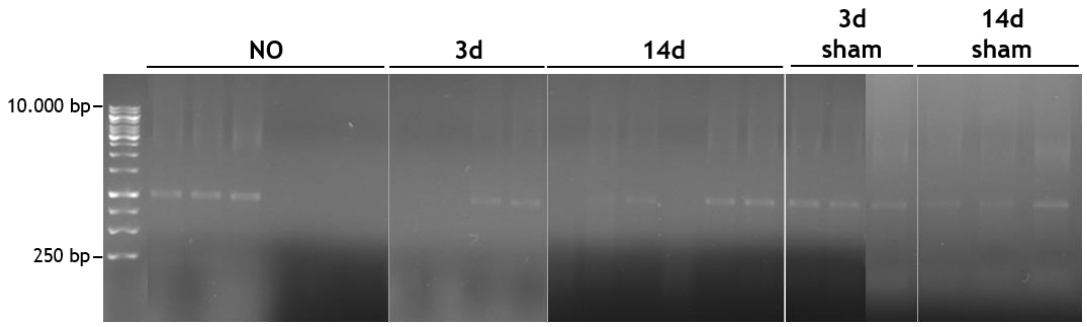
Supplementary Figure 1. Representative brightfield microscopic images of dendritic cells (DC) differentiated *in vitro*, in the presence of IL-4 and GM-CSF, from bone-marrow isolated from the femurs of non-operated, 3 days and 14 days post-bone injury rats (NO, 3d, 14d, respectively). Scale bar: 150 µm.



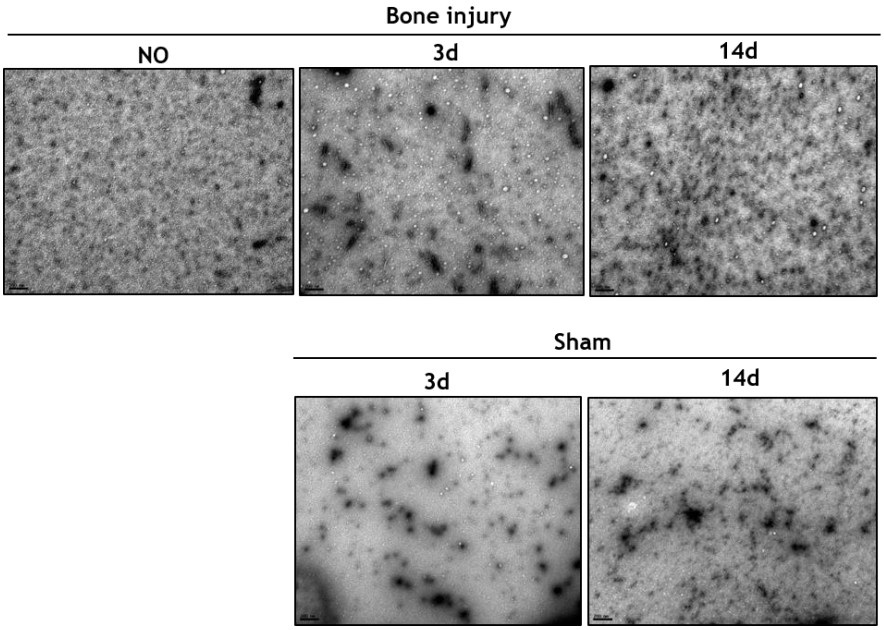
Supplementary Figure 2. The expression of the activation marker CD86 on DC decreased with time after injury. Dendritic cell (DC) populations isolated from bone marrow (BM) of non-operated (NO), 3 days (3d) and 14 days (14d) post-injury animals were immunostained for the surface markers CD11b/c (myeloid lineage marker) and CD86 and analysed by flow cytometry. The percentages of CD11b/c+ and CD86+ cells in the DC populations analysed are depicted. Box plots represent min-to-max distribution of n= 2 to 4 animals per group. P-values are indicated, as determined by Kruskal-Wallis, followed by Dunn's multiple comparisons testing.



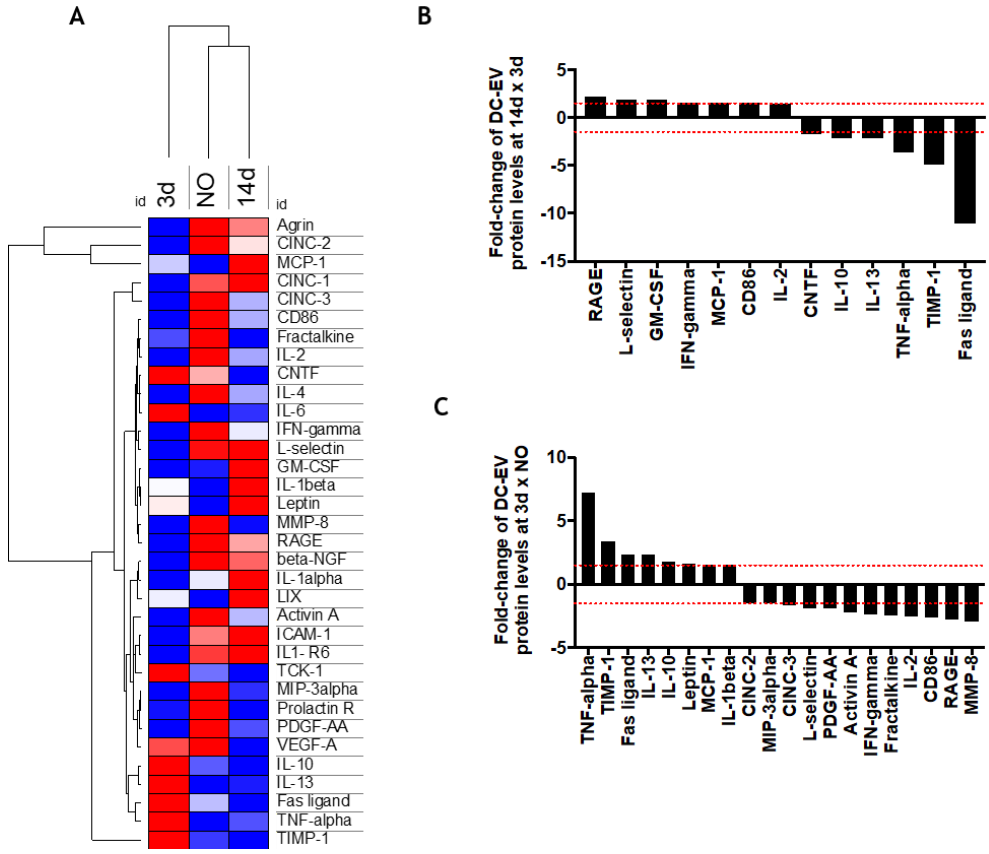
Supplementary Figure 3. The systemic inflammatory response to bone injury is reflected in the levels of pro-inflammatory cytokines circulating in the plasma of animals at different timepoints after injury. The levels of TNF-α, IL-1β and IL-6 in the plasma of non-operated (NO), 3 days (3d) and 14 days (14d) post-injury animals were quantified by ELISA. Quantifications were normalized for the protein concentration of conditioned media samples. Box plots represent min-to-max distribution of n= 6 to 8 animals per group. P-values are indicated, as determined by Kruskal-Wallis, followed by Dunn's multiple comparisons testing.



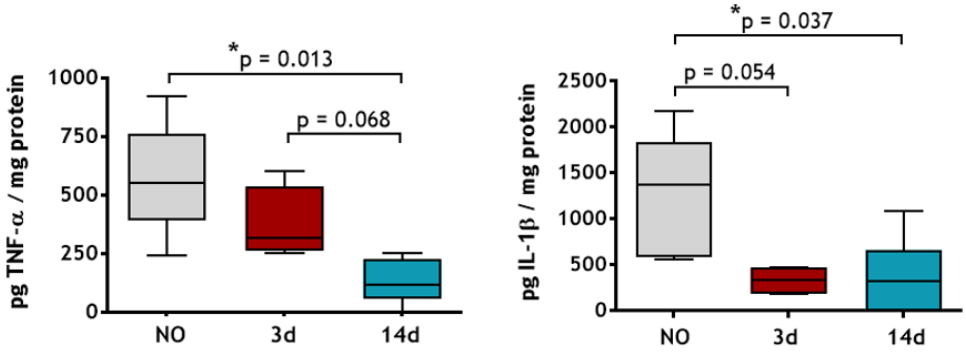
Supplementary Figure 4. RNA samples of whole bone-marrow isolates of rats at different timepoints after injury presented signs of degradation. Agarose gel electrophoresis was performed to assess the integrity of RNA isolated from bone-marrow of non-operated (NO), 3 days (3d) and 14 days (14d) post-injury and sham-operated animals (3d sham, 14d sham, respectively).



Supplementary Figure 5. EV were not detected in the supernatants of DC-EV ultracentrifugation. Representative TEM micrographs of ultracentrifugation (UC) supernatants (UC control) collected after EV isolation from culture media of dendritic cells (DC) differentiated from bone-marrow (BM) of non-operated (NO), 3 days (3d) and 14 days (14d) post-injury and sham-operated animals (3d sham, 14d sham, respectively). Magnification: 50 000x. Scale bar: 200 nm.



Supplementary Figure 6. Time after bone injury affects the content of DC-EV. (A) Heatmap of cytokine/chemokines protein levels in EV secreted by dendritic cells (DC) from non-operated (NO), 3 days and 14 days post-injury rats. The Euclidean hierarchical clustering method was used to group animal groups and cytokines/chemokines. (B) Fold-change of cytokines/chemokines protein levels in EV from DC at 14 days post-injury, relative to EV from DC at day 3. (C) Fold-change of cytokines/chemokines protein levels in EV from DC at 3 days post-injury compared to EV from DC of NO animals. Only cytokines with an absolute fold-change higher than 1.5 are presented. Dashed lines indicate fold-changes thresholds of -1.5 and 1.5.



Supplementary Figure 7. TNF-alpha was depleted in total conditioned media from DC of 14 days-post injury animals. The levels of TNF-α and IL-1ß in total conditioned media from dendritic cells (DC) of non-operated (NO) rats, and at day 3 (3d) and day 14 (14d) after bone injury were assessed by ELISA. Quantifications were normalized for the protein concentration of conditioned media samples. Box plots represent min-to-max distribution of n= 3 to 6 animals per group. P-values are indicated, as determined by Kruskal-Wallis, followed by Dunn's multiple comparisons testing.