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New strategies for mesenchymal stromal cell recruitment: a role for exosomes in bone regeneration?

Andreia Machado da Silva

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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New strategies for mesenchymal stromal cell recruitment: a role for exosomes in bone regeneration?

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Aos meus pais,

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URL: http://onlinelibrary.wiley.com/doi/10.5966/sctm.2016-0033/abstract

4. Daniel M. Vasconcelos, Raquel M. Gonçalves, Catarina R. Almeida, Inês O. Pereira, Marta I. Oliveira, Nuno Neves, **Andreia M. Silva**, António C. Ribeiro, Carla Cunha, Ana Rita Almeida, Cristina C. Ribeiro, Ana M. Gil, Elizabeth Seebach, Katharina L. Kynast, Wiltrud Richter, Meriem Lamghari, Susana G. Santos, Mário A. Barbosa (2016), Fibrinogen scaffolds with immunomodulatory properties promote in vivo bone regeneration. Biomaterials 111:163e178, doi: 10.1016/j.biomaterials.2016.10.004.
URL: http://www.sciencedirect.com/science/article/pii/S0142961216305415

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URL: http://www.sciencedirect.com/science/article/pii/S1473050216300830

6. Maria I. Almeida, **Andreia M. Silva**, Daniel M. Vasconcelos, Catarina R. Almeida, Hugo Caires, Marta T. Pinto, George A. Calin, Susana G. Santos, Mário A. Barbosa (2016), miR-195 in human primary mesenchymal stromal/stem cells regulates proliferation, osteogenesis and paracrine effect on angiogenesis. Oncotarget, 7(1):7-22, doi: 10.18632/oncotarget.6589.
URL:http://www.impactjournals.com/oncotarget/index.php?journal=oncotarget&page=article&op=view&path[]=6589&pubmed-linkout=1

7. João P. Barbosa, Ana R. Neves, **Andreia M. Silva**, Mário A. Barbosa, Maria S. Reis, Susana G. Santos (2016), Nanostructured lipid carriers loaded with resveratrol modulate human dendritic cells. International Journal of Nanomedicine, 11:3501-3516, doi: 10.2147/IJN.S108694.
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8. Heidi Schwarzenbach, **Andreia M. Silva**, George Calin, Klaus Pantel (2015), Data Normalization Strategies for MicroRNA Quantification. Clinical Chemistry, 61(11):1333-1342, doi: 10.1373/clinchem.2015.239459.
URL: http://clinchem.aaccjnls.org/content/61/11/1333.long

9. Marc D. Bullock, **Andreia M. Silva**, Pinar Kanlikilicer-Unaldi, Justyna Filant, Mohammed H. Rashed, Anil K. Sood, Gabriel Lopez-Berestein, George A. Calin (2015), Exosomal Non-Coding RNAs: Diagnostic, Prognostic and Therapeutic Applications in Cancer. Non-Coding RNA, 1(1):53-68, doi:10.3390/ncrna1010053.
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Index of contents

Abstract ..... **XIX**

Sumário ..... **XXIII**

List of abbreviations ..... **XXVII**

I. Introduction **31**

1. Bone repair and regeneration **33**
1.1. The socio-economic impact of bone disorders 33
1.2. Bone tissue and mechanisms of bone repair 35
1.2.1. Physiological bone repair: the importance of the immune system 37
1.2.1.1. Immune cells in bone repair 40
1.2.1.2. Cytokines and growth factors in bone repair 45
1.2.2. Therapeutic approaches for bone repair 48
1.2.2.1. Biomaterial-based therapies for bone repair: the role of immunomodulatory biomaterials 50
1.2.2.2. Cell therapies for bone repair: the Mesenchymal Stem Cells paradigm 52
1.2.2.3. Gene therapies for bone repair: a focus on microRNAs 55

2. Extracellular Vesicles: Immunomodulatory messengers in the context of tissue repair/regeneration **59**
Graphical abstract 59
Abstract 60
2.1. Introduction 60
2.2. EV in Tissue Repair and Regeneration 62
2.2.1. EV as Immunomodulatory Messengers: Implications for Tissue Repair and Regeneration 63
2.2.2. EV as Cell Proliferation and Differentiation Mediators in Tissue Repair and Regeneration 66
2.2.3. EV Carry ECM Remodelling Messages: Their Influence on Tissue Repair 68
2.3. EV as Therapeutic Vehicles in Tissue Repair and Regeneration 69
2.3.1. Application of EV as New Drugs 70
2.3.2. EV as Novel Drug-delivery Vehicles 72
2.4. Conclusion 76

References 77

3. Aims of the thesis **99**

II. Original research work **101**

1. Dendritic Cell-derived Extracellular Vesicles mediate Mesenchymal Stem/Stromal Cell recruitment **103**
Abstract 103
1.1. Introduction 104

1.2. Results 105
1.2.1. EV isolated from Dendritic Cells are enriched in exosomes 105
1.2.2. DC-derived EV are internalized by MSC without negatively impacting MSC proliferation or differentiation 106
1.2.3. DC-derived EV promote MSC recruitment and migration 110
1.2.4. DC-derived EV are rich in mediators that can promote cell migration 112
1.3. Discussion 116
1.4. Methods 120
Acknowledgments 125
References 126
Supplementary information 131

2. Extracellular Vesicles from bone marrow-derived dendritic cells promote mesenchymal stem cells recruitment: the impact of bone injury 135
Graphical abstract 135
Abstract 136
2.1. Introduction 137
2.2. Methods 138
2.3. Results 142
2.3.1. Bone injury triggers a systemic immune and inflammatory response 142
2.3.2. Bone injury affects the secretion of EV by BM-derived DC and Macrophages 144
2.3.3. Bone injury influences the capacity of DC-EV to promote MSC migration 148
2.3.4. Cytokine/chemokine content of DC-EV changes with time after bone injury 149
2.4. Discussion 150
Acknowledgments 156
References 156
Supplementary information 162

3. Profiling the circulating miRnome reveals a temporal regulation of the bone injury response 167
Graphical abstract 167
Abstract 168
3.1. Introduction 169
3.2. Methods 170
3.3. Results 174
3.3.1. Expression profile of circulating miRNAs changes over time upon bone injury 174
3.3.2. Let-7 family of miRNAs is up-regulated at day 14 post-injury 177
3.3.3. Detected miRNAs are predicted to control cellular processes important for bone regeneration 180
3.3.4. Detected miRNAs profile suggests changes in miRNA biogenesis pathways 183
3.4. Discussion 187
Acknowledgments 192
References 192
Supplementary information 198

III. General discussion and future perspectives 203

References 213

Abstract

Musculoskeletal conditions are the second largest contributor to disability worldwide, significantly impacting quality-of-life throughout the life-course (WHO, 2018). In particular, the skeletal system may be affected by acute trauma and a variety of disorders that disrupt the homeostatic bone remodelling process.

Bone and immune system share the bone marrow compartment and many molecular mediators, including Extracellular Vesicles (EV). Moreover, when bones are affected by acute injury, an inflammatory response takes place, that is controlled in time, and crucial for an effective bone healing. Importantly, immune cells secrete several paracrine mediators able to influence cell recruitment and differentiation. In our previous work we showed that Dendritic Cells (DC) were able to promote Mesenchymal Stem/Stromal Cells (MSC) recruitment *in vitro* by a paracrine communication mechanism, an effect that was impaired by DC exposure to a pro-inflammatory environment.

For this doctoral dissertation work, we hypothesized that MSC recruitment could be mediated by DC-derived EV, and influenced by their cargo. So herein we aimed to: (i) determine the potential of DC-EV to promote MSC recruitment *in vitro,* characterizing the content of their cargo; (ii) determine the impact of bone injury pre-conditioning of precursor cells, on the capacity of bone marrow-derived macrophages and DC to secrete EV that promote MSC recruitment and differentiation, using a rat model of critical size bone defect; (iii) characterize the cargo of EV circulating systemically upon bone injury, namely in terms of microRNAs (miRNAs), to uncover new biomarkers and therapeutic targets for improving bone healing.

To accomplish our aims we started by investigating the ability of EV, secreted by human peripheral blood monocyte-derived DC, to recruit MSC. We found that isolated DC-EV populations were enriched for exosomes, which could be internalized by human MSC. Importantly, DC-EV *per se* promoted MSC recruitment in a dose-dependent manner. On the other hand, the proliferative capacity of MSC and their differentiation potential were not affected by DC-EV stimulation. Importantly, DC-EV cargo content was characterized for the presence of cytokines and chemokines, and was found to be enriched in chemotactic mediators, including osteopontin, and to contain also metalloproteinase-9. These findings are reported in the scientific article *Silva et al. (2017), Scientific Reports, 7*.

The impact of bone injury on the capacity of bone marrow (BM)-derived macrophage- and DC-EV to promote MSC recruitment and differentiation was then investigated using an *in vivo* model of critical size bone defect, surgically performed in the femurs of adult rats.

Animals were analysed at days 3 and 14 after injury, corresponding respectively to the acute phase and the resolving phase of the inflammatory response that occurs upon injury. Non-operated (NO) animals were used as controls. The systemic response to bone injury resulted in increased proportions of myeloid cells at day 3 in blood, and particularly in spleen and draining (inguinal and popliteal) lymph nodes, which returned to basal levels by day 14. In addition, BM-derived DC and macrophages (Mac) were differentiated *ex vivo*, and the EV they secreted were collected and characterized. Importantly, EV from DC at day 3 post-injury impaired rat BM-MSC recruitment *in vitro,* compared to EV from NO animals, while EV from DC at day 14 post-injury, significantly promoted MSC recruitment. Interestingly, Mac-EV were not efficient in recruiting MSC. On the other hand, neither DC-EV nor Mac-EV significantly affected the differentiation potential of rat MSC. Comparing the cargo of EV from DC at different timepoints after bone injury, we found that EV from DC at 14 days post-injury contained several chemotactic mediators, including Monocyte Chemoattractant Protein-1. Moreover, EV from 14 days post-injury DC were markedly depleted of the pro-inflammatory cytokine TNF-α and the metalloproteinase inhibitor TIMP-1, compared to DC-EV from day 3 post-injury. These results are currently in preparation for publication.

For the third part of this thesis, we attempted to characterize the miRNA content of EV circulating in plasma at different timepoints after critical size bone defect injury in rats. However, technical limitations in the recovery of sufficient plasma-derived EV of appropriate quality for miRNA array screening, precluded this analysis. Alternatively, extracellular miRNAs circulating systemically in plasma were profiled. RT-qPCR array analysis revealed a major down-regulation of miRNAs expression in plasma at day 3 post-injury compared to NO controls, which was partially recovered by day 14 after injury. From the miRNAs analysed, let-7a, let-7d and miR-21 were significantly up-regulated in plasma at day 14 compared to day 3 post-injury, being promising biomarkers for bone healing monitoring. In addition, this expression profile was not found in sham operated animals at the same timepoints. These miRNAs were also found upregulated in splenocytes of bone-injured animals at 14 days post-injury, compared to day 3. The bioinformatics analysis of the plasma miRnome, revealed that miRNAs differently expressed at day 14 post-injury relative to day 3 were involved in biological processes such as cell development, proliferation and migration. Thus, we tested the capacity of rat plasma to influence rat MSC proliferation, and found that plasma from 3 and 14 days post-injury could significantly promote cell proliferation relative to control. In accordance with the miRNA expression profile found, bioinformatics analysis also predicted that the miRNA processing enzymes Dicer and Ago2, as well as the Ephrin receptor B6 (EphB6), were inhibited at day 3 post-injury, with Ago2 becoming activated at day 14. Interestingly, the levels of Dicer and Ago2 were increased in splenocytes from day 14 post-injury, comparing with day 3 post-injury and NO animals, with EphB6 following the

same tendency. Smad2/3 was also found to be significantly decreased at day 14 post-injury splenocytes, compared to day 3 and NO animals. Splenocytes from sham operated animals did not show the same protein expression profile. These findings are reported in the scientific article *Silva et al. (2018), Theranostics*.

Overall, the work developed in the scope of this thesis contributed to further dissect the crosstalk between the immune system and bone-forming cells. Particularly, we demonstrated that EV are the main mediators of MSC recruitment by DC, and importantly, that this effect depends on the pre-conditioning of DC precursors *in vivo*. Moreover, we found three miRNAs candidates circulating in plasma after bone injury that can be used as new theranostics tools for improved bone healing, a finding with potential for clinical translation in the short-to-mid-term period. Furthermore, in the future, these miRNAs can be explored for DC-EV functionalization, further boosting their potential role in promoting endogenous or transplanted MSC recruitment to the place of injury, and bone tissue repair and regeneration. These findings will impact the development of new strategies for the treatment of non-healing bone traumas, and other bone-affecting diseases.

Sumário

As doenças e lesões músculo-esqueléticas constituem a segunda causa mais comum de incapacidade física em todo o mundo, afetando profundamente a qualidade de vida das populações ao longo de toda a sua vida (OMS, 2018). Em particular, o sistema esquelético pode ser afetado por traumas agudos e por uma diversidade de doenças que alteram significativamente a remodelação óssea que ocorre em homeostasia.

Naturalmente, o tecido ósseo e as células do sistema imunitário partilham o microambiente da medula óssea e vários mediadores moleculares, incluindo as Vesículas Extracelulares (EV, do inglês, *extracellular vesicles*). Para além disso, quando o osso é afetado por um trauma agudo, ocorre uma resposta inflamatória, controlada no tempo, que é crucial para que a regeneração óssea ocorra de forma eficaz. Por outro lado, as células imunes secretam vários mediadores parácrinos capazes de modular o recrutamento e a diferenciação celular. No nosso trabalho anterior, demonstrámos que as células dendríticas (DC, do inglês *dendritic cells*) têm capacidade para promover o recrutamento das células mesenquimais estaminais ou do estroma (MSC, do inglês *mesenchymal stem/stromal cells*) *in vitro,* através de mecanismos de comunicação parácrina. Vimos também que o recrutamento das MSC é afetado pelo pré-condicionamento das DC num ambiente pró-inflamatório.

Deste modo, o trabalho desenvolvido nesta tese de Doutoramento foi baseado na hipótese de que o recrutamento das MSC pelas DC poderia ser mediado pelas EV que as DC secretam, e dependente do conteúdo destas vesículas. Neste contexto, os seguintes objetivos foram definidos: (i) investigar o potencial das EV secretadas pelas DC na promoção do recrutamento das MSC *in vitro*, caracterizando também o conteúdo das EV; (ii) investigar a influência do pré-condicionamento de células precursoras, na capacidade de DC e macrófagos derivados da medula óssea para secretar EV capazes de promover o recrutamento e diferenciação de MSC, utilizando um modelo de lesão de tamanho crítico, em rato; (iii) caracterizar o conteúdo de EV circulantes no organismo após uma lesão óssea, em particular do seu conteúdo em microRNAs (miRNAs), para encontrar novos biomarcadores e alvos terapêuticos para promover a regeneração óssea.

Para atingir os objetivos propostos, começou-se por investigar a capacidade das EV, secretadas por DC derivadas de monócitos de sangue periférico humano, para promover o recrutamento de MSC. Os resultados obtidos mostraram que as populações de EV de DC isoladas eram enriquecidas em exossomas, que podiam ser internalizados por MSC

humanas. Foi ainda demonstrado que as EV derivadas de DC promovem *per se* o recrutamento de MSC, de uma forma dependente da dose de EV. No entanto, a capacidade proliferativa das MSC, bem como o seu potencial de diferenciação, não foram alterados pela estimulação com estas EV. Desta forma, o conteúdo das EV secretadas pelas DC foi caracterizado, nomeadamente para a deteção de citocinas e quimiocinas, e descobriu-se que as EV eram enriquecidas em mediadores quimiotáticos, como a osteopontina, contendo também metaloprotease-9. Estes resultados foram publicados no artigo científico *Silva et al. (2017), Scientific Reports, 7.*

De seguida, foi investigado o impacto de uma lesão óssea na capacidade das EV secretadas por DC e macrófagos (Mac) derivados da medula óssea para promover o recrutamento e diferenciação das MSC, usando para tal um modelo *in vivo* de lesão óssea de tamanho crítico induzido cirurgicamente em fémures de ratos adultos. Os animais lesionados foram depois analisados aos dias 3 e 14 após a cirurgia, que correspondem à fase aguda e à fase de resolução da resposta inflamatória que ocorre após a lesão, respetivamente. Animais não operados foram usados como controlo. Descobriu-se que a resposta sistémica ao trauma ósseo resultou num aumento da proporção de células mielóides ao dia 3, no sangue e, em particular, no baço e nos nódulos linfáticos (inguinais e poplíteos) drenantes, regressando a níveis normais ao dia 14 após a lesão. DC e Mac derivados da medula óssea foram também diferenciados *ex vivo*, e as EV secretadas foram isoladas e caracterizadas. Os resultados obtidos demonstraram que as EV secretadas pelas DC do dia 3 após a lesão inibiam o recrutamento *in vitro* de MSC da medula óssea de rato, comparativamente às EV de animais não operados, enquanto que as EV secretadas pelas DC do dia 14 após lesão promoviam significativamente o recrutamento das MSC. Os resultados obtidos indicam ainda que as EV secretadas pelos Mac não foram eficientes na indução do recrutamento das MSC. Por outro lado, nem as EV secretadas pelas DC nem pelos Mac foram capazes de afetar significativamente a diferenciação das MSC de rato. Deste modo, comparando o conteúdo das EV de DC de diferentes tempos após a lesão, descobriu-se que as EV de DC do dia 14 continham vários mediadores moleculares quimiotáticos, incluindo a Proteína Quimiotática de Monócitos-1 (do inglês, *Monocyte Chemoattractant Protein*-1). Para além disso, EV de DC do dia 14 após a lesão tinham quantidades muito reduzidas da citocina pró-inflamatória Fator de Necrose Tumoral-α (do inglês, *Tumor Necrosis Factor*-α) e do Inibidor Tecidual de Metaloproteases-1 (do inglês, *Tissue Inhibitor of Metalloproteinase-1*), comparativamente às EV de DC do dia 3 após a lesão. Estes resultados estão a ser preparados para serem submetidos para publicação.

Para o terceiro capítulo desta tese pretendia-se caracterizar o conteúdo das EV circulantes no plasma de ratos a diferentes tempos após indução da lesão óssea. Contudo, limitações técnicas no isolamento de VE do plasma em quantidade e qualidade apropriadas,

impediram a análise do perfil global de miRNAs destas vesículas, por hibridação em *microarray*. Em alternativa, caracterizou-se o perfil de miRNAs extracelulares em circulação sistémica no plasma. Uma análise por *array* de RT-qPCR revelou que ao dia 3 após a lesão óssea ocorre uma sub-expressão generalizada de miRNAs no plasma, em comparação com animais controlo não operados, e que é parcialmente revertida ao dia 14 após a lesão. Dos miRNAs analisados, o let-7a, let-7d e miR-21 estavam significativamente sobre-expressos no plasma ao dia 14 após a lesão, em relação ao dia 3, sendo por isso potenciais biomarcadores para monitorização da regeneração óssea e também alvos terapêuticos. De notar que não foi encontrado um perfil de expressão semelhante para estes miRNAs no plasma de animais submetidos a cirurgia mas sem indução de defeito ósseo (controlo *sham*). Verificou-se que estes miRNAs estavam também sobre-expressos em células do baço de animais ao dia 14 após a lesão óssea, comparativamente com o dia 3. Uma análise bioinformática do perfil global de expressão de miRNAs revelou que os miRNAs diferencialmente expressos ao dia 14 após a lesão em relação ao dia 3, estão envolvidos em processos biológicos como o desenvolvimento celular, proliferação e migração. Por este motivo, a capacidade do plasma de rato para influenciar a proliferação de MSC de rato foi testada, revelando que plasma dos dias 3 e 14 após a lesão promovia um aumento significativo da proliferação celular em relação ao controlo negativo. Tal como esperado pelo perfil de expressão de miRNAs encontrado, a análise bioinformática previu que as enzimas Dicer e Ago2, responsáveis pelo processamento de miRNAs, assim como o receptor EphB6 (do inglês, *Ephrin receptor B6*), estariam inibidas ao dia 3 após a lesão, sendo que a Ago2 estaria ativada ao dia 14. Os resultados obtidos mostraram que os níveis de Dicer e Ago2 estavam aumentados nas células do baço ao dia 14 após a lesão, em relação ao dia 3 e aos controlos não operados, com a EphB6 seguindo a mesma tendência. Os níveis da proteína Smad2/3 estavam significativamente diminuídos nas células do baço ao dia 14 após a lesão, relativamente ao dia 3 e aos controlos não operados. Para além disso, este padrão de expressão proteica não foi encontrado nos controlos *sham*. Estes resultados foram publicadas no artigo científico *Silva et al. (2018), Theranostics.*

Globalmente, o trabalho desenvolvido no âmbito desta tese contribui para um melhor conhecimento da inter-comunicação entre o sistema imunitário e as células que formam o tecido ósseo. Em particular, demonstramos que as EV secretadas pelas DC são os principais mediadores do recrutamento de MSC e, mais importante, que este efeito depende do pré-condicionamento dos precursores de DC num contexto pró-inflamatório *in vivo.* Para além disso, revelamos três miRNAs candidatos em circulação no plasma após uma lesão óssea, que poderão ser usados como ferramentas teranósticas para a melhoria da regeneração óssea, uma descoberta com grande potencial para transferência para a clínica a curto ou médio prazo. Para além disso, no futuro, estes miRNAs podem ser utilizados para

a funcionalização de EV de DC, potenciando o seu papel promissor na promoção do recrutamento de MSC endógenas ou transplantadas para locais de lesão, e promovendo a reparação e regeneração do osso. Estas descobertas irão com certeza influenciar o desenvolvimento de novas estratégias para o tratamento de traumas ósseos que não regeneram, bem como de outras doenças que afetam o osso.

List of abbreviations

a.u.	Arbitrary units
ACAN	Aggrecan
ADAM	A disintegrin and metalloproteinase
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
Ago	Argonaute
ALP	Alkaline phosphatase
APC	Antigen presenting cell
BC	Bottom compartment
BI	Blood
BM	Bone marrow
BMP(s)	Bone morphogenetic protein(s)
BSA	Bovine serum albumin
CCR2	CC chemokine receptor type 2
CD	Cluster of differentiation
CEBPB	CCAAT/enhancer binding protein-β
CHSJ	Centro hospitalar São João
CM	Conditioned media
COX	Cyclooxygenase
CTX	C-terminal crosslinking telopeptide of type I collagen
DC	Dendritic cell(s)
DGAV	Direção-Geral de Alimentação e Veterinária
DGCR8	DiGeorge syndrome critical region gene 8
DLS	Dynamic light scattering
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EE	Efficiency of loading
EPC	Endothelial progenitor cells
EphB6	Ephrin receptor B6
ESC	Embryonic stem cells
ESCRT	Endosomal sorting complex required for transport
EV	Extracellular Vesicles
FasL	First apoptosis signal receptor ligand
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FDR	False discovery rate
FGF	Fibroblast growth factor
FMI	Forward migration index
FoxP3	Forkhead box P3
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GOI	Gene of interest
H&E	Haematoxylin and eosin

HA	Hydroxyapatite
HMGB1	High mobility group box 1
i.p.	Intraperitoneal
i.v.	Intravenous
IFN	Interferon
IL	Interleukin
IPA	Ingenuity pathway analysis
LN	Lymph nodes
LPS	Lipopolysaccharide
Ly6G	Lymphocyte antigen 6 complex locus G6D
M-CSF	Macrophage colony-stimulating factor
Mac	Macrophage(s)
MAFIA	Macrophage Fas-induced apoptosis
MCP-1	Monocyte chemoattractant protein-1
miRNA(s)	microRNA(s)
MMP	Metalloproteinase
mRNA	Messenger RNA
MSC	Mesenchymal stem/stromal cells
MSP	Muscle-specific peptide
MyD88	Myeloid differentiation primary response 88
MYOD1	Myogenic differentiation 1
MYOG	Myogenin
NF-κB	Nuclear Factor-κB
NK	Natural Killer (cells)
NO	Non-operated
NTA	Nanoparticle Tracking Analysis
OCN	Osteocalcin
OPG	Osteoprotegerin
OPN	Osteopontin
P/S	Penicillin/G-streptomycin
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PIIINP	Collagen III N-terminal propeptide
PK	Proteinase K
RA	Rheumatoid arthritis
RAGE	Receptor for advanced glycation endproducts
RANKL	Receptor activator of nuclear factor κB ligand
RFU	Relative fluorescence units
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RPMI 1640	Roswell Park Memorial Institute 1640 media
RT	Room temperature
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
RUNX2	Runt-related transcription factor 2
RVG	Rabies viral glycoprotein
SDF-1	Stromal cell-derived factor-1
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide electrophoresis gel
SFM	Serum-free media
SHH	Sonic hedgehog

siRNA	Small interfering RNA
SOX9	(Sex-determining region Y)-box 9
Sp	Spleen
SPM	Specialized pro-resolving mediators
STAT	Signal transducers and activators of transcription
TC	Top compartment
TCP	Tricalcium phosphate
TCR	T cell receptor
TEM	Transmission electron microscopy
TGF-β	Transforming growth factor-β
TIMP	Tissue inhibitor of metalloproteinases
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
TRAP	Tartrate-resistant acid phosphatase
T_{reg}	Regulatory T cells
Tspan8	Tetraspanin8
UC	Ultracentrifugation
USC	Urine-derived stem cells
VEGF	Vascular endotelial growth factor
WHO	World Health Organization
α-MEM	Minimum essential medium alpha modification
μCT	Micro-Computed tomography

I. Introduction

1. Bone repair and regeneration

1.1. The socio-economic impact of bone disorders

Musculoskeletal disorders remain among the top causes for years lived with disability, particularly in the developed world. Bones in particular are affected by a myriad of diseases and injuries, which alter their structure and metabolism. Although many of such conditions can be treated to achieve the full repair and, ultimately, regeneration of the affected bone, the treatments are usually long lasting or even chronic, constituting a burden both for the patients and healthcare providers. Moreover, for some musculoskeletal conditions the treatments currently available are ineffective and a significant proportion of patients does not achieve proper bone healing. These encompass: (1) non-healing fractures, (2) cancer metastasis to bone, (3) bone metabolic imbalances, (4) inflammatory disorders and (5) genetic diseases.

Non-healing fractures include delayed bone unions, where fracture healing takes twice the expected time, and non-unions, where there is an absence of fracture healing within 9 months after the trauma [1]. Bone fractures are highly incident traumas worldwide, accounting for at least 129 million new cases in 2013, according to the results of the Global Burden of Disease and Injury study [2]. This study also reported that fractures were one of the top injuries contributing to years of life lived with disability [2]. This becomes even more relevant because it is estimated that 1.9-10% of bone fractures usually progress to a non-union [1]. Importantly, data from 2006 to 2008 estimated that the cost of standard treatment of non-union tibia shaft fractures, in the USA, was twice the cost of the same fractures without non-union [3]. Furthermore, non-healing fractures greatly extend the expected time of treatment, which carry major secondary costs, mainly due to patients' absence from work [4]. Importantly, bone metabolic diseases, specially osteoporosis and autoimmune disorders, like rheumatoid arthritis, greatly increase the risk of bone fracture [5], adding the costs of fracture treatment to those of disease management. Of note, several cancers, like breast, prostate and lung, metastize to bone, towards which their cells have a particular tropism [6]. Upon metastization, cancer cells often lead to osteolysis, creating large bone defects that increase bone fragility and susceptibility to fracture [7].

Bone metabolic imbalances result from alterations in the rate of bone formation and resorption, leading to an abnormal increase or decrease in bone mass. Osteopenia and osteoporosis are the most common examples of bone metabolic imbalances, characterized by a progressive loss of bone mass and microstructure. Osteoporosis has higher incidence in women than men, and is more prevalent after 50 years of age [8]. In 2010, ~12 million women and ~3 million men had a diagnosis of osteoporosis only in Europe (data for

Germany, France, United Kingdom, Italy and Spain). In the more advanced stage of osteoporosis, there is an enormous susceptibility to bone fractures, even due to low energy traumas. Indeed, in 2000, osteoporosis was in the origin of more than 8.9 million fractures worldwide, registering higher incidence in Europe [8]. Furthermore, it is predicted that in Europe alone, the number of osteoporosis-related fractures will rise from 2.46 million in 2010 to 3.17 million by 2025 [8]. Importantly, the direct economic burden of osteoporosis in Europe in 2010 was estimated at €30.7 billion, with fractures accounting for nearly 95% of this amount (data for Germany, France, United Kingdom, Italy and Spain). Moreover, indirect costs were estimated to more than double the burden of osteoporotic fractures, to nearly €78 billion [8].

Inflammatory disorders leading to bone injury affect primarily the joints, and only at later stages the bone tissue. The most prevalent are osteoarthritis and rheumatoid arthritis. Osteoarthritis leads to bone hyperthrophy, with the formation of osteophytes and sclerosis of subchondral bone [5]. Data from the year 2000 shows that osteoarthritis was more prevalent in elderly women than in men of the same age rank, being estimated at the time that in the population over 80 years of age, 25 000 women per 100 000 inhabitants were affected by osteoarthritis in developed countries (including Western Europe, North America, Japan, Australia and New Zealand) [5]. On the other hand, in rheumatoid arthritis the highly pro-inflammatory environment leads to erosion of bone starting from the affected joints [5]. Overall, rheumatoid arthritis affects less people than osteoarthritis, with an estimated prevalence of 0.5-1% in white individuals worldwide [9], with women being again more affected than men [5]. According to data from the year 2000, rheumatoid arthritis was most prevalent in developed countries (Western Europe, North America, Japan, Australia and New Zealand), affecting ~2700 women per 100 000 inhabitants, among the population over 80 years-old [5]. But this disease may start earlier, at 20-40 years of age for young-onset rheumatoid arthritis [10], impacting people's productive life.

Bone integrity may also be compromised by several genetic defects, although their incidence is relatively rare. The most common genetic disorders affecting bone are osteopetrosis and osteogenesis imperfecta. Osteopetrosis is characterized by a pathological increase in bone mass, with the most common form of the disease affecting 1 in 20 000 births [11]. On the other hand, osteogenesis imperfecta is characterized by low bone mass and growth, having an estimated incidence of 1 in 15 000-20 000 births [12]. Importantly, both diseases lead to an increase in the risk of bone fracture.

All together, these conditions require not only medical and financial resources for their treatment, but they lead to many other indirect costs. Bone injuries are very frequent, responsible for a high degree of disability, and often managed with treatments that are long-lasting or even chronic. Therefore, they are prominent causes of absence from work and for

requesting disability pensions [5], and thus a major burden for Healthcare and Social Security Systems worldwide, and are expected to keep increasing. Indeed, the ageing of populations in the majority of developed countries, and the increase in life expectancy in many developing countries is leading to a higher incidence of bone-related diseases [5, 8]. This was already recognized in the beginning of 2000, leading the World Health Organization (WHO) to declare the decade of 2000 to 2010 the Bone and Joint Decade, to raise awareness about these disorders and to promote the development of new strategies that increase the effectiveness of treatments and reduce costs [13]. In this context, it is important to keep researching for the development of novel therapeutic approaches that more efficiently promote bone repair and regeneration, which motivated the development of this thesis. In particular, due to the central role of bone fractures in the panorama of bone disorders, this thesis was focused on cellular interplay and mediators of bone fracture healing.

1.2. Bone tissue and mechanisms of bone repair

Bone is a unique tissue in the body, organized in anatomically distinct areas that participate in different functions, such as skeletal growth, mechanical support and hematopoiesis. As depicted in Figure 1, bone tissue can be organized in trabecular or lamellar bone, that is lined internally by the endosteal membrane. On their outer surface, bones are wrapped by the periosteum. In humans (and other higher mammals), lamellar bone can be further organized in Haversian systems, which are concentric lamellae of bone surrounding a central canal composed of blood vessels longitudinally oriented [14]. Interconnected with the bone tissue, there are plenty of blood vessels, responsible for bone irrigation. Bone blood supply is mainly guaranteed by the periosteal circulation, which irrigates the periosteum and the most external regions of the bone, and the medullary circulation, which is across the bone marrow and irrigates the most internal regions of the bone [14]. At the molecular level, bone tissue is constituted by an extracellular matrix (ECM) composed by an organic phase and a mineral phase, and that supports a diversity of cells (Figure 1). The organic phase of bone matrix is mainly constituted by type I collagen, decorated with a plethora of non-collagenous proteins (e.g., proteoglycans, bone sialoproteins, osteocalcin, osteopontin), whereas the mineral phase is constituted by hydroxyapatite, crystals of calcium and phosphate [15]. The cellular fraction embedded within this matrix is mainly constituted by osteoblasts, osteocytes, osteoclasts (Figure 1) and resident populations of immune cells, like osteomacs, a resident macrophage population. In addition, populations of multipotent mesenchymal stromal/stem cells (MSC) and more

committed osteoprogenitor cells can be found in the endosteum [16], and with particular abundance in the periosteum [17]. MSC may also be found in the bone marrow, with some authors also suggesting their distribution as pericytes in the perivascular niche of sinusoid vessels of the bone marrow, an issue that is still controversial [18]. Of note, bones also harbour the hematopoietic system in their marrow.

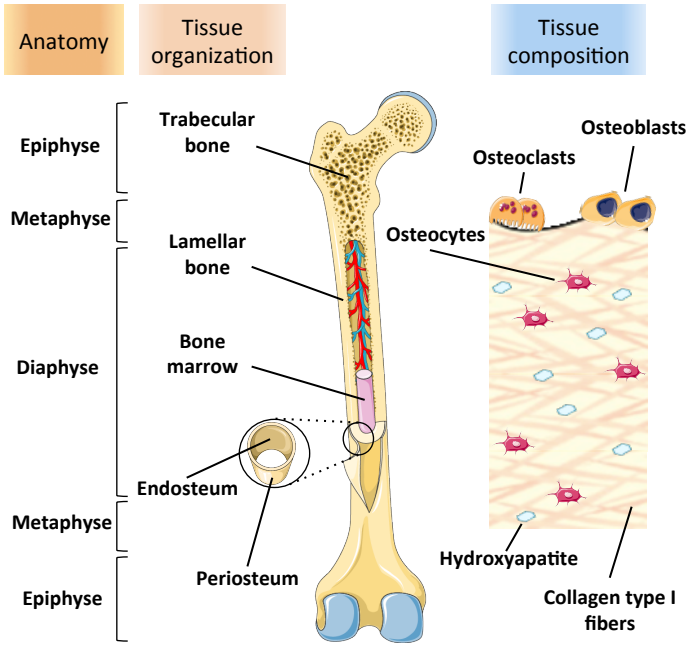


Figure 1. Bone anatomy and composition. Bones have distinct anatomical regions - epiphysis, metaphysis and diaphysis - that differently contribute to their structure, from growth to their overall mechanical behaviour. Throughout the bone, tissue organization is different, comprising trabecular (or spongy) bone and lamellar (or cortical) bone. Bone marrow is lodged inside long bones, and is where hematopoiesis takes place, giving rise to immune cells and osteoclast precursors. Bone tissue surfaces are wrapped in specialized membranes, the periosteum and the endosteum, that harbour osteoprogenitor cell populations crucial for osteogenesis. Importantly, interconnected with the different bone structures, there are plenty of blood vessels. Bone tissue is composed by a unique extracellular matrix constituted by an organic and inorganic phase, that is maintained throughout life by the concerted action of bone-forming osteoblasts and bone-resorbing osteoclasts.

MSC and other osteoprogenitor cells within the bone tissue give rise to osteoblasts upon differentiation into the osteogenic lineage. These osteoblasts will then become quiescent osteocytes when entrapped in the middle of mineralized ECM (Figure 1). On the other hand, osteoclasts are originated by the differentiation of myeloid progenitor cells, derived from hematopoietic stem cells in the bone marrow. Importantly, osteoblasts are cells responsible for bone formation, and osteoclasts are cells responsible for bone resorption, which make these two cell types key players in the maintenance of bone size, structure and mineral content in homeostasis [19]. Indeed, these two complementary metabolic processes of bone synthesis and resorption drive the bone remodelling that occurs continuously

throughout life. At the molecular level, the balanced action of osteogenesis and osteoclastogenesis is maintained by tight communication pathways established between the different intervenient cell populations, including through cell-secreted Extracellular Vesicles (EV) [20], and may be controlled by many different mediators, including hormones (such as vitamin D_3, parathyroid hormone and estrogen) [21–23], cytokines, growth factors [24] and non-coding RNAs, namely microRNAs (miRNAs) [25].

When a bone injury occurs, this balanced metabolism of synthesis and resorption is disrupted, and normal bone structure is affected. But, if injury is not too extensive, healthy bones can be repaired or even completely re-established to their original form by normal physiological mechanisms of bone repair and regeneration.

1.2.1. Physiological bone repair: the importance of the immune system

Bone tissue repair and regeneration is reported to occur along three main phases: inflammation, repair and remodelling (Figure 2). These phases partially overlap, and are dependent on each other, relying on several mechanisms of cell-to-cell communication [26]. The average duration of each phase is known in more detail for rodents, but in humans a somewhat different time-frame is reported [27].

When a bone injury occurs, there is a disruption of bone tissue, the surrounding soft tissue and blood vessels, leading to cell death and the release of danger signals that trigger an inflammatory response. The first line of events in response to injury aims to stop bleeding, activating platelets and the blood coagulation cascade [28]. Consequently, the fracture gap is filled in with a provisional fibrin matrix, which results from the enzymatic conversion of circulating fibrinogen, constituting the hematoma. This matrix is highly infiltrated by local and blood-derived immune cells (mainly innate immune cells), and characterized by low oxygen levels and low pH [27]. At the time of injury, other cell populations, such as tissue resident immune cells, endothelial cells, bone-lining and stromal cells are also activated [27]. These activated cells intensively secrete mediators, including cytokines and chemokines, that induce the recruitment of other circulating immune cells, namely polymorphonuclear neutrophils, monocytes and lymphocytes [27]. The recruited immune cells further contribute to the cocktail of cytokines and chemokines secreted at the injury site, which promote the recruitment of fibroblasts and osteoprogenitor cells, including MSC [28]. At the same time, revascularization and angiogenesis is promoted, in order to re-establish the blood supply to the bone [27]. These events occur in the first 7 days after injury in rats, and constitute the acute phase of the inflammatory response to bone injury [27]. This acute inflammatory response is crucial, not only for the removal of potential pathogens and clearing of the wound

area, removing necrotic cells, but also to condition bone stem and progenitor cells, activate mature bone-remodelling cells (osteoblasts and osteoclasts), initiate vascularization, and promote turnover of the bone ECM.

During the acute inflammatory response, there is secretion of a wide variety of cytokines, chemokines and growth factors, that ultimately mediate these processes, being the most relevant interleukin (IL)-6, IL-1β, tumour necrosis factor (TNF)-α, macrophage colony-stimulating factor (M-CSF), receptor activator of nuclear factor κB ligand (RANKL), transforming growth factor (TGF)-β and bone morphogenetic proteins (BMP) [27]. The levels of all these immune mediators will then change dynamically in time, along the whole bone repair process, although the actual range of concentration of many of these cytokines *in situ* remains to be elucidated. This temporal variation of cytokines and growth factors is coordinated with different cellular processes taking place along time. For instance, levels of IL-6, TNF-α and IL-1β peak very early in the acute inflammatory phase, then subside, and are again up-regulated at later stages of bone repair (Figure 2) [24].

The acute inflammatory response established upon bone injury is self-limited and enters into the resolution phase around day 14 after injury [29]. At this stage, mediators responsible for inflammation resolution, such as specialized pro-resolving mediators (SPM), are reported to be secreted by neutrophils and macrophages, favouring the end of the inflammatory response. SPM are a family of lipid mediators that comprise resolvins, protectins, maresins and lipoxins [30]. These mediators have been described as beneficial for tissue repair and regeneration in different models of injury, including bone [31]. Work from our team also demonstrated the role of biomaterials incorporating resolvin D1 in promoting bone repair *in vivo* [32].

By the time of resolution of the inflammatory phase, there is as extraordinary increase in the vascularization of the injured site, an activation of local fibroblasts, the deposition of new ECM, and the replacement of lost bone tissue cells due to the activation of cell proliferation and differentiation of osteoprogenitor cells, including MSC [27]. These events constitute part of the repair phase that ultimately will restore bone structure.

In this step of bone repair, many of the processes that occur during bone ontogeny are recapitulated, rendering healthy bone healing a regenerative event [29]. In this way, the formation of new bone may occur by two main mechanisms: intramembranous ossification or endochondral ossification. Intramembranous ossification is characterized by the direct differentiation of MSC into the osteoblastic lineage, whereby functional osteoblasts deposit bone matrix and promote its mineralization into immature woven bone [14]. This is a “non-templated” ossification process, starting as early as 3 to 7 days after injury in rats, without the formation of a transient cartilage scaffold. On the other hand, endochondral ossification is initiated at day 7 to 10 after injury in rats, by the synthesis of a cartilage template by MSC,

the bone callus, that is then mineralized into woven bone and later on replaced by mature bone tissue [14, 29]. The occurrence of each one of these processes is mainly determined by the mechanical conditions of the injured site [33], leading to complete ossification by day 28 to 35 after injury in rats [27]. In fracture patients this stage is reached later on, by week 8 to 16 after injury [27].

Finally, the woven bone tissue formed during the repair stage is remodelled into functional mature bone that resembles the pre-injury bone tissue, by the concerted action of osteoblasts and osteoclasts [29]. In rats, this process takes 5 to 8 weeks, however it can be extended up to several years in humans [27].

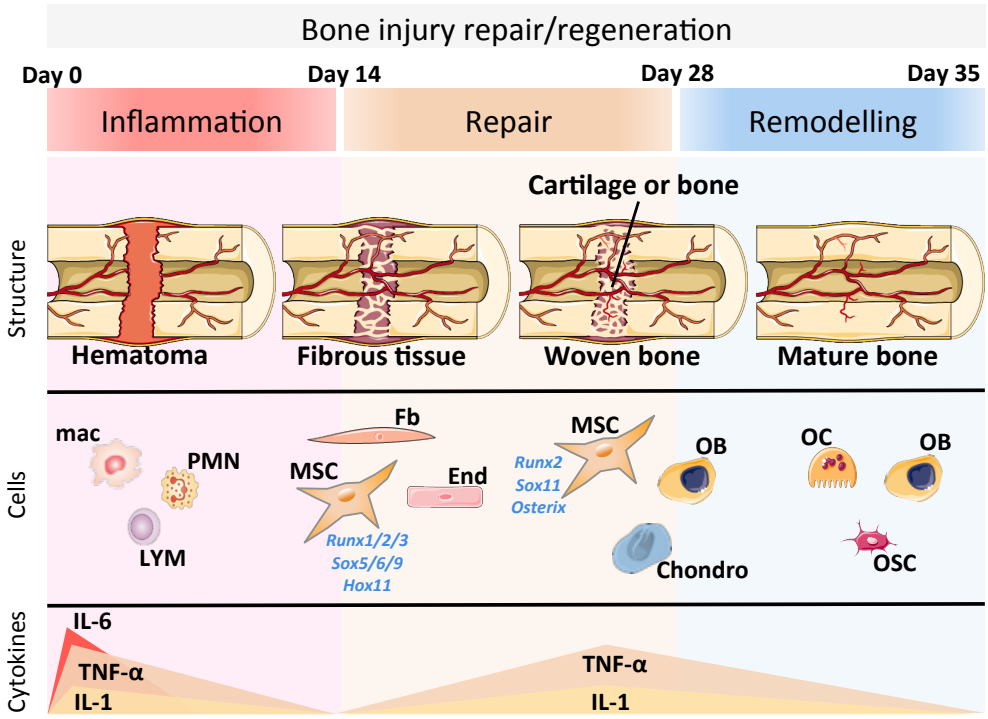


Figure 2. Progression of bone repair and regeneration after acute injury. Bone injuries trigger an inflammatory response at the injured site that promotes the recruitment of circulating immune cells, endothelial cells, fibroblast and osteoprogenitor cells, including MSC. In rats, this phase occurs in the first 2 weeks after injury and is characterized by an intense secretion of immune mediators. In the subsequent two weeks, osteogenesis is initiated and immature woven bone tissue is deposited at the injured site either by intramembranous ossification or endochondral ossification. The main transcription factors controlling MSC differentiation during bone repair are indicated in blue. The immature bone formed is then remodelled into functional mature bone by the concerted action of anabolic osteoblasts and catabolic osteoclasts. Mac, macrophages; PMN, polymorphonuclear cells; LYM, lymphocytes; Fb, fibroblasts; End, endothelial cells; MSC, mesenchymal stromal/stem cells; Chondro, chondroblasts; OB, osteoblasts; OC, osteoclasts; OSC, osteocytes. Based on [28, 29, 34].

For its relevance in the scope of this thesis, the roles of inflammation and the immune system, particularly of main immune cell populations, in the regulation of bone tissue development, homeostasis and response to acute traumatic injury is further discussed below.

1.2.1.1. Immune cells in bone repair

Although inflammation was historically regarded as an impairment for effective bone tissue repair and regeneration, the last two decades were pivotal in changing this perspective. In fact, early observations of alterations in bone tissue of patients suffering from inflammatory disorders [35], and pioneer work implicating immune cells in the regulation of bone metabolism [36, 37], suggested that immune cells and mediators are important players in bone tissue biology. Consequently, in 2000 a new field in bone research was proposed, named osteoimmunology [38], dedicated to explore the communication and interface between immune cells and bone-remodelling cells. Since then, many studies have been further elucidating the role of the immune system in bone formation and integrity. In addition, the importance of the initial inflammatory response that is triggered by bone trauma, for its proper repair and regeneration, has also been increasingly recognized (reviewed in [39]).

One of the first immune cell populations arriving at the bone injury site are polymorphonuclear neutrophils, attracted by cytokines and chemokines released by the damaged tissue cells [27, 40]. Early investigations suggested a role for neutrophils in bone repair, with neutrophil depletion improving mechanical properties of healed bone in a rat osteotomy model, but without changing callus volume during the repair process [41]. However, more recently and contrarily to earlier results, specific neutrophil depletion in a tibial osteotomy mouse model by administration of an anti-Ly6G antibody prior to lesion induction, reduced the percentage of new bone tissue formed by day 21 after injury, also decreasing bone bending stiffness [42]. On the other hand, neutrophils might contribute to bone tissue repair by the early deposition of a fibronectin-rich provisional ECM, necessary for the progression from the inflammatory phase to the repair phase [43].

With the progression of the inflammatory response to bone injury, macrophages assume a more important role in the communication with bone tissue cells. In fact, macrophages are the immune cell type for which the interactions with bone are most well described. Bone tissue may be affected by two major distinct populations of macrophages: the bone-resident macrophages, that migrate to bone in the pre-natal period [44], designated osteomacs, and the macrophages derived from circulating monocytes that are recruited from blood to the bone by inflammatory mediators [45]. As detailed below, many studies on the macrophage-bone relationship are focused on osteomacs, while others report observations for systemic monocyte-derived macrophages. However, the differences in the relative contribution of each population to bone tissue development, maintenance and repair, if any, are not yet clearly elucidated [45].

Nowadays, it is recognized that macrophages affect both bone tissue development, as well as its repair after an injury. In the work of Vi *et al.* it was shown that the systemic

depletion of myeloid cells in mice (deficient in cells expressing the myeloid marker gene lysozyme-M) did not affect skeletal development during embryonic stages. However, along time after birth, macrophage-depleted animals had increasingly shorter bones than controls, and at adulthood, not only bone size was affected by the absence of macrophages, but also their mineral density was decreased, presenting an osteoporosis-like appearance [46]. Moreover, the induction of a bone fracture in the tibia of these macrophage-deficient animals resulted in the development of smaller fracture calluses, with less chondrocytic regions, less bone deposition, but increased fibrous tissue than control animals. Interestingly, the *in vitro* osteogenic differentiation of bone marrow cells from macrophage-deficient mice was markedly reduced compared to bone marrow cells from control animals, suggesting a role for macrophages in the promotion of osteogenic differentiation of osteoprogenitor cells. Of note, similar results for progression of bone endochondral ossification were obtained by Schlundt *et al.,* using a model of macrophage depletion by administration of clodronate liposomes [47]. Curiously, Schlundt *et al.* also reported an enhanced intramembranous ossification in the absence of macrophages, in the periphery of the injury [47].

In another study using a rat model of bone lesion by osteotomy, a splenectomy was performed simultaneously at the time of the injury. In these animals, fracture healing by endochondral ossification was delayed comparing to controls, due to an inhibition of fibroblasts differentiation and of chondrogenesis. This delay was accompanied by a marked reduction in macrophage infiltration at the injury site in rats that underwent splenectomy, at all timepoints after injury analysed. Furthermore, there was a reduced expression of alkaline phosphatase (ALP), osteocalcin, collagen type 1 A1 and collagen type 2 A1 at the injury site, compared to non-splenectomized rats, as well as a reduced expression of osteoprotegerin (OPG) and RANKL (that can be produced by osteoblasts), suggesting an impairment of osteoblasts activity associated with the absence of macrophages [48].

In earlier studies, the contribution of osteomacs for bone tissue development and repair had also been suggested. Osteomacs were first identified by Chang *et al.*, as cells arranged in canopy-like structures over osteoblast-like cells in bone surfaces at 4 weeks after birth, and which were present until 6 months-old [49]. Moreover, in accordance with the observations of Vi *et al.,* the systemic depletion of macrophages in the drug-inducible macrophage Fas-Induced apoptosis (MAFIA) mouse model, led to the disappearance of this osteomac canopy structure from endosteal bone surface, and, more strikingly, of the interconnected osteoblast layer, compared to control animals. This suggests that osteomacs are essential to maintain the osteoblast layer at the bone surface. In addition, macrophage depletion from mice calvaria cell isolates significantly reduced osteoblast maturation and mineralization *in vitro* [49]. In a later study, Alexander *et al.* observed a marked decrease in deposition of new woven bone in MAFIA mice subjected to a tibial injury that naturally heals

by intramembranous ossification, comparing to vehicle-treated mice, even if macrophage depletion was delayed until 3 days after lesion [50]. These observations were further validated using a model of macrophage depletion by administration of clodronate liposomes. On the other hand, wild-type mice subjected to the same injury and treated with M-CSF, a cytokine involved in the proliferation and differentiation of myeloprogenitors, had increased deposition and mineralization of new bone, an effect accompanied by an increase in the number of macrophages at the injured site [50]. Interestingly, osteomacs could be found closely associated with mature osteoblasts on osteoid surface up to day 9 after injury, forming their typical canopy structure [50]. Of note, abundant osteomacs could also be found associated with areas of bone formation in bone samples from osteoarthritis patients [49]. Similarly, depletion of synovial-lining macrophages in mice reduced TGF-β-mediated osteophyte formation [51].

Interestingly, in the work of Alexander *et al.*, it was also possible to distinguish two populations of macrophages infiltrating the injury site: Mac-$2^{-/low}$ osteomacs and Mac-2^{high} inflammatory macrophages. Importantly, at days 5 and 7 after fracture, Mac-2^{high} macrophages were mainly present in the periphery of the injury, distant from osteoblasts on bone surface and regions of intense new bone deposition, where mainly Mac-$2^{-/low}$ osteomacs were found, suggesting the pro-inflammatory phenotype of macrophages may be deleterious for osteoblasts activity [50]. Accordingly, Schlundt *et al.* found a reduction in the pro-inflammatory $CD68^+CD80^+$ M1 macrophages in the injured site of an osteotomy mouse model, from day 1 to day 3 post-injury. By day 7, the osteotomy gap was invaded by pro-regenerative $CD68^+CD206^+$ M2 macrophages, and no M1 macrophages could be found. Curiously, the induction of the M2 macrophage phenotype *in vivo,* by the implantation of collagen scaffolds carrying IL-4 and IL-13 at the osteotomy gap, improved bone healing, increasing callus and bone volume [47].

A role for dendritic cells (DC) in bone repair and regeneration has also been suggested in a few studies. Wang *et al.* demonstrated that TLR4 knockout in $CD11c^+$ DC led to increased bone volume in a mouse model of calvarial defect, at day 28 after injury, an effect that was not observed for TLR4 knockout in $lysozime^+$ myeloid cells [52], suggesting a specific role for DC in bone repair. On the contrary, immature DC have been suggested as novel precursors of osteoclasts *in vitro* and *in vivo* [53–55]. For instance, the transplantation of DC into osteopetrotic mice (oc/oc mouse model) led to a rescue of their osteopetrosis phenotype, with $TRAP^+$ multinucleated cells, derived from transplanted DC, being detected at bone surfaces [55]. Nonetheless, it should be noted that the real contribution of DC for osteoclastogenesis is still a matter of debate, due to possible cross-contamination of *in vitro* cell cultures and cell suspensions transplanted into animal models with osteoclasts and/or

monocytic precursors [56]. Therefore, further studies are required to clarify the role of both immature and mature DC on bone development and repair.

Moreover, DC are the most potent Antigen Presenting Cells (APC), able to activate naïve T cells and consequently the adaptive immune response. Interestingly, activated T cells were demonstrated to induce the differentiation of DC into osteoclasts, via the RANKL that they secrete [57], implicating the adaptive immune system in the indirect regulation of bone metabolism. Other studies have been dissecting the contribution of T cells and B cells, the main effectors of adaptive immunity, to bone repair and regeneration, with T cells being by far more extensively explored. Using $Rag1^{-/-}$ mice lacking mature functional lymphocytes, in 2011, Toben and colleagues showed that knockout mice had accelerated femur fracture healing, compared with wild-type animals subjected to injury [58]. On the other hand, in the following year, Nam *et al.* reported an opposite role for the adaptive immune system in bone healing, using also a $Rag1^{-/-}$ mice model [59]. Nonetheless, these contradictory observations may have been impacted by the induction of different bone lesions in both studies. Besides, other reports have been correlating other subsets of lymphocytes with impaired bone repair. For instance, Reinke *et al.* showed that bone healing could be affected by changes in the levels of $CD8^+$ T cells alone. In fact, the volume and mineral density of mice femurs undergoing osteotomy were the highest for animals depleted of $CD8^+$ T cells by antibody therapy, decreasing with increasing percentages of these lymphocytes in mice bone marrow [60]. This study also found that delayed healing in tibia fracture patients was positively correlated with higher levels of peripheral blood effector memory $CD8^+$ T cells ($CD3^+CD8^+CD11^{++}CD57^+CD28^-$) [60]. An earlier work also implicated the γδ T cell subset in bone fracture repair [61]. In this study, $TCR\gamma^{-/-}$ mice lacking functional γδ T cells showed improved tibia fracture union at early timepoints after the lesion, with bone callus expressing bone- and cartilage-related proteins at higher levels than wild-type mice. Nonetheless, no differences could be found for the bone biomechanical properties of both mice, at week 28 week post-injury [61], implicating that the regulatory action of γδ T cells upon bone healing might be restricted to the early stages of this process. All together, these observations might suggest that the activity of specific cell types of the adaptive immune system seems to impair bone repair and regeneration.

Importantly, the dynamics of lymphocyte activity at a bone injury site was addressed by the work of Könnecke and colleagues [62]. Using a physiological mouse model of femur fracture healing by endochondral ossification, they showed that lymphocyte infiltration into the fracture region is timely controlled during repair. As early as 3 days post-lesion, T and B cells were present throughout the whole injured area. Upon inflammation resolution (day 7 post-lesion), lymphocyte infiltration was markedly reduced, being absent from the cartilagenous callus already formed, and prevailing close to the endosteal tissue near the

fracture. Curiously, upon mineralization of the fracture callus, lymphocytes repopulated the fracture gap, locating nearby the newly formed woven bone but still remaining absent in the cartilage regions of the callus. Of note, the percentage of T cells was also evaluated in the contralateral femur of injured animals, being increased along time after injury, from day 1 to day 21. This supports the occurrence of a systemic inflammatory response to injury, with non-injured contralateral femurs resembling injured femurs regarding adaptive immune response to the lesion. Moreover, during this period, the ratio of $CD8^+/CD4^+$ cells decreased, with $CD4^+$ T cells prevailing over $CD8^+$ cells in the bone marrow, further supporting a deleterious role of these cells for bone healing [62]. Interestingly, the induction of a femur fracture in a similar mouse model was reported to be accompanied by a significant increase in the percentage of ($CD3^+CD4^+$) $CD25^+FoxP3^+$ regulatory T cells (T_{reg}) in the injured femur tissue at day 10 post-fracture, compared to bone tissue of non-injured animals [63]. Importantly, this increase was accompanied by a reduction in the percentage of $CD69^+$ activated ($CD3^+CD4^+$) T cells, although not statistically significant. Of note, the changes found for the T_{reg} levels (but not activated T cells) along time after fracture, were also observed in the contralateral non-fractured femur of injured mice, but not in their spleen [63].

In line with the observations from Al-Sebaei *et al.* [63], Kong and colleagues showed that T cell activation (with anti-CD28) *in vivo* lead to bone loss due to increased osteoclastogenesis, mediated by T cells-secreted RANKL [37]. Together, both studies suggest that the pro-inflammatory activation status of T cells might be a key factor determining their commonly deleterious role in bone repair.

Regarding the contribution of B cells to bone formation, a pioneer study by Marusici *et al.* suggested these cells could be involved both in endochondral and intramembranous ossification [64]. In this study, μMT mice, carrying an immunoglobulin heavy chain mutation that renders them B cell-deficient, were used, with endochondral ossification mimicked by the subcutaneous implantation of a BMP-2-loaded clot, and intramembranous ossification mimicked by bone marrow ablation in the tibia. B cell depletion was associated with improved endochondral ossification, since at day 12 post-implantation, BMP-2-induced ossicles were larger in B cell-deficient mice than in control animals. On its turn, intramembranous ossification was accelerated in μMT mice at early timepoints of bone marrow reconstitution, however, at day 10 after the marrow depletion, tibia of knockout and control animals were similar, with μMT mice still registering a higher number of trabeculae [64]. Later studies further addressed the role of B cells in bone repair using more physiological *in vivo* models of bone injury. Despite the description of B cell participation in endochondral ossification reported by Könnecke *et al.*, Raggatt and colleagues could not find an association between these lymphocytes neither with physiological bone modelling nor with intramembranous ossification [65]. Indeed, B cells were scattered in the bone marrow of actively growing mice,

rather than preferentially associated with bone-lining active osteoblasts. In addition, in mice subjected to an osteotomy, only few B cells could be detected at the injury site at 4 days post-surgery. More strikingly, the repair of the bone lesion in μMT mice progressed as in wild-type animals [65].

In summary, the roles of immune cell and the mediators they secrete in bone repair and regeneration are increasingly recognized. So far, macrophages are the immune cell population most extensively characterized for their role in bone metabolism in homeostasis, upon injury and during treatment, namely involving biomaterial implantation. In fact, macrophages have been demonstrated to affect bone development, (although they do not seem necessary for bone formation during embryonic life [46]) and bone fracture healing, either by endochondral or intramembranous ossification. Importantly, these effects are dependent on their inflammatory status, with highly pro-inflammatory macrophages being deleterious for new bone formation. Furthermore, they seem to promote osteoblast differentiation, maturation, and matrix mineralization, affecting bone anabolism by acting at multiple stages of the process. On the other hand, controversial roles in bone repair have been described for neutrophils, dendritic cells, T cells and B cells, with many studies overlooking the activation/polarization profile of these cells. Further studies in this field are thus still needed to clarify the details of the crosstalk between the different populations of immune cells with bone cells.

1.2.1.2. Cytokines and growth factors in bone repair

Bone injury leads to the production and secretion of cytokines and growth factors that shape the reparative/regenerative response, by recruiting and activating the intervening cell populations (Table 1).

The inflammatory cytokines most widely described as involved in the bone repair process are IL-6, TNF-α and IL-1-β, which are highly expressed right after injury both by local tissue cells, in response to damage, and by recruited activated immune cells in the hematoma [24]. At early stages after injury, immune cells at the lesion site are major contributors for the cytokine and growth factor expression pattern observed, and thus the action of immune cells described above is at least partially mediated by these mediators. At later stages, particularly during bone remodelling, osteoblasts and osteoclasts become important players in cytokine secretion [24].

In fact, the delayed bone injury healing reported by Xiao *et al.* in splenectomised rats was also accompanied by reduced levels of systemic and local TNF-α, IL-1β and IL-6, compared to animals with spleen. IL-6 has been reported to have a role in the first weeks

post-bone injury, as demonstrated in a mouse model of bone osteotomy [66], and by its presence at higher levels in plasma of hip fracture patients 1 year after fracture, correlated with impaired functional recovery [67]. Moreover, work using a TNF-α receptor-deficient mouse model, showed that abrogation of TNF-α signalling delayed endochondral ossification 2 to 3 weeks, compared to normal animals [68]. Besides its expression in the inflammatory phase of bone healing, TNF-α levels also increase at later stages, peaking around week 4 after injury. This implies that TNF-α impacts the activity of the cells with major roles at this stage, such as MSC, osteoblasts and osteoclasts. Indeed, several *in vitro* and *in vivo* works have been describing a dual role for TNF-α, which seem to promote both osteogenesis and osteoclastogenesis, depending on its concentration [69] and the TNF receptor it binds to (reviewed in [70]). Similarly to what was reported for TNF-α, knockout of the receptor IL1R1, and of the IL-1 signalling pathway molecule MyD88, allowed bone repair in the transgenic mice subjected to a otherwise critical size defect in the calvaria, demonstrating that IL-1 is a negative regulator of bone healing [71]. A previous study further implicated IL-1 in bone formation, with $IL1R^{-/-}$ mice presenting shorter bones with lower mass, compared to wild-type mice [72].

Table 1. Effect of the cytokines and growth factors most involved in bone repair upon bone-forming and bone-resorbing cells.

Cytokine or growth factor	Experimental model	Action on bone cells
IL-6	*In vitro* models IL-6-/- mouse model	Promotes MSC osteogenic differentiation [73] Promotes mineralization [66] Impairs osteoclast activity [66]
TNF-α	*In vitro* models TNF-α transgenic mice TNF-α receptor -/- mouse model Rodent models RA bone explants	Dual role: promotes/inhibits MSC and pre-osteoblasts osteogenic differentiation [69, 74] Promotes osteocyte apoptosis [69] Dual role: promotes/inhibits bone resorption [69] Promotes osteoclastogenesis, in the presence of RANKL [75]
IL-1β	*In vitro* models IL-1-/- mouse model IL-1 receptor -/- mouse model	Dual role: promotes/inhibits MSC proliferation and osteogenic differentiation [76, 77] Promotes pre-osteoblasts proliferation and osteogenic differentiation [77] Promotes osteoclastogenesis [78]
IFN-γ	*In vitro* models IFN-γ-/- mouse model IFN-γ receptor 1-/- mouse model	Dual role: Promotes/inhibits MSC osteogenic differentiation [79, 80] Promotes mineralization [79] Dual role: inhibits osteoclastoegenesis *per se*, but promotes T cell-mediated osteoclastogenesis [81]
IL-17	*In vitro* models IL-17-/- mouse model	Promotes MSC and pre-osteoblasts osteogenic differentiation [59, 74] Promotes osteoclastogenesis [82]

Table 1 (continuation)

Cytokine or growth factor	Experimental model	Action on bone cells
IL-8	*In vitro* models Ectopic bone formation mouse model	Promotes MSC recruitment [83] Promotes MSC proliferation and osteogenic differentiation [84] Promotes osteoclastogenesis [85]
TGF-β	*In vitro* models Tgfb1-/-Rag2-/- mouse models	Promotes MSC and pre-osteoblasts recruitment [86, 87] Inhibits MSC osteogenic differentiation [74, 87] Inhibits mineralization [87, 88] Dual role: promotes/inhibits osteoclasts differentiation and recruitment [89]
IL-10	*In vitro* models IL-10-/- mouse model	Promotes bone formation and mineralization [90] Inhibits bone resorption [91]
VEGF	*In vitro* models Vegfr1 and Vegfr2 deletion mouse model $VEGF^{120/120}$ mutant mice Osx-Cre $VEGFA^{fl/fl}$ mouse model Murine fracture and bone defect models Rabbit bone defect model	Promotes MSC recruitment [92] Promotes MSC and pre-osteoblasts differentiation [93, 94] Dual role: promotes/inhibits mineralization [95, 96] Promotes osteoclastogenesis [97] Promotes osteoclast survival, migration and activity [95, 97, 98]
BMPs		
BMP-2	*In vitro* models *In vivo* models using BMP-2-overexpressing MSC *In vivo* injury animal models using recombinant BMP-2 Clinical trials	Promotes MSC osteogenic differentiation [87, 99] Promotes fracture union [100, 101]
BMP-7	*In vitro* models $Prx1\text{-}BMP\text{-}7^{c/c}$ mouse model Clinical trials	Promotes MSC osteogenic differentiation [102] Promotes cartilage integrity [103] Promotes fracture union [104]
BMP-3	*In vitro* models Bmp3-/- mouse model BMP3-overexpressing transgenic mice	Counteracts BMP-2 action [105] Decreases mineralization [105, 106]
RANKL	*In vitro* models RANKL-/- mouse model	Promotes osteoclastogenesis [107, 108]
M-CSF	*In vitro* models op/op mouse model	Promotes osteoclastogenesis [109, 110] Supports mature osteoclasts [110]
OPG	*In vitro* models OPG-/- mouse model	Inhibits osteoclastogenesis [111, 112]

Several growth factors are also essential for bone homeostasis and repair after injury (Table 1). Some of the most important belong to the TGF-β superfamily, namely the members of the BMP family. The role of BMP-2 has been particularly explored in bone repair, being absolutely necessary at the early steps of the process for bone healing to

proceed [113]. Several members of the BMP family, including BMP-2 and BMP-7, have been used in clinical trials (www.clinicaltrials.gov), and those have even been FDA approved for clinical use in specific situations and with determinate carriers, as discussed in more detail bellow.

The importance of cytokines/chemokines in bone repair was further supported in a knockout mice model for the CC chemokine receptor type 2 (CCR2), in which the endochondral repair of a tibia fracture was delayed at day 21 after injury compared to control mice. This was accompanied by a reduction in the number of macrophages at the fracture site and impaired vascularization. Curiously, the resorbing capacity of osteoclasts in this model was also impaired [114]. Of note, different cytokines/chemokines known to be secreted at places of fracture injury, in response to the lesion, have been described in the promotion of MSC recruitment, with some impacting also their proliferation and differentiation (Table 1). This will be further addressed in section 1.2.2.2.

Interestingly, besides describing the contribution of different (cellular and non-cellular) immune mediators in the regulation of bone tissue, many of the studies discussed above also support a common key idea, crucial for successful bone tissue repair and regeneration: acute inflammation starts the reparative process, but the inflammatory and immune responses have to be balanced and tightly regulated in time after injury, in order to have a major beneficial role in bone repair and regeneration, which is achieved by the crosstalk between different cell populations, including via EV. In line with this, our previous work showed that the capacity of DC to promote MSC migration *in vitro* was strongly dependent on the inflammatory status of these cells, with immature DC promoting MSC migration to a higher extent than TNF-α-stimulated DC [115]. Moreover, this MSC migration impairment upon DC stimulation with TNF-α could be rescued by treatment of DC with the anti-inflammatory compound resveratrol [115]. This idea of temporal regulation of inflammation upon bone injury is further supported by the fact that treatments administered during bone injury based on non-steroidal anti-inflammatory drugs, inhibitors of the enzyme cyclooxygenase (COX)-2 involved in the production of pro-inflammatory mediators, markedly impaired bone healing (reviewed in [116]).

1.2.2. Therapeutic approaches for bone repair

Many different treatments are available or under investigation to promote bone repair after acute trauma or disease. In the clinical setting, the first approach for the treatment of mild fractures is the stabilization of the fractured site using casts and splints. For more

severe fractures, stabilization may be achieved using internal splints, plates and screws, surgically implanted [27]. After that, the injured bone tissue, particularly in healthy patients, will repair and regenerate along time, following the physiological mechanisms of bone repair mentioned above, in section 1.2.1 [27, 14]. In parallel with mechanical stabilization of the fracture, and mostly in cases where normal bone repair is impaired or does not occur, complementary treatment strategies are necessary.

The strategy most applied to treat delayed or non-union fractures is the transplantation of autologous bone grafts [117]. Indeed, this is the gold-standard treatment for the most severe cases of bone injury, with autologous grafts having all the properties required for new bone formation, namely osteogenic, osteoconductive and osteoinductive capacity [117]. However, clinical application of this strategy is limited by the availability and size of bone grafts, and the morbidity associated with donor site. Alternatively, allogeneic grafts from deceased patients and bone banks have been used, but their availability is also limited, and there is increased risk of graft rejection and disease transmission [117].

To overcome the constraints of bone transplantation, alternative therapies have been intensively investigated to promote bone repair. These include biophysical treatments, the administration of synthetic drugs or recombinant proteins with known roles in bone metabolism, implantation of biomaterials, cell therapies and gene therapies, as detailed below.

Biophysical approaches were tested in clinical studies for the treatment of non-healing fractures, namely stimulation by electromagnetic fields, low-intensity pulsed ultrasonography and extracorporeal shock wave therapy (discussed in [118]). The different studies published so far suggest that all these methods may promote bone healing of non-union or delayed fractures, however it is also recognized by the scientific community that the studies are in general limited and of low quality [118], and further investigations are still needed to confirm the real benefits of such strategies.

Interestingly, a few proteins with known functions in bone homeostasis have been explored for their therapeutic potential to enhance bone healing. These proteins include BMPs [100, 119, 120], fibroblast growth factor (FGF)-2 [121] and platelet-derived growth factor (PDGF) [122]. Food and Drug Administration (FDA) approved BMP-2 for clinical application in 2004 [123], but clinical evidence for improved bone repair with the use of BMP-2, versus conventional treatment, is not very convincing. In one of the first clinical trials applying recombinant BMP-2 for human treatment, patients with open tibial shaft fractures were treated with intramedullary nail fixation and standard care, combined with the implantation of a collagen sponge loaded with BMP-2 at different doses. From the patients reaching the end of the study (421), 44% had reduced risk of fracture management failure, were subjected to fewer invasive secondary interventions and presented faster fracture

healing at 1 year after the treatment, compared with patients receiving standard care only. In addition, 58% of the patients receiving BMP-2 had fracture union, compared to only 38% in the group undergoing standard care, with higher doses of BMP-2 leading to significantly higher percentage of patients with healed fractures [100]. However, later clinical trials did not find any improvements in bone healing upon BMP-2 administration, when compared to the standard treatment [119, 120]. Thus, it remains unclear at the moment the real benefits of BMP-2 treatment for bone fractures. BMP-7 was also used in a clinical trial for the treatment of tibial non-union fractures, leading to bone healing in a percentage of patients similar to that of patients treated with bone autograft [104], nevertheless, this recombinant protein is not approved yet for clinical use by FDA.

Considering the hormonal regulation of bone metabolism [22, 23], hormone-based treatments were also tested for bone repair. A clinical study was conducted where the effectiveness of a systemic parathyroid hormone treatment for osteoporotic fractures was tested in women, with hormone administration decreasing the time of bone healing for different types of fracture, compared to the placebo-treated group [124, 125].

1.2.2.1. Biomaterial-based therapies for bone repair: the role of immunomodulatory biomaterials

Another approach to promote bone healing relies on the development of biomaterials for bone repair, with many different materials being prepared in very different formulations, and tested *in vivo* and *in vitro* mainly for their osteoconductive and osteoinductive properties. The ideal biomaterial for bone repair should be: osteoconductive, functioning as a scaffold for growth of new bone tissue; osteoinductive, promoting the differentiation of osteoprogenitor cells into mature osteoblasts; osteogenic, carrying the osteoprogenitor cells that will form the new bone tissue; and easily vascularized, while conferring mechanical stability to the injury site during bone repair [126]. The most common formulations currently under research include organic cements, bioceramics, biocomposites and polymeric hydrogels.

Polymethyl methacrylate organic cements have been extensively explored in the orthopaedic field [127], and are still one of the most currently used materials in the clinics, both as cements to secure prosthesis of other materials, and as bone defect fillers themselves [128]. Also, bioceramics have been particularly explored in the clinical setting as bone substitute materials, including hydroxyapatite (HA), tricalcium phosphate (TCP) and glass ceramics, that resemble the inorganic phase of bone matrix [129]. On the other hand, several types of hydrogels have been developed for bone applications, although their clinical application has been less explored than bioceramics. Hydrogels developed for bone repair

may be synthesized using both natural and synthetic polymers. Some of the most commonly used polymers encompass gelatin, collagen, hyaluronic acid, alginate and chitosan [130]. Importantly, most of the materials explored so far for bone repair have been modified by loading of proteins [131] and cells with known capacity to enhance bone repair, rendering them osteoinductive and even osteogenic biomaterials.

In more recent years, some authors have proposed that biomaterials for bone repair should also be immunomodulatory, being able to modulate the inflammatory response that occurs after bone injury by interacting with and conditioning the activity of immune cells, in order to improve healing. Many biomaterials have an intrinsic immunogenicity that has been suggested to underlie or contribute to their effects on bone regeneration. In an interesting work from Graney and colleagues, the inflammatory response of monocyte-derived macrophages induced by ceramic scaffolds for bone repair was evaluated *in vitro*. TCP-HA scaffolds, commonly used in the clinics, promoted a down-regulation of most M2a and M2c macrophage gene markers relative to the other ceramic scaffolds tested (baghdadite and strontium-hardystonite-gahnite), and known to contribute to the repair of large bone defects under load more successfully than TCP-HA [132]. Importantly, work from our team has demonstrated that the immunomodulatory properties of a biomaterial may be modulated. Work in our group started by investigating *in vitro* the macrophage response to chitosan [133], to then modify it using fibrinogen, a pro-inflammatory and pro-healing protein that is part of the provisional ECM formed upon injury. We showed the impact of using fibrinogen to modify chitosan *in vitro* on immune cell activation [134, 135] and crosstalk with MSC [136], and also on osteoclast activity [137]. These materials were then tested *in vivo*, in a critical size bone defect model in the femur, and showed that the incorporation of fibrinogen in chitosan scaffolds greatly improved their capacity to promote bone repair, and promoted systemic changes in immune cells and mediators [138]. Importantly, this work was one of the first investigating the systemic response to biomaterials, analysing immune cells in blood and draining lymph nodes [138]. More recently, a scaffold composed only of fibrinogen was also tested by us in the same animal model, followed by analysis of the local and systemic inflammatory and immune responses. In this work, there was an increase in the local expression of IL-6 and IL-8 compared with animals with empty bone defects, at 6 days after surgery (inflammatory phase of bone repair), which correlated with a tendency for increased percentages of T cells, and decreased percentages of B, NK, NKT and myeloid cells systemically, namely in blood, spleen and draining lymph nodes. These changes were accompanied by the formation of new and functional bone tissue at 2 months after scaffold implantation [139].

A very recent study from our team further demonstrated that the incorporation of immunomodulatory compounds in biomaterials is beneficial to promote bone repair [32].

Indeed, the incorporation of resolvin D1, a SPM naturally produced to actively resolve inflammation, in a chitosan scaffold, led to improved bone repair in a rat model of critical size femoral defect at 2 months after scaffold implantation, in comparison with rats implanted with chitosan scaffolds alone [32]. Bone biomaterials may also be functionalized with cytokines and growth factors capable of modulating immune cells phenotype. In the work by Hu *et al.* it was demonstrated that heparin-modified gelatin microspheres functionalized with IL-4 improved bone repair in a rat model of periondontal fenestration defect, with simultaneous modulation of the polarization of macrophages in the lesion into the M2 phenotype [140].

1.2.2.2. Cell therapies for bone repair: the Mesenchymal Stem Cells paradigm

One of the most simplistic cellular therapies tested so far for bone healing is the transplantation of autologous bone marrow [141, 142]. In the early work of Hernigou *et al.*, 60 patients with non-union fractures of the tibia shaft were treated with autologous bone marrow aspirates collected from the iliac crests and then concentrated [141]. Strikingly, in 95% of the treated patients a bone union was achieved, as evidenced by the formation of a callus at 12 weeks (on average) after the treatment. Furthermore, the elapsed time until bone union was achieved was inversely correlated with the number of fibroblast colony-forming units, and thus progenitor cells, in concentrated bone marrow aspirates. This suggests that recruitment of endogenous osteoprogenitor cells to injured bones could positively impact bone healing, since it would allow an optimal concentration of osteoprogenitors at the site to drive bone formation.

Another approach very much explored to improve bone repair of non-healing fractures is the transplantation of more defined populations of stem cells, administered alone in the host, systemically or at the injury site, or entrapped in an appropriate biomaterial that is implanted in the region to be healed. The stem cells populations most studied so far for bone repair, i*n vitro* and *in vivo,* comprise endothelial progenitor cells (EPC) and MSC, as following described.

Reestablishment of vascularization in a bone lesion is crucial for its proper healing, since it not only avoids tissue necrosis, but also allows the recruitment of immune cells and osteoprogenitor cells necessary for the healing process [27]. In one of the first studies using endothelial cells therapies for bone regeneration, EPC isolated from rat bone marrow were impregnated in gel-foam scaffolds implanted in a segmental femur defect in rats [143]. Animals bearing scaffolds with EPC showed more callus formation than animals treated with scaffold alone, having bridged bone ends by week 3 post-implantation, a feature that was not observed in the control group not even at 10 weeks. Furthermore, by week 10, the defects of

EPC-treated animals showed ossifications across the whole gap, with formation of new trabecular and cortical bone [143]. Many reports have been demonstrating the positive effects of EPC on bone healing, but interestingly, most of them follow an approach where both EPC and MSC are transplanted together to the bone injury site. Indeed MSC seem to have a supportive role for EPC-driven vascularization, whereas MSC osteogenic differentiation is favoured by mediators and cells arriving through the vessels network created by EPC [144].

MSC are the most obvious stem cell population for applications in bone repair and regeneration, as they are known to be osteogenic since their discovery by Friedenstein and colleagues [145]. Indeed, MSC are adult multipotent cells, which are able to differentiate into multiple cell lineages, including bone and cartilage [146], two tissue types crucial for proper bone repair and regeneration. These cells can be found and easily isolated from multiple tissues, being the most common bone marrow, adipose tissue, synovial tissues and extraembryonary tissues (placenta, umbilical cord and umbilical cord blood). In the bone, MSC are thought to be localized not only in the bone marrow, specially near blood vessels as pericytes [147], but also to be part of the pool of osteoprogenitor cells in the bone-lining membranes periosteum and endosteum [148].

A great amount of investigations were conducted in the last decades assessing the regenerative potential of MSC for bone repair and regeneration, using rodent, rabbit and sheep animal models of bone fractures or defects [149], but most interestingly, several human clinical trials have also been conducted for this purpose (mainly phase I-II). In fact, ~19% of the MSC-based clinical trials registered in the US National Institutes of Health database in 2015, were targeted for the treatment of bone and cartilage diseases, accounting for the largest percentage of the reported clinical trials [150]. At the moment, 25 clinical trials are registered for the evaluation of MSC in fracture treatment [151]. Results of one of the completed trials are already available and show that transplantation of bone marrow MSC into the gap of non-union fractures of lower limbs long bones, promoted bone union in 3 out of 5 patients, in a timeframe of 6 to 12 months [152]. Another report in the scope of this clinical trial demonstrated that implantation of MSC combined with a platelet lysate in the gap of non-union fractures of the tibia or femur, led to bone union in 4 out of 7 patients. However, a control group treated with the platelet lysate derivative only was not included in this study [153]. Importantly, in both studies no adverse side effects of MSC transplantation were registered, supporting the safety of such therapeutic strategy for bone repair. In addition, other studies demonstrated positive effects of MSC in the treatment of other bone diseases such as osteogenesis imperfecta [154, 155].

A role for MSC intervention in bone repair is further suggested by detection of MSC circulating in the peripheral blood of fracture patients, likely mobilized in response to injury

[156]. Alm and colleagues detected circulating MSC in 22% of the (elder) patients with hip fracture analysed, and in 46% of the (younger) patients with tibia or ankle fracture, by 39 h to 144 h after fracture [156]. Although these are not impressing results regarding MSC endogenous mobilization, they could not find MSC in the blood of patients with hip osteoarthritis. In a later investigation, Hoogduijn and colleagues could also detect MSC in the blood of hip fracture patients [157]. However they could not detect these cells in patients with major trauma in other solid organs, leading the authors to conclude that MSC found in the blood would likely be derived from the disrupted bone marrow, that allowed MSC release into the circulation, rather than their active mobilization in response to injury [157]. On the contrary, Wiegner *et al.* recently reported that pelvis fracture in multiple trauma patients was inversely correlated with the number of MSC found circulating in blood [158]. Whether MSC are truly mobilized in response to bone injury, or if they enter circulation as a result of bone marrow and blood vessels disruption is still a matter of debate.

Regardless of being systemically transplanted or recruited endogenously, MSC have been widely suggested to home to places of injury *in vivo* [159], including bone injury [160], mostly by a transendothelial migration process very similar to chemotactic immune cell migration [161]. In our own work, we demonstrated *in vitro* that dendritic cells, macrophages, NK cells and T cells could promote MSC migration via paracrine factors [115, 136]. Moreover, MSC migration *in vitro* was shown to be increased towards several chemokines and growth factors [162]. Among these, one of the chemokines more commonly implicated in MSC migration, including during bone repair, is stromal cell-derived factor (SDF)-1, which binds CXCR4 receptor on target cells. In a study by Kitaori *et al.*, the repair of a femoral osteotomy defect in mice was shown to be impaired in SDF-$1^{+/-}$ and $CXCR4^{+/-}$ animals compared to wild type. Moreover, the transplantation of a bone graft from $CXCR4^{+/-}$ mice (and thus expressing SDF-1 normally) into SDF-$1^{+/-}$ animals partially recovered bone formation, whereas the opposite did not shown any improvements in bone repair, further demonstrating the crucial role of this chemokine in bone healing [163]. Later on, Liu *et al.* proved the chemotactic effect of SDF-1 upon MSC during bone repair by implanting a scaffold with SDF-1 in a rat model of periodontal defect. Indeed, SDF-1 led to improved bone repair, and host-derived MSC could be found at the injured site [164]. Importantly, some of the cytokines mostly expressed upon bone injury, such as TNF-α and IL-6, were shown to promote stromal/stem cells migration *in vitro,* correlating with improved bone healing when administered *in vivo* in animal models of bone fracture [165]. Glass *et al.* demonstrated that conditioned media from human fracture explant cultures could promote migration of muscle-derived stromal cells *in vitro*, an effect that was abolished in the presence of anti-TNF-α and anti-IL-6 antibodies. Importantly, the action of these cytokines promoting stromal stem cells recruitment was dependent on the dose administered, with levels >1pg/mL of TNF-α, and

>1ng/mL of IL-6 actually inhibiting cell migration [165]. Of note, many of these mediators may be secreted by cells in a soluble form, but may also be found enclosed inside EV, according to database repositories of the content of EV from different origins (e.g, Vesiclepedia).

The exact mechanism through which MSC benefits bone repair is not known, however, they have the potential for a pleiotropic action at several levels. In addition to their osteogenic and chondrogenic potential, MSC have immunomodulatory properties, being capable of conditioning the activity of many of the immune cells populations described as participating in bone healing, exerting an action that is mainly immunomodulatory/immunosuppressive [166]. Thus, the role of MSC in bone repair might also be mediated by their active contribution for the resolution of the inflammatory phase that occurs after bone injury. Very interestingly, it is nowadays recognized that the pro-regenerative action of MSC in bone repair is not completely dependent on their presence at the place of injury, but instead may be mediated paracrinally by their secretome. Indeed, in a recent clinical study, Katagiri and colleagues showed that TCP scaffolds soaked with MSC conditioned media could significantly increase the area of new bone tissue formed in patients undergoing maxillary sinus floor elevation, in comparison with patients receiving control TCP scaffolds [167]. The extent of the bone regenerative potential of MSC secretome in comparison with cells themselves, including of the EV they secrete, still needs to be further confirmed, particularly in a clinical setting. This is the focus of many studies currently under development, as discussed in detail for MSC-derived EV in section 2.

1.2.2.3. Gene therapies for bone repair: a focus on microRNAs

Another strategy explored to improve bone healing is the delivery of genes of interest (GOI) to the injured site, which can encode proteins with important roles in bone metabolism and repair, or to be regulatory RNAs. The GOI most explored to enhance bone repair include BMP [168], Wnt proteins [169], chemokines and growth factors [170, 171], osteogenic transcription factors [172], immunomodulatory enzymes (e.g., cyclooxygenase-2) [173] and miRNAs [174]. These GOI may be delivered by the direct and local injection of the vectors carrying them [175], or by their previous *ex vivo* transfection into cells, such as autologous or allogeneic MSC, that are then transplanted [176]. In both gene therapy strategies, the GOI is commonly carried in viral vectors, such as adenoviruses, retroviruses, lentiviruses, recombinant adeno-associated viral vectors and baculoviruses [176], and more than one GOI can be combined in the gene therapy designed [92, 177], in order to modulate different stages of the bone repair process. Interestingly, miRNAs have been regarded as valuable GOI for bone repair gene therapies, since they are regulatory RNAs with a pleiotropic action,

being able to simultaneously influence different aspects of the bone repair and regeneration cascade [174, 178].

miRNAs make part of the large family of regulatory non-coding RNAs, which are RNAs transcribed from the genome but not translated into proteins. They have a small size of ~22 nucleotides, and are responsible for the control of gene expression, mainly at the post-transcriptional level [179]. miRNA genes are transcribed as long pri-miRNA transcripts, which are then processed by the Drosha-DGCR8 ribonuclease complex into smaller hairpin miRNA precursors (Figure 3). These pre-miRNAs are exported from the nucleus into the cytoplasm, where they are cleaved by Dicer ribonuclease into double-stranded miRNAs (Figure 3). Finally, the double strand is separated and the functional mature small miRNAs are loaded into the RNA-induced silencing complex (RISC) via Argonaute-2 (Ago2) proteins, where they bind complementary messenger RNA (mRNA), preventing its translation or promoting its degradation (Figure 3) [180]. Importantly, one single miRNA is able to bind several complementary target mRNAs, having a pleiotropic action by affecting the expression of multiple genes at once. Furthermore, the same mRNA may be targeted by several miRNAs, allowing a more stringent control of the expression of a specific target mRNA [181]. miRNAs may act inside the cell that produces them, but may also be secreted, being found circulating systemically in body fluids, associated with protein complexes containing Ago proteins or enclosed inside EV that protect them from degradation [182]. Inside the target cell, miRNAs may regulate virtually all cell functions, including survival, proliferation, migration and differentiation [179].

Importantly, miRNAs were recently implicated in the response to bone injury. In a wide screening of miRNA expression in bone callus of a mouse model of femur fracture, Hadjiargyrou *et al.* demonstrated that injury induced a down-regulation of 40.6% of the detected miRNAs, and an up-regulation of 33.3% of them, relatively to intact contralateral bones. Moreover, these alterations in miRNA expression at the callus varied along time after fracture, further revealing a controlled miRNA expression accompanying the bone regenerative process [183]. In a more focused approach, Waki and colleagues analysed local miRNA expression in healing and non-healing femur fractures in rats. Interestingly, they found that the miRNA profile differed between both groups and along time after fracture, with a set of 5 miRNAs (miR-140-3p, miR-140-5p, miR-181a-5p, miR-181d-5p and miR-451a) being significantly up-regulated in healing fractures at the resolution of the inflammatory phase (14 days post-injury) [184]. Altered miRNA expression upon fracture was also reported in humans. Comparing miRNA expression in bone samples from osteoporotic hip fracture patients and control hip osteoarthritis patients, Garmilla-Ezquerra and colleagues found that miR-187 was significantly down-regulated upon fracture, whereas miR-518f was up-regulated [185]. Interestingly, bone fractures lead to changes in miRNA expression not only

locally at the injury site, but also systemically, with several clinical studies reporting sets of differently expressed miRNAs in the serum of osteoporotic fracture patients compared with osteoarthritis or healthy fracture patients [186–188]. Moreover, miRNAs have been extensively studied *in vitro* for their role in osteogenic differentiation and osteoclastogenesis, with several specific miRNAs being identified as promoters or inhibitors of such processes (reviewed in [25]). For instance, in our own work we have demonstrated that miR-195 is an inhibitor of MSC osteogenic differentiation, impairing also their proliferation and angiogenic capacity [178]. These observations further support the active participation of miRNAs in bone repair and regeneration. In addition, several proof-of-concept *in vivo* studies have been validating the suggested biological role of these miRNAs, either through their overexpression or inhibition [178, 189] .

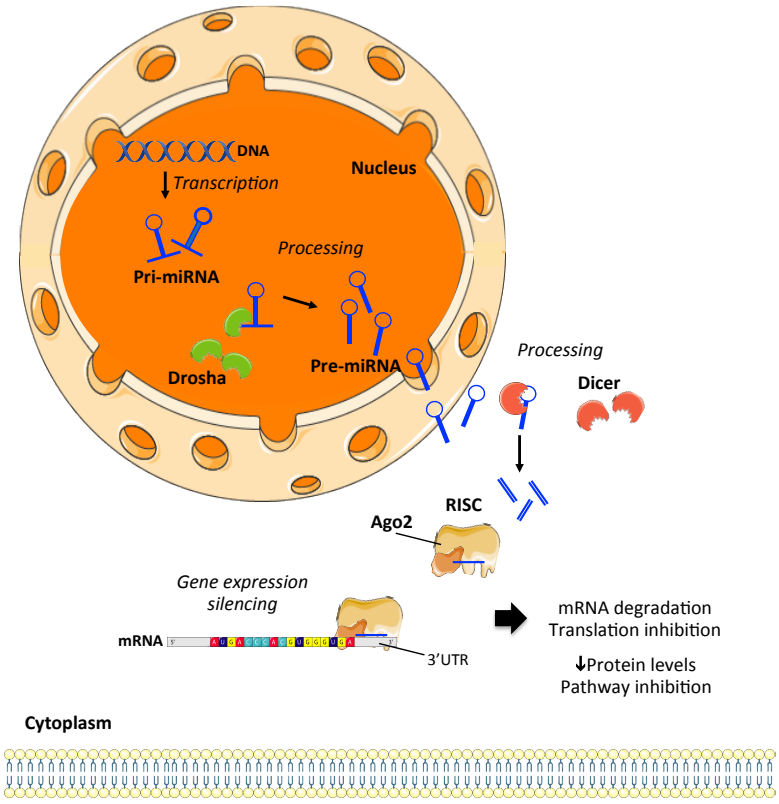


Figure 3. Pathway of microRNAs biosynthesis and mode of action. microRNAs (miRNAs), small regulatory non-coding RNAs, are originated from longer non-coding RNAs, the pri-miRNAs, transcribed from the DNA in the cell nucleus. Pri-miRNAs are then fragmented into smaller hairpin RNAs, the pre-miRNAs, by the action of enzymatic complexes that contain the ribonuclease Drosha. Pre-miRNAs are then actively transported to the cell cytoplasm by exportin-5, where they are further processed. The stem-loop of the pre-miRNAs is cleaved by Dicer, originating double-stranded miRNAs. The double strand is then separated and the mature functional miRNAs are loaded into the RNA-induced silencing complex (RISC) by the action of Argonaute-2 (Ago2) proteins. Once loaded in the RISC, miRNA may bind complementary messenger RNAs, particularly at their 3'-untranslated region (3'UTR), preventing their translation or promoting their degradation. When a miRNA is up-regulated, the levels of their target mRNAs are decreased, the protein levels encoded by the mRNAs are diminished, and the pathways where these proteins participate are inhibited. On the contrary, when a miRNA is down-regulated, the levels of their target mRNAs are increased, and the pathways where the mRNA-encoded proteins participate are activated. Based on [180].

Besides their role upon osteogenesis and osteoclastogenesis, miRNAs are also described as important regulators of other biological processes that impact bone repair. For instance, they regulate inflammation and immune cells development and function [190, 191]. Considering the multi-target action of miRNAs, it is likely possible to further improve bone repair by choosing for gene therapies miRNAs that are capable of regulating biological processes occurring in the different phases of the repair/regeneration cascade.

As described above, the events that occur during bone repair and homeostatic bone remodelling are very well articulated in time and in space, which is only possible if tight pathways of communication are established between all cell populations involved. The crosstalk between these cells may occur by different mechanisms, including direct cell contact, however paracrine communication is particularly relevant. Many of the mediators that establish this crosstalk are known and intensively studied, as is the case of cytokines and miRNAs, however the role of cell-secreted EV in bone repair and normal remodelling is vastly underexplored. Importantly, many of the mediators described above can be found enclosed inside EV. Thus, in section 2 we revisit the physiological role of EV, highlighting their potential contribution for bone repair and regeneration.

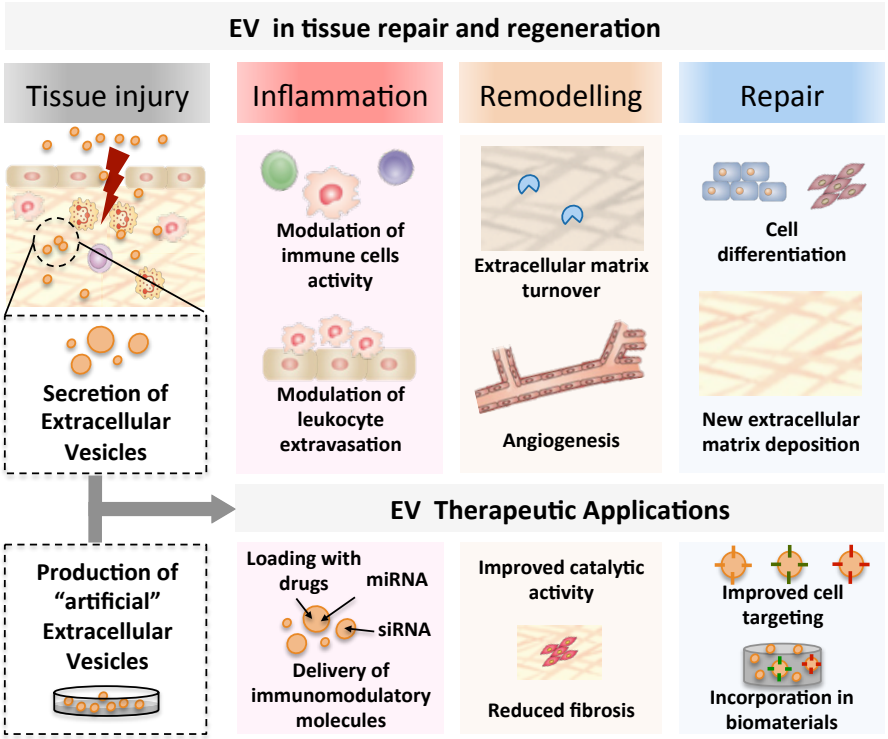
2. Extracellular Vesicles: Immunomodulatory messengers in the context of tissue repair/regeneration

The content of this section constitutes the review article published in the scope of this thesis:

Article I

Andreia M. Silva, José H. Teixeira, Maria I. Almeida, Raquel M. Gonçalves, Mário A. Barbosa, Susana G. Santos (2017), **Extracellular Vesicles: Immunomodulatory messengers in the context of tissue repair/regeneration.** European Journal of Pharmaceutical Sciences 98:86-95, doi: 10.1016/j.ejps.2016.09.017.

Graphical abstract



Abstract

Inflammation is a complex and highly regulated biological process, crucial for a variety of functions in the human body, from host response against infectious agents to initiation of repair/regeneration of injured tissues. In the context of tissue repair, the action of different immune cell populations and their interplay with tissue specific cells, including stem cells, is still being uncovered. Extracellular Vesicles (EV) are small membrane vesicles secreted by cells in a controlled manner, which can act locally and systemically. The ability of EV to influence tissue repair and regeneration has been proposed as a physiologically intelligent and targeted strategy of cell communication. Herein, the role of EV in tissue repair is reviewed, summarising first their contribution to the regulation of immune cell function, and discussing the implications for the resolution of inflammation during repair. Next, the impact of EV on cell proliferation and differentiation, and on extracellular matrix remodelling, key aspects of the subsequent phases of tissue repair, is addressed. Finally, EV-based therapies are discussed, focusing on the application of naturally produced EV, and the use of EV as delivery vehicles.

Keywords: Extracellular Vesicles; Exosomes; Immune cells; Inflammation; Mesenchymal Stem Cells; Drug delivery

2.1. Introduction

In the last decade, the importance of inflammation and immune cells action in triggering tissue repair or regeneration has been increasingly recognized in different systems, such as bone [27, 28], skin [192], skeletal muscle [193], cardiac muscle [194], and the peripheral and central nervous system [195]. Also, a very recent report elegantly demonstrates the importance of a mature peritoneal macrophage population for tissue repair, in a model of liver injury [196]. A sequential arrival of immune cells at the injury location and their concerted action, tightly regulated in time, appear to be required for successful regeneration, namely in bone (reviewed in [27]). Unbalanced immune responses are considered deleterious for tissue regeneration. Taking bone as a model, it was reported that regeneration was delayed in the absence of effective TNF-α signalling, in a mouse model of tibia fracture [68]. Moreover, bone loss is a common symptom in patients with chronic inflammatory disorders, such rheumatoid arthritis [197]. Thus, while the inflammatory response is necessary for complete tissue repair, it has to be tightly and timely controlled, and that likely relies on close cell-to-cell communication.

The concerted action of immune cells and tissue cells may be achieved not only by contact-dependent cell-to-cell communication, but also in a paracrine manner, via secreted immune mediators, which include free proteins (cytokines, chemokines, growth factors) and Extracellular Vesicles (EV) [198]. The designation of EV encompasses different populations of vesicles, mainly exosomes, microvesicles and apoptotic bodies, distinguishable by their size, composition and origin in the cell [199]: exosomes are smaller (50–200 nm), formed in multivesicular bodies and seem to benefit from a more controlled release mechanism; microvesicles are bigger vesicles (200 nm–1 μm) and originate by budding from the plasma membrane; and apoptotic bodies correspond to portions of cells (>1 μm) that disintegrate from the parent cell during cell apoptosis (for a review of EV nomenclature and characterization please see [200]). Although several attempts of standardization have been made in this field [200, 201], the methods currently employed to isolate each population have varying degrees of success in terms of population purity. Also, not all studies follow the nomenclature guidelines proposed by the International Society for Extracellular Vesicles, which makes the real identity of EV populations found in different reports sometimes still unclear. Thus, in this article the designation “EV” will be preferred.

In broad terms, EV are composed by a lipid bilayer that encloses and protects biomolecules such as proteins, nucleic acids (both RNA and DNA) and bioactive lipids, but their detailed composition depends on their biogenesis mechanism (revised in [202]). In general, different types of phospholipids, cholesterol and sphingomyelin compose the lipid fraction of EV's membrane, with different types of vesicles carrying distinct proportions of each component (revised in [203]). EV membrane is further decorated with proteins involved in membrane trafficking, adhesion and cell communication (e.g., tetraspanins, ESCRT proteins, Rab proteins, integrins, ligands for cell surface receptors). In their lumen, EV have been reported to enclose a wide variety of proteins, such as enzymes (e.g., GAPDH, pyruvate kinase, phospholipases), signalling and signal transduction proteins (e.g., cytokines, growth factors, 14-3-3 proteins) and cytoskeleton proteins (e.g., actin, tubulin), among others [203, 204]. Moreover, several kinds of RNA molecules can also be found inside EV, with an enrichment of small non-coding RNAs, mainly vault RNA, Y-RNA, transfer RNA [205], and miRNA [206]. Messenger RNA [206], long non-coding RNA [207] and fragments of DNA [208] have also been found inside EV.

EV are secreted by virtually all cell types and can be found in circulation, likely mediating biological actions in locations distant from their origin [209]. These vesicles are reported to interact with target cells by different mechanisms. They can either bind receptors on the cell membrane, triggering intracellular signalling or internalization and, when internalized (either receptor mediated or not), EV release their content in the endocytic

pathway. Alternatively EV can also fuse with the plasma membrane and deliver their content to the cell cytosol directly [210].

EV have been most studied so far in the fields of cancer research and immune response [211]. In fact, EV are being regarded as important regulators of immune cell activity, and consequently capable of modulating inflammatory responses, which are crucial for the outcome of tissue repair and regeneration processes. At the same time, a limited number of in vivo studies have been associating the administration of EV with tissue function recovery in different models of injury [212], but the mechanisms underlying this beneficial effects have not yet been revealed.

In this work we review the potential contribution of EV for tissue repair and regeneration, in terms of their described roles and therapeutic potential. We start by addressing described EV functions that are relevant for the different stages of tissue regeneration. Then, the therapeutic potential of natural, modified and artificial EV for inflammation control and enhanced tissue repair and regeneration is discussed.

2.2. EV in Tissue Repair and Regeneration

Tissue repair and regeneration develop along three main phases: inflammation, repair and remodelling, which partially overlap, and are dependent on each other [26]. Injury activates tissue cells, including epithelial, stromal and resident immune cells that induce recruitment of other circulating immune cells, starting an inflammatory response, the first phase of tissue repair/regeneration. This reaction is crucial for removal of pathogens and damaged cells from the injury site, as well as for extracellular matrix (ECM) turnover. After clearance of dead and/or infected cells inflammation needs to resolve [213], in order to allow for tissue regeneration to progress into the remodelling and repair phases, mainly characterized by enhanced angiogenesis, fibroblast activation and deposition of new ECM, and tissue cell replacement by proliferation and differentiation. Interestingly, EV have been assigned with functions that might play important roles in the different processes taking place in each of the main stages of tissue repair/regeneration [212] (Figure 1).

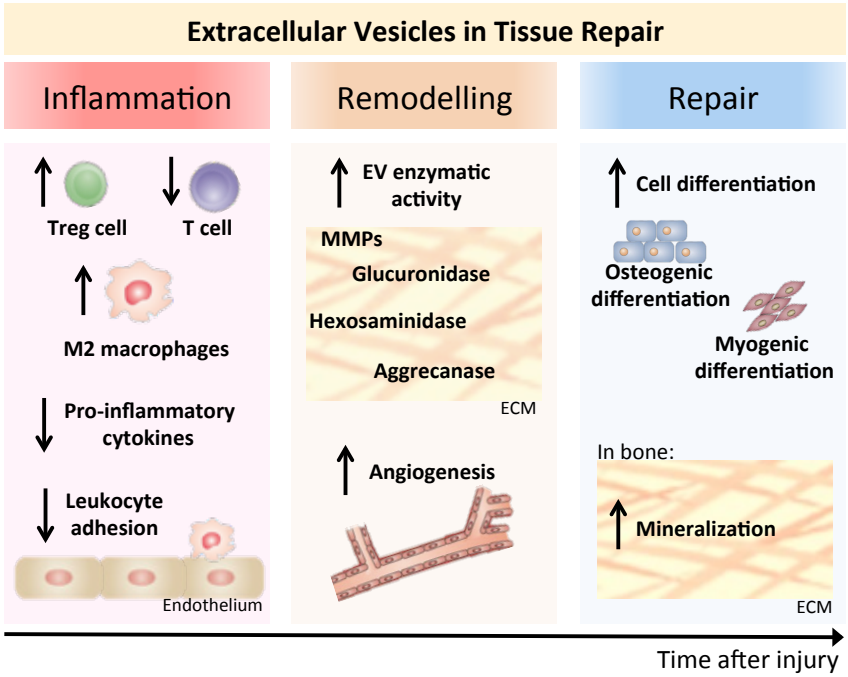


Figure 1. Extracellular Vesicles contribute to tissue repair and regeneration. EV from multiple cell sources, namely leukocytes and MSC, have been reported to participate in the different stages of tissue repair and regeneration. EV may contribute for the resolution of inflammation, triggered by the injury, by modulating infiltration of immune cells into the damaged tissue, inhibiting highly pro-inflammatory processes, activating regulatory immune cells subsets, and promoting alternative pathways of activation. Along with inflammation resolution, the damaged extracellular matrix (ECM) is renewed by the action of proteases carried by EV, contributing to the preparation of a microenvironment more permissive for the success of a later stage of tissue remodelling. At the same time, EV promote angiogenesis at the site under repair, creating a port of entry for recruited cells responsible for tissue remodelling, including stem or progenitor cells. During the final stage of tissue remodelling, EV may be associated with the promotion of cell differentiation, and maturation of the newly formed tissue.

2.2.1. EV as Immunomodulatory Messengers: Implications for Tissue Repair and Regeneration

Immune cells are able to modulate the activity of other immune and tissue-resident cells. This has been reported to take place by direct contact, and by the action of paracrine mediators, some likely contained in EV. In fact, EV have been reported both as immune activators and immune suppressors (Table 1).

Table 1. Role of extracellular vesicles in immune cell activity modulation. EV from other cell populations and from immune cells themselves, impact on immune cell biology. The outcome of EV action on immune cell function is dependent on EV origin, and may have either an activating (pro-inflammatory) or inhibitory (immunosuppressive) role dependent on the context they are produced.

Macrophage

- **Activation and Polarization**

- EV derived from pathogen-infected cells elicit macrophage pro-inflammatory responses [214, 215].

- THP-1 monocyte cell line stimulated with alcohol release EV that stimulate differentiation of naive THP-1 cells into M2-like macrophages [216].

- EV released by ESC-derived MSC activate differentiation of THP-1 cells into a M2-like macrophage phenotype [217].

- EV from LPS stimulated MSC drive THP-1 polarization into M2 phenotype [218].

DC

- **Inhibition**

- DC release EV containing miRNA that inhibit gene expression in recipient DC *in vitro* [219].

- Bone marrow DC release EV containing miR-155 that leads to increased levels of serum TNF-α and IL-6, and decreased levels of miR-155 target genes in several organs of miR-155-deficient mice injected with LPS [220].

- Bone marrow DC exchange EV containing miR-155 and miR-146a, which are functionally active *in vivo* in the recipient cells and modulate response to endotoxins [220].

T cell

- **Activation/Antigen presentation**

- DC-EV activate $CD8^+$ T cells in absence of the APC [221].

- DC-EV primed with tumor peptides activate specific $CD8^+$ T cells in mice and suppress tumor growth [222].

- EV released by infected cells contain pathogens antigens and elicit T cell activation [214, 215].

- **Inhibition**

- Breast milk EV inhibit T cell activation *in vitro* [223].

- EV derived from IL-10-overexpressing DC inhibit T cell proliferation in a mixed lymphocyte reaction *in vitro* [224].

Memory T cell

- **Activation**

- EV derived from IL-10-overexpressing DC delay onset and suppress progression of collagen- induced arthritis in mice [224].

NK cell

- **Inhibition**

- Placenta-derived EV reduce NK cells cytotoxicity [225].

In the context of tissue repair and regeneration, inflammation resolution is a key step where EV have been reported to be involved. A recent study from Saha *et al.* demonstrates that EV have the capacity to elicit inflammation resolution after cell exposure to a tissue injury-like scenario [216], instead of an infection-like scenario. The authors demonstrated that exposure of THP-1 monocyte cell line to alcohol promotes secretion of EV that transfer molecules, such as miR-27a, into naïve THP-1 cells, stimulating their differentiation into M2-like macrophages, which is important for inflammation resolution in the context of tissue repair [216]. This work unravels important clues regarding physiological mechanisms of the body to resolve tissue damage, particularly the effect that locally activated immune cells, may have in the priming and control of systemic naïve immune cells, arriving to the injury location. Moreover, immune cell-derived EV were reported to contribute to brain tissue repair and

regeneration *in vivo* due, at least in part, to inflammation modulation [226]. The authors reported that EV derived from DC stimulated with low levels of Interferon-γ were enriched in anti-inflammatory miRNAs, and reduced the levels of reactive oxygen species in microglia of rat hippocampal slices cultures induced into oxidative stress. When administered *in vivo*, those EV increased myelination in rats with acute lysolecithin-induced demyelination. However, EV beneficial role *in vivo* was likely due to their action in other cell populations besides microglia, since they were also enriched in miRNAs responsible for oligodendrocytes differentiation and were actually preferentially internalized by these cells [226].

Inflammation resolution at injury sites is also influenced by immune cells crosstalk with endothelial cells. EV derived from activated immune cells act upon endothelial cells by promoting the secretion of pro-inflammatory cytokines and pro-coagulation factors [227], actions that might be important for hemostasis right after injury. In addition, platelet-derived EV promote expression of cell adhesion molecules in endothelial cells, facilitating monocyte adhesion, at least *in vitro* [228], thus potentially contributing to leukocyte infiltration towards the injured tissue. Conversely, EV from polymorphonuclear leukocytes carry anti-inflammatory cues, such annexin 1, that control leukocyte adhesion to endothelial cells, likely contributing to inflammation resolution in late phases of innate immune responses [229]. On the other hand, EV from endothelial cells were reported to suppress monocyte activation, due to their anti-inflammatory miRNA content [230].

In the last decades, multipotent mesenchymal stromal cells (MSC) have been widely reported to have immunomodulatory properties, efficiently regulating immune cells activity [231]. Besides their multi-lineage differentiation capacity, this property of MSC has motivated the exploitation of these cells, not only as promoters of tissue repair/regeneration, but also for the treatment of inflammation-driven diseases, with very promising results both in the laboratorial and pre-clinical settings [232]. More recently, numerous studies have suggested that MSC beneficial effect is mainly due to their secretome, including MSC-secreted EV (reviewed recently in [233]). MSC-EV administration was reported to lead to an outcome similar to MSC cell transplantation in a mouse model of stroke, mitigating post-ischemia peripheral blood lymphopenia and favouring brain remodelling [234]. Also, Zhang *et al.* demonstrated an immunosuppressive role for EV isolated from Embryonic Stem Cells (ESC)-derived MSC cultures, which may be envisaged as leading to inflammation resolution [217]. The same EV increased the levels of the anti-inflammatory cytokine IL-10 in human monocytes, concomitantly with decreasing levels of pro-inflammatory cytokines IL-1β and IL-12p40. The authors further demonstrate that THP-1 cells exposed to these MSC-derived EV could induce the differentiation of $CD4^+CD25^+FoxP3^+$ T_{reg} cells [217]. *In vivo*, EV from ESC-derived MSC were shown to globally reduce acute inflammation in a model of myocardium tissue damage by ischemia, contributing significantly to the preservation of heart function.

This effect was observed both locally, with reduced neutrophils and macrophages infiltration into heart tissue, and systemically, with reduced numbers of systemic white blood cells [235]. Interestingly, pre-conditioning of MSC may impact the immunosuppressive action of their EV on immune cells. EV from MSC previously stimulated with LPS were more effective than EV from unstimulated MSC in driving M2 polarization of THP-1 cells, and promoting inflammation resolution in a model of diabetic cutaneous wound, enhancing wound closure [218].

MSC-derived EV can also modulate endothelial cells activation and permeability. In a rat model of kidney injury the injection of Wharton Jelly MSC-derived EV reduced tubular lesions resulting from ischemia and reperfusion when compared with vehicle-treated animals. This effect was accompanied by a reduction in inflammation in the injured kidney characterized by a short-term decrease in $CD68^+$ macrophages infiltration into the tissue and a local decrease in TNF-α levels and increase in IL-10. Simultaneously expression of the chemokine fractalkine was inhibited in endothelial cells suggesting an MSC effect dependent on the modulation of leukocyte infiltration to the injury site [236].

Considering the studies discussed in this section, it is clear that EV function is highly dependent on the microenvironmental context of the cells that produce them. Thus, it would be important to clarify the action of EV produced in the injury microenvironment, which remains largely to be uncovered. Knowing the conditions that lead to the production of EV capable of fostering inflammation resolution, instead of further promoting a pro-inflammatory response, could allow for the design of new EV-based therapeutics with the capacity of controlling the inflammation process to promote tissue repair and regeneration.

2.2.2. EV as Cell Proliferation and Differentiation Mediators in Tissue Repair and Regeneration

Beyond their role in immune cell function mediation and regulation, EV have additional properties that could be important in subsequent stages of tissue repair/regeneration. EV may also be involved in the re-definition of the final regenerated tissue by influencing their repopulation with functional differentiated cells (Figure 1). Some studies suggest that immune cells, including macrophages and DC are able to recruit MSC via a paracrine action [115, 136], a process likely involving EV [115].

Interestingly, immune cells-derived EV may contribute to tissue repair/regeneration by promoting stem cell differentiation. EV from monocytes activated with LPS were shown to induce *in vitro* osteogenic gene expression programs in MSC, increasing RUNX2 and BMP-2 mRNA levels, but in a lower extent than monocytes conditioned media. Osteocalcin was also up-regulated, and to a greater extent than for monocytes conditioned media [237]. Similar

effects were obtained with platelet lysate-derived EV, which promoted MSC osteogenic differentiation in a dose-dependent manner, increasing matrix mineralization after 17 days of repeated stimulation in comparison with 10% platelet lysates [238].

In several recent studies EV from bone marrow MSC were also implicated in osteogenesis. Qin *et al.* showed that MSC-derived EV promote the expression of osteogenesis gene markers, such as RUNX2, ALP, OCN and OPN, increasing mineralization in human osteoblasts cultures, mainly through the transfer of miR-196a into osteoblasts [239]. Also, EV from human induced pluripotent stem cell-derived MSC were combined with β-TriCalcium Phosphate (TCP) scaffolds, enhancing osteogenesis and angiogenesis in a dose-dependent manner, in the calvarial defect of an osteoporosis rat model [240]. Importantly, a study using $CD9^{-/-}$ mice, which show reduced levels of EV and impaired bone repair, demonstrates that fracture repair was rescued by human MSC produced EV [241]. Another recent study suggests that local injection of MSC-derived EV can regenerate cartilage in a rat model, although the authors did not include controls such as the EV-depleted ultracentrifugation supernatant [242]. However, none of these studies specifically evaluated if enhanced repair was due to promotion of cell recruitment and osteogenic differentiation, or also due to the immunomodulatory role of MSC-EV.

MSC-EV were also suggested to promote myogenesis. The stimulation of mouse C2C12 myoblasts with human MSC-derived EV increased the expression of the myogenesis markers MYOD1 and MYOG most likely due to the transfer of myogenesis-controlling miRNAs [243]. A recent study also showed that EV derived from MSC that were stimulated to differentiate into the osteogenic lineage by chemical induction or transient transfection with RUNX2 promote differentiation of unstimulated MSC [244]. In vivo MSC-derived EV have been associated with improved tissue function upon administration in different systems many times accompanied by cell differentiation [245]. However their direct role on cell differentiation *in vivo* was not dissected yet and thus the mechanisms underlying their beneficial action important for tissue repair are still unknown.

EV have also been implicated in angiogenesis, an important process occurring during the repair stage of the response to injury. Indeed, endothelial progenitor cells secrete EV that actively promote angiogenesis either *in vitro* and *in vivo* by transferring mRNAs related to angiogenic gene expression programs [246]. Most importantly, and in a continuum with their role during the inflammatory stage, immune cells themselves influence endothelial cell activity and may promote angiogenesis *via* EV. In a study using a mouse model of hind limb ischemia, animals treated with EV from a T cell line had higher vascular density and blood flow in injured limbs than vehicle-treated mice, in a process strongly regulated by the morphogen SHH (sonic hedgehog) carried by the vesicles [247].

Similarly, cord-blood MSC-derived EV promoted angiogenesis *in vitro* and in a rat model of skin burn through the delivery of the morphogen Wnt4 to endothelial cells, however it is not clear if the effect observed is mainly due to protein or RNA transfer to target cells [248]. Moreover, EV derived from induced pluripotent stem cells-derived MSC were shown to promote cutaneous wound healing in mice, which was associated to a local enhancement of vascularization and collagen synthesis. These EV induced proliferation and migration of both endothelial cells and human fibroblasts *in vitro* [249]. Similar effects were recently observed in a rat model of wound healing, with EV from human urine-derived stem cells (USC) [250], reported to have MSC-like properties and identity. USC-derived EV increased proliferation of HUVEC cells, their 2D-migration and tube formation *in vitro*. The authors also report accelerated wound closure upon local injection in full-thickness wounds inflicted in rats, which was accompanied by an increased vascularization at the lesion site [250].

2.2.3. EV Carry ECM Remodelling Messages: Their Influence on Tissue Repair

There is no tissue repair or regeneration without new ECM production, deposition, and its remodelling. Here, EV have also been implicated in direct degradation of different ECM components, which might be important to prepare the injury microenvironment for the deposition of a provisional matrix occurring in the beginning of the repair stage (Figure 1). EV of different cell origin, particularly cancer cells-derived exosomes, have been shown to carry metalloproteinases such as MMPs, ADAMs and ADAMTs [251], but these proteins are also found in immune cells-derived EV. For instance, MMP-9 and MMP-10 are found in different subpopulations of EV from neutrophils [252]. More important, previous studies suggest that monocyte exposure to pro-inflammatory cues increases the secretion of MMP-containing EV [253]. EV have additional proteolytic activity for specific ECM fibres. For instance, EV derived from synovial fibroblasts isolated from rheumatoid arthritis patients have aggrecanase [254], hexosaminidase [255] and glucuronidase [256] activity. However, despite the evidences of EV involvement in ECM degradation, the relevance of their enzymatic activity in ECM turnover during tissue repair has not yet been fully explored.

Coordinated with ECM turnover, fibroblasts at the injury site are activated to produce a provisional ECM for cell repopulation. EV may be important players in the regulation of fibrosis during tissue repair, with previous reports implicating them in the regulation of fibroblasts activity *in vivo*. Borges *et al.* demonstrated that EV isolated from tubular epithelial kidney cells grown in hypoxic conditions promoted proliferation of fibroblasts and activated them to express higher levels of type 1 collagen, F-actin, α-smooth muscle actin and TGF-β1, as opposed to EV from epithelial cells grown in normoxia [257]. In addition, in a mouse

model of hypoxic injury by ureteral obstruction, EV isolated from injured kidneys were enriched in TGF-β1 mRNA, with the authors suggesting that, *in vivo*, injured epithelial cells subjected to the hypoxic environment of the injury trigger fibroblast activation for tissue repair, in a process likely dependent on TGF-β1 mRNA transfer. On the other hand, MSC-derived EV may have a protective role against excessive and deleterious fibroblast activity. Indeed, human umbilical cord MSC-derived EV were shown to reduce fibrosis in a rat model of hypoxic heart injury by coronary artery ligation [258], and to decrease scar formation, via reduced myofibroblast accumulation, in a skin wound healing mouse model [259]. The signals carried by EV that determine the balance between excessive fibrosis and scar tissue formation, and controlled provisional ECM deposition and remodelling are only now starting to be uncovered. A recent report shows the crucial role of specific miRNA (miR-21, -23a, -125b and -145) in suppressing myofibroblast formation by inhibiting the TGF-β2/SMAD2 pathway [259]. Another recent report indicates that in human umbilical cord MSC-derived exosomes, the presence of 14-3-3ζ protein mediated the self-regulation of the Wnt- β-catenin signalling, modulating the remodelling phase of cutaneous repair [260]. Uncovering the signals underlying EV action will be a key factor in promoting successful tissue repair.

Also, in bone repair/regeneration, EV may be able to directly contribute to ECM formation. Indeed, a specific type of vesicles usually found associated with ECM fibres and that contain calcium phosphate, may function as starting sites of mineralization in the new forming bone, contributing to calcified matrix synthesis during endochondral ossification [261, 262]. In this way, EV from different origins, and likely present at the injury environment across different time-points, may contribute to different stages of ECM remodelling, initially implicated in the removal of the damaged matrix, while at later stages contributing to its final maturation.

2.3. EV as Therapeutic Vehicles in Tissue Repair and Regeneration

Biological properties of EV can mimic the effect of different pharmaceutical drugs currently used in the clinics to improve or accelerate tissue repair, behaving as drugs themselves upon administration. In addition, EV can be used as drug delivery vehicles, taking advantage of their natural biocompatibility and cell targeting.

In a reductionist perspective, EV and liposomal vehicles are alike: both are composed by a lipidic layer, which can be functionalized with glycoprotein receptors and ligands, that carry enclosed a cargo with therapeutic action. But unlike liposomes, where each component of the vesicle has to be added step-by-step until a complex structure is achieved, EV are

naturally assembled inside cells, and thus can be obtained as ready “off-the-cell” products (Figure 2).

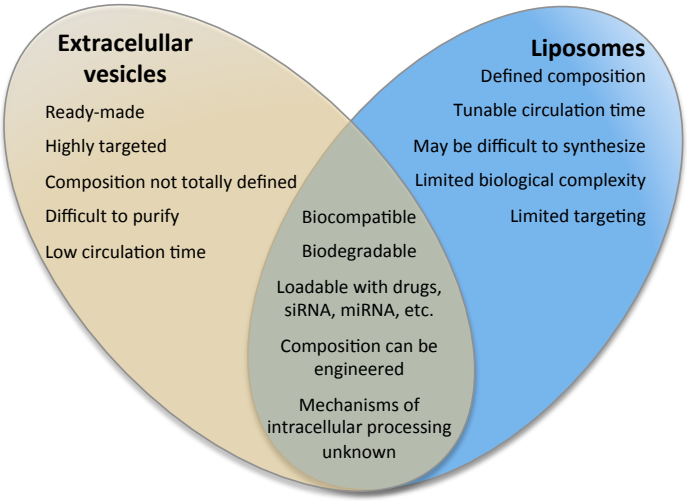


Figure 2. Extracellular Vesicles versus liposomes: major advantages and disadvantages of each carrier for drug delivery. Liposomes are FDA-approved drug delivery systems considered the gold standard in nanomedicine. Currently, they can be synthesized in laboratory for the inclusion of drugs, nucleic acids (e.g., siRNA, microRNA, etc.) and other compounds, and their core and surface composition may be modified for improved circulation time and cell targeting. EV, share many similarities with liposomes and are now being regarded as advantageous as drug delivery vehicles. Unlike liposomes, EV are already naturally assembled as complex and targeted biological structures. Although numerous in vivo studies have been suggesting the beneficial role of EV administration in a variety of diseases and conditions, very few clinical trials were performed with human subjects.

2.3.1. Application of EV as New Drugs

EV pharmaceutical potential has been recognized in the last years, with an increasing number of *in vivo* animal studies and a few human clinical trials using EV. These unravelled the first details on EV pharmacokinetics and pharmacodynamics properties, establishing EV safety profile and effectiveness in the resolution of few disorders.

Although EV have high therapeutic potential, already explored in different animal models, covering a wide variety of diseases and conditions, including tissue repair/regeneration [263], several technical hurdles still require close attention. In particular, standard protocols for EV isolation and EV administration *in vivo* are still missing, making it difficult to compare the effects ascribed to EV in different reports. These inconsistencies delay translation of EV application into human clinical trials.

For most of the in vivo studies using EV in murine animal models, vesicles are usually administered by intravenous (i.v.) or intraperitoneal (i.p.) injection, in a dosing scheme of multiple administrations every few days (usually two to three) for long term treatments

(approximately 1 month) [247], or of single-dose administration for acute effects evaluation (usually up to 3 days) [235].

Previous *in vitro* studies suggest that EV have specific cell targets, at least to some extent. However, the biodistribution of EV upon systemic administration only more recently started being explored. Animal studies suggest that EV are readily cleared from circulation upon injection (within a timeframe of minutes) into diverse body locations, such as liver, adipose tissue, lung, muscle and kidney [264]. In one of the first studies dedicated to the topic, Rana *et al.* demonstrated that EV enriched in specific tetraspanins and integrins are differently internalized by specific cell types [265]. For instance, EV enriched in tetraspanin 8 (Tspan8) preferentially bind endothelial cells, whereas EV enriched in Tspan8 and integrin-β4 are more readily internalized by lymph node stromal cells. Furthermore, they also showed that these EV are taken up differently by specific organs when injected in mice. Not surprisingly, the work by Saunderson *et al.* also suggests that EV biodistribution after administration is dependent on their cell of origin, which will influence the profile of receptors and ligands they present on their surface and thus determines the preferential sites for EV binding and uptake [266]. These findings are further supported by a more recent study that extensively addresses the factors influencing EV biodistribution [267]. In this study, EV from mouse muscle cells, melanoma cells and immature DC injected in mice homed to similar body locations. However, the percentage of uptaken vesicles in each of these locations greatly varied depending on their cell of origin. Muscle-derived EV homed preferentially to the liver, melanoma-EV to the gastrointestinal tract and DC-derived EV to the spleen, with the authors speculating that EV follow the same homing pattern as their parent cells. This EV property is a great advantage, when comparing for instance with liposomes envisaging clinical application, since more targeted therapies are likely to be achieved with exosomes than with engineered liposomes. Nonetheless, it also raises the necessity to carefully select the EV cell of origin to achieve successful effects in the context of tissue repair and regeneration.

To date there is no evidence that EV administration upon tissue injury preferentially homes to injury sites, compromising the dose-effect outcome of EV clinical application. To overcome this issue, in addition to direct EV administration, researchers have used a delivery system for a more controlled released of EV *in situ*. For instance, Qin *et al.* used a commercially available hydrogel (HyStem-HP) to deliver MSC-derived EV in a critical size bone defect of rat calvaria in order to evaluate EV osteogenic potential [239]. In fact, EV are now regarded as promising factors to be incorporated in biomaterials, to which they can likely be adhered using gels, or by active binding to molecular linkers attached to the biomaterials' surface. This approach has long been applied for the incorporation of biological functional molecules in biomaterials in order to improve tissue regeneration [268]. The biological

functions of EV allied to this approach of biomaterial construction will likely lead to the development of biomimetic biomaterials, with improved function to promote tissue repair and regeneration.

So far the evaluation of EV clinical potential in human clinical trials is very limited, and the number of patients included in these studies is also very small, so strong conclusions regarding EV therapeutic efficiency and safety should not be taken. Nevertheless, important aspects have to be pointed out from these studies, namely regarding EV production, dosing and routes of administration. Indeed, in all of them EV were isolated following Good Manufacturing Practices, by ultracentrifugation and on top of sucrose gradients, to ensure highly pure vesicle preparations, devoid of soluble contaminants/effect confounders. Subcutaneous and intradermal injections seem to be the preferential routes of administration, whereas in animal studies i.v. and i.p. are preferred sites of injection, likely leading to stronger effects shorter after administration than in humans. On the other hand, the quantity of EV that should be administered to achieve a clinical outcome is still very debatable, since the clinical trials performed so far used different methods of EV quantification, and ultimately did not lead to striking evident benefits for the patients thus far. In addition, pharmacodynamics, pharmacokinetics and biodistribution parameters associated with EV administration are still lacking in human-based studies. EV clinical use has been particularly explored in the field of cancer immunotherapy [211]. The first EV-based clinical trials reported the use of EV from autologous monocyte-derived DC that were loaded with tumour peptides for the treatment of melanoma patients. These studies demonstrated for the first time the feasibility of EV injection for human treatments, with good tolerance of the therapy, only mild adverse effects (such as erythema, pain and pruritus at the site of injection), and without auto-immune reactions, but with limited beneficial effects for the patients within the range of doses administered [269]. The benefits of EV- based therapies were further supported by phase I and phase II clinical trials for cancer treatment, and one ongoing study for the treatment of type I diabetes [270]. These clinical trials demonstrate the feasibility of EV-based therapies, but so far no completed clinical trial has evaluated the potential of EV in tissue repair and regeneration, and thus the clinical application of EV in this field stills remains to be proven.

2.3.2. EV as Novel Drug-delivery Vehicles

Beyond the delivery of their natural cargo, EV therapeutic properties for tissue repair/regeneration may be further boosted by transforming them in drug delivery vehicles for other natural or synthetic compounds of interest. Their potential as drug-delivery vehicles is

currently being addressed in terms of capacity for loading with exogenous drugs, cell targeting, and ability to cross biological barriers.

The feasibility of loading EV with drugs, which remain active upon *in vivo* delivery, has been demonstrated. In the work from Zhuang *et al.*, curcumin, a natural anti-inflammatory compound, and the JSI124 STAT3 inhibitor were encapsulated in EV derived from a T cell lymphoma cell line by simple incubation of EV with the drugs. Loaded EV further purified by sucrose gradient centrifugation were then administered intranasally to three different animal models of inflammation-driven brain injury, leading to an anti-inflammatory effect, mainly characterized by a reduction in the number and activation of inflammatory $CD45.2^{+}$ microglial cells [271]. These results demonstrate the possibility of boosting naturally produced EV with inflammation-modulatory compounds, supporting their application in the treatment of tissue injury. Also the drugs paclitaxel and doxorubicin were loaded onto EV and retained their anti-tumour activity *in vivo* [272, 273]. Moreover, EV have been engineered for an improved delivery of components similar to their natural cargo, namely microRNAs and siRNAs. Indeed, the study of Alvarez-Erviti *et al.* demonstrated for the first time the capacity of EV to transfer genetic material, namely siRNAs, to target cells *in vivo* [274]. Since then, few other studies supported the capacity of EV from different origin, including cell lines and biological fluids, to functionally deliver siRNAs to target cells [275–277]. Similarly, miRNAs can be efficiently delivered by EV to target cells, modulating their behaviour [219]. In the context of inflammation modulation, Akao *et al.* demonstrated that THP-1 cells transfected with chemically-modified miR-143 release, *in vitro* and *in vivo*, EV enriched in this microRNA [278]. In a recent study, MSC transfected with miR-181c released EV capable of controlling inflammation following burn injury in rats, comparing to EV from control-transfected MSC [279]. The authors suggested a role for vesicular miR-181c in suppressing TLR4 signalling, which led to reduced pro-inflammatory cytokines (TNF-α and IL-1β) and increased IL-10. Taken together these studies set the ground for the development of new strategies of tissue repair by incorporation of exogenous microRNAs into EV, able to regulate immune cell function and resolve inflammation in an injury scenario. The direct loading of proteins into EV was also previously reported, with catalase being incorporated into EV. Importantly, these catalase-loaded EV were able to reduce inflammation induced by oxidative stress, in the brain of mouse models of Parkinson disease [280].

However, the efficiency of loading (EE) of EV with compounds of interest may differ significantly from liposomes. For nucleic acids such as siRNA, EE as low as 0.05% for EV [281] was reported, while other authors have shown >90% EE for specific liposomes formulations [282]. Several technical approaches have been employed recently to improve EV loading, such as extrusion, electroporation, sonication and freeze-and-thaw cycles (reviewed in [283]). Also, in comparison with liposomes, EV loading greatly benefits from the

possibility of incorporation of specific cargo into the vesicles during their biogenesis, via modification of parent cells to express the nucleic acids and proteins of interest and specifically direct them into EV. In addition, the low efficiencies of drug loading into EV may be circumvented by improved rates of cargo retention and more efficient and/or functional delivery of functional cargo to target cells. Indeed, EV can deliver siRNA to *in vitro* cell cultures at levels comparable to liposomes due to improved cell targeting [274]. Similarly, EV loaded with doxorubicin were demonstrated to have an improved anti-tumour activity comparing to phosphatidylcholine-cholesterol liposomes loaded with the same quantity of the drug, likely due to a slower rate of drug release and higher retention in tumour tissue [284].

As mentioned above and in Section 2.3.1, EV have a certain degree of target specificity, which constitutes an advantage over liposomes. In fact, the biodistribution of "naked" liposomes is mainly determined by their capacity to perfuse though tissues with permeable blood vessels, and their surface has to be functionalized with targeting molecules to modify their biodistribution. From a practical point of view, the use of EV as drug carriers may thus appear advantageous over liposomes, since the synthesis of complex functionalized and targeted liposomes is time-consuming, difficult to control and cost demanding, whereas EV are more readily obtained from cell cultures supernatants or biofluids.

Interestingly, different populations of EV, such as exosomes and microvesicles, have different surface compositions, in terms of lipids, proteins and also their glycosylation [203], thus consisting of vehicles for drug delivery with intrinsic distinct targeting, and also pharmacokinetic and pharmacodynamics properties.

In order to improve the targeting of specific cell populations, EV surface may be further functionalized. In the ground-breaking study of Alvarez-Erviti *et al.*, specific targeting of neuronal and muscle cells by DC-derived EV was achieved by overexpressing a fusion protein of the exosomal component Lamp2b with the neuron-specific rabies viral glycoprotein (RVG) peptide or a muscle-specific peptide (MSP), respectively, in EV parent cells [274]. Indeed, RVG-EV loaded with siRNA against GAPDH were able to significantly knockdown this gene in neuro2A neuroblast cells, but not C2C12 myoblast cells. Conversely, MSP-EV knocked-down GAPDH in C2C12 cells, but not neuro2A cells. This proof-of-concept study opens the way for the modification of EV known to have functions in tissue repair, in order to target them to specific cells in the harsh environment established after injury. EV targeting might be improved by the incorporation of other viral proteins on their surface, namely lentivirus proteins, since they are highly specialized cell targeting entities. Although the biosafety of this approach is questionable, EV and viral particles share common biogenesis pathways inside the cells, which would be an easy method to produce targeted EV [285]. Another important aspect is the natural capacity of EV to cross biological barriers. The work

from Zhuang *et al.* demonstrates the capacity of EV of smaller size (theoretical size of 30–100 nm), with origin in a cell type non-related with the nervous lineage, to readily cross the blood-brain barrier, being found in mice olfactory bulb and, more specifically, internalized in microglial cells [271]. This capability of EV for ready and efficient drug delivery across biological barriers, with little or no modifications to their constitution, is hardly achieved by the most commonly used vehicles for drug delivery.

Although only recently EV started to be explored as drug-delivery vehicles, they are very promising alternatives to other commonly studied carriers. Unfortunately, one of the features that characterizes EV as good delivery vehicles is also one of major drawbacks in their clinical use: the fact that EV are readily synthesized by parent cells, also means that numerous molecules are incorporated in the vesicles that are not identified, or even relevant for the treatments, turning EV into semi-black boxes. Since EV composition is not known in detail, it is hard to predict their effect *in silico*, which may lead to unexpected deleterious effects once administered in clinical practice [286]. Furthermore, the mechanisms that control EV biogenesis and function are not yet known in detail, which associated with sub-optimal methods of EV isolation, largely increases the likelihood of batch-to-batch variations in EV composition, setting back the clinical translation approval of EV application.

Perhaps the most practical approach to overcome these drawbacks would be to produce “artificial” EV devoid of all the components not necessary for their function. Conceptually, this might be achieved by the modification of liposomal-based vehicles to better resemble natural EV. Thus, artificial vesicles may be designed that mimic the molecular composition of natural EV, in order to improve cell targeting and biological properties. This could be achieved by: (i) modifying the lipid composition of the liposomes to resemble different populations of EV (e.g., incorporation of cholesterol or sphingomyelin) [287]; (ii) incorporating carbohydrate moieties or even synthetic polymers (such as polyethylene glycol) in the liposomes' membrane [288, 289]; (iii) incorporating molecules on liposomes' surface responsive to pH alterations or degradable by enzymes present at target sites [290]; (iv) coating the liposomes' surface with antibodies, ligands and receptors bound by specific target cells [291]; (v) incorporating jointly inside EV nucleic acids, enzymes and signalling molecules. Very simple formulations of artificial EV were already attempted [292], yet new technical advances in the liposome field, and further knowledge about the composition and loading mechanisms of EV are still needed, until a formulation of multifunctional artificial EV is achieved.

2.4. Conclusion

EV are biological complexes with ascribed functions that are importantly linked to key steps, which occur sequentially during repair/regeneration processes. Either *in vitro* or *in vivo*, EV have been reported as contributing to control inflammation, promote cell recruitment and differentiation, and participate in ECM remodelling. In addition, their natural composition confers to them a modest but intrinsic specificity for target cells, and their effects are likely dependent on their cell of origin, allowing an additional level of control of their action via parent cell conditioning. Considering these aspects altogether, EV may be regarded as advantageous drug delivery vehicles in comparison with lipidic nanoparticles, like liposomes, used in nanomedicine formulations currently employed in the clinics. However, EV complexity may also come with pleiotropic actions, which can be advantageous or deleterious from the therapeutic perspective. In addition, it should be emphasized that the techniques most commonly applied for EV isolation, namely ultra- centrifugation and polymeric precipitation, are still not suited for production of large quantities of EV, and lead to the co-precipitation along with EV of soluble proteins and chemical supplements often used in cell cultures, originating a complex EV pellet whose composition is not completely defined. Ultimately, functions currently assigned to EV may be biased by other components of EV pellets. Consequently, there still persists a necessity to include in all experiments appropriate controls, discriminating between biological roles of EV and of the soluble contaminants in EV pellets, and to encourage standard protocols of EV isolation techniques, appropriate to EV source, and that allow researchers to obtain EV pellets majorly devoid of confounding soluble contaminants.

In summary, the use of EV in tissue engineering strategies would largely overcome the disadvantages associated with cell therapies and tissue engineering with implantation of live cells. However, current knowledge of EV biology, content and function needs to be deepened before widespread clinical application of EV in therapy, including for inflammation modulation, promoting improved tissue repair and regeneration. Considering the rapidly evolving field of EV, we anticipate that new clinical trials will be launched in the coming years, which will reveal the EV effectiveness for the treatment of human disease and injury, namely in the tissue regeneration field.

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3. Aims of the thesis

The hypothesis underlying this thesis was that the capacity of immune cell populations to promote the recruitment of MSC could be mediated by the EV they secrete. We also hypothesized that bone injury would affect EV cargo, as well as the secretion of other mediators, such as miRNAs, modulating MSC recruitment to injury locations, and thus conditioning tissue repair and regeneration.

To test our hypothesis, the main goals of this thesis were:

1) To determine the potential of DC-secreted EV to recruit MSC *in vitro.*

In order to achieve this objective, human-derived DC and MSC were used. EV secretion, isolation and characterization methodologies were set up. The ability of DC-secreted EV to promote MSC recruitment was evaluated by 2 distinct approaches and the protein content of EV was characterized, to uncover the potential molecular mediators of recruitment. The results obtained are presented in Chapter II.1, and show the ability of DC-EV *per se* to recruit MSC in a dose-dependent fashion.

2) To evaluate the impact of bone injury on the EV produced *ex vivo* by bone marrow-derived DC and macrophages, and their ability to promote MSC recruitment and differentiation.

To accomplish this goal, an *in vivo* critical size femoral bone injury model was used. Animals were evaluated at day 3 and 14 post-injury for the systemic response and bone marrow cells from the injured limb were used to differentiate DC and macrophages. The secreted EV were isolated, characterized and tested for their capacity to promote MSC recruitment and osteogenic differentiation. DC-derived EV from non-operated and 14 days post-injury animals revealed to be the most efficient in recruiting MSC, with the ones from cells isolated at day 3 inhibiting recruitment. These results are presented in Chapter II.2.

3) To determine the effect of bone injury on the composition of circulating EV and miRnome.

The same critical size bone defect model and timepoints were used in this part of the work. Given the technical limitations in the amount and quality of EV obtained from plasma, the circulating miRnome was characterized in total plasma. The results point to the let-7 family of miRNAs and miR-21 as being temporally regulated upon injury in plasma and also in splenocytes. Moreover, at day 3 post-injury there was a downregulation of miRNAs, which correlated with reduced levels of the miRNA processing machinery. These results are described in Chapter II.3.

II. Original research work

1. Dendritic Cell-derived Extracellular Vesicles mediate Mesenchymal Stem/Stromal Cell recruitment

The content of this section is published in the following original research article:

Article II

Andreia M. Silva, Maria I. Almeida, José H. Teixeira, André F. Maia, George A. Calin, Mário A. Barbosa, Susana G. Santos (2017), **Dendritic Cell-derived Extracellular Vesicles mediate Mesenchymal Stem/Stromal Cell recruitment.** Scientific Reports, 7:1667, doi:10.1038/s41598-017-01809-x.

Abstract

Orchestration of bone repair processes requires crosstalk between different cell populations, including immune cells and mesenchymal stem/stromal cells (MSC). Extracellular vesicles (EV) as mediators of these interactions remain vastly unexplored. Here, we aimed to determine the mechanism of MSC recruitment by Dendritic Cells (DC), hypothesising that it would be mediated by EV. Primary human DC-secreted EV (DC-EV), isolated by ultracentrifugation, were characterized for their size, morphology and protein markers, indicating an enrichment in exosomes. DC-EV were readily internalized by human bone marrow-derived MSC, without impacting significantly their proliferation or influencing their osteogenic/chondrogenic differentiation. Importantly, DC-EV significantly and dose-dependently promoted MSC recruitment across a transwell system and enhanced MSC migration in a microfluidic chemotaxis assay. DC-EV content was analysed by chemokine array, indicating the presence of chemotactic mediators. Osteopontin and matrix metalloproteinase-9 were confirmed inside EV. In summary, DC-EV are naturally loaded with chemoattractants and can contribute to cell recruitment, thus inspiring the development of new tissue regeneration strategies.

1.1. Introduction

Bone repair and regeneration requires a timely controlled inflammatory response[1]. An impaired pro-inflammatory response may compromise bone regeneration[2], while excessive inflammation leads to increased bone destruction[3]. Resolution of inflammation during bone repair is dependent on the communication between immune cells and other cell populations in the bone microenvironment, including multipotent mesenchymal stromal/stem cells (MSC). Cell-to-cell communication may occur *via* direct contact or be mediated by cell-secreted factors, many of which likely carried by Extracellular Vesicles (EV).

Different EV populations are produced and released by cells, including apoptotic bodies, large microvesicles (200 nm–1 μm), and nanometric exosomes (30–200 nm), which carry proteins (e.g. cytokines) and nucleic acids (DNA, mRNA, microRNA) capable of modulating the activity of target cells[4]. Exosomes, that originate in multivesicular bodies inside the cells, are actively loaded and secreted[5], and show some degree of cell targeting[6,7]. They are secreted by virtually all cells, and can be found in biofluids. Therefore, exosomes may act in locations distant from those where they were produced and released[8]. EV have ascribed functions both in homeostasis and pathological conditions[9], being most studied in the cancer field, for their potential use in cancer therapy[10], and as immune mediators[9]. Thus, EV likely also impact the contribution of immune cells to tissue repair processes[9, 11]. As part of their immumodulatory activity, DC exosomes were shown to promote granulocyte migration, containing enzymes that participate in synthesis of chemotactic molecules[12]. *In vitro* and *in vivo* studies suggest beneficial roles for EV in tissue repair[13, 14], likely through inflammation modulation.

MSC have been intensively explored for their potential use in stem cell therapies for tissue repair and regeneration, including in several ongoing clinical trials[15]. They are particularly interesting for bone tissue regeneration due to their immunomodulatory properties, potential to differentiate along osteogenic and chondrogenic lineages, and supportive role for other cells in the microenvironment[13]. MSC have been shown to home into locations of active inflammation[16]. However, cell mobilization and retention at injury locations is usually ineffective. Thus enhancing endogenous or transplanted cell recruitment and engraftment could improve current MSC-based therapies.

Our previous work showed that DC promote MSC migration *in vitro*, through the secretion of paracrine factors[17]. Other studies demonstrated the same capacity for other immune cells, such as macrophages, NK cells and T cells[18]. Also, monocyte/macrophages and their secreted EV have been shown to enhance MSC osteogenic differentiation[19].

Thus, in the current work we hypothesized that the paracrine chemoattractant effect of DC upon MSC was largely mediated by DC-secreted EV. To test our hypothesis, we used

EV secreted by primary human DC and investigated their biologic effect on MSC. The results presented here confirm our hypothesis, showing that DC-derived EV were internalized by MSC, and promoted their migration in a dose-dependent manner. Interestingly, DC-derived EV did not impact neither MSC proliferation nor osteogenic differentiation. The molecular composition of EV revealed that they were enriched in molecules involved in cell recruitment, like osteopontin, and also contain the matrix metalloproteinase (MMP)-9.

1.2. Results

1.2.1. EV isolated from Dendritic Cells are enriched in exosomes

In order to isolate a population of EV enriched in exosomes, supernatants from primary human monocyte-derived DC, cultured with EV-depleted FBS, were processed via the classical differential (ultra)-centrifugation method. EV obtained were characterized for vesicle morphology and presence/absence of characteristic membrane protein markers (Fig. 1). For each EV preparation the amount of protein was quantified, and protein concentration was used as a measure for the amount of EV to be used in all the functional assays. TEM analysis revealed the presence of vesicles with a lipid bilayer and cup-like morphology. Vesicles sub-populations with different sizes could be distinguished, all of them with a diameter under 200 nm (Fig. 1a). For an accurate analysis of size distribution, EV suspensions were analysed by NTA. Results obtained show EV with an average diameter in solution of ~150 nm, and including subpopulations of different sizes (Fig. 1b). Similar results were obtained by DLS, with a polydispersity index of 0.36 ± 0.06 for EV suspensions. In terms of protein markers, both western blotting and flow cytometry results indicate that the EV population isolated contained CD63 (Fig. 1c,d). The antigen presenting cell marker HLA-DR was also detected at high levels on isolated EV, in agreement with their DC origin (Fig. 1c), while the endoplasmic reticulum marker calnexin was absent from EV (Fig. 1d). Overall, our data indicate that the EV population isolated was highly enriched in exosomes.

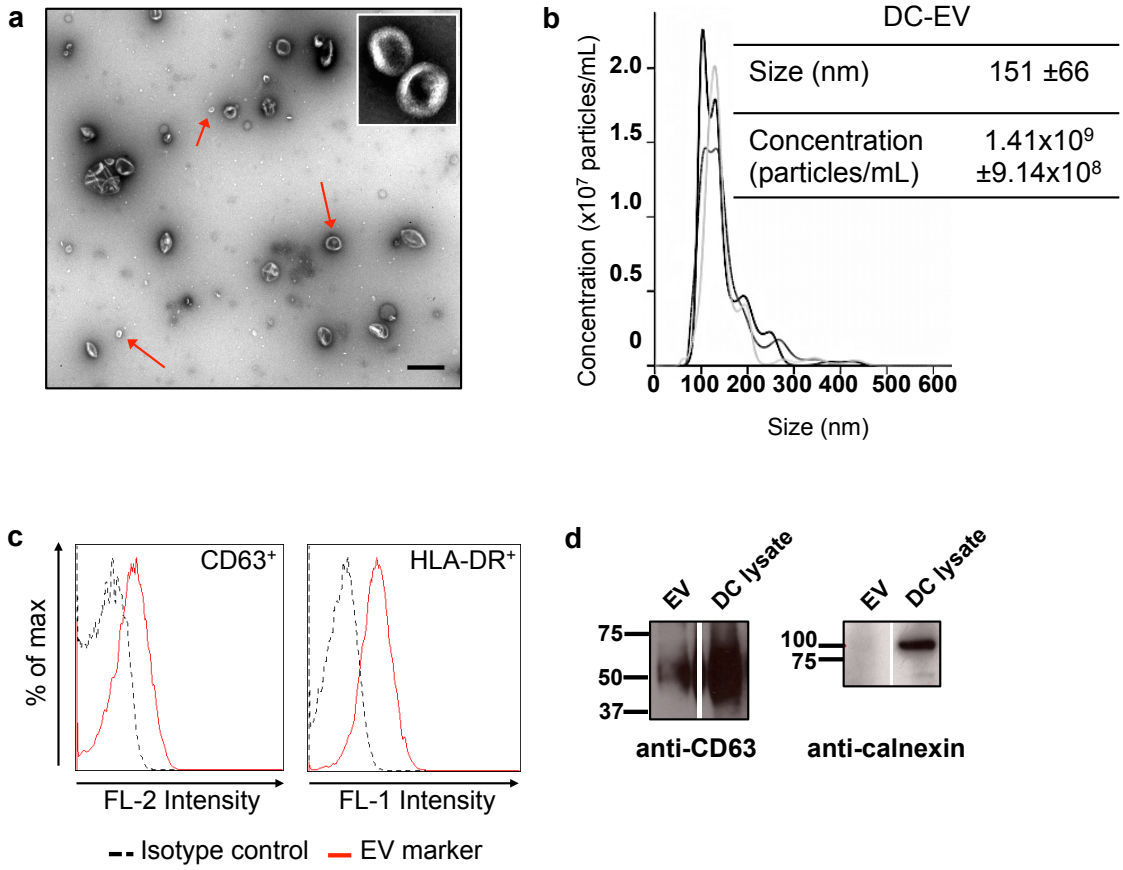

Figure 1. Characterization of exosome-enriched EV isolated from human monocyte-derived dendritic cells cultures. (**a**) Morphology of the isolated EV as imaged by transmission electron microscopy. Red arrows point to some EV of different sizes (scale bar: 500 nm). Image inset shows imaged EV in finer detail, at a magnification of 100,000x. (**b**) Characterization of concentration versus size distribution of isolated EV, by nanoparticle tracking analysis. Plot is representative of EV size and concentration analysis with NanoSight NS300. (**c**) Representative plots of conventional flow cytometry for the identification of the exosomal marker CD63 and the antigen-presenting cell marker HLA-DR on DC-derived EV, after immunostaining of DC-EV coupled to beads with CD63-PE and HLA-DR-FITC antibodies. (**d**) Representative western blots for the detection, as indicated, of the exosomal marker CD63 (non-reducing conditions) in isolated EV and DC whole lysates, and the endoplasmic reticulum marker calnexin (reducing conditions). Full gel scans are available in Supplementary Fig. S5. For all techniques n ≥ 3.

1.2.2. DC-derived EV are internalized by MSC without negatively impacting MSC proliferation or differentiation

The most ascribed functions of DC-derived EV (DC-EV) are related to immune responses. Here we investigated their potential role in linking inflammation with repair, by interacting with MSC and influencing their behaviour.

First we examined the capacity of DC-EV to interact with MSC, by labelling them with the lipophilic dye PKH26 before co-culturing with MSC. Upon incubation with cells, labelled DC-EV could be detected inside MSC, suggesting their internalization (Fig. 2a, left panel).

Importantly, to preclude that PKH26 micelles or other form of labelled structure could lead to artefacts in internalization detection, both protein pellets from ultracentrifuged cell-free media and PBS (EV vehicle) were labelled in the same way and incubated with MSC, leading to no detectable staining inside the cells, even upon 6 h of incubation (Fig. 2a, right panel).

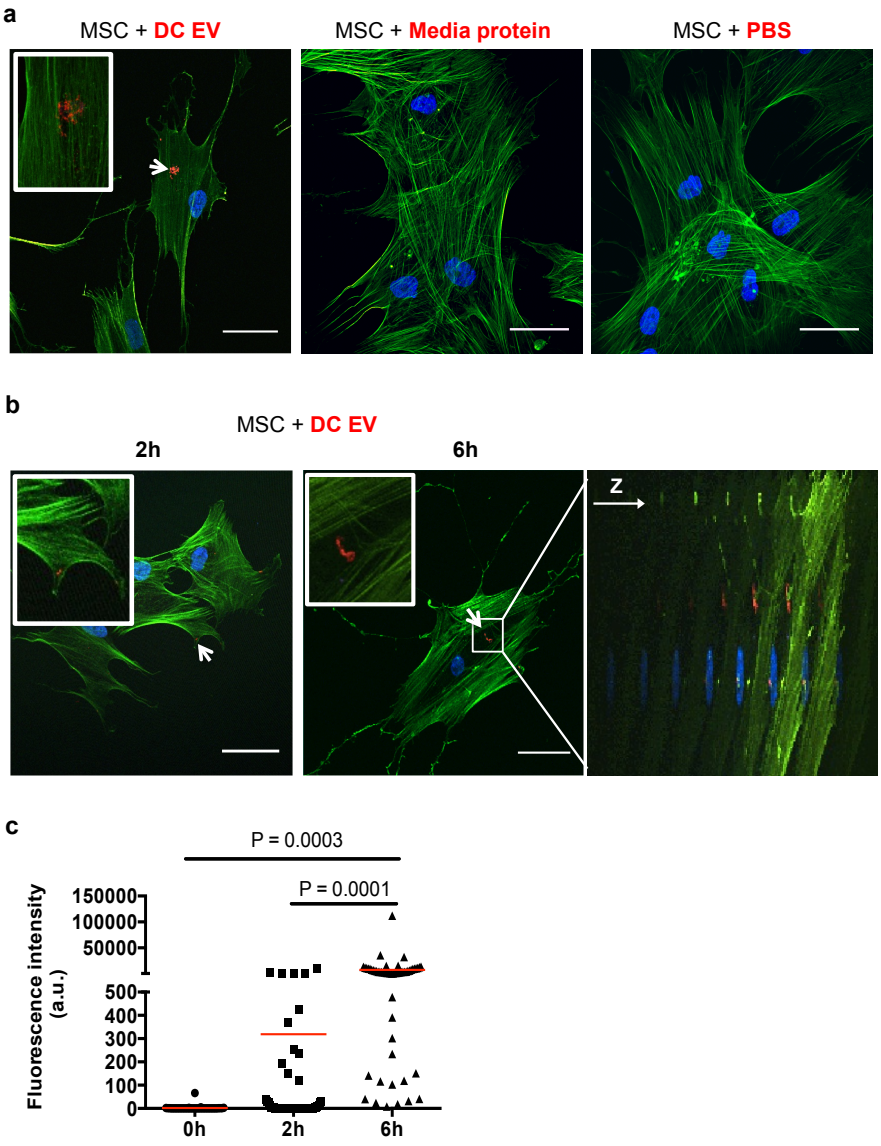

Figure 2. DC-derived EV are internalized by MSC. DC-derived EV were labelled with the lipophilic dye PKH26 before being co-incubated with MSC. Cells were labelled for actin cytoskeleton in green and nuclei in blue. (**a**) MSC with internalized red-labelled EV after 6 h of co-incubation (left panel). Specificity of EV labelling and internalization were accessed adding to MSC protein pellets of ultracentrifuged cell-free media (middle panel) or PBS (right panel) red-labelled as for EV, with no red staining detected. (**b**) MSC with internalized red-labelled EV after 2 h (left panel) and 6 h (middle panel) of co-incubation. Right panel shows the z-axis of an image stack, at 6 h. White arrows point to internalized EV, which are highlighted in picture insets. Z-stacks of images were acquired by laser scanning confocal microscopy and processed on Fiji software. Scale bar: 50 µm. (**c**) Quantification of DC-EV internalization by measuring the fluorescence levels (a.u. = arbitrary units) of the red channel in cell ROIs previously defined according to phalloidin-AF488 staining. Red dash represents the mean and p-values are indicated as determined by 2-way ANOVA, followed by uncorrected Fisher's multiple comparisons testing.

Following the kinetics of DC-EV uptake, after 2 h of co-incubation, internalized DC-EV start to be detected inside MSC, in small scattered dots, close to the cell membrane (Fig. 2b, left panel). After 6 h of incubation, abundant DC-EV could be detected inside MSC, mostly clustered and well inside the cell cytoplasm, as evidenced by the z-direction panel (Fig. 2b, right panel). The amount of fluorescence detected inside cells was quantified, showing a significant increase in the levels of fluorescence for internalized EV at 6 h of co-incubation, when compared to control and to 2 h of co-incubation (Fig. 2c).

To determine if DC-EV had an impact on MSC metabolism, we exposed the cells to EV, total conditioned media (CM) or the EV-depleted 100K supernatant (UC control), and monitored their metabolic activity for up to 14 days. The results obtained show no significant differences in MSC metabolic activity, in presence of DC-EV or the 100K supernatant (Fig. 3a). To further confirm these results, we evaluated MSC proliferation using the Ki67 marker. Results are illustrated in Fig. 3b and show dividing cells labelled for Ki67 in the nucleus. Quantification of proliferating MSC across different experiments showed no significant reduction of MSC proliferation in presence of DC-EV (Fig. 3c).

Next, we tested if DC-EV influenced MSC fate decision, inducing higher osteogenic or chondrogenic differentiation. At the gene expression level, results obtained indicate that EV stimulation promoted a slight increase in the expression of ALP and RUNX2 genes (osteogenic lineage markers), and also SOX9 (chondrogenic lineage marker), relatively to unstimulated MSC, at 6 h after stimulation (Fig. 3d). However, this increase was non-significant and below 1.5 times, much lower than promoted by osteogenic stimulation. Moreover, DC conditioned media, and even EV-producing media that was never in contact with cells (Media; contains EV-depleted FBS and DC-differentiating cytokines), promoted a higher increase in ALP and RUNX2 expression (Fig. 3d). To clarify these results, we cultured MSC in presence of the different stimuli for 14 days and looked at the ALP activity staining. Representative images are shown in Fig. 3e and confirm the results obtained by gene expression, with more ALP activity in presence of DC-EV than in basal conditions, but less than osteogenic stimulation. Also here DC conditioned media was capable of stimulating ALP activity to comparable levels as DC-EV (Fig. 3e). Together, these results indicate that any factors that may interfere with MSC osteogenic differentiation were not preferentially associated with DC-EV.

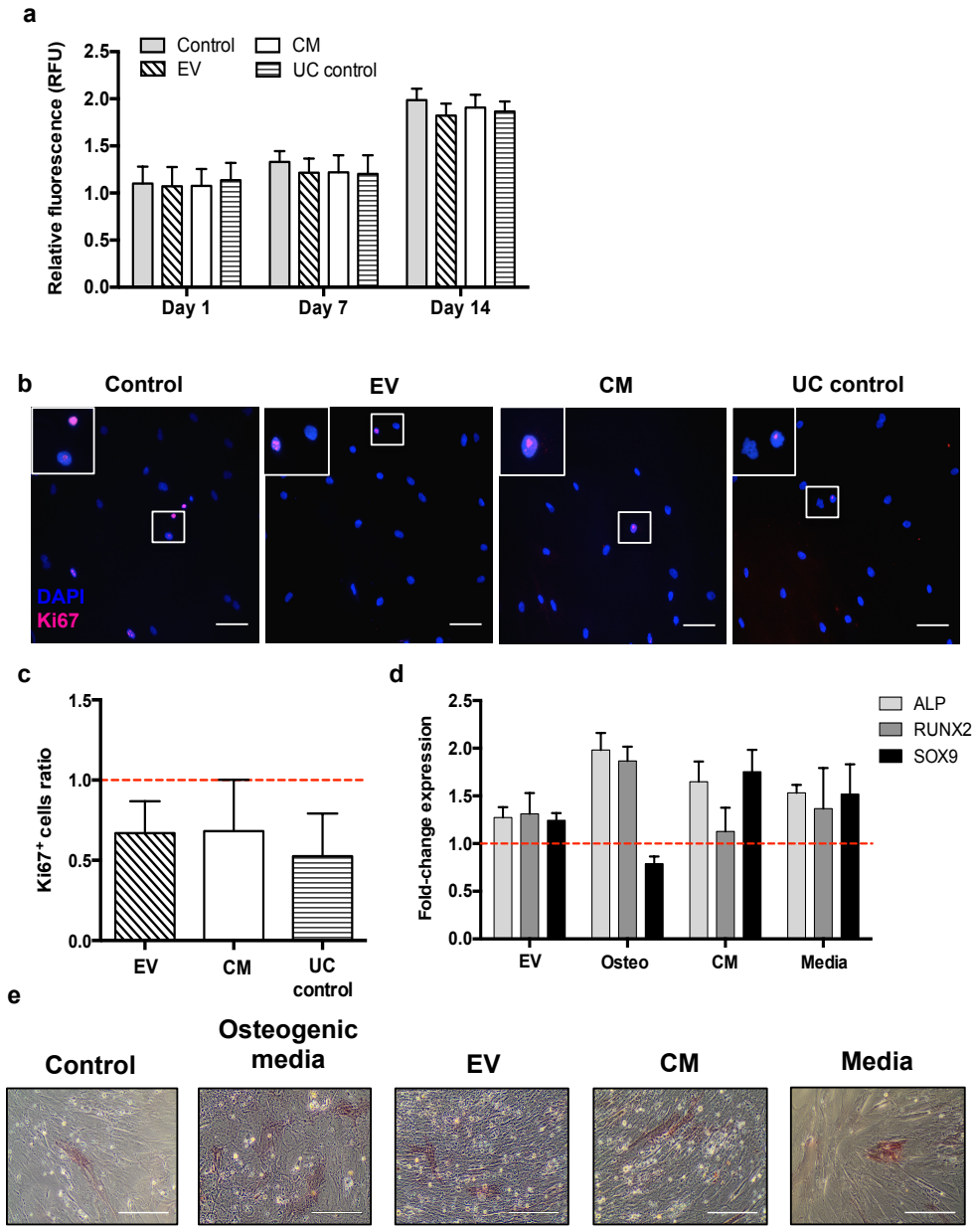

Figure 3. Stimulation of MSC with DC-derived EV is not cytotoxic and do not affect significantly their proliferation nor differentiation capacity. (**a**) MSC were incubated alone, with DC-derived EV (10 µg of protein), total conditioned media or 100 K ultracentrifugation supernatant (UC control) for 14 days. At days 1, 7 and 14, MSC were incubated with resazurin and conversion to resorufin was determined by fluorescence measurement (RFU = relative fluorescence units). Relative fluorescence at each timepoint normalized to day 0 is depicted. (**b**) MSC were incubated alone (Control), with 50 µg protein from EV, total conditioned media (CM), or 100 K ultracentrifugation supernatant (UC control) for 24 h, before staining for the Ki67(-AlexaFluor 647; pink) proliferation marker and DAPI for nuclei (blue). Scale bar: 100 µm. (**c**) Stained cells were imaged by fluorescence microscopy in 20 random fields, the total number and the number of $Ki67^+$ cells were counted, and are represented as a ratio to non-stimulated control condition (dashed line) (n = 4 different DC donors). All bar graphs represent average ± SD. n = 3 different DC donors. (**d**) Fold-change gene expression by RT-qPCR (average ± SD) of osteogenic markers (ALP and RUNX2) and a chondrogenic marker (SOX9) of MSC stimulated with DC-derived EV, osteogenic media (osteo: supplemented with Dexamethasone $1x10^{-7}$ M, β-glycerophosphate $1x10^{-2}$ M and ascorbic acid $5×10^{-5}$ M), conditioned media (CM), or protein extracted from complete media (including cytokines) that was not in contact with cells (media), comparatively to MSC in basal conditions (dashed line; n = 2 different DC donors). (**e**) ALP activity staining (pink/red), associated with early osteogenic differentiation, of MSC cultured in the presence of the indicated stimuli for 14 days. Although ALP activity staining is similar in stimulated conditions, only osteogenic media lead to morphological changes of MSC characteristic their osteogenic commitment. Scale bar: 1 mm.

1.2.3. DC-derived EV promote MSC recruitment and migration

Since EV are secreted biological messages that can travel in body fluids, it is likely that they may participate in chemotactic processes and exert their function on cells located far from their origin. So, in order to investigate their potential to recruit MSC, different amounts of DC-EV were placed in the bottom of tissue culture wells and transwell inserts with seeded MSC were placed on top (Fig. 4a). This setup allows the establishment of an EV gradient from the bottom to the top compartment. The number of MSC that migrated in response to DC-EV stimuli or controls were quantified across 9 different DC donors. The results are presented as migration index relative to control, and clearly show that DC-EV were able to stimulate MSC recruitment in a dose dependent manner (Fig. 4b). A statistically significant increase in MSC migration was observed for as little as 10 µg of EV, when compared with control conditions (Fig. 4b). On the other hand, the same amount of protein from total DC conditioned media (CM) or EV-producing media that had not been in contact with DC (containing EV-depleted FBS and DC-differentiating cytokines), did not recruit significantly more MSC than the negative control (Fig. 4b), further confirming the specificity of MSC recruitment by DC-EV. As a control for the effect of gradient on MSC migration, 50 µg of EV were equally distributed on the top and bottom compartments of the transwell migration system (EV No gradient), disrupting the chemotactic EV gradient. In these conditions, MSC migration was similar to CM control conditions, and lower than in the presence of a gradient, for the same amount of EV (50 µg, EV Gradient), indicating some chemotactic role for EV on MSC migration.

To further validate the capacity of EV to promote MSC migration, 10 µg of DC-EV were added to a microfluidic chamber, and the migration pattern of MSC seeded across the EV gradient was followed by time-lapse video microscopy (Fig. 4c, Supplementary Fig. S1 and Supplementary Video S1). Different parameters of cell migration were then determined by image analysis (Fig. 4d–g). Under the influence of 10 µg of EV gradient, the length of the cell migration pathways was statistically significantly higher than for UC or negative control (Fig. 4d). This result confirmed the previous transwell assay results on the capacity of DC-EV to specifically promote MSC migration, in doses as low as 10 µg.

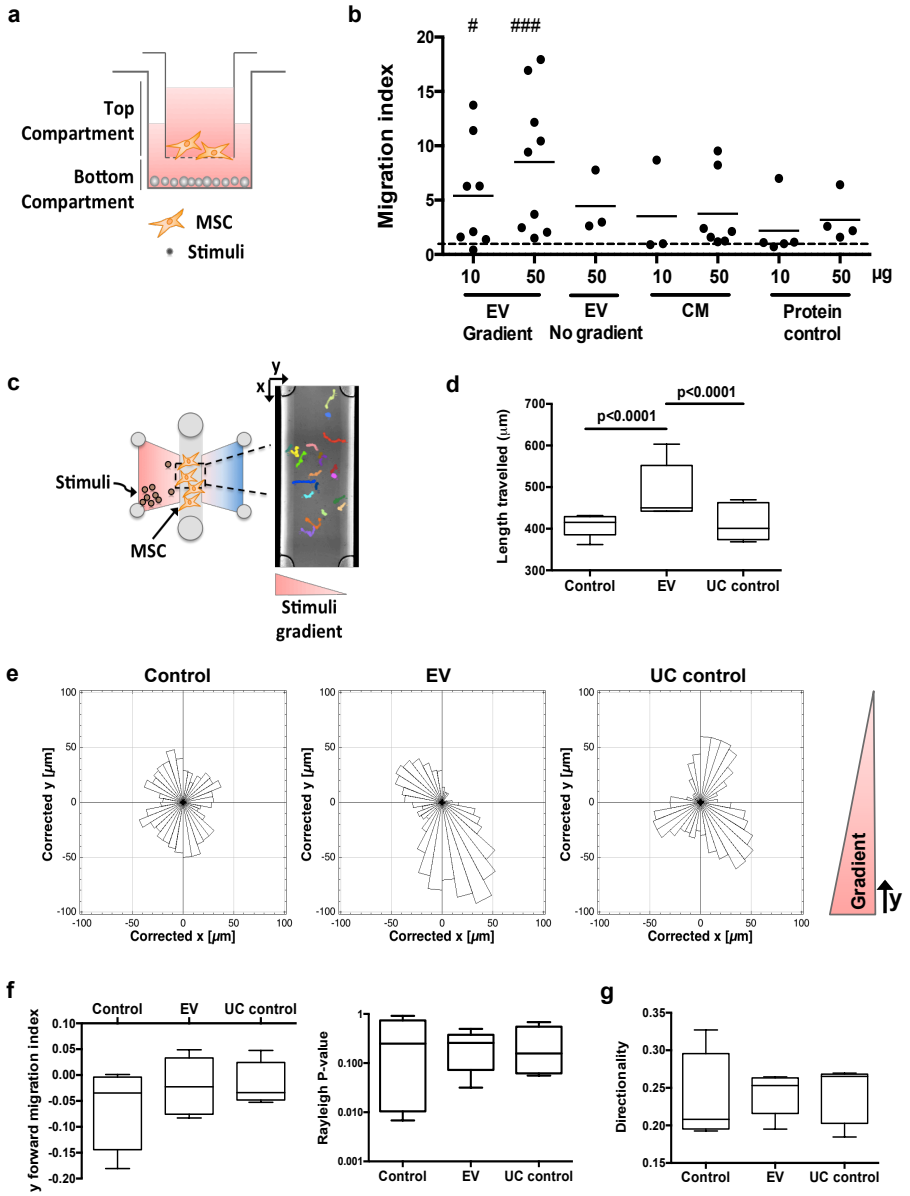

Figure 4. DC-derived EV specifically promote MSC migration. (**a**) Representation of the transwell system set up for MSC recruitment assay. EV or control stimuli were added to the bottom compartment, acting as chemoattractants for MSC seeded inside the gelatin-coated transwell insert. For gradient disruption, 50 µg EV were diluted in media that was then equally distributed in the top and bottom compartments (no gradient). (**b**) MSC were allowed to migrate for 8 h, and then stained with DAPI. Total number of migrated cells adhered to the outer surface of the insert membrane were counted and migration index calculated to non-stimulated control. The dashed line represents the migration index of non-stimulated control. Each dot represents an independent experiment (n = 3 to 9 DC donors) and the mean value of cells migrated is indicated as a dash. For statistical analysis Kruskal-Wallis test, followed by uncorrected Dunn's multiple comparison test was used, $^{\#}P = 0.0152$ and $^{\#\#\#}P = 0.0002$, compared to non-stimulated control. (**c**) Representation of the chamber setup for MSC chemotaxis assay. Stimuli were added on the left side of the chamber and a gradient was formed across the central channel, where MSC were seeded (n = 5 DC donors). For each condition, MSC were followed for 13 h by time-lapse video microscopy, and cell pathways were then determined by manual tracking, as represented by the coloured tracks overlay on the exemplificative central channel image. See also Supplementary Fig. S1. (**d**) Box plots represent total length of the pathways travelled by MSC. Statistical analysis used 2-way ANOVA, followed by uncorrected Fisher's multiple comparison test, p-values are indicated. (**e**) Representative rose diagram of MSC direction of migration (x and y position normalized for cell's initial position). (**f**) MSC forward migration index in gradient y-direction (left panel), and p-value of cell direction uniformity as determined by Rayleigh test (right panel). (**g**) Directionality of MSC migration. All box plots are min to max, with median value indicated.

Analysing the directionally of MSC migration in response to the gradient, despite the tendency for MSC to migrate in direction of EV (y coordinates < 0) comparing to control, or 100 K supernatant (UC control) (Fig. 4e), there were no statistically significant differences for this DC-EV concentration, as quantitatively assessed by the forward migration index across the y axis (yFMI; axis of gradient establishment), and the p-value of the Rayleigh test for uniformity of the pathways followed by MSC (Fig. 4f). Similarly, while directionality of MSC migration was slightly higher for conditions with stimuli (EV and UC control), it was not significantly different from control conditions (Fig. 4g).

1.2.4. DC-derived EV are rich in mediators that can promote cell migration

Considering the chemotactic action of DC-EV on MSC, we then investigated their protein content. We focused on the analysis of cytokines, chemokines and metalloproteinases, as they are some of the most important modulators of cell migration. An antibody array was used to screen the content of DC-EV. Total conditioned media was used for comparison, in order to obtain the molecules that are enriched inside EV (Supplementary Fig. S2 shows an original array image). The results obtained are illustrated in Fig. 5a, and indicate that some mediators are much enriched and others much depleted in DC-EV. Interestingly, IL-5, osteopontin (OPN), FGF-7 and MCP-1 appeared highly enriched in EV (fold-change >2), while IP-10, IL-6, CXCL1 and IL-4 were depleted from EV preparations (fold-change <−1.5).

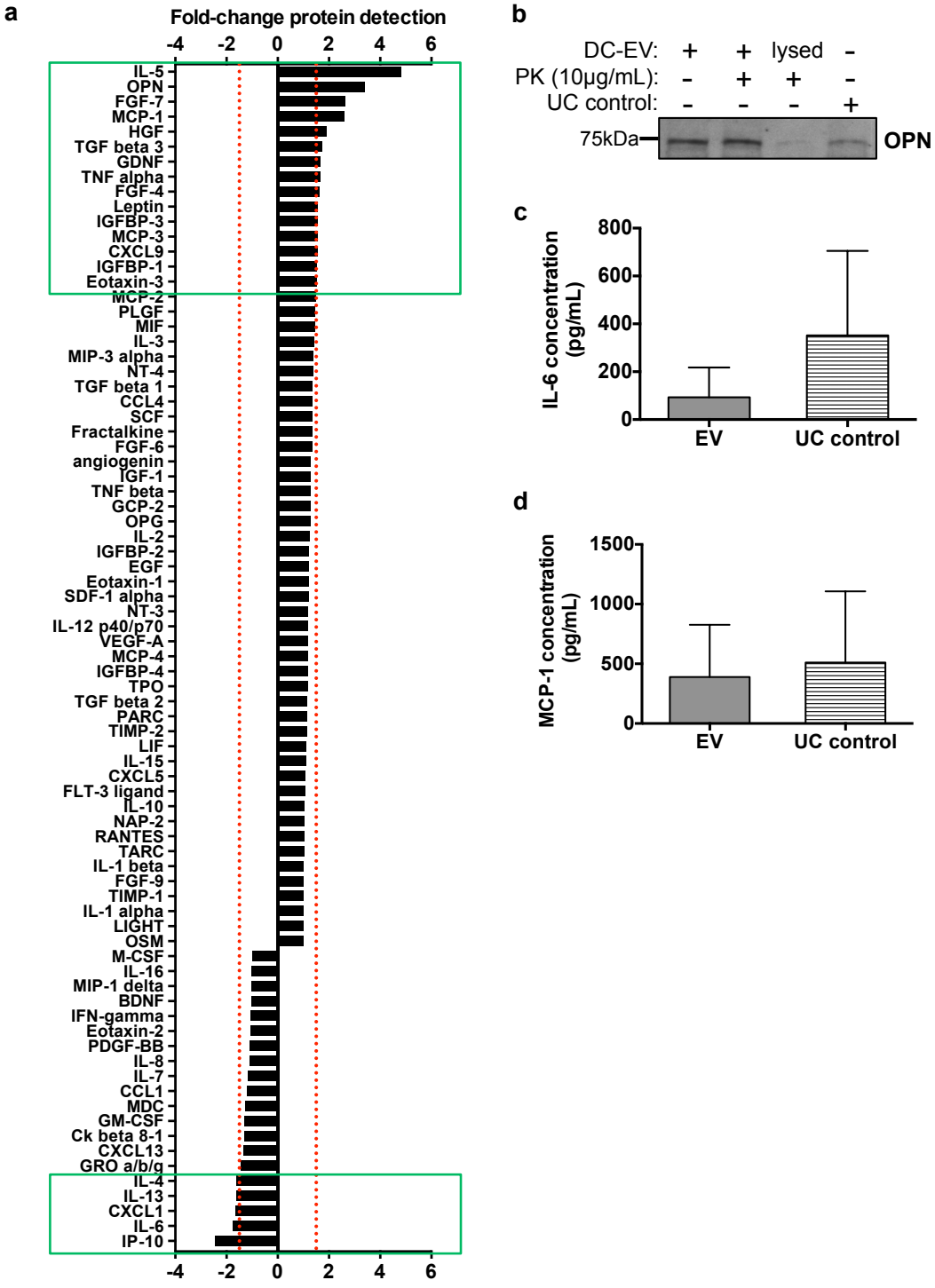

Figure 5. DC-derived EV are enriched in mediators with reported chemoattractant properties. (**a**) Chemokines and growth factors enriched/depleted in DC-EV comparatively to DC conditioned media, as assayed by antibody array. Red dashed lines represent the fold-change thresholds of 1.5 and −1.5. Mediators with an absolute fold-change ≥1.5 are highlighted by the green box. See also Supplementary Fig. S2. (**b**) Detection of osteopontin (OPN) in the DC-derived EV fraction (DC-EV) and 100 K supernatant (UC control) by western blot. Proteinase K (PK) digestion, before or after vesicle lysis, were performed as indicated. Full gel scan is available in Supplementary Fig. S5. Relative concentration (average ± SD) of (**c**) IL-6 and (**d**) MCP-1 in DC- derived EV (after proteinase K digestion) and 100 K supernatant (UC control), as determined by ELISA. For all assays, EV, 100 K supernatant and conditioned media from 3 different DC donors were pooled together.

The array results were validated by alternative approaches for chemokines selected from those most differently enriched or depleted in DC-EV (Fig. 5b–d). When probing DC-EV for osteopontin the results obtained confirm the array data, showing an enrichment in EV, relative to the 100 K supernatant (UC control). Moreover, the results indicate that osteopontin was present inside DC-EV, as it was detectable even after EV treatment with proteinase K (Fig. 5b). Importantly, when EV were lysed before proteinase treatment, osteopontin was not detectable, as it is no longer protected from degradation (Fig. 5b). ELISA assays confirmed that IL-6 was highly depleted from DC-EV, in comparison with the 100 K supernatant (Fig. 5c). The enrichment of MCP-1 in EV, at least relatively to the 100 K supernatant, could not be confirmed (Fig. 5d). The observed similar concentration of MCP-1 in EV and 100 K supernatant (UC control) suggests that it may be secreted in both fractions, *i.e*. as a soluble mediator, and associated with EV.

Our previous results indicated that MMP-9 was produced abundantly by DC and could be involved in MSC recruitment by these cells[17]. Thus, we first determined if MMP-9 and MMP-2, the two most common gelatinases, were present in media collected from the transwell migration assays described in the previous section (Fig. 4a and b). As shown in Fig. 6a, when DC-EV were added to the bottom compartment, a clear increase in the levels of both proenzyme and active MMP-9 was detected. This pattern was replicated in media from the top compartment, but with lower amounts of MMP-9, as expected. MMP-9 could also be found in total DC conditioned media, but not in media that had not been in contact with these cells. On the other hand, MMP-2 was only detected when MSC were present, mainly in the top compartment and at relatively constant levels in all conditions, indicating that it is likely produced by MSC.

Knowing that MMP-9 was present in the EV fraction we then proceeded to investigate if MMP-9 was inside EV, by two independent approaches. First, we stained for MMP-9 and detected it by imaging flow cytometry (Fig. 6b, bottom panel), in a pool of permeabilized DC-EV coupled to latex beads. A representative cytometry profile is illustrated in Fig. 6b (top panel) and shows that DC-EV stain for MMP-9 at higher levels than isotope controls or MMP-9 stained beads that had been incubated with the 100 K supernatant (UC control), which indicates specific MMP-9 staining. However, this could not preclude that MMP-9 could pellet in the ultracentrifugation, or be associated with the surface of EV. So, in order to clarify if MMP-9 was indeed inside the vesicles, gelatin zymography of undigested versus proteinase K-digested EV was performed. Results are illustrated in Fig. 6c and show that active form of MMP-9 was present in EV, and still detectable after proteinase K digestion, but was no longer detectable when EV were disrupted before proteinase digestion. Interestingly, the pro-MMP-9 form, most abundant in 100 K supernatant, but also detectable in the EV fraction,

was not resistant to proteinase K digestion, indicating that it may be extraexosomal, or be associated on the outer side of EV.

Together these results show that DC-EV are enriched in chemotactic mediators and MMP-9, all likely contributors to MSC migration.

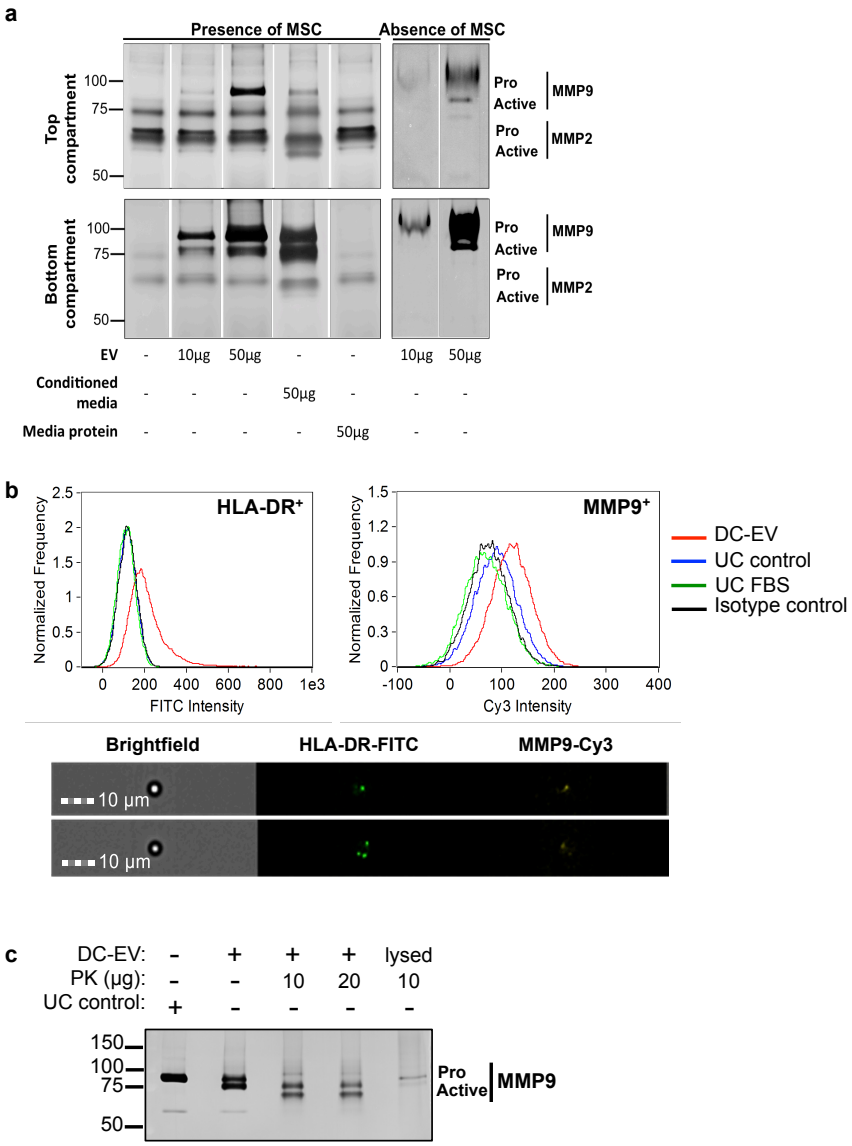

Figure 6. DC-derived EV contain MMP-9 capable of promoting MSC migration. (**a**) Detection of MMP-9 and MMP-2 activity by gelatin zymography in media collected from transwell assays. Cell culture media from top and bottom compartments of MSC recruitment transwell assays was collected separately. Protein was precipitated from these media (Presence of MSC) and from DC-EV pellets (Absence of MSC), resolved by gelatin zymography, and proteolytic activity was detected as clear bands on Coomassie Brilliant Blue stained gels. Bands corresponding to the proenzyme and the active form of each MMP are indicated at the corresponding molecular weight next to gels images. (**b**) Representative imaging flow cytometry plots (top panel) and images (bottom panel) of MMP-9 and HLA-DR detection in DC-derived EV coupled to beads. Beads with EV were stained with HLA-DR-FITC antibodies before being permeabilized and stained using anti-MMP-9 followed by Cy3-secondary antibodies, being detected as defined dots in the periphery of the beads. (**c**) Gelatin zymography detection of MMP-9 in DC-derived EV (DC-EV), and the 100 K ultracentrifugation supernatant control (UC control). Proteinase K (PK) digestion, with or without previous vesicle lysis, were performed as indicated. Pro-MMP-9 and active MMP-9 are indicated at the corresponding molecular weight. Full gel scans are available in Supplementary Fig. S6.

1.3. Discussion

In the current study we investigated for the first time the influence of DC-EV on different aspects of MSC biology, in particular their migration, and related that effect with EV content.

In order to improve the regenerative processes there is a need to enhance our understanding on the coordination of the different stages of repair. In particular the signals that contribute to the articulation between inflammatory and repair phases are crucial, as reduced or exacerbated inflammatory responses can be profoundly deleterious to repair[20].

Cells can secrete a variety of EV with different functions, namely exosomes, microvesicles and apoptotic bodies, distinguished by their size, composition, biogenesis pathway and function[4]. So, we first characterized the population of EVs isolated by differential (ultra)centrifugation from DC cultures, according to the minimal criteria parameters recommended by the International Society of Extracellular Vesicles, in order to confirm their enrichment in exosomes[21]. Overall, morphology and size distribution of the isolated vesicles, the presence of the endosomal protein marker CD63, and the absence of a protein marker characteristic of the endoplasmic reticulum (calnexin), indicate that our methodology allows, according to these criteria, the isolation of vesicle populations enriched in exosomes of endosomal origin. Moreover, although CD63 has been described in other sub-populations of DC-EV in a very recent study[22], the EV population isolated here is similar to the one described by those authors as the “100 k pellet”, both in terms of NTA profile and protein markers.

The mechanism of EV action likely depends on the way they interact with target cells. The main forms of interaction reported are EV-mediated activation of cell surface receptors, triggering signal transduction pathways; fusion with the cell membrane, releasing EV content to the cell cytoplasm; or internalization, delivering EV content already inside the cell[23]. Although other mechanisms of interaction cannot be ruled out, our results clearly show that MSC internalize DC-EV, which are found in clusters, well inside the cytoplasm of the cells. This vesicle distribution upon internalization was previously reported[19,24], suggesting an active endocytosis of the vesicles, that are incorporated in different compartments of the endocytosis pathway along MSC cytoplasm, and consequently lead to an active processing of the molecules they carry[23]. Despite the accumulation of DC-EV inside MSC no deleterious effects were observed in their metabolism, capacity to proliferate or differentiate towards osteogenic or chondrogenic lineages.

At the functional level, DC-EV appear to specifically promote MSC recruitment, as they did not impact significantly other aspects of MSC biology, such as their proliferation or differentiation. This action somehow correlates with the biological role of DC in

immunological surveillance, and their transient presence at injury locations. On the other hand, recent reports suggest that MSC-derived EV can promote their own osteogenic differentiation[25–29]. Also, EV derived from monocytes/macrophages and platelet lysates, were reported to stimulate MSC osteogenic differentiation[19,24], while osteoclasts secrete EV containing microRNA that inhibit MSC osteoblastic differentiation[30].

Of note, we had previously shown that DC interact with MSC, promoting their recruitment trough paracrine factors[17]. In this work, we demonstrate for the first time that the paracrine MSC migration by DC is mainly mediated by a functional exosome-enriched EV population secreted by DC. The EV-depleted 100 K supernatant was used to account for possible effects due to soluble proteins secreted by DC that could co-precipitate with isolated EV. Indeed, MSC migration increases with increasing quantities of chemoattractant DC-EV, which work even in the absence of the EV producing cells. A similar pattern of MSC migration was previously reported for comparable quantities of exosomes (5 µg and 50 µg) derived from human platelet lysates[24], but DC-derived EV appear able to promote higher recruitment of MSC across the transwell membrane. Another recent study indicates that iPS-derived MSC produce EV capable of promoting BM-MSC migration, in a wound closure assay[31]. The authors also report that iPS-MSC-derived EV enhance MSC differentiation and proliferation[31]. Importantly, DC-EV effect on MSC migration was specific for the isolated EV, and migration was not significantly promoted by the same amount of protein from any of the controls tested: (i) total conditioned media, which contains both EV and soluble mediators; (ii) ultracentrifuged protein pellets derived from cell-free EV-producing media, which contains FBS proteins (although EV depleted) and DC differentiating cytokines; or (iii) ultracentrifugation supernatant, which contains the soluble proteins secreted by DC but is depleted from EV. Furthermore, this effect was observed using two different assays, several independent DC donors and two independent MSC donors, supporting an overall new mechanism of MSC migration modulation by DC-derived EV.

Cell migration may be affected by changes to either their chemokinesis, the movement of cells without a defined direction due to the presence of a stimuli, and/or their chemotaxis, the directed movement of cells towards a given stimuli[32]. In previous reports, cell-secreted chemical mediators were shown to specifically induce higher chemokinesis on their target cell[33,34]. On the other hand, a recent study shows that exosomes play a crucial role in directional cell movement[35]. Although we could observe a tendency for MSC directional movement towards DC-EV, it did not reach statistical significance, likely due to the sensitivity of the assay for the gradient that was established with the small concentration of EV used (10 µg). In addition, the presence of an EV-directing gradient appeared to be important to promote MSC migration, since an equal amount of DC-EV without a gradient was not capable of significantly promoting MSC migration in the transwell system. Together,

our results indicate that the presence of EV likely has effects on both MSC chemotaxis and chemokinesis. Considering the reported capacity of MSC to migrate to injury sites[36], these effects of DC-EV could be important for MSC mobilization in a clinical context.

In order to further understand the mechanisms underlying the observed effect on MSC recruitment and identify candidate molecular mediators, we then explored the content of DC exosomes. Among the mediators mostly enriched in DC exosomes, OPN and MCP-1 were identified. The enrichment of OPN inside DC-derived EV was confirmed by Western Blot, which also showed that proteinase K was not able to degrade OPN, while it was protected by EV. Importantly, the secretion of OPN greatly increases in injury situations, and different studies suggest its capacity to promote MSC migration. Indeed, similar quantities of recombinant OPN were shown to promote human MSC migration in chemotaxis chambers to a level just slightly lower than in our study[37], an effect also demonstrated in rat bone marrow-derived MSC[38]. In addition, some studies have described intracellular roles for OPN. An early study with foetal rat calvaria stromal cells found an association between increased cell migration and increased levels of intracellular OPN, which accumulated close to the cell membrane, together with CD44[39]. These findings were later confirmed for murine osteoclasts[40] and macrophages[41]. Thus, it is possible that OPN inside DC-EV may modulate MSC migration upon EV internalization, without the need for its binding to a receptor on MSC surface.

On the other hand, MCP-1 enrichment in DC-derived EV comparing to ultracentrifugation supernatant could not be confirmed by ELISA, as it was found in both at similar levels. Nonetheless MCP-1 appeared to be carried inside the vesicles or may be associated on their surface. Contradictory roles for MCP-1 effects on human MSC migration have been reported. Dwyer *et al*. demonstrated a dose-dependent increased migration of iliac crest-derived MSC in the presence of recombinant MCP-1 and conditioned media from breast tumor cells, which was rich in this chemokine[42]. On the other hand, Ringe *et al*. stated that MCP-1 had no chemotactic effect on iliac crest-derived MSC. However, among the three MSC donors those authors tested, one does not express CCR2 (MCP-1 receptor), and the other two show migration in the absence of a chemoattractant to be the highest among all the experimental conditions[43]. Most interestingly, this chemokine was also demonstrated to promote murine adult bone marrow-derived MSC migration into the heart of transgenic mice specifically overexpressing MCP-1 in this organ, whereas only trace amounts of cells were found in other organs[44]. This work also reported HGF as a potent MSC chemoattractant, a growth factor also enriched in the DC-derived EV reported here.

Of note, SDF-1 was not found at high levels inside DC-derived EV. This chemokine is often considered the gold standard mediator of stem cell migration, including MSC, and in particular in injury pro-inflammatory environments[45,46,] and thus our results support the

relevance of alternative mediators for MSC migration. Also cytokines, such as IL-6, and IL-4 and GM-CSF (both added exogenously for DC differentiation) were found to be depleted inside EV, thus providing an important control for EV purity and specificity of action.

In addition to chemoattractant mediators, we also studied the contribution of metalloproteinases, namely gelatinases, to MSC migration in our *in vitro* model. MMPs are a family of secreted enzymes that are described to promote cell migration and invasion via degradation and remodelling of extracellular matrix components. However, they can potentially also have intracellular activity, as they are able to cleave several intracellular proteins, including cytoskeletal proteins[47], although the functional outcome of such processes is not yet completely uncovered. Our previous results suggested a role for MMP-2 and MMP-9 in MSC recruitment by DC[17]. In agreement with those results, we found an increase in MMP-9, namely pro-MMP-9, in media of the transwell migration experiments, when DC-derived EV were present, and detectable MMP-2 only when MSC were present. However, in this setup we could not confirm the cell origin of MMPs, since MSC secrete higher levels of MMPs upon stimulation with different cytokines[48]. Thus, we further tested the presence of MMP-9 inside DC-derived EV. The presence of MMPs in EV, namely MMP-2 and MMP-9, has been previously described for several cell populations, including neutrophils[49] and MSC[50]. Our results indicate that the EV fraction is positive for MMP-9, as detected by flow cytometry. Moreover, Western blot analysis confirmed that active forms of MMP-9 were found inside EV, as they were resistant to proteinase K digestion, while pro-MMP-9 was likely mainly extraexosomal, either soluble or associated with vesicles membrane. Thus, EV contain functional MMP-9 that can contribute to degrade the gelatin coating of the transwell inserts, facilitating MSC migration. Interestingly, MMP-9 is also able to cleave osteopontin into fragments with different biological activity, some of which particularly prone in the promotion of cell migration and invasion, as demonstrated *in vitro* for hepatocellular carcinoma cells[51]. Although these were amongst the most represented molecules in our screening, we cannot rule out that other chemotactic mediators contained in EV could be responsible for the increased MSC migration. Further clarifying this would require knock-down experiments evaluating the molecule or combination of molecules without which migration in response to DC-EV could no longer be observed. The DC-derived EV population enriched in exosomes constitutes nanosized carriers, likely containing several chemotactic mediators, some of which able to interact with each other in order to promote enhanced cell migration. This supports a local role for EV in promoting healing, which could be manipulated both by modulating the cargo and secretion of EV derived from the immune cells, or by the delivery of exogenous EV.

In the context of future new regenerative therapies development, we here demonstrate the capacity of EV from naïve DC to promote MSC migration, which could be

exploited for the local recruitment of either endogenous or transplanted cells towards injury sites. Also, the potential for EV from different cell populations to lead MSC to perform different functions, should be explored. This could support time-controlled changes to the injury microenvironment, that can enhance the regenerative response. Further studies addressing the use of EV in promoting tissue repair are granted and necessary to explore their full potential.

1.4. Methods

ETHICAL APPROVAL

Human biological samples used in this work and all the procedures followed to obtain them were conducted according to the principles of the Declaration of Helzinki. Surplus buffy coats for monocytes isolation were from healthy blood donors and donated by Immunohemotherapy Department of Centro Hospitalar de São João (CHSJ, Porto, Portugal). MSC were isolated from discarded human bone marrow tissues of patients undergoing total hip arthroplasty[18], and obtained from the Orthopaedics Department of CHSJ. All blood donors and patients gave informed written consent. Experimental protocols were approved by CHSJ Ethics Committee for Health. No information on age, sex, or any other identifying element was provided to the researchers, so all samples were analysed anonymously.

MSC ISOLATION AND CULTURE

MSC were isolated from bone marrow tissue of femur or tibia of orthopaedic patients as previously reported[18]. MSC identity was confirmed according to the criteria defined by the International Society for Cellular Therapy[52]. For each experiment, MSC were cultured in MSC growth medium - low glucose Dulbecco's modified Eagle's medium (DMEM) + Glutamax (Invitrogen), supplemented with 10% heat inactivated MSC-compatible Foetal Bovine Serum (FBS, HyClone™ or Gibco™) and 1% penicillin G-streptomycin (P/S; Invitrogen). Cells were maintained in a humidified incubator, at 37 °C and 5% CO_2. MSC from two different donors were used, from passage 5 to 9.

DC DIFFERENTIATION AND EV PRODUCTION

Monocytes were isolated from buffy coats of healthy blood donors using RosetteSep™ human monocyte enrichment antibody cocktail (STEMCELL Technologies), as previously described[53]. DC cultures were set up with 60–100×10^6 human peripheral blood monocytes, plated at 20×10^6 cells per 100 mm diameter petri dishes, in DC differentiating

media - RPMI-1640 + Glutamax (Invitrogen) cell culture media, with 10% heat inactivated FBS (Lonza) and 1% P/S (Invitrogen), further supplemented with 50 ng/mL IL-4 and GM-CSF (Immunotools). After 4 days of differentiation, cells were washed with PBS and re-cultured for 3 additional days in EV producing media. This media was obtained from ultracentrifuged (100,000×g, for 2 h at 4 °C) RPMI supplemented with 10% FBS, which was then diluted to 1% FBS, and further supplemented with 1% P/S, IL-4 and GM-CSF, as above. Absence of bovine-EV in the ultracentrifuged RPMI + 10% FBS media was confirmed by transmission electron microscopy (Supplementary Fig. S3). Cells were maintained in a humidified incubator, at 37 °C and 5% CO_2. DC cultures in EV-producing media were monitored by optical microscopy and no changes in cell morphology or general culture condition were observed (Supplementary Fig. S4).

EV ISOLATION

DC-derived EV were isolated from cell culture media by differential centrifugation: cell culture media was collected and centrifuged at 2,000×g, for 20 min, at 4 °C; the supernatant was recovered and centrifuged again, now at 10,000×g, for 20 min, at 4 °C; the supernatant was again recovered and ultracentrifuged, at 100,000×g, for 2 h, at 4 °C, in a Beckman Optima L80-XP ultracentrifuge. EV pellets were resuspended in PBS and supernatant (100 K supernatant; UC control) collected, and stored at −80 °C. A similar volume of EV producing media, that was never in contact with cells, was also ultracentrifuged as described above. Protein pellets from ultracentrifugation were resuspended in PBS and stored at −80 °C, as for DC-EV. For each EV or control sample, protein was quantified directly using *DC*™ Protein Assay (Bio-Rad), and the calculated protein concentration was used as a measure for functional assays.

EV MORPHOLOGY AND SIZE CHARACTERIZATION

EV morphology was analysed by transmission electron microscopy (TEM). DC-derived EV were adsorbed onto 400-mesh formvar nickel grids and then negatively stained with uranyl acetate 5% for 30 sec. Samples were imaged in a Jeol JEM 1400 electron microscope.

For size and concentration characterization of EV suspensions, 10 µg of DC-derived EV by nanoparticle tracking analysis (NTA) in a NanoSight NS300 with NTA3.0 software. Size distribution and EV suspension polydispersity was further characterized by dynamic light scattering (DLS) in a Zetasizer Nano ZS device.

FLOW CYTOMETRY ANALYSIS OF EV

For flow cytometry analysis, EV were first coupled to 4 µm size aldehyde/sulphate latex beads 4%(w/v) (Invitrogen). Sonicated beads were incubated with DC-derived EV at 1:1 ratio (vol:protein quantity), first for 1 h at room temperature, and then overnight at 4 °C, under mild agitation. Protein pellets from cell-free EV producing media processed as above or 100 K supernatant were adsorbed to beads for negative controls. Functional groups remaining on the beads were blocked by incubation with glycine 100 mM for 30 min at room temperature, under mild agitation. EV were immunostained with anti-human CD63 PE-conjugated, or anti-human HLA-DR FITC-conjugated antibodies (all from Immunotools), and then fixed with paraformaldehyde 4%(w/v). For MMP9 detection, bead-coupled EV were fixed, permeabilized (Triton X-100 0.2%(v/v), 5 min), and incubated with primary antibody (Abcam), followed by Cy3-conjugated secondary antibody. Immunostaining with matching isotypic antibodies were used as control. Samples were analysed on a FACS Calibur flow cytometer (BD Biosciences) or ImageStream®X imaging flow cytometer (Amnis, EMD Millipore). Beads with coupled EV were gated according to forward and side scatter parameters with BD CellQuest software (BD Biosciences) or Inspire software (Amnis, EMD Millipore), respectively, and 20,000 events were recorded. Data was analysed using FlowJo Software (TreeStar, Inc) or IDEAS software (Amnis, EMD Millipore), respectively.

WESTERN BLOTTING

Protein content of DC lysates, EV pellets and 100 K supernatants was quantified as above and the same amount of protein (7 µg) prepared in non-reducing (for CD63) or reducing (for calnexin and osteopontin) conditions. Samples were denaturated at 65 °C for 15 min, resolved on SDS-PAGE 10% polyacrylamide gels (Bio-Rad), and then transferred to nitrocellulose membranes. Membranes were blocked and probed overnight with the primary antibody anti-human CD63 (BD Pharmingen™), anti-human calnexin (Abcam) or anti-human osteopontin (Rockland), followed by HRP-conjugated secondary antibody (GE Healthcare). Membranes were incubated with ECL substrate and chemiluminescent signal detected upon exposure to autoradiographic films (all from GE Healthcare). Full scans of the films are available in Supplementary Fig. S5.

EV INTERNALIZATION ASSAY

DC-derived EV were labelled with PKH26 0.5 µM dye (Sigma-Aldrich), for 5 min, at 37°C, and free dye washed in VivaSpin® centrifugal columns (10 kDa cut-off). Labelled EV were co-cultured with MSC (1.5×10^3 cell/mL). Pellets of cell-free EV producing media ultracentrifuged as above, and PBS were labelled with PKH26 and used as negative controls. At different timepoints, cells were fixed with paraformaldehyde 2%(w/v), stained

with FITC-conjugated phalloidin (Life Technologies), and mounted on slides with fluoroshield with DAPI. Images were acquired by laser scanning confocal microscopy (Leica TCS SP2) using the same settings for all conditions tested, and Z-stack images analysed using Fiji software. For internalization quantification, cells were defined as ROI according to phalloidin staining (green channel) and then fluorescence intensity PKH26 (red channel) determined on Fiji software.

MSC PROLIFERATION ASSAYS

MSC (20×10^3 cells/mL) cultured in EV-free media were stimulated for 24 h with 50µg of indicated stimuli. MSC were then immunostained for Ki67 (ThermoFisher Scientific), followed by fluorochrome-conjugated secondary antibody, and mounted with fluoroshield with DAPI. Secondary antibody alone was used as negative control. Images from 20 random locations were acquired and analysed using Fiji software for number of nuclei and $Ki67^+$ cells counting.

Cell proliferation over time was assessed indirectly with resazurin cell viability assay according to manufacturer's protocol. Briefly, MSC (1.5×10^4 cells/mL) cultured in EV-free media were stimulated with 10 µg DC-derived EV or appropriate controls, and at each timepoint, incubated with resazurin (10 µg/mL, 4 h, 37 °C). Media fluorescence was measured at 590 nm (Synergy HT plate reader (Biotek)).

MSC DIFFERENTIATION ASSAYS

MSC (4×10^4 cells/mL) cultured in EV-free media were stimulated as indicated for 6 h (gene expression), or until day 14 (ALP activity staining). Basal media was supplemented with ultracentrifuged FBS, and 10 µg of stimuli were added as indicated. Osteogenesis inducing media was further supplemented with Dexamethasone 1×10^{-7} M, β-glycerophosphate 1×10^{-2} M, ascorbic acid 5×10^{-5} M (all from Sigma-Aldrich). For gene expression analysis, MSC RNA was extracted with TRIzol® (Invitrogen) following manufacturer's protocol. RNA concentration was determined using a NanoDrop ND-1000 device (ThermoFisher Scientific), and its integrity confirmed by agarose gel electrophoresis. RNA (2 µg) was then digested with TURBO DNA-free Kit (Life Technologies) according to manufacturer's instructions, and used for cDNA synthesis using random hexamers and SuperScript® III Reverse Transcriptase (all from Life Technologies). RT-qPCR was performed in a iQ5 Real-Time PCR Detection System (Bio-Rad) using cDNA, gene specific primers and iQ SYBR Green Supermix (Bio-Rad). Gene expression was considered for Cq threshold cycles <35. GAPDH was used as reference gene and relative gene expression was calculated by the ΔCq method (according to MIQE guidelines). For ALP activity staining, MSC were fixed with paraformaldehyde 4% (w/v) and incubated with a Fast Violet B salt

solution (0.25 mg/mL) containing Naphthol AS-MX phosphatase alkaline solution (diluted 1:25) (all from Sigma-Aldrich), for 45 min at room temperature. Cells were washed and imaged on an Olympus CKX41 microscope.

TRANSWELL MIGRATION ASSAY

Transwell assays were set up in 24-well plates using inserts with membranes of 8 µm pore size (BD Falcon™). Insert membranes coated with gelatin (1% (w/v), 1 h at 37 °C) were seeded with 0.2×10^6 MSC (TC, top compartment) and indicated stimuli were placed on the bottom wells for experiments with gradient (BC, bottom compartment), using serum-free DMEM. For gradient disruption control, 50 µg of EV were diluted in serum-free DMEM and media equally distributed by the top and bottom compartments. DC total conditioned media and protein pellets from cell-free EV producing media, processed as above, were used as controls. After 8 h, insert membranes were fixed with paraformaldehyde 4% (w/v), non-migrated cells removed with cotton swabs, membranes cut from the inserts and mounted using fluoroshield with DAPI. Migrated cells were counted in the entire insert membranes using an epifluorescence microscope, and migration index calculated for normalization across experiments as the ratio of migrated MSC in the test condition to the MSC migrated in the respective control condition, serum free media.

2D CHEMOTAXIS ASSAY

Chemotaxis assays were performed with Collagen IV-coated µ-Slide Chemotaxis microfluidics devices (ibidi® GmbH), as recommended by the manufacturer. Briefly, 1×10^4 MSC in EV-free media were seeded in the central channel of the chemotaxis chamber and allowed to adhere for 1 h. Afterwards, a chemotactic gradient was established by addition of 10 µg of indicated stimuli to the right side of the chamber. Cell movement in the central channel was recorded by time-lapse video microscopy (see Supplementary Video S1) in an IN Cell Analyzer 2000 (GE Healthcare), using a Nikon 10x/0.45 NA Plan Fluor objective, with controlled temperature and CO_2. Brightfield images were acquired every 10 min during 13 h, and processed using IN Cell Investigator software (GE Healthcare). Movement of 20 cells per condition was tracked by image analysis with MTrackJ plugin (Supplementary Fig. 1), and chemotaxis parameters determined using the Chemotaxis Tool plugin (both for Fiji software[54]).

EV CHEMOKINES CONTENT ANALYSIS

DC-derived EV pellets or total conditioned media of 3 different DC donors were pooled (1:1:1) to 60 µg of protein, followed by hybridization on a semi-quantitative RayBio® C-Series human cytokine antibody array (RayBiotech), following manufacturer's protocol. Full

membrane scans are available in Supplementary Fig. 2. IL-6 and MCP-1 were validated by ELISA assay in DC-derived EV pellets and 100 K supernatant pooled as above. Standard ABTS ELISA development kit (PeproTech) was used, following manufacturer's protocol, and cytokine concentration was determined by comparison with a standard curve.

GELATIN ZYMOGRAPHY

Gelatinase activity of MMPs was analysed on conditioned media collected separately from TC and BC compartments of transwell assay, and processed as described before[17], or directly on EV pellets and 100 K supernatants. Where indicated, EV and 100 K supernatant samples were incubated with proteinase K (10 µg/mL, 15 min, 37 °C, Sigma-Aldrich), for soluble proteins digestion, before or after EV lysis. Protease was heat-inactivated (60 °C, 10 min) in the presence of phenylmethylsulfonyl fluoride 5 mM. Protein was quantified and the same amount (4 µg) resolved in gelatin-containing zymography gels as previously described[17], followed by gel incubation overnight with MMP substrate buffer ($CaCl_2$ 10 mM in Tris 50 mM buffer, pH7.5). Gels were stained with Coomassie Brilliant Blue R-250 (Sigma-Aldrich), washed until clear bands were visible, and imaged in a calibrated GS-800 densitometer (gel full scans are available in Supplementary Fig. S6).

STATISTICAL ANALYSIS

Statistical analysis was performed on Prism v6.0c software. Gaussian distribution was tested by D'Agostino-Pearson omnibus normality test. For non-normal data, Kruskal-Wallis followed by Dunn's multiple comparison test was used. For DC-EV internalization assay, resazurin, and chemotaxis assays, 2-way ANOVA followed by multiple comparisons testing was used. Statistical significance was considered for $p\text{-value} < 0.05$.

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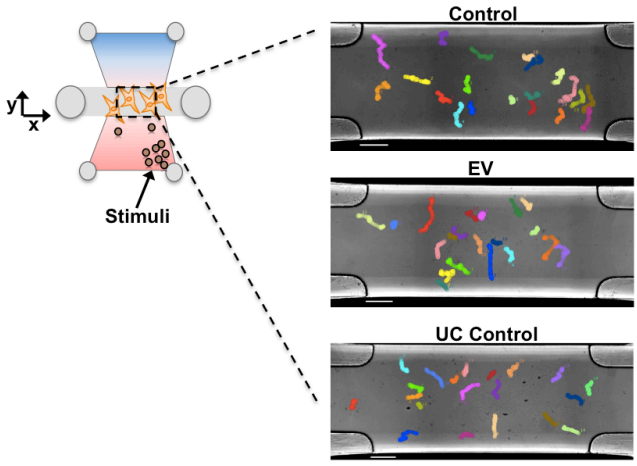
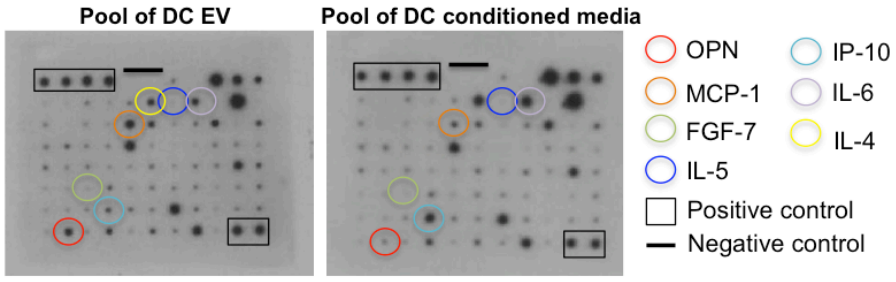
Supplementary information

Supplementary Figure S1



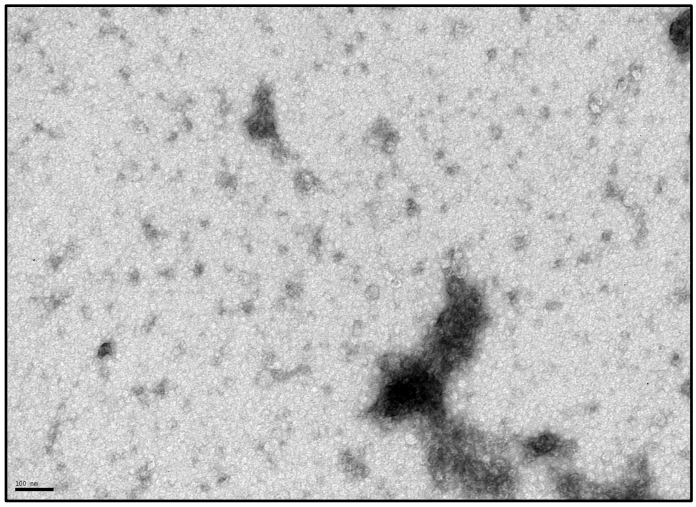
Supplementary Figure S1. MSC tracking in the chemotaxis devices (Related to Figure 4). Exemplificative images of the pathways manually tracked for MSC seeded in the central channel of the chemotaxis microfluidics devices, for unstimulated control condition and upon EV and 100K ultracentrifugation supernatant gradient formation. Scale bar: 200µm.

Supplementary Figure S2



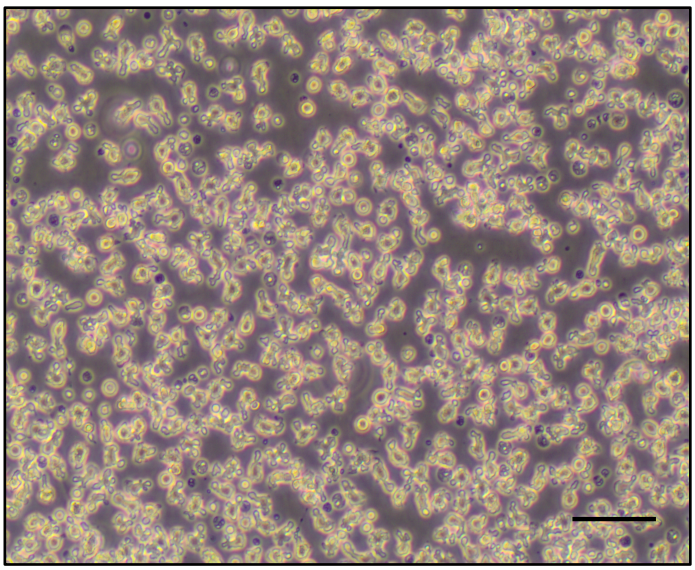
Supplementary Figure S2. Characterization of DC exosomes protein content by cytokine antibody array hybridization (Related to Figure 5). Scans of membranes hybridized with DC-EV pellets and conditioned media pools. Spots corresponding to selected chemoattractants with the highest absolute fold-change are highlighted, along with membrane positive and negative internal controls.

Supplementary Figure S3



Supplementary Figure S3. Transmission electron microscopy analysis of ultracentrifuged RPMI+10% FBS media used for DC cultures. Transmission electron microscopy micrograph of EV-producing media used for DC culture, showing dark protein agglomerates but not EV of bovine origin. Scale bar: 100 nm.

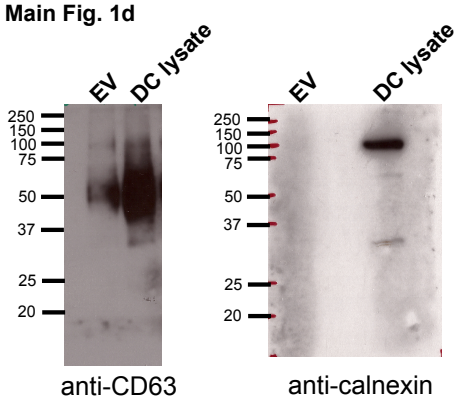
Supplementary Figure S4



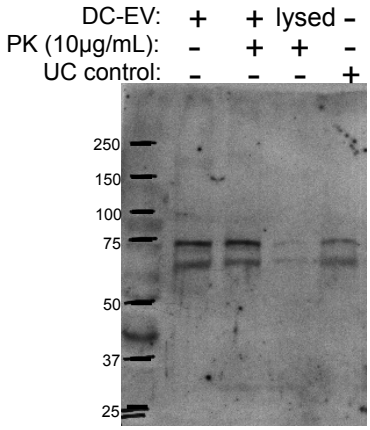
Supplementary Figure S4. Overall aspect of DC after 3 days in culture in ultracentrifuged RMPI+1% FBS. DC maintain their integrity with reduced cell death in the decreased serum conditions used. Scale bar: 50 µm.

Supplementary Figure S5

Main Fig. 1d

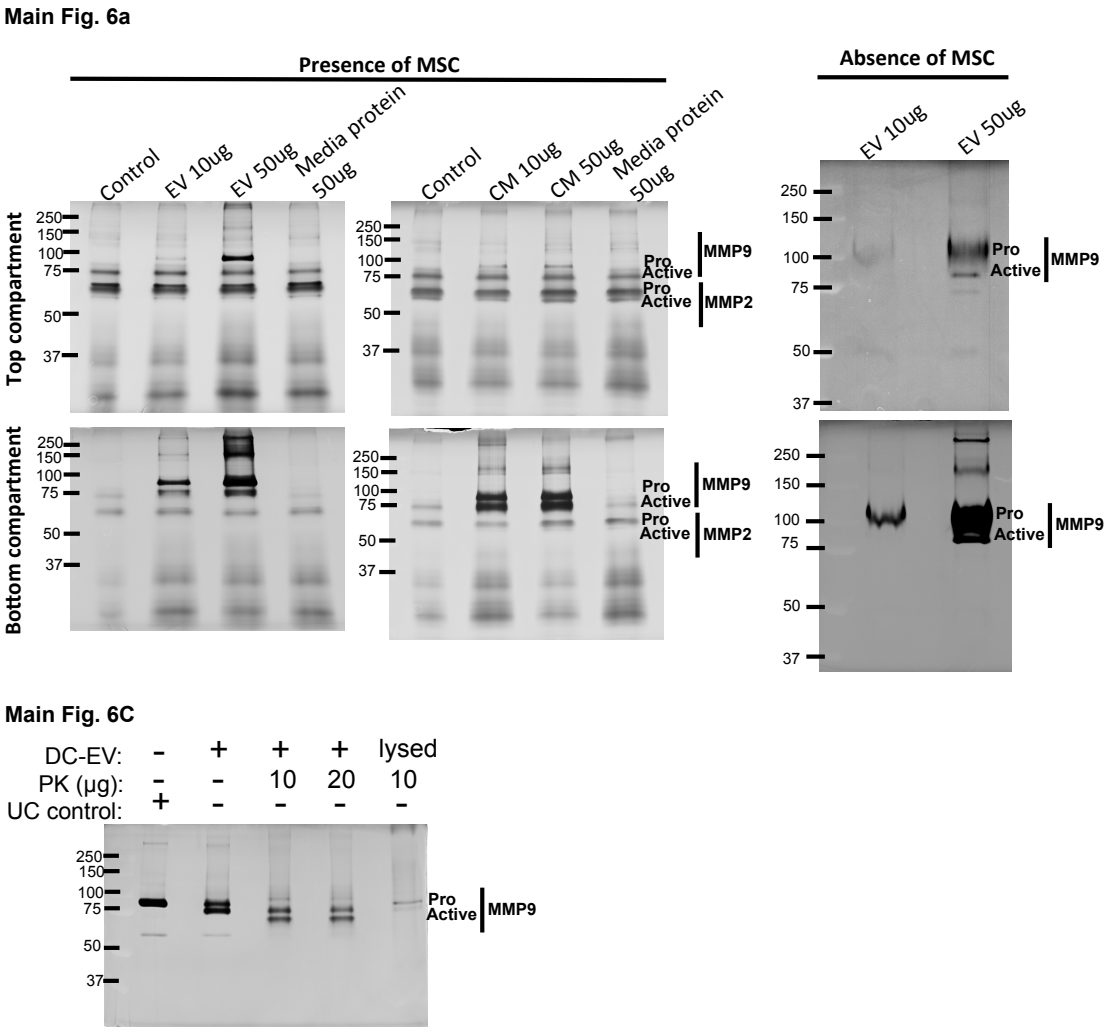


Main Fig. 5b



Supplementary Figure S5. Full-length scan of Western blots. Correspondence with cropped figures is indicated.

Supplementary Figure S6



Supplementary Figure S6. Full-length scan of gelatin zymography gels. Correspondence with cropped figures is indicated.

Supplementary Video S1

Supplementary Video S1 is available online at doi:10.1038/s41598-017-01809-x

Supplementary Video S1. MSC tracking on images acquired by time-lapse video microscopy (Related to Figure 4). An exemplificative time-lapse video is shown for MSC exposed to DC-derived EV gradient. Migrating MSC were tracked manually using the MTrackJ plugin for Fiji, and tracked pathways are shown in colour along time.

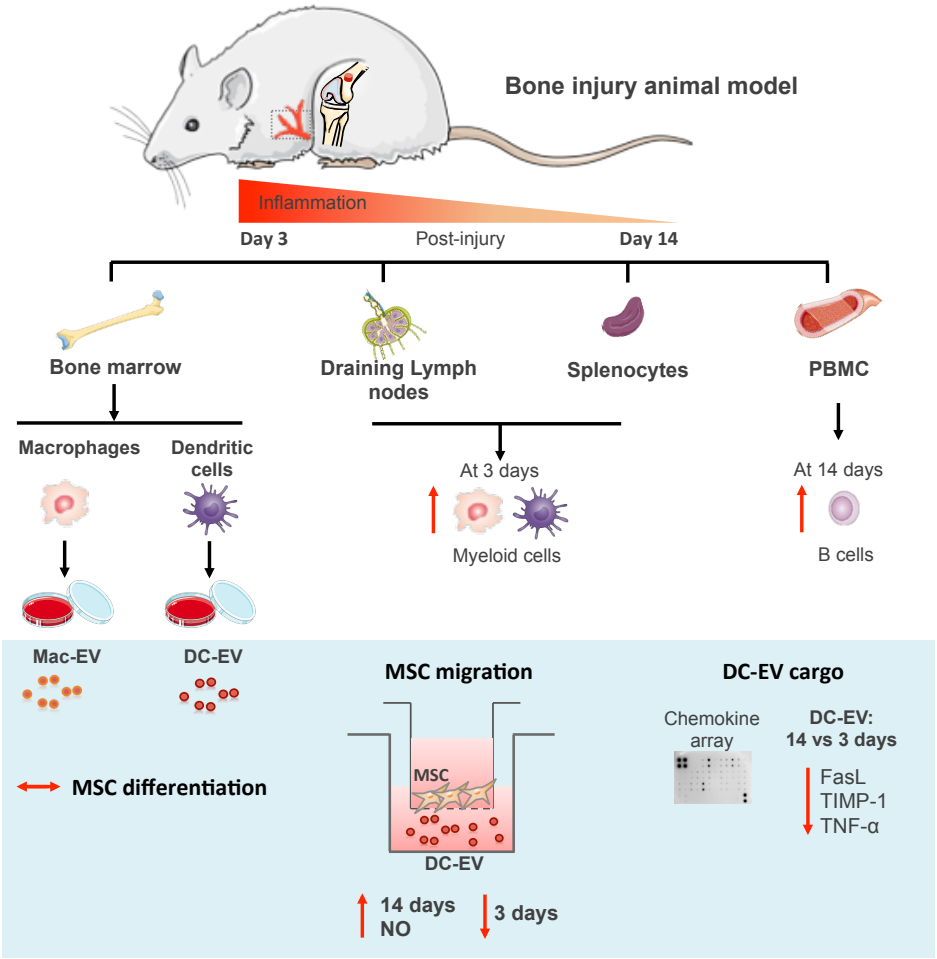
2. Extracellular vesicles from bone marrow-derived dendritic cells promote mesenchymal stem cells recruitment: the impact of bone injury

The results of this chapter are compiled in a manuscript that is currently in preparation for publication:

Article IV

Andreia M. Silva, José H. Teixeira, Ana R. Ferreira, Maria I. Almeida, Daniel M. Vasconcelos, Joana Oliveira, Carla Cunha, Daniela Vasconcelos, Nuno Neves, Mário A. Barbosa, Susana G. Santos. **Extracellular vesicles from bone marrow derived dendritic cells promote mesenchymal stem cell recruitment: the impact of bone injury.** *In preparation.*

Graphical abstract



Abstract

Bone injury triggers a local and systemic inflammatory response, transient in time, but crucial for successful repair/regeneration. Immune cells recruited to the place of injury are able to modulate the activity of bone cells, including multipotent mesenchymal stromal/stem cells (MSC), involved in the formation of new bone tissue. The crosstalk between these cells may occur paracrinally, mediated by secreted factors that can be found soluble or inside Extracellular Vesicles (EV). In this work, we hypothesized that the inflammatory environment upon injury would condition the immune cells at the lesion site, and their secreted EV, influencing their action upon MSC. Thus, using an *in vivo* model of femur injury, we aimed to investigate the effect of EV secreted by dendritic cells (DC) and macrophages (Mac) differentiated from bone marrow of injured bones, on the recruitment and differentiation of MSC.

A critical size bone defect rat model was surgically performed, and animals euthanized at 3 days and 14 days after injury. Non-operated (NO) animals were used as controls. First, systemic inflammatory response to injury was evaluated by flow cytometry, revealing an increase in CD11b/c^{+} myeloid cells in draining lymph nodes and spleen at day 3 post-injury, comparing with NO animals, and returning to basal levels at day 14.

At the local level, bone marrow of injured bones was isolated and differentiated to DC and Mac. EV were isolated from those cell cultures, containing vesicle subpopulations that resemble exosomes. Interestingly, EV from day 3 post-injury DC significantly impaired MSC recruitment in a transwell migration assay, whereas EV from DC at day 14 promoted it. A similar trend for MSC migration was observed upon stimulation with Mac-EV, but differences were not significant. Importantly, fibroblast recruitment was not increased by EV. On the other hand, EV did not impact MSC differentiation. In accordance with these results, EV from day 14 post-injury DC were depleted of TIMP-1, TNF-α and Fas ligand, containing the chemokine MCP-1.

In conclusion, this work showed that bone injury impacts the ability of bone marrow DC-EV to promote MSC recruitment, supporting the use of EV as vehicles of physiological signals for improved bone repair/regeneration.

Keywords: exosomes; *in vivo* bone defect; inflammation; bone repair/regeneration

2.1. Introduction

Bones are organs capable of fully repairing/regenerating upon injury, at least in healthy conditions. This process develops along time after lesion in three well coordinated main stages: inflammation, repair and remodelling [1, 2]. The inflammatory phase is triggered immediately by the lesion and is characterized by an intense secretion of cytokines and chemokines, which in turn promote the systemic recruitment of immune cells to the hematoma formed at the lesion site [1]. In fact, the inflammatory response to bone injury comprises not only a local reaction at the place of injury, but occurs also sistemically, leading to alterations in the levels of plasma cytokines and immune cell populations in distinct anatomical locations [3, 4].

Recently, inflammation has been increasingly recognized as a process of utmost importance for successful bone healing, with immune cells and chemokines directly modulating both bone-forming cells, such as mesenchymal stem/stromal cells (MSC) and osteoblasts, and bone-resorbing cells, the osteoclasts [2]. Indeed, *in vivo* depletion of myeloid cells is reported to impair bone development and repair after fracture [5], and macrophages have been suggested to promote osteogenesis [5–7]. Contrarily, both conventional and γδ T cells seem to be deleterious for bone healing [8–10]. Furthermore, impaired cytokine and chemokine signalling was also previously reported to negatively impact bone development and repair/regeneration [11–13]. On the contrary, highly pro-inflammatory environments have been reported to impair bone healing [14], and to lead to bone loss, a feature particularly frequent in patients with chronic inflammatory diseases [15]. Thus, a balanced inflammatory response to injury, that resolves along time, is needed for proper bone healing, relying on tightly controlled cell-to-cell communication.

Extracellular vesicles (EV) are cell-secreted vesicles that play important roles in cell communication, including in tissue repair and regeneration [16]. They encompass a family of different vesicle types, including microvesicles and exosomes [17]. In particular, exosomes comprise the smallest set of cell-secreted vesicles, carrying a cargo of functional proteins, RNA and DNA for delivery into target cells [18]. Importantly, this cargo varies according to the physiological status of the secreting cell [19], including the inflammatory conditions of their environment [20]. When EV reach their target cell, they are able to control different cell functions, such as cell activation, proliferation, survival, migration and differentiation [21], being reported as mediators of the immune response and immune cells crosstalk with stem cells *in vitro* and *in vivo*, in different models of tissue injury [16, 22–25]. However, the role of EV during bone repair and regeneration is still largely unexplored.

In our previous work, we have contributed to demonstrate the capacity of immune cells to crosstalk with bone cells, reporting that human dendritic cells (DC) are able to

promote human MSC recruitment *in vitro* [26], an effect that can be exclusively mediated by DC-secreted EV [27]. Importantly, this recruitment capacity of DC depends on their inflammatory status, being impaired by DC pre-conditioning with the pro-inflammatory cytokine TNF-α [26]. For this reason, we hypothesized that the inflammatory environment that occurs upon bone injury could condition the immune cells at the lesion site and the EV they secrete, influencing their action upon MSC. Thus, using an *in vivo* model of critical size femur defect, it was our aim to determine the capacity of EV secreted by DC and macrophages (Mac) derived from bone marrow of injured femurs, at different timepoints of the inflammatory phase after lesion, in promoting the recruitment of MSC *ex vivo*.

2.2. Methods

BONE INJURY ANIMAL MODEL

All animal procedures were performed in accordance to the ethical requirements on animal welfare and experimentation, and approved by the Portuguese official authority regulating laboratory animal sciences (DGAV).

An *in vivo* model of critical size femur defect was performed in three-months-old male Wistar-Han rats (N= 6 to 8 animals per group; Supplementary Figure S1), as previously described [28]. Briefly, a lateral arthrotomy of rats' right knee was performed, muscles surrounding the femur were retracted, and a cylindrical defect (with 3 mm diameter and ~4 mm depth) was surgically drilled in the anterolateral wall of the lateral condyle of the femur. Surgeries were performed under general anesthesia with volatile isoflurane. After surgery, analgesia was provided by subcutaneous administration of buprenorphine (0.05 mg/kg) twice a day.

Operated animals were then sacrificed at 3 days after bone injury, corresponding to the acute inflammatory phase of bone healing, and at 14 days after bone injury, the end of the resolution of that inflammatory phase. Non-operated (NO) animals were used as control.

BLOOD AND ORGAN COLLECTION AND PROCESSING

Rats were maintained in general anesthesia with volatile isoflurane and whole blood was collected by cardiac puncture, into tubes with citrate-phosphate-dextrose solution (Sigma-Aldrich) at a 1:5 ratio (vol solution:vol blood). Blood was centrifuged at 1200 x*g* (without acceleration/deceleration), for 20 min at room temperature (RT), and leukocytes buffy coat was collected. Buffy coat was then diluted in phosphate buffered saline (PBS), overlaid on equal volume of Lymphoprep (Axis-Shield), and centrifuged at 800 x*g* (without

acceleration/deceleration), for 30 min at RT, for peripheral blood mononuclear cells isolation (PBMC).

Animals were then dissected for collection of spleen, inguinal and popliteal lymph nodes draining the side of the injury, and the injured femur (Supplementary Figure S1).

Half of the spleen was injected with collagenase I 1 mg/mL (Sigma-Aldrich), and crushed on a 100 µm cell strainer for single cell isolation. Red blood cells on cell suspension were then lysed by incubation with lysis buffer (NH_4Cl 150 mM in Tris 10 mM) for 8 min, at 37 °C. Draining lymph nodes were crushed in the same way for single cell isolation.

Injured femurs were used for bone marrow collection. Bone epiphyses were cut at the metaphysis region and bone marrow was recovered by diaphysis flushing with PBS. Cell suspension was filtered through a 100 µm cell strainer for bone debris removal, and red blood cells were lysed as described above.

FLOW CYTOMETRY

PBMCs, spleen and lymph nodes cell suspensions were characterized for the presence of T cells, B cells, NK cells, NKT cells and myeloid cells by immunophenotyping of specific cell surface markers. First, cells were incubated with anti-rat CD32 (clone D34-485, 20 µg/mL, BD biosciences) diluted in staining buffer (BSA 0.5%, sodium azide 0.01% in PBS), for 5 min, on ice, to block Fc receptors. Then, cells were incubated with the following fluorophore-conjugated anti-rat antibodies diluted in staining buffer, for 30 min, on ice: anti-TCR-PerCP (clone R73, 4 µg/mL), anti-CD4-APC (clone OX-35, 4 µg/mL), anti-CD8a-V450 (clone OX-8, 8 µg/mL), anti-CD45R-PE (clone His24, 8 µg/mL), anti-CD161a-FITC (clone 10/78, 2 µg/mL), anti-CD11b/c-PE-Cy7 (clone OX-42, 1.6 µg/mL), anti-RT1B (MHC class II)-PerCP (clone OX-6, 16 µg/mL), anti-CD40-FITC (clone HM40-3, 40 µg/mL), anti-CD86-PE (clone 24F, 16 µg/mL) (all from BD Biosciences), and anti-CD163-AlexaFluor 647 (clone ED2, 4 µg/mL, Bio-Rad). Cells were then washed with staining buffer, and fixed with paraformaldehyde 1%. Cells stained with matching isotype antibodies were used as control. Samples were analysed in a flow cytometer FACSCanto (Benton Dickinson), with acquisition of 10 000 events in gate for each sample. Data analysis was performed with FlowJo software.

HISTOLOGICAL ANALYSIS

Half of the spleen was fixed in formalin 10% (v/v) for 24 h and processed by paraffin embedding. Paraffin sections of 3 µm thickness were sequentially cut, deparaffinized in xylol, and rehydrated in a series of ethanol of decreasing concentration. Sections were stained with haematoxylin and eosin (H&E) by incubation in Gill's Haematoxylin, followed by incubation in alcoholic Eosin Y. Slides were mounted and imaged in an Axiovert 200M microscope (Zeiss).

CELL ISOLATION AND CULTURE

Rat DC were differentiated from femur bone marrow (Supplementary Figure S1) by culturing cell suspensions for 7 days, in 10-cm tissue culture-treated Petri dishes ($10x10^6$ cells/dish), using Roswell Park Memorial Institute (RPMI) 1640 media (Corning) with 10% fetal bovine serum (FBS, Biowest), 1% penicillin/streptomycin (P/S) and 1% fungizone (basal media), further supplemented with murine IL-4 25 ng/mL and GM-CSF 25 ng/mL (Immunotools). Rat Mac were differentiated from femur bone marrow in similar conditions (Supplementary Figure S1), by culturing cell suspensions in 10-cm non-treated Petri dishes in the same basal media, but supplemented with rat M-CSF 10 ng/mL (Immunotools).

Rat MSC were derived from bone marrow flushed from femurs and tibias of Wistar-Han rats, as previously described [29]. MSC were selected by adhesion and cryopreserved for later use. Selected cells were $CD29^+$, $CD90^+$ and $CD45^-$, and able to differentiate into the osteogenic, chondrogenic and adipogenic lineages [29]. MSC were cultured in minimum essential medium alpha modification (α-MEM) containing 10% MSC-qualified FBS (both from ThermoFisher Scientific) and 1%P/S, and used until passage 7 in this work.

Rat dermal fibroblasts were isolated from underarm skin after tissue digestion, as previously described [29]. Isolated cells were $CD29^+$, $CD90^+$, and $CD45^-$, were unable to differentiate into the chondrogenic lineage and had very limited osteogenic and adipogenic potential. Fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM; ThermoFisher Scientific) with 15% FBS (Biowest) and 1%P/S, and used until passage 7 in this work.

All cell cultures were maintained in a humidified incubator, at 37 °C and 5% CO_2.

EV PRODUCTION, ISOLATION AND CHARACTERIZATION

Rat bone marrow-derived DC and Mac differentiated for 7 days were washed three times with PBS, and cultured for 3 days in EV producing media. This media was previously prepared by ultracentrifugation (100 000 x*g*, for 2 h at 4 °C) of RPMI media supplemented with 10% FBS (Biowest) and 1% P/S, which was diluted to 1% FBS EV-free for cell culture, and further supplemented with cytokines, as above. Cells were maintained in a humidified incubator, at 37 °C and 5% CO_2.

EV secreted by DC and Mac were isolated from cell culture media by differential centrifugation, as previously described [27]. Briefly, cell culture media was collected, centrifuged at 2 000 x*g* (20 min, at 4 °C), then at 10 000 x*g* (20 min, at 4 °C), and finally ultracentrifuged at 100 000 x*g* (2 h, at 4 °C, in a Beckman Optima L80-XP ultracentrifuge). Ultracentrifugation (UC) supernatant (UC control) was collected, EV pellets resuspended in PBS, and samples saved at -80 °C until further use. Protein of EV and UC control samples

was quantified using *DC*™ Protein Assay (Bio-Rad), and the calculated protein concentration was used as a quantitative measure for functional assays.

EV isolated and UC supernatants were characterized by transmission electron microscopy (TEM) and nanoparticle tracking analysis (NTA), as previously described [27]. Briefly, samples were adsorbed onto formvar nickel grids, and negatively stained with uranyl acetate 5% prior to TEM imaging, using a Jeol JEM 1400 electron microscope. For NTA, protein concentration of EV suspensions and UC supernatants was quantified as described above, and 10 µg were used for the analysis in a NanoSight NS300 device.

MSC DIFFERENTIATION ASSAY

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% FBS (Biowest) and 1% P/S, and cells stimulated in the following day for 48 h with 10 µg of EV secreted by DC or Mac derived from NO, 3 days and 14 days post-injury animals, as indicated. The same amount of UC supernatant was used as control, and unstimulated cells in basal media were used as negative control. Total RNA was then extracted with TRIzol® (Invitrogen) following manufacturer's protocol. RNA concentration was determined using a NanoDrop ND-1000 device (ThermoFisher Scientific), and its integrity confirmed by agarose gel electrophoresis. RNA (2 µg) was then digested with TURBO DNA-free Kit according to manufacturer's instructions, and used for cDNA synthesis with random hexamers and SuperScript® III Reverse Transcriptase (all from Life Technologies). RT-qPCR was performed in an iQ5 Real-Time PCR Detection System (Bio-Rad) using cDNA, gene specific primers and iQ SYBR Green Supermix (Bio-Rad). Primer sequences are available in Supplementary Table 1. Gene expression was considered for Cq<35. GAPDH was used as reference gene and relative gene expression was calculated by the ΔΔCq method (in accordance to MIQE guidelines).

CELL MIGRATION ASSAY

The effect of EV on MSC and fibroblasts migration was evaluated by transwell migration assays, using inserts with membranes of 8 µm pore size (BD Falcon™) set up in 24-well plates. Insert membranes were coated with gelatin 1% (w/v) (1 h at 37 °C), seeded with 0.08x10^6 MSC or fibroblasts (TC, top compartment) and placed on bottom wells containing different stimuli (BC, bottom compartment), as indicated, establishing a stimuli gradient. Assays with MSC were set up using serum-free (SFM) α-MEM, whereas DMEM was used for assays with fibroblasts. Serum-free media without any further supplementation was used as negative control, to assess basal levels of cell migration. After 12 h, insert membranes were fixed with paraformaldehyde 4% (w/v), cleaned of non-migrated cells with cotton swabs, cut from the inserts, and mounted using Fluoroshield with DAPI mounting

media. Insert membranes were imaged on a high content imaging IN Cell Analyzer 2000 device (GE Healthcare), using a Nikon 20x/0.45 NA Plan Fluor objective, and images were analysed on IN Cell Developer software. The number of migrated cells was automatically counted in the entire membrane and migration index calculated for normalization across experiments as the ratio of migrated MSC in the test condition, to the MSC migrated in the respective SFM control condition.

EV CHEMOKINE CONTENT ANALYSIS

DC-EV or respective UC supernatant from three different animals were pooled for each group (NO, 3 days and 14 days post-injury), to a final protein quantity of 60 µg. Samples were then hybridized on individual semi-quantitative RayBio® C-series rat cytokine antibody membrane arrays (C2 array, RayBiotech), following manufacturer's protocol. Membranes were imaged on a ChemiDoc™ device (Bio-Rad) and images were analyzed for determination of dots intensity using Protein Array Analyzer plugin for Fiji.

STATISTICAL ANALYSIS

Analysis of data collected was performed on GraphPad Prism v6.0 software. Normality distribution of data was tested by D'Agostino and Pearson normality test. Presence of outliers was tested by the ROUT method with Q=0.5%. For comparisons between multiple groups, Kruskal-Wallis test was performed for non-parametric data, followed by Dunn's multiple comparison test. Tests were run for a significance level α of 0.05.

2.3. Results

2.3.1. Bone injury triggers a systemic immune and inflammatory response

Bone injury is reported to trigger a local inflammatory response characterized by timely controlled variations in the immune cell populations at the lesion site [2]. In order to determine if this local response to bone injury was accompanied by a systemic inflammatory and immune response, a rat model of bone injury was performed. Cells were isolated from blood (Bl), draining inguinal and popliteal lymph nodes (LN), and spleen (Sp) from non-operated (NO) rats, and animals at day 3 and day 14 after bone injury. LN cells were counted and at day 3 there were significantly more cells in the draining LN than at day 14 (Figure 1A). Also, the number of cells at day 14 was significantly lower than for NO animals (Figure 1A).

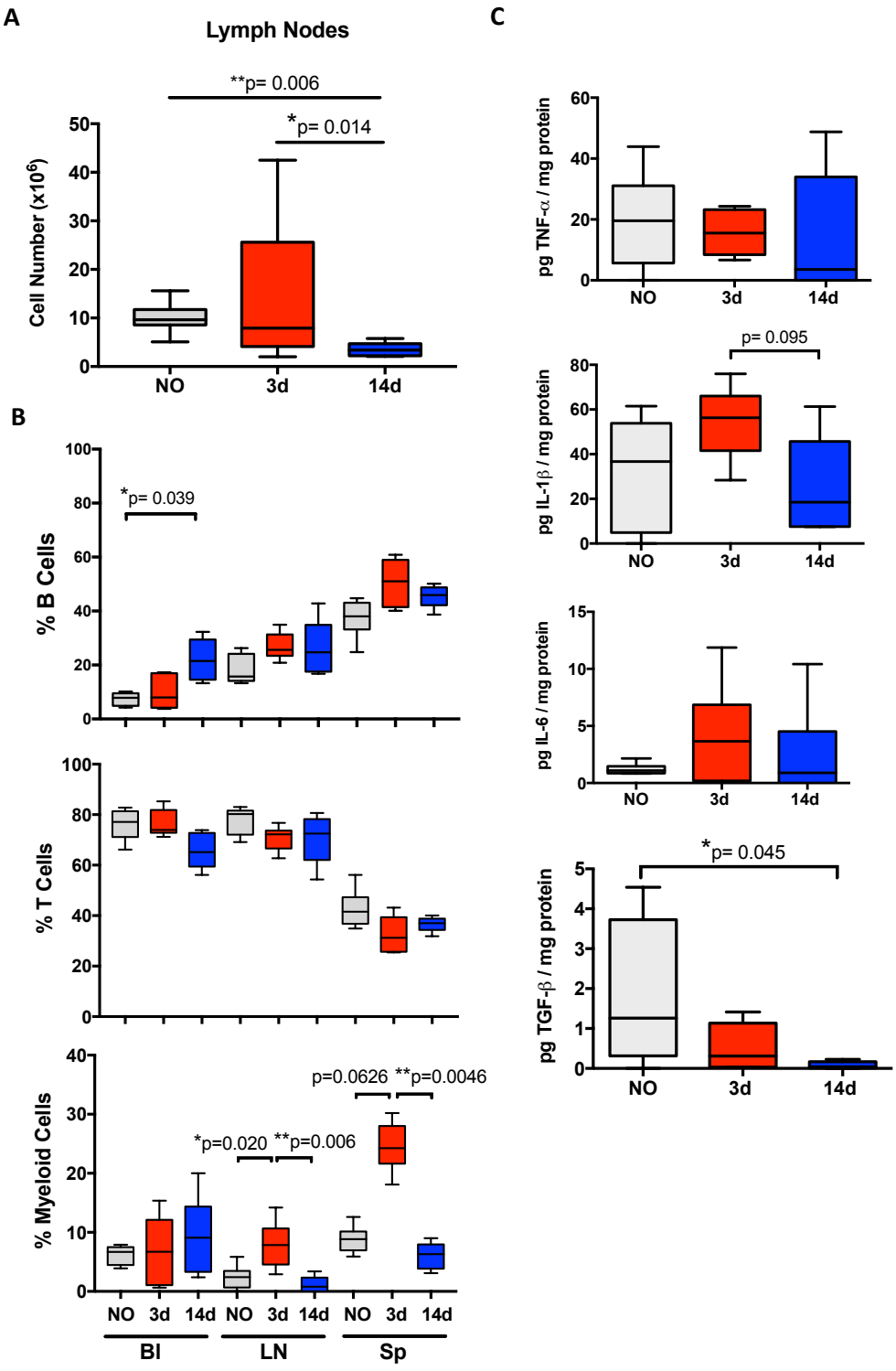


Figure 1. Bone injury causes a systemic response. (**A**) Numbers of cells in draining lymph nodes of non-operated (NO) rats, and injured rats at day 3 (3d) and day 14 (14d) after bone injury were counted. (**B**) Immune cell proportions at systemic level were analysed in blood (Bl), draining lymph nodes (LN) and spleen (Sp) of non-operated NO rats, and injured rats at day 3 and day 14 after bone injury. Percentage of B cells (CD45R+), T cells (TCR+) and myeloid cells (CD11b/c+) were determined by multicolour flow cytometry. (**C**) Levels of TNF-α, IL-1β, IL-6 and free active TGF-β circulating in the plasma of NO, 3 days and 14 days post-injury rats was quantified by ELISA. Quantifications were normalized for the protein concentration of plasma 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 test, followed by Dunn's multiple comparisons testing.

Based on the presence of characteristic cell surface markers, populations of myeloid cells (CD11b/c), T cells (TCR) and B cells (CD45R), NK and NKT cells (CD161a) in Bl, LN and SP were identified by flow cytometry. Representative dot plots, used in our gating strategy are illustrated in Supplementary Figure S2A. The results obtained and quantified across different animals are presented in Figure 1B. The proportion of B cells (top graph) was significantly increased in the blood of animals at 14 days post-injury, compared to NO animals. A concomitant decrease in the proportion of T cells could be observed in the blood (middle graph), albeit not statistically significant. Interestingly, bone injury affected mainly CD11b/c$^+$ myeloid cell populations (bottom graph), with significant increases in the proportion of CD11b/c$^+$ myeloid cells at day 3 after injury, compared to 14 days in LN and Sp, and also to NO animals in LN. Importantly, the proportion of CD11b/c$^+$ cells in LN nodes and Sp at 14 days returned to levels similar to NO control animals (Figure 1B, bottom graph), revealing a temporal regulation of the systemic inflammatory response to bone injury. In line with these results, histological analysis of spleens from injured and non-operated animals revealed inflammatory infiltration at days 3 and 14 after bone injury, compared to the spleen of NO animals (Supplementary Figure S3).

The systemic inflammatory response to bone injury was further evaluated by the analysis of pro-inflammatory cytokines and growth factors, circulating in plasma of injured and non-operated animals. According to the results in Figure 1C, plasma levels of the pro-inflammatory cytokines IL-6 and IL-1β were increased at day 3 after injury, compared to day 14 and NO animals, although no statistical differences could be found. In addition, similar levels of TNF-α were found in all time points. On the other hand, the levels of free active TGF-β decreased along time after injury, being significantly different between day 14 and NO animals.

2.3.2. Bone injury affects the secretion of EV by BM-derived DC and Macrophages

Immune cells recruited to sites of bone injury are reported to communicate with bone cells, modulating their action during bone repair [5, 30]. To investigate the impact of bone injury we differentiated DC and Mac from whole bone marrow cells isolated from NO and injured animals, by culturing them in presence of appropriate cytokines cocktails (Figure 2). Morphologically, cells cultured in DC differentiation conditions showed the characteristic stellate morphology, while those in macrophage differentiation conditions had mixed morphologies with stretched and round cells, characteristic of macrophage cultures (Figure 2A). Both cell populations expressed the myeloid lineage marker CD11b/c (Figure 2B). The

expression of CD86 on DC decreased with time after injury, albeit not significantly, while on macrophages CD86 was kept constant, when compared with NO-derived cells. In line with these results, the levels of pro-inflammatory cytokines TNF-α and IL-1β secreted by DC and Mac cultures were lower for 3 and 14 days post-injury groups, compared with NO animals, and TNF-α was significantly lower in the supernatants of both cell populations from 14 days post-injury, when compared to NO-derived cells (Figure 2C). The levels of free active TGF-β were not significantly different in supernatants of DC along time post-injury (Figure 2C), however it was significantly lower in supernatants of Mac from 14 days post-injury, compared to NO-derived cells.

Cell culture media from DC and Mac cultures, previously depleted of FBS-derived EV, was then collected for cell-secreted EV isolation. A protocol of EV isolation by ultracentrifugation was followed, allowing for the enrichment of exosomes populations. The EV isolated from DC (DC-EV) and Mac (Mac-EV) culture media were characterized by transmission electron microscopy and results showed the presence of vesicles with a cup-shape morphology (Figure 3A), typical of exosomes, for all conditions tested. A more detailed analysis of these EV by nanoparticle tracking analysis also revealed that the majority of the vesicles in the EV population isolated had a mean diameter of 150 nm, within the expected size range for exosomes (Figure 3B). No significant differences were observed in the size of EV from cells of injured or non-operated animals (Supplementary Figure S4).

Importantly, bone injury seemed to affect the capacity of BM-derived immune cells to secrete EV, particularly in DC. Indeed, the concentration of EV recovered from DC culture media was reduced for cells from day 3 post-injury, relative to EV derived from cells of NO animals (Figure 3C). Furthermore, EV secretion was recovered from day 3 to day 14 post-injury. A similar secretion pattern was found for BM-derived macrophages cultures, although differences were only slight. Of note, the concentration of particles in EV samples was much higher than in ultracentrifugation supernatants (UC control), supporting the efficiency of the protocol followed for the isolation of cell-secreted EV.

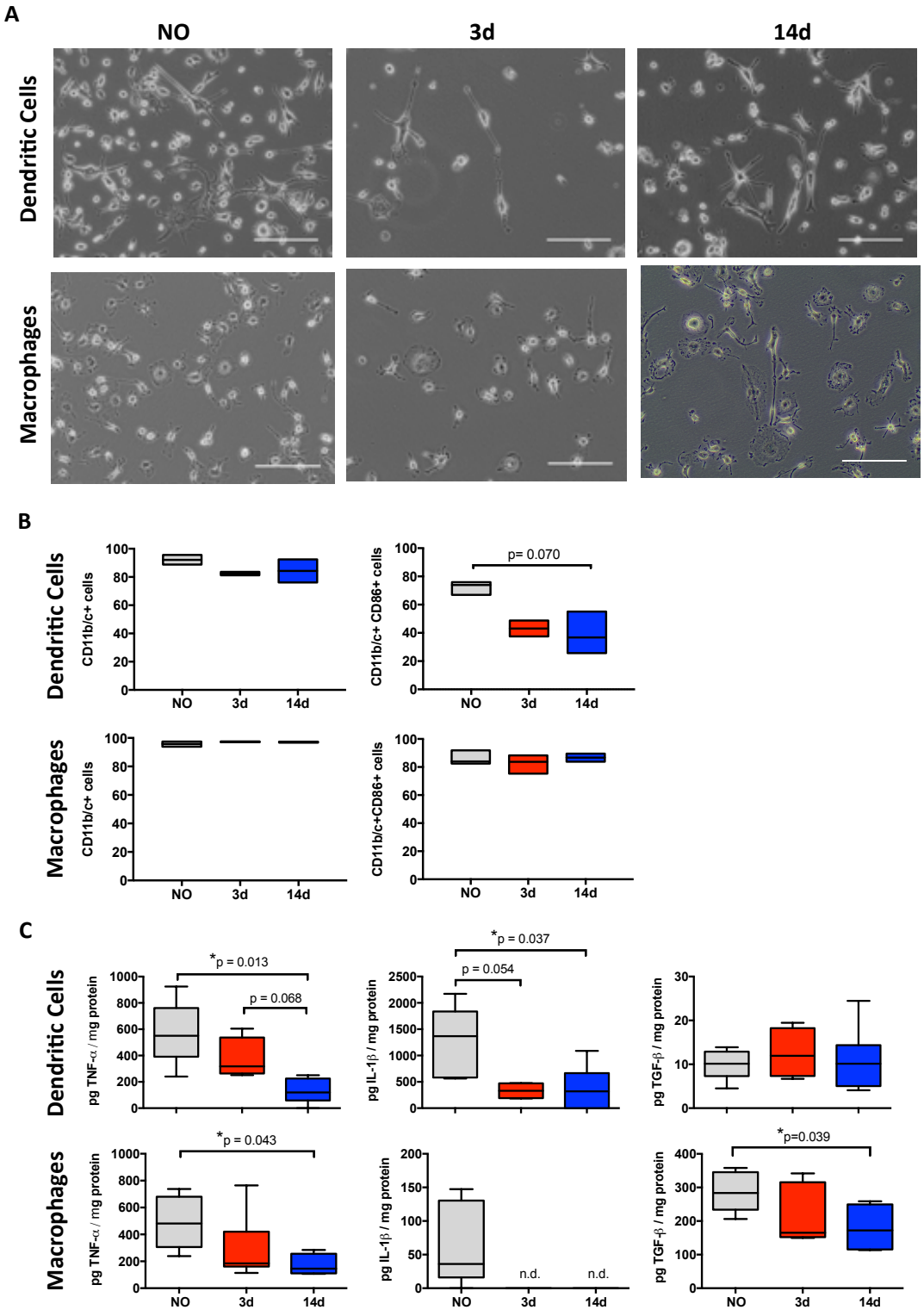


Figure 2. Bone marrow cells can be differentiated into dendritic cells and macrophages in vitro. (**A**) Representative brightfield microscopy images of bone marrow cells isolated from non-operated (NO) rats, and at day 3 (3d) and day 14 (14d) after bone injury induction, and differentiated into dendritic cells (DC) in the presence of IL-4 and GM-CSF, or macrophages (Mac) in the presence of M-CSF, both for 7 days. (**B**) Percentage of CD11b/c+ and CD11b/c+CD86+ cells in the DC and Mac populations isolated. (**C**) Levels of TNF-α, IL-1β and free active TGF-β in total conditioned media collected at day 10 of DC or Mac culture, as quantified by ELISA. Quantifications were normalized for the protein concentration of conditioned media samples. Box plots represent min-to-max distribution of n= 2 to 6 animals per group. P-values are indicated, as determined by Kruskal-Wallis, followed by Dunn's multiple comparisons testing.

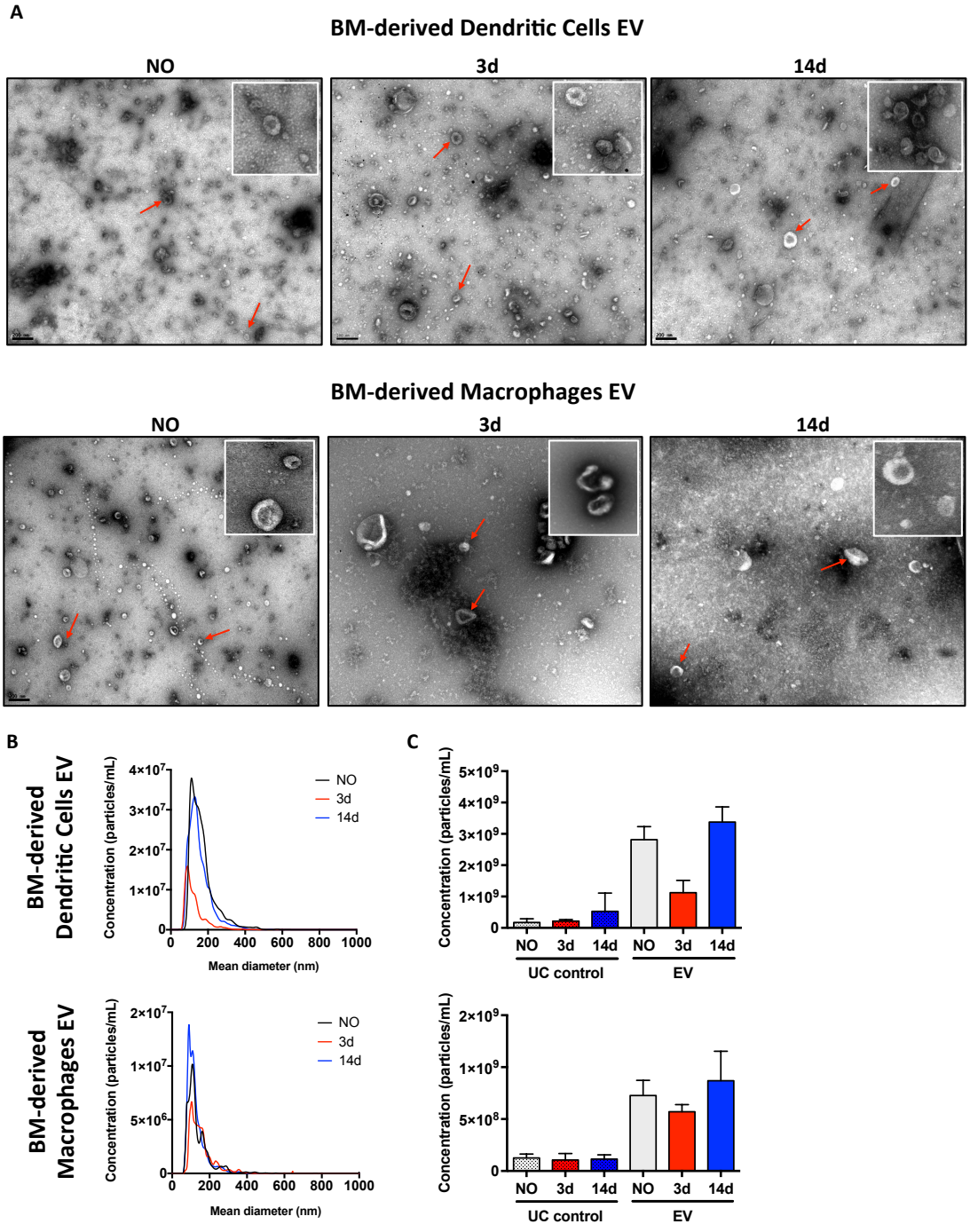


Figure 3. Bone injury affects the secretion of EV by bone marrow-derived DC and macrophages. (**A**) Exemplificative TEM micrographs of EV isolated from dendritic cells (DC) and macrophages (Mac) derived from bone marrow (BM) of non-operated (NO) rats, and animals at day 3 (3d) and day 14 (14d) after bone injury. Magnification: 50 000x. Scale bar: 200 nm. EV are indicated by red arrows. Details of EV are shown in figure insets, at a magnification of 100 000x. EV populations isolated from DC (n=3) and Mac cultures (n=3) were characterized for size distribution (**B**) and concentration (**C**). Statistical significance was tested by Kruskal-Wallis test, followed by Dunn's multiple comparisons testing, and no significant differences between groups were found.

2.3.3. Bone injury influences the capacity of DC-EV to promote MSC migration

We previously demonstrated that human DC-EV are able to promote human MSC recruitment [27], and MSC are known to migrate to places of injury [31]. Thus, we further investigated the capacity of EV secreted by DC and Mac, differentiated from bone marrow at different timepoints after bone injury, to mediate MSC recruitment (Figure 4). A transwell migration assay was used for this study as reported before [27], whereby DC-EV or Mac-EV (or respective UC supernatants) were added to the bottom compartment of the system, acting as stimuli for MSC migration (Figure 4A).

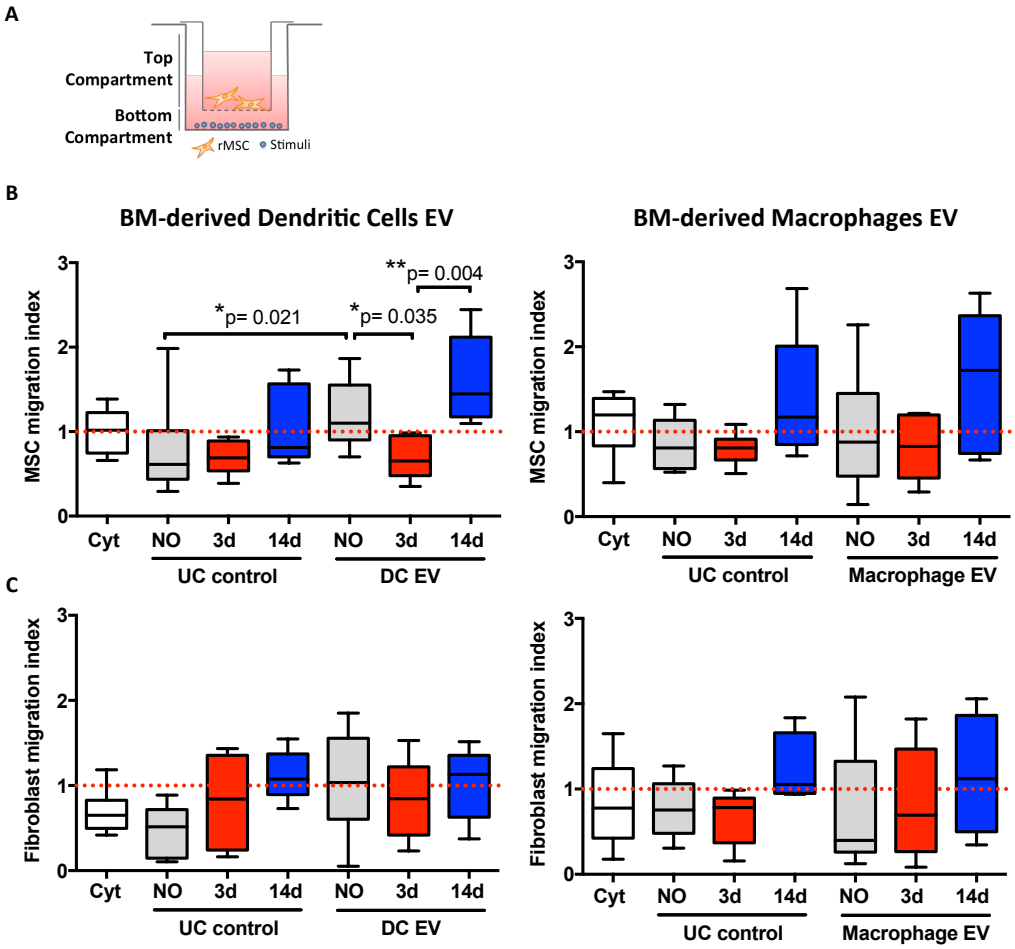


Figure 4. Bone injury affects the capacity of EV from bone marrow-derived dendritic cells to promote MSC recruitment. (**A**) Schematic representation of the transwell system, set up to evaluate the paracrine effect on cell recruitment of EV from bone marrow (BM)-derived dendritic cells (DC) and macrophages (Mac), from non-operated (NO), 3 days (3d) and 14 days (14d) post-injury rats. MSC or fibroblasts were seeded inside the transwell insert (top compartment) and allowed to migrate towards stimuli added on the wells (bottom compartment). (**B**) Migration index of MSC in the presence of the indicated stimuli, relative to unstimulated control condition (absence of stimuli in the bottom compartment; dashed red line). (**C**) Migration index of fibroblasts in the presence of the indicated stimuli, relative to unstimulated control condition (dashed red line). Supernatants from EV ultracentrifugation (UC control) were used as controls. Cell migration in the presence of DC-differentiating cytokines (IL-4 and GM-CSF) and Mac cytokine (M-CSF) was also evaluated as a control (cyt). Box plots represent min-to-max distribution of n= 5 to 6 animals per group. P-values were determined by Kruskal-Wallis test, followed by Dunn's multiple comparisons test, and are indicated.

The results obtained were quantified across different animals and are illustrated in Figure 4B. DC-EV from animals at different times post-injury had significantly different capacity to promote MSC recruitment. DC-EV from 3 days post-injury significantly impaired MSC recruitment, when compared with those from NO and 14 days post-injury cells (Figure B, left graph). Importantly, DC-EV from NO animals promoted a significantly higher MSC recruitment, than the supernatant from EV ultracentrifugation. Mac-derived EV, showed a similar pattern, but no significant differences were observed (Figure 4B, right graph).

In order to assess the specificity of the effects induced by EV on MSC recruitment, transwell migration assays were alternatively set up using fibroblasts, a cell type of mesenchymal origin also present at the bone injury site, and often leading to fibrosis [2]. Interestingly, none of the DC and Mac-derived EV promoted significant recruitment of fibroblasts, suggesting specificity in MSC recruitment (Figure 4C). Moreover, the impact of DC and Mac-derived EV on MSC differentiation into the different lineages was also investigated. There was no significant promotion of MSC differentiation by any of the EV samples tested (Supplementary Figure S5).

2.3.4. Cytokine/chemokine content of DC-EV changes with time after bone injury

To further investigate potential candidate molecules involved in the striking effect of DC-EV upon MSC recruitment, the protein cargo of EV from DC differentiated from NO, 3 days and 14 days post-injury animals, was investigated for the presence of chemokines and cytokines.

The results are illustrated in Figure 5A, and hierarchical clustering of EV cytokines/chemokines content revealed a high similarity between EV from DC of NO and 14 days post-injury animals. Indeed, EV from DC at day 14 post-injury were enriched in chemokines, such as CINC-2, MCP-1 and CINC-1, which were absent in EV from DC of 3 days post-injury animals (Figure 5A). Moreover, EV from DC at day 3 after injury were enriched in Tissue Inhibitor of Metalloproteinase (TIMP)-1, TNF-α, Fas ligand (FasL), IL-13 and IL-10, compared to EV from day 14 post-injury and NO controls (Figure 5B). In a more detailed comparison of the content of DC-EV from both timepoints after injury (Figure 5B, top graph), EV from 14 days post-injury DC were highly depleted of FasL, TIMP-1 and TNF-α (fold-change<1.5), containing slightly higher levels of Receptor for Advanced Glycation Endproducts (RAGE), L-selectin and MCP-1 (fold-change>1.5) than EV from 3 days post-injury DC.

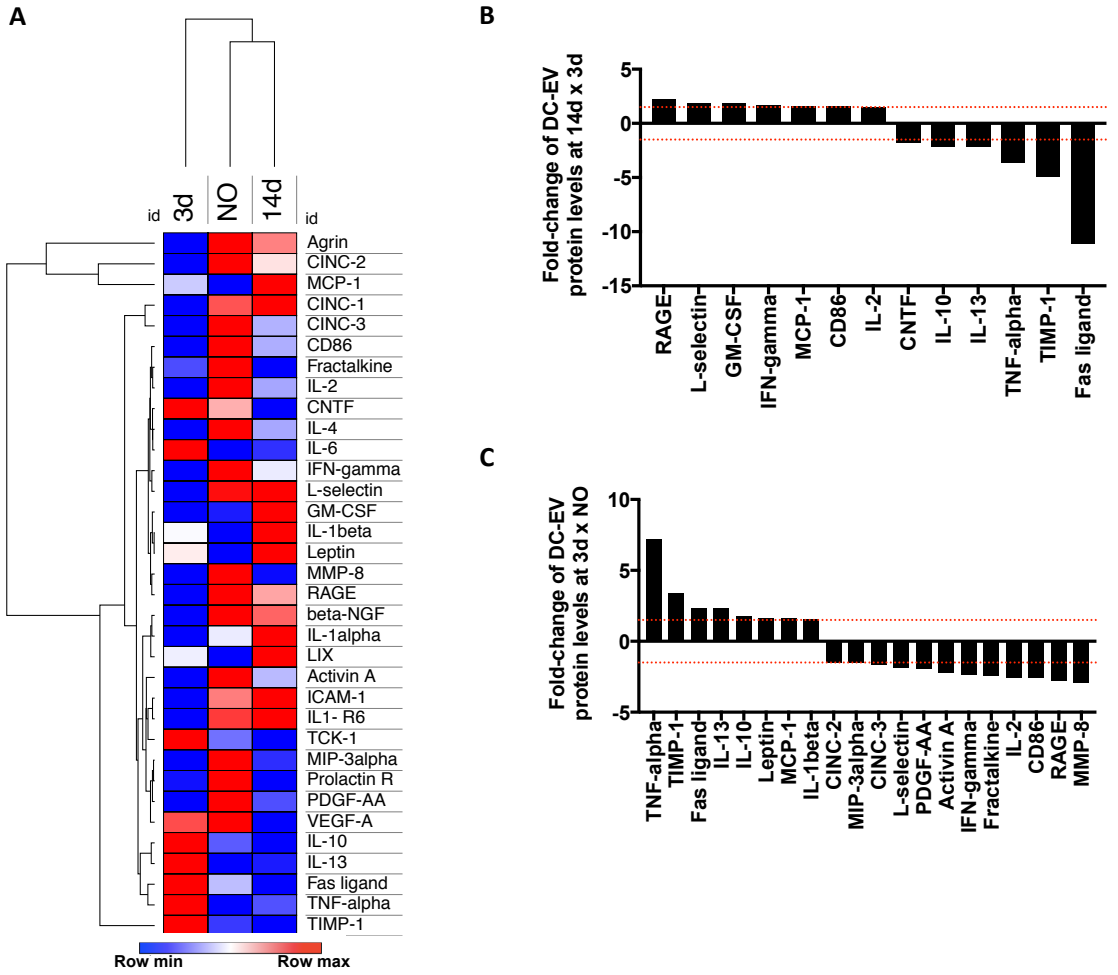


Figure 5. Time after bone injury influences the cytokine and chemokine content of EV from bone marrow-derived EV. (**A**) Heatmap of cytokines/chemokines protein levels in EV secreted by dendritic cells (DC) derived from bone marrow (BM) of non-operated (NO) rats, and rats at day 3 (3d) and day 14 (14d) after bone injury. Animal groups and cytokines/chemokines were grouped by Euclidean hierarchical clustering. (**B**) Fold-change of cytokines/chemokines protein levels in EV from BM-derived DC at 14 days post-injury, relative to EV from BM-derived DC at day 3. Only cytokines/chemokines with an absolute fold-change>1.5 are represented. (**C**) Fold-change of cytokines/chemokines protein levels in EV from BM-derived DC at 3 days post-injury, relative to EV from BM-derived DC of NO rats. Fold-changes thresholds of -1.5 and 1.5 are indicated by dashed red lines.

2.4. Discussion

When bone injury occurs, an inflammatory response takes place that is relatively well characterized and suggested to contribute for a microenvironment that supports bone tissue remodelling and repair along time [2]. Moreover, this local inflammatory reaction is accompanied by a systemic response that is reflected in the blood and plasma, but also in the draining lymph nodes, and spleen. In the current study we characterize the cellular populations at the systemic level in the acute inflammatory phase (day 3) and at the inflammation resolution phase (day 14) that occur after bone injury. We then focused on the

effect of bone injury in EV secreted by immune cell populations obtained from the bone marrow of the injury site at the same timepoints.

The inflammatory phase of bone injury healing is controlled in time, with an acute immune response occurring in the first 3 to 5 days post-injury, that is then resolved until days 10 to 14 post-injury [32]. In the current study, we found significantly increased numbers of cells in draining LN at day 3, when compared to day 14, supporting that this earlier timpepoint was within the acute inflammatory response, while at day 14 we see resolution of inflammation, with LN contracting. Bone injury is reported to increase the levels of pro-inflammatory cytokines in the lesion area, that promote an alteration on the proportion of local immune cells populations, leading to the infiltration of neutrophils within the first 24 h, followed by recruitment of monocytes and lymphocytes [1]. Importantly, these local alterations in the proportions of immune cell populations, induced by the injury, are also accompanied by changes in the proportion of immune cells populations at the systemic level. Tätting *et al.* found that changes in immune cell populations in the marrow of injured bone, in a mouse model of tibia metaphyseal injury, was accompanied by similar changes in non-injured contralateral tibia and humerus [33]. Also, previous work from our team also reported alterations in the proportion of different immune cells populations in blood, lymph nodes and spleen, in the same rat model used here and in response to biomaterial implantation, at day 6 post-injury, comparing with NO animals [4]. Interestingly, these changes were still detectable in lymph nodes of injured animals at 2 months after injury [28]. In the current study, we found a systemic increase in the proportions of myeloid cells at 3 days post-injury, which resolved back to levels similar to NO animals by day 14 post-injury. This is in accordance with our previous studies, where we showed that the proportion of myeloid cells suffered the most significant changes, decreasing by 6 days after bone injury (end of the acute inflammatory phase [32]), and highlights the dynamic changes observed overtime at the systemic level. In the current study, there was not an overall increase in plasma levels of pro-inflammatory cytokines, at least in the analysed timepoints. Also, in our previous work, plasma levels of IL-1β were similar for 6 days post-injury, compared to NO animals [4]. In contrast with that work, we were not able to detect any increase in the levels of the TGF-β along time after injury, a growth factor involved in bone remodelling [34]. Nonetheless it should be noted that in the study of Vasconcelos *et al.*, total TGF-β was analysed, whereas our study focused on the analysis of free active form of TGF-β [4]. Importantly, a recent study reported an inverse correlation between the serum levels of free active TGF-β1 and bone volume in a mouse model of tibial plateau fracture, at 8 weeks after the lesion. This effect was likely due to the presence of osteoclasts at the injury site [35].

In this study, bone marrow from injured femurs was used for DC and Mac differentiation. We focused our attention in these two cell populations, because Mac have been suggested to actively contribute to bone formation by conditioning osteoblasts and MSC, whereas dendritic cells are major players in the activation of adaptive immunity, in particular T cells, whose function in bone repair has also been recently further explored [2]. Since whole bone marrow cells were used for immune cell differentiation, the proportion of different immune cells populations present locally at the site of the bone defect was not analysed, neither their variation along time after injury. Nonetheless, the phenotype of DC and Mac differentiated from injured femurs cells, and thus pre-conditioned by its inflammatory microenvironment, was analysed, in comparison with NO animals [2]. Surprisingly, DC and Mac from day 3 after injury did not show a marked pro-inflammatory profile, a phenotype that was further evident for DC from day 14 post-injury. Indeed, only less than half of the CD11b/c$^+$ DC population obtained expressed the activation marker CD86, and secreted lower levels of the pro-inflammatory cytokines IL-1β and TNF-α, compared to cells from day 3 post-injury. The direct contribution of DC for bone repair remains largely unexplored. Nonetheless, Vinish *et al.* previously reported that DC-depleted mice with burn wounds had delayed wound healing compared to wild-type animals, presenting lower re-epithelialization, revascularization and granulation tissue formation, accompanied by low levels of TGF-β at the wound, at day 3 after burn [36]. In an earlier study, cardiac repair was also shown to be impaired in a mouse model of myocardial infarct depleted of DC, an effect that was associated with a time-sustained overexpression of pro-inflammatory cytokines in the injured heart [37]. Also the phenotype we found for Mac from injured bones is in line with previous reports. Indeed, a previous study dedicated to characterizing the phenotype of immune cells in the marrow of a mouse model of tibia methaphysial fracture, reported that the proportion of myeloid CD86$^+$ cells was not altered at 3 days after injury, compared to non-injured animals, but there was an increase in the proportion of CD206$^+$ myeloid cells, an hallmark of M2-polarized macrophages. Similarly, our results showed that the proportion of CD11b/c$^+$ CD86$^+$ cells in Mac cultures did not vary with time after injury, with these cells secreting low levels of the pro-inflammatory cytokines typically secreted by M1 macrophages, particular those from day 14 animals.

We were able to successfully isolate EV from the culture media of DC and Mac differentiated from bone marrow cells at different timepoints after bone injury, and from NO animals. The morphological characterization of these vesicles indicates that the EV population isolated is enriched in exosomes [38]. Interestingly, bone injury affected EV secretion by bone marrow-derived DC and Mac, in a time-post-injury-dependent fashion, and impacting not only the quantity of EV released, but also their functional properties. To the best of our knowledge the profile of EV secretion *in vivo* upon bone injury, or *in vitro* by cells

isolated from injured bones, remains elusive. Nonetheless, a previous report suggested that the concentration of EV in synovial fluid collected from osteoarthritis patients is very similar to that in healthy subjects [39]. On the other hand, hypoxia and oxidative stress, expected to occur during the acute inflammatory response to bone injury [2], were previously reported to promote EV secretion in cardiomyocytes [40] and monocytes [22], respectively. In our model, we did not verify this effect, since in cells, particularly DC, differentiated from BM at day 3 post-injury, the secretion of EV was reduced, returning to levels similar to those of NO animals, or even slightly higher, at day 14 post-injury.

Considering the importance of immune cells and inflammation in successful bone repair, we further explored the EV-mediated crosstalk between DC and Mac derived from different timepoints post-injury, and MSC, a cell population capable of differentiating in osteogenic and chondrogenic lineages, that are responsible for bone tissue formation. Interestingly, DC-EV from different times post injury had opposing effects in terms of promoting MSC migration. In our previous work, we had demonstrated that human DC-derived EV were able to specifically promote human MSC recruitment [27]. In our current work, we show for the first time that the capacity of DC-EV to promote MSC recruitment depends on time after bone injury when DC precursors were isolated, and thus potentially on cell pre-conditioning in an inflammatory environment. Indeed, DC derived from bone marrow at 3 days post-injury, characterized by the local acute inflammatory response, inhibited MSC recruitment, while DC-EV from bone marrow cells isolated at day 14 post-injury, the inflammation resolution phase, promoted MSC recruitment to levels slightly higher than control conditions. This is supported by our early work, whereby we showed that the capacity of DC to promote MSC migration was impaired by cells pre-conditioning with TNF-α [26]. Importantly, we further demonstrate that the capacity of DC to promote recruitment is specific for particular cell populations, since they were not able to affect fibroblasts migration. Interestingly, the effect of DC-derived EV does not repeat with Mac-derived EV, which are unable to significantly recruit MSC.

Moreover, MSC stimulation with DC- and Mac-EV did not significantly affect their differentiation potential. This is in accordance with our previous results, whereby human MSC stimulation with EV from human naïve DC only mildly affected the osteogenic and chondrogenic differentiation of the cells, compared to unstimulated basal conditions [27]. However, previous work by Ekström *et al.* reported that EV isolated from human monocytes cell cultures stimulated with LPS promoted MSC osteogenic differentiation [24]. Similarly, EV derived from human platelet lysates were reported to promote MSC osteogenic differentiation, when used in low amounts for cell stimulation [25].

Considering the effect of DC-EV on MSC recruitment, we further characterized the content of these EV, secreted by DC derived from different timepoints after bone injury, for

the presence of cytokines, chemokines and other molecules, potentially implicated in cell migration. Our analysis was focused on DC-EV because they impacted MSC migration more significantly than Mac-EV. According to our data, EV from DC at day 14 post-injury contained slightly higher levels of MCP-1 than EV from DC at 3 days post-injury, a chemokine that is reported to promote rat MSC recruitment in different *in vitro* models [41, 42]. Indeed, Wang *et al.* showed that brain tissue extracts from rats subjected to a brain ischemic injury, and that contained MCP-1, were able to promote rat MSC recruitment *in vitro*, an effect that was partially abrogated in the presence of anti-MCP-1 antibodies [41]. In addition, EV from DC at 14 days post-injury were also enriched in RAGE, a receptor implicated in the transduction of signals that promote cell migration [43]. For instance, RAGE has been described as a promoter of DC migration, including *in vivo*, by binding the high mobility group box 1 nuclear protein (HMGB1) [44, 45]. Lin *et al.* also demonstrated that HMGB1 promotes MSC migration, although the mechanism involved in cell migration was not dependent on HMGB1 binding by RAGE, but rather by TLR [46]. RAGE was also suggested as an activator of pro-inflammatory signalling cascades in immune cells, amplifying and perpetuating inflammation [43], which does not seem to be the case in our study, at day 14 post-injury, as both cell surface markers of activation and pro-inflammatory cytokines are lower at that timepoint. Moreover, the soluble form of RAGE may act as a decoy receptor for inhibitors of osteoclast biogenesis and activity, thus indirectly promoting osteoclastogenesis [47]. Furthermore, soluble RAGE was found to be elevated in the serum of osteoporosis female patients, in comparison with normal subjects [48]. In this way, it is possible that EV from DC at 14 days post-injury, might contribute to bone tissue repair, not only by acting upon MSC, but rather on osteoclasts, whose action is required during the repair and remodelling phases of bone repair/regeneration. Interestingly, rather than being highly enriched in chemotactic molecules, EV isolated from 14 days post-injury DC were highly depleted from cell migration inhibitory proteins, including those reported for MSC [49], such as TIMP-1. Importantly, in our previous work we have shown that DC-EV contain enclosed MMPs, likely contributing for the effect of these EV in promoting MSC recruitment [27]. In the current work, the EV content for MMPs, namely gelatinases, MMP-2 and MMP-9, could not be addressed by the antibody membrane array, and is currently being further explored. Nonetheless, the collagenase MMP-8 was depleted in EV from DC at 3 days post-injury, compared to DC from NO animals, which is line with the effect of those EV on MSC recruitment. Importantly, granulocyte-derived MMP-8 was previously reported to promote hematopoietic stem cell mobilization [50]. Moreover, MMP-8 has been implicated in inflammation resolution in wound repair [51] and arthritis [52], being elevated in the serum of non-union fracture patients [53], although we could not find any difference in the levels of this MMP in DC-EV from day 3 and day 14 post-injury. Furthermore, EV from 14 days post-injury DC were also highly depleted in TNF-α,

which we previously implicated in impaired MSC recruitment by DC [26]. In contrast, TNF-α alone in low doses, was previously reported to promote rat MSC recruitment in a transwell *in vitro* migration assay [54]. MSC pre-conditioning with TNF-α also have a similar effect, promoting MSC migration [55]. In addition, 14 days post-injury DC-EV were also highly depleted in FasL. This molecule is known for its role in mediating cell apoptosis, upon binding of receptor Fas on target cells membrane [56], an effect that can be solely mediated by cell-secreted EV [57]. Nonetheless, FasL has also been described as having immunomodulatory [56] effects and influencing bone cells biology [58], which might have important implications during bone repair and regeneration. In the work of Miwa *et al.*, FasL was shown to have a pro-inflammatory role, contributing to the recruitment of neutrophils [59] and the production of pro-inflammatory cytokines [60]. On the other hand, FasL and, importantly, FasL-bearing EV, have been more often ascribed with immunosuppressive functions, being a key molecule in the establishment of immunoprivileged environments, as in the brain, intervertebral discs and uterus during pregnancy [56]. Indeed, EV derived from engineered DC overexpressing FasL, and thus enriched in this ligand, were demonstrated to inhibit inflammation in murine models of delayed-type hypersensitivity and collagen-induced arthritis, when administered locally or systemically *in vivo* [61]. In our injury model, the increase in DC-derived EV from day 3, compared to NO, and the subsequent reduction of FasL from day 3 to day 14 post-injury, suggests FasL may have a predominantly pro-inflammatory role, contributing to the recruitment of neutrophils and other immune cells into the injury site at the early acute inflammatory phase, decreasing at day 14 post-injury, with the resolution of the inflammatory phase of bone repair. Interestingly, FasL secreted by MSC was previously implicated in the capacity of these cells to induce apoptosis of osteoclasts [62]. Moreover, low levels of FasL are reported to promote MSC proliferation, a process that occurs at the early stage of bone repair that follows inflammatory phase resolution [63]. On the other hand, FasL is reported to inhibit osteogenic differentiation [64], and thus a temporal regulation of FasL is likely to occur during bone healing.

In summary, with this work we demonstrated that bone injury leads to changes in DC-secreted EV, including their content, which ultimately impact their capacity to promote MSC recruitment *in vitro*. In particular, we demonstrate that the pro-inflammatory environment that establishes after bone injury and that pre-conditions BM-derived DC is deleterious for their capacity to promote MSC recruitment via EV, pinpointing the importance of inflammation resolution for improved bone repair/regeneration, and suggesting naïve DC-derived EV as a new biological drug for the treatment of impaired bone fracture healing.

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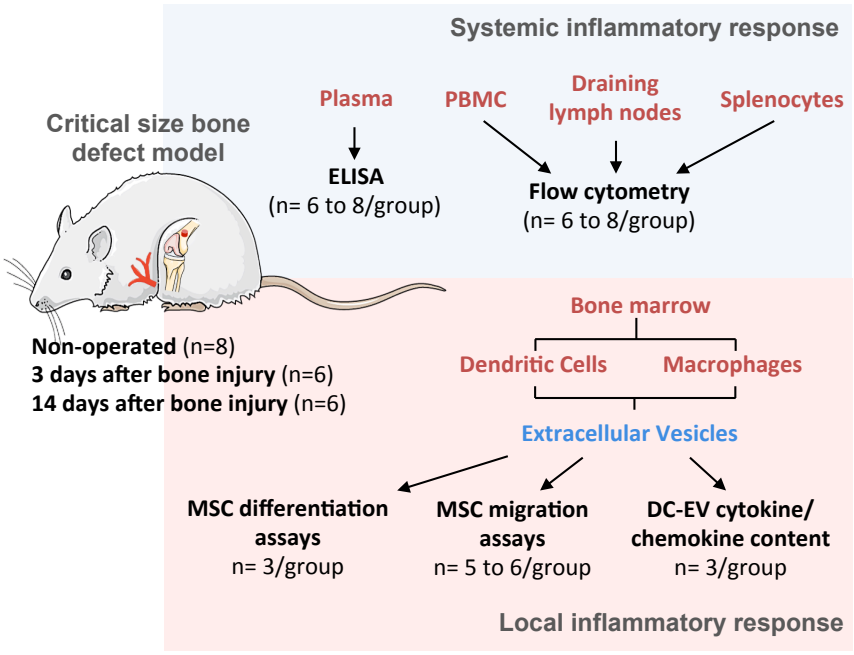
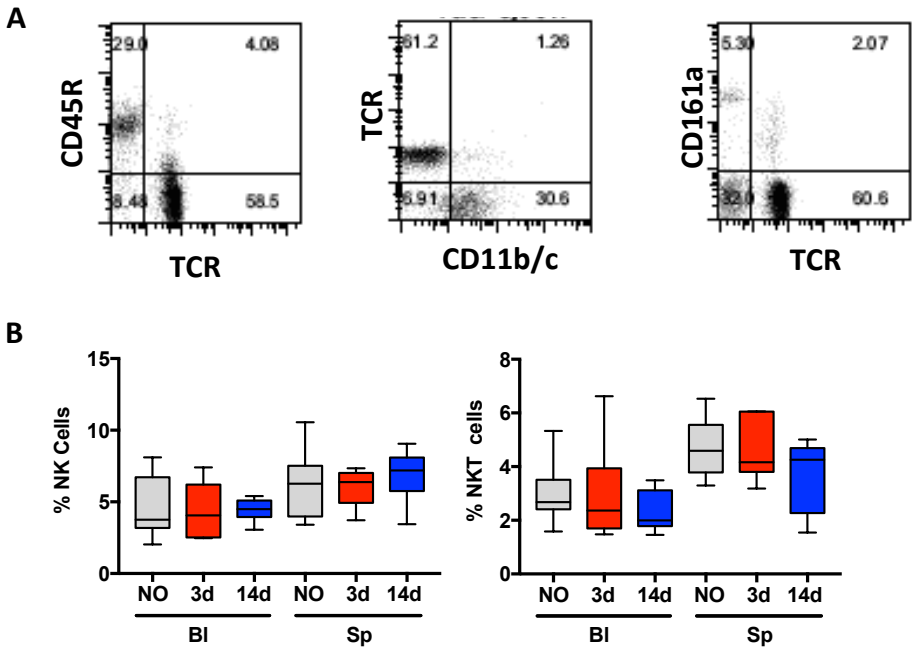
Supplementary information

Supplementary Figure S1



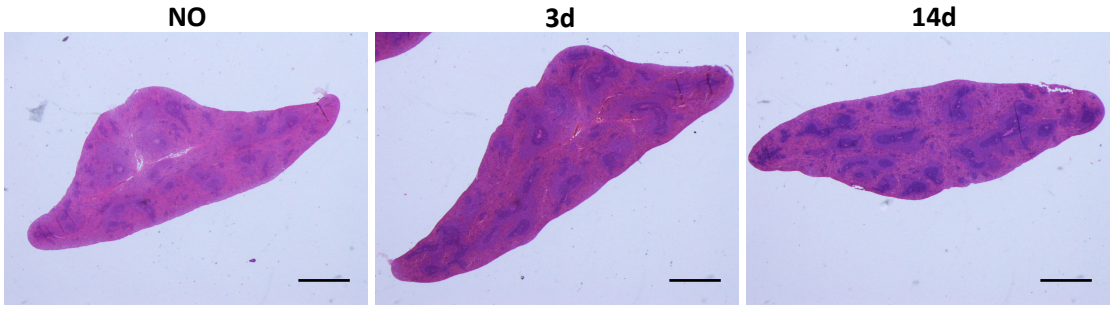
Supplementary Figure S1. Diagram of the study design implemented. A critical size bone defect was surgically performed in Wistar-Han rats. Six animals per group were euthanized at day 3 and day 14 after bone injury. A group on non-operated rats containing 8 animals was used as control. Upon euthanasia, blood, lymph nodes draining the bone injury site (popliteal and inguinal), splenocytes and injured femurs were collected. Plasma and PBMCs were isolated from blood, single cells were isolated from lymph nodes and spleen, and bone marrow was flushed from femurs. Plasma, PBMC, lymph node cells and splenocytes isolated from all animals were used to evaluate the systemic inflammatory response to bone injury. Bone marrow cells of each animal were divided for differentiation into dendritic cells and macrophages, which were then used for Extracellular Vesicles production (EV). EV isolated were then used in functional assays evaluating their capacity to influence MSC migration and differentiation, and their cytokines/chemokines content was analysed by antibody array. Due to limitations in the quantity of EV samples isolated, only a subset of animals per group could be used in the assays performed.

Supplementary Figure S2



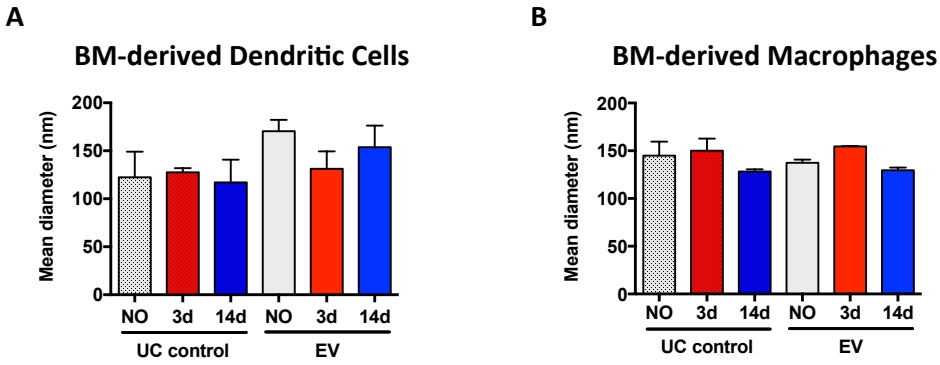
Supplementary Figure S2 (related to Figure 1). Representative flow cytometry dot plots and histograms used to gate immune cell populations analysed. (**A**) Gating strategy for flow cytometry analysis: B cells were CD45R+ and TCR-, T cells were TCR+ and CD161a-, myeloid cells were CD11b/c+ and TCR-, NK cells were CD161a+ and TCR-, and NKT cells were CD161a+ and TCR+. (**B**) Percentages of NK and NKT cells in blood and spleen of the animals at the different timepoints. Percentages in draining lymph nodes were bellow 1% and so are not presented.

Supplementary Figure S3



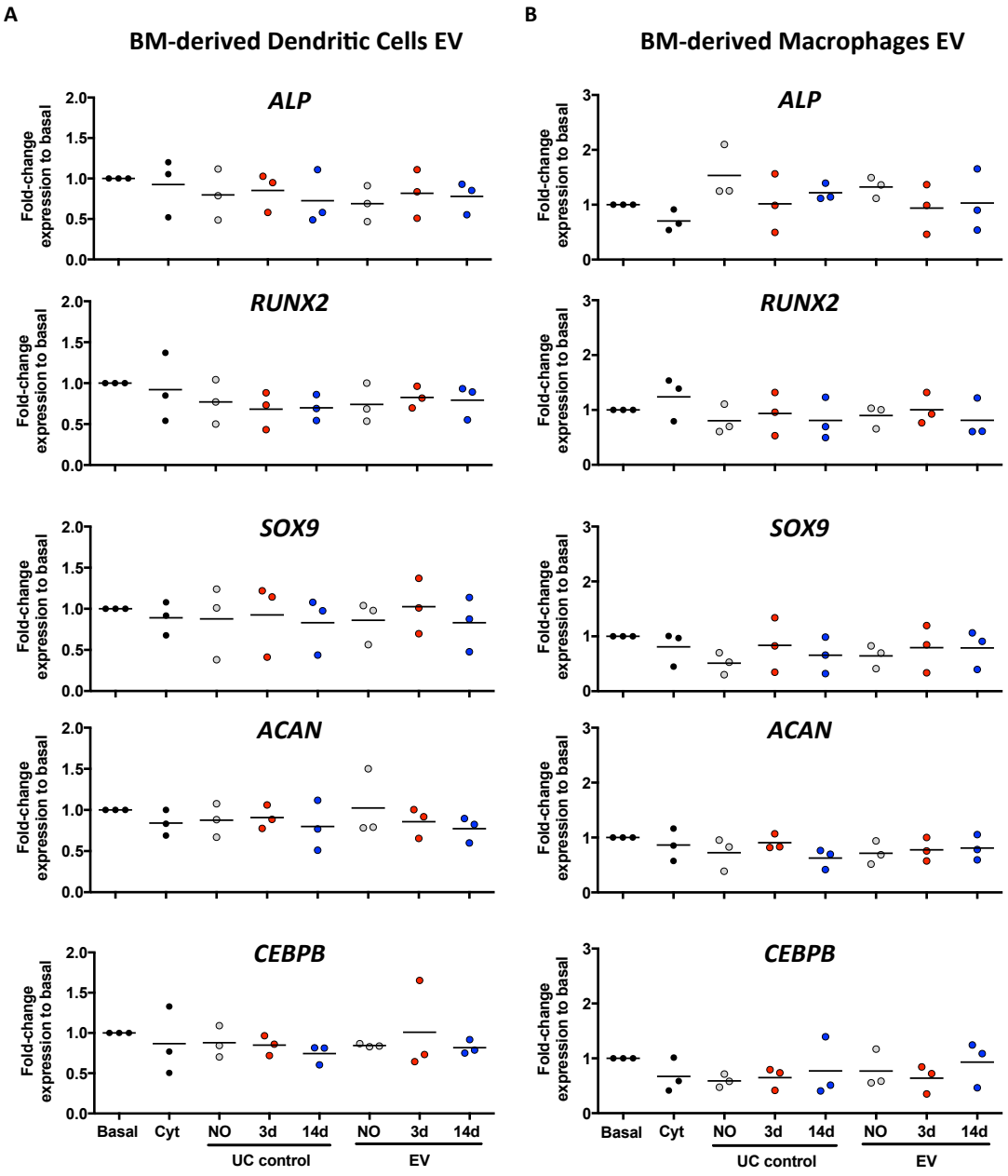
Supplementary Figure S3 (related to Figure 1)**. Histological analysis of rat spleens reveals inflammatory infiltrations upon bone injury.** Hematoxilyn and eosin staining of sections from spleen of non-operated (NO) rats, and rats at 3 days and 14 days after bone injury. Scale bar: 1mm.

Supplementary Figure S4



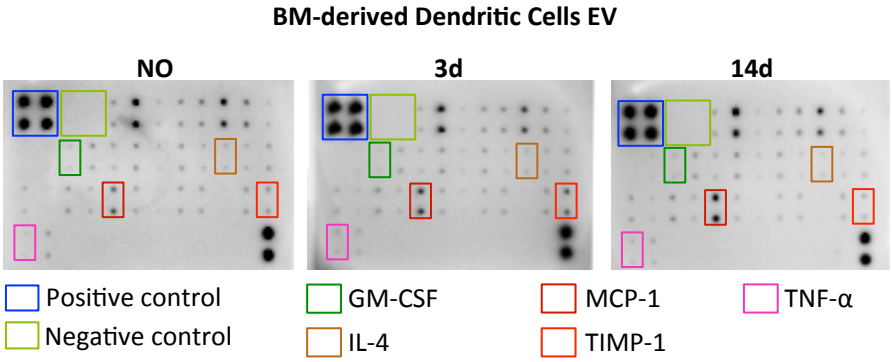
Supplementary Figure S4 (related to Figure 3)**. Bone injury does not affect the size of EV secreted by DC and macrophages.** Mean diameter of EV secreted by (**A**) dendritic cells (DC) and (**B**) macrophages (Mac) derived from bone marrow of non-operated (NO), 3 days (3d) and 14 days (14d) post-injury rats, showing a size in the range of exosomes populations. Supernatant of EV ultracentrifugation (UC control) was used as control and few vesicles could still be detected, having a size similar to EV suspensions analysed.

Supplementary Figure S5



Supplementary Figure S5. EV secreted by bone marrow-derived dendritic cells and macrophages have distinct effects on MSC differentiation. (**A**) Fold-change gene expression of osteogenesis markers *ALP* and *RUNX2*, chondrogenesis markers *SOX9* and *ACAN*, and adipogenesis marker *CEBPB* in MSC (from top to bottom graphs), after stimulation with EV from bone marrow (BM)-derived dendritic cells (DC) from non-operated (NO) rats, and 3 (3d) and 14 days (14d) post-injury rats. Supernatants from EV ultracentrifugation (UC control) were used as controls for EV action specificity. MSC were also stimulated with IL-4 and GM-CSF (cyt) to discard a possible effect of DC-differentiating cytokines on cell differentiation. (**B**) Fold-change gene expression of *ALP, RUNX2, SOX9, ACAN* and *CEBPB* in MSC (from top to bottom graphs), upon stimulation with EV from BM-derived macrophages (Mac) from NO, 3 days and 14 days post-injury rats. Supernatants from EV ultracentrifugation (UC control) were used as controls. A possible effect of Mac-differentiating cytokine on MSC differentiation was assessed by cell stimulation with M-CSF (cyt). Three independent experiments were performed, with each dot representing a different animal. Mean fold-change expression is represented by a dash for each condition tested. Statistical significance was tested by Kruskal-Wallis test, followed by Dunn's multiple comparisons testing, and no differences were found.

Supplementary Figure S6



Supplementary Figure S6 (related to Figure 5). Photographs of antibody array membranes used to characterize the cytokines/chemokines content of EV from dendritic cells (DC) derived from bone marrow of non-operated (NO) rats, and rats at day 3 (3d) and day 14 (14d) after bone injury. The location of antibodies recognizing relevant candidate cytokines/chemokines are indicated by coloured boxes. Membrane positive and negative internal control spots are also indicated.

Supplementary Table 1. Sequences of primers used for gene expression analysis by RT-qPCR presented in Supplementary Figure S5.

Gene	Primer sequence
ALP	Forward: 5'- GACAAGAAGCCCTTCACAGC -3' Reverse: 5'- CTGGGCCTGGTAGTTGTTGT -3'
RUNX2	Forward: 5'- CCGATGGGACCGTGGTT -3' Reverse: 5'- CAGCAGAGGCATTTCGTAGCT -3'
SOX9	Forward: 5'- CTGAAGGGCTACGACTGGAC -3' Reverse: 5'- TACTGGTCTGCCAGCTTCCT -3'
ACAN	Forward: 5'- CTTGGGCAGAAGAAAGATCG -3' Reverse: 5'- GTGCTTGTAGGTGTTGGGGT -3'
CEBPB	Forward: 5'- CAAGCTGAGCGACGAGTACA -3' Reverse: 5'- AGCTGCTCCACCTTCTTCTG -3'
PPARG	Forward: 5'- CTGATGCACTGCCTATGAGC -3' Reverse: 5'- TGGACTCCATAGTGGAAGCC -3'

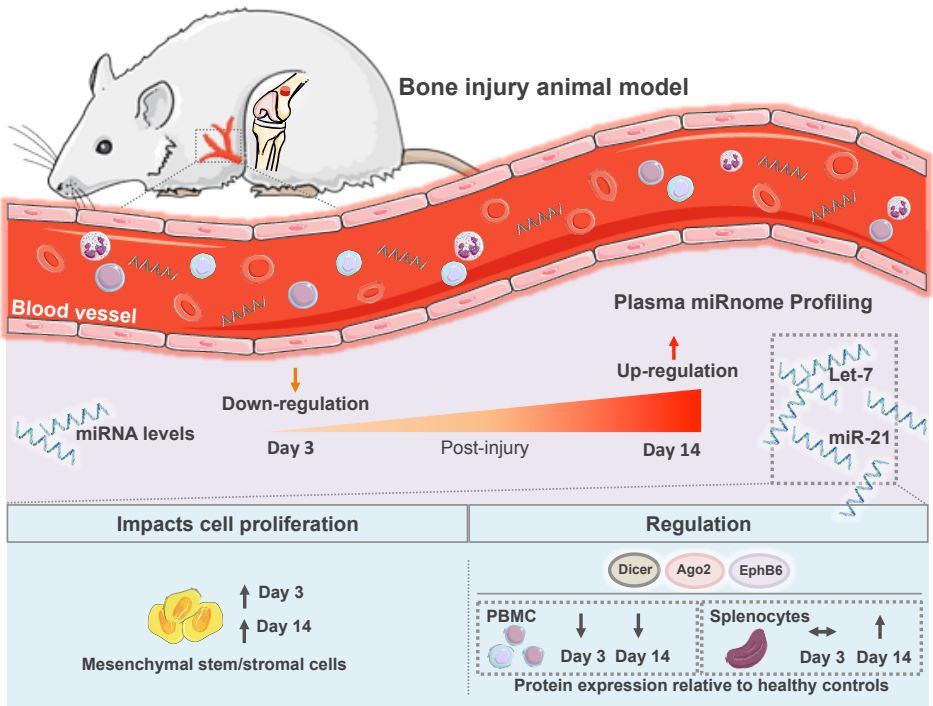
3. Profiling the circulating miRnome reveals a temporal regulation of the bone injury response

The content of this chapter is the result of a collaborative work developed at Dr. George Calin's lab in The Univeristy of Texas – MD Anderson Cancer Center, and is published in the following original research article:

Article III

Andreia M. Silva, Maria I. Almeida, José H. Teixeira, Cristina Ivan, Joana Oliveira, Daniel Vasconcelos, Nuno Neves, Cláudia Ribeiro-Machado, Carla Cunha, Mário A. Barbosa, George A. Calin, Susana G. Santos (2018), **Profiling the circulating miRnome reveals a temporal regulation of the bone injury response.** Theranostics. *Accepted for publication.* doi:10.7150/thno.24444.

Graphical abstract



Abstract

Bone injury healing is an orchestrated process that starts with an inflammatory phase followed by repair and remodelling of the bone defect. The initial inflammation is characterized by local changes in immune cell populations and molecular mediators, including microRNAs (miRNAs). However, the systemic response to bone injury remains largely uncharacterized. Thus, this study aimed to profile the changes in the plasma miRnome after bone injury and determine its biological implications.

Methods: A rat model of femoral bone defect was used, and animals were evaluated at days 3 and 14 after injury. Non-operated (NO) and sham operated animals were used as controls. Blood and spleen were collected and peripheral blood mononuclear cells (PBMC) and plasma were separated. Plasma miRnome was determined by RT-qPCR array and bioinformatics Ingenuity pathway analysis (IPA) was performed. Proliferation of bone marrow mesenchymal stem/stromal cells (MSC) was evaluated by Ki67 staining and high-throughput cell imaging. Candidate miRNAs were evaluated in splenocytes by RT-qPCR, and proteins found in the IPA analysis were analysed in splenocytes and PBMC by Western blot.

Results: Bone injury resulted in timely controlled changes to the miRNA expression profile in plasma. At day 3 there was a major down-regulation of miRNA levels, which was partially recovered by day 14 post-injury. Interestingly, bone injury led to a significant up-regulation of let-7a, let-7d and miR-21 in plasma and splenocytes at day 14 relative to day 3 after bone injury, but not in sham operated animals. IPA predicted that most miRNAs temporally affected were involved in cellular development, proliferation and movement. MSC proliferation was analysed and found significantly increased in response to plasma of animals days 3 and 14 post-injury, but not from NO animals. Moreover, IPA predicted that miRNA processing proteins Ago2 and Dicer were specifically inhibited at day 3 post-injury, with Ago2 becoming activated at day 14. Protein levels of Ago2 and Dicer in splenocytes were increased at day 14 relative to day 3 post-bone injury and NO animals, while in PBMC, levels were reduced at day 3 (albeit Dicer was not significant) and remained low at day 14. Ephrin receptor B6 followed the same tendency as Ago2 and Dicer, while Smad2/3 was significantly decreased in splenocytes from day 14 relative to NO and day 3 post-bone injury animals.

Conclusion: Results show a systemic miRNA response to bone injury that is regulated in time and is related to inflammation resolution and the start of bone repair/regeneration, unravelling candidate miRNAs to be used as biomarkers in the monitoring of healthy bone healing and as therapeutic targets for the development of improved bone regeneration therapies.

Keywords: inflammation, bone tissue repair, plasma, microRNA, biomarker

3.1. Introduction

Inflammation has been increasingly recognized as an important stage at the onset of tissue repair and regeneration [1,2]. Upon injury, danger signals are released that initiate an inflammatory response characterized by the local secretion of chemokines, cytokines and growth factors, and the recruitment of immune cells (namely, neutrophils and monocytes) to the lesion site, where they contribute to the remodelling of the damaged tissue. Our recent studies on the inflammatory response to biomaterials for bone repair/regeneration suggest that the immune response initiated at the injury site is reflected at the systemic level, leading to alterations on the levels of blood cell populations and circulating cytokines [3,4]. A balanced immune response to injury is crucial for the successful repair/regeneration of damaged tissues; however its temporal regulation is still poorly understood. Indeed, early impairment of immune response compromises bone repair upon injury, whereas a prolonged and exacerbated activity of the immune system may delay or prevent bone healing (e.g., non-union bone fractures) [5,6]. Cytokines and microRNAs (miRNAs) are suggested to be major regulators of this inflammatory phase during tissue repair [7].

miRNAs are non-coding RNAs of small size (~22 nucleotides) responsible for the control of gene expression, mainly at the post-transcriptional level [8]. miRNA genes are physiologically transcribed in cells as long transcripts that are then processed by enzymes such as Drosha and Dicer [9] and loaded into the RNA-induced silencing complex via Ago2, where they target complementary messenger RNA (mRNA), preventing its translation or promoting its degradation [10]. This implies that an alteration in the level of a specific miRNA is usually accompanied by an opposite change in the levels of proteins encoded by their target mRNAs. Importantly, one single miRNA is capable of binding to several mRNAs [11], thus affecting the expression of multiple genes at once. On the other hand, different miRNAs may target the same mRNA [12], potentiating a stronger effect upon the expression of a specific protein. miRNAs have been described as important regulators of cell functions such as proliferation, survival, differentiation and metabolic activity [8], being implicated as well in the onset and progression of different diseases, particularly cancer [13]. Cells secrete miRNAs, which can be found circulating systemically in virtually all body fluids, associated with protein complexes containing Ago proteins or enclosed inside extracellular vesicles that protect them from degradation. For this reason, miRNAs have been extensively explored for their potential as diagnostic and prognostic non-invasive biomarkers in injury and disease [14–16].

Tissue injury has been reported to impact the miRNA expression profile in different organs, namely heart [17], liver [18] and kidney [19]. Furthermore, previous work has demonstrated that these local alterations in miRNA expression are accompanied by changes

in the circulating miRnome [20], potentially influencing the response to injury. Changes in miRNA expression at the lesion site were previously described for bone injury, with expression changing with time after injury [21–23]. In addition, a defined set of miRNAs were found to be differently expressed in the serum of osteoporotic fracture patients compared with osteoarthritis or healthy control fracture patients [24–26]. However, a detailed characterization of the systemic alterations occurring in the circulating miRnome over time after bone injury in normal physiological conditions remains to be explored. Thus, this work aimed to dissect the plasma miRNA expression profile over time after bone injury. This is of great importance for monitoring fracture healing and for the development of more efficient bone tissue regeneration strategies.

To achieve this goal, an *in vivo* rat model of bone injury was used and miRNA expression in plasma was profiled following bone injury at the acute inflammatory phase (3 days) and at the inflammation resolution phase (14 days). A timely controlled miRNA expression pattern was found, with a general down-regulation of miRNA expression at day 3 that started recovering and up-regulating specific miRNAs at day 14. Bioinformatics analysis predicted a regulation of cell development, proliferation and migration over time after injury. Plasma collected at days 3 and 14 post-injury stimulated an increased proliferation of MSC *in vitro*. A time-dependent regulation of the miRNA processing proteins Ago2 and Dicer was also predicted, and their protein levels were confirmed to be altered in splenocytes and PBMC over time after injury. To the best of our knowledge this is the first study showing a temporal characterization of the systemic miRNA profile upon bone injury.

3.2. Methods

BONE INJURY ANIMAL MODEL

A critical size bone defect *in vivo* model was performed as previously described [3]. Briefly, three-month-old male Wistar-Han rats (N = 6 animals per group) were subjected to a lateral arthrotomy of the right knee, muscles were retracted and a cylindrical defect (with 3 mm diameter and ~4 mm depth) was surgically drilled in the anterolateral wall of the lateral condyle of the femur. For sham operated animals the same procedure was followed, but no cylindrical defect was created (N = 3 animals per timepoint). Surgeries were performed under general anesthesia with volatile isoflurane. After surgery, buprenorphine subcutaneous analgesia (0.05 mg/kg) was provided twice a day to operated animals, for 2 days. Animals were sacrificed at 3 and 14 days after surgery, which correspond to the acute inflammatory phase, triggered by the injury, and to the resolution of inflammation, respectively [27]. Non-operated (NO) animals were used as control.

All procedures were performed in accordance with the ethical requirements on animal welfare and experimentation and were approved by the institutional animal ethics committee and the Portuguese official authority regulating laboratory animal sciences (DGAV).

BLOOD AND ORGAN COLLECTION AND PROCESSING

Animals were maintained under general anesthesia with volatile isoflurane and whole blood was collected by cardiac puncture into tubes containing anticoagulant citrate-phosphate-dextrose solution (Sigma-Aldrich) at a 1:5 ratio (Vol solution: Vol blood). Within 30 min of collection, blood was centrifuged at 1200 ×g (without acceleration/deceleration) for 20 min at room temperature (RT), and plasma and buffy coat layers were collected separately. Plasma was further centrifuged at 2500 ×g for 15 min at 4 ºC, to remove cell debris. Supernatant was collected and preserved at -80 °C until further use. Buffy coat was diluted in phosphate buffered saline, overlaid on an equal volume of Lymphoprep (Axis-Shield), and centrifuged at 800 ×g (without acceleration/deceleration) for 30 min at RT for peripheral blood mononuclear cells (PBMC) isolation.

Animals were then dissected for collection of spleen, liver, and inguinal and popliteal draining lymph nodes. Liver was preserved in neutral buffered formalin 10% (v/v). Spleen was injected with 1 mg/mL collagenase I (Sigma-Aldrich), and crushed on top of a 100 µm cell strainer for single cell isolation. Red blood cells in cell suspension were then lysed by incubation with lysis solution (NH_4Cl 150 mM in Tris 10 mM) for 8 min, at 37 °C. Draining lymph nodes were crushed as above for single cell isolation.

RNA ISOLATION

For plasma total RNA isolation, 400 µL of plasma were mixed with TRIzol® in a 1:2 ratio (Vol plasma: Vol TRIzol®), and then samples were spiked with 25 fmol of exogenous *C. elegans* miRNA-39 mirVana miRNA mimics (Ambion). Chloroform was added, samples were centrifuged and the colourless upper phase containing the RNA was collected. RNA was precipitated with absolute ethanol and then purified using the PureLink kit columns (Life Technologies), following the manufacturer's protocol.

For RNA isolation from splenocytes, at least 1×10^6 cells were lysed with TRIzol®, followed by spiking with 25 fmol of exogenous *C. elegans* miRNA-39 mirVana miRNA mimics (Ambion), as above. RNA was then isolated following the manufacturer's protocol.

RT-QPCR ANALYSIS OF MIRNA EXPRESSION IN PLASMA AND SPLENOCYTES, AND MICROARRAY PROFILING OF PLASMA RNA

The expression of circulating mature miRNAs in rat plasma was analysed with Rat miRBase Profiler HC (Plates 1 and 2) miScript™ miRNA PCR Array, following the

manufacturer's protocol. Briefly, total plasma RNA was used to synthetize cDNA using miScript II RT kit with miScript HiSpec Buffer, which includes a mix of forward primers specific for each rat miRNA included on miRBase v.20. RT-qPCR was then performed with synthesized cDNA and miScript SYBR Green PCR kit in a CFX384 Real-Time PCR Detection system (Bio-Rad). RNA quality and efficiency of the reaction were assessed by the positive control wells included in the RT-qPCR arrays. RNA samples analysed showed Cq = 19±2 (**Figure S1A**), which according to the manufacturer's guidelines indicates good quality of RNA and efficient reactions. Only miRNAs with Cq < 35 cycles that were expressed in at least 75% of the animals of at least one experimental group were considered for the analysis. miRNA expression data was normalized by the ΔCq method using endogenous and spiked miRNAs. Heat maps were generated using Morpheus software (Broad Institute, available online at https://software.broadinstitute.org/morpheus/), a platform designed for visualization and analysis of data in a matrix format. Hierarchical clustering of the fold-change heat map was computed using Euclidean distance and complete linkage method.

Detection of single miRNA (rno-let-7a-5p, rno-let-7d-5p, rno-miR-21-5p and *C. elegans* miR-39-5p) expression was performed by RT-qPCR using total RNA from plasma and splenocytes and miScript™ Primer assays (Qiagen), following the manufacturer's instructions.

BIOINFORMATICS ANALYSIS

The expression pattern of specific miRNA genes at 3 days post-injury versus NO, 14 days post-injury versus NO, and 14 days post-injury versus 3 days was analysed using Venn diagrams generated with Venny 2.1 online software [28]. For the analysis, only miRNAs with an absolute fold-change >1.5 that were expressed in at least 75% of the animals of at least one experimental group were considered.

The miRNA expression patterns obtained were correlated with biological pathways using Ingenuity Pathway Analysis (IPA) software (QIAGEN Inc., https://www.qiagenbio-informatics.com/products/ingenuity-pathway-analysis) [29]. IPA determines miRNA target genes and performs statistical tests for the enrichment of those genes in canonical pathways in its database, which was hand-curated and includes not only gene interactions predicted *in silico* but also those experimentally demonstrated and previously published in the scientific literature.

MSC ISOLATION AND CULTURE

Rat MSC were isolated from rat femur bone marrow, as previously described [30]. Briefly, bone marrow was flushed from bones, filtered through a 100 µm cell strainer, red blood cells were lysed as above, and cells were seeded in minimum essential medium alpha

modification (α-MEM) containing 10% MSC-qualified fetal bovine serum (FBS; both from ThermoFisher Scientific). MSC were selected by adhesion and cryopreserved for later use. Resulting cells were CD29+, CD90+ and CD45-, and were able to differentiate into the osteogenic, chondrogenic and adipogenic lineages [30]. For each experiment, a vial of MSC was thawed and cells maintained in MSC media as above in a humidified incubator at 37 °C and 5% CO_2. MSC of passage 5 were used for this work.

MSC PROLIFERATION ASSAY

Rat MSC were seeded at 3000 cells/cm^2 and grown in MSC media until 60-70% confluence. Then, MSC were stimulated with rat total plasma diluted 1:3 (v:v) in α-MEM supplemented with heparin 3 U/mL (B. Braun) to avoid coagulation. Serum-free media and MSC media (with 10% FBS) were used as controls. After 48 h, cells were fixed, immunostained for Ki67 (diluted 1:150; clone SP6, ThermoFisher Scientific) followed by AlexaFluor647-conjugated secondary antibody, and nuclei stained with DAPI 1 µg/mL. Secondary antibody alone was used as negative control. The bottom of the wells where MSC were seeded were imaged in an IN Cell Analyzer 2000 (GE Healthcare) using a Nikon 20x/0.45 NA Plan Fluor objective, capturing 30 sequential, non-overlapping images that mapped mainly the centre of the well, corresponding to a coverage of 60% of the total well area. Acquired images were analysed using IN Cell Developer software. For the analysis, the total number of nuclei in an image was counted, and the Ki67 staining intensity levels inside the nuclei were determined. MSC were considered Ki67-positive for fluorescence levels above the threshold found in cells immunolabelled with secondary antibody only (negative control).

WESTERN BLOTTING

PBMC and spleen cells were lysed with RIPA buffer supplemented with phosphatase and protease inhibitors, and protein was quantified by DC Protein Assay kit (Bio-Rad).

The same amount of protein (20 µg) of each sample was prepared in reducing loading buffer, denatured at 95 °C for 5 min, and loaded in 10% polyacrylamide SDS-PAGE gels. Resolved proteins were wet-transferred to nitrocellulose membranes, and membranes blocked with non-fat dry milk 5% solution. Membranes were probed for 1 h at room temperature, or overnight at 4 °C with the following primary antibodies: anti-Dicer (clone F-10), anti-Ago2 (clone B-3), anti-EphB6 (clone D-7), anti-Smad2/3 (clone C-8) (all from Santa Cruz Biotechnology), and anti-Akt (Cell Signalling Technology). Membranes were probed with HRP-conjugated secondary antibody (GE Healthcare), incubated with ECL substrate, and chemiluminescence signal was detected with autoradiographic films (all from GE Healthcare). Films were scanned on a GS-800 calibrated imaging densitometer (Bio-Rad),

protein bands were quantified on Fiji, and relative protein levels were calculated using total Akt bands as normalizer.

HISTOLOGICAL ANALYSIS

Liver was fixed in formalin 10% (v/v) for 24 h and processed for paraffin embedding. Paraffin sections of 3 µm thickness were sequentially cut, deparaffinized in xylol, and rehydrated in a series of ethanol solutions of decreasing concentration. Sections were stained with haematoxylin and eosin (H&E) by incubation in Gill's Haematoxylin, followed by incubation in alcoholic Eosin Y. Slides were mounted and imaged using an Axiovert 200M microscope (Zeiss).

STATISTICAL ANALYSIS

Data analysis was performed using GraphPad Prism v6.0 software. Normal distribution of data was tested by D'Agostino & Pearson omnibus normality test and found to be non-parametric. Presence of outliers was tested by the ROUT method with Q = 0.5%. For comparison of miRNA expression between two groups, the non-parametric Mann-Whitney test was computed, along with the False Discovery Rate for each miRNA, calculated for multiple comparison correction by the Benjamini and Hochberg method. For comparisons between multiple groups, the non-parametric Kruskal-Wallis test was used, followed by uncorrected Dunn's multiple comparison test. Tests were run for a significance level α of 0.05.

3.3. Results

3.3.1. Expression profile of circulating miRNAs changes over time upon bone injury

To profile the circulating non-coding RNAs potentially involved in bone injury, the plasma miRnome of animals 3 and 14 days after bone injury was compared to that of NO animals using RT-qPCR array (**Figure 1**). From 653 rat miRNAs included in the array, 66 miRNAs were successfully detected in 75% of the animals of at least one experimental group, as represented in the **Figure S1B** heatmap of miRNA expression in plasma of all animals included in the study. Results obtained are illustrated in **Figure 1A** as the fold-change of miRNA expression levels at each timepoint after injury, relative to basal levels of NO controls (fold-change = 1, black). A trend to lower levels of expression, particularly at day 3, was observed and is denoted by the predominant green colour representation in the heatmap. The hierarchical clustering of the miRNA expression profiles at each timepoint indicates a higher similarity of circulating miRnome between day 14 post-injury and NO

animals, which differentiates from day 3 post-injury (**Figure 1A**). A detailed analysis of the miRNAs detected in each timepoint revealed that 68% of the circulating miRNAs detected at day 3 post-injury were down-regulated relative to NO controls (fold-change <-1.5), whereas only 33% of the miRNAs detected at 14 days after injury were down-regulated (**Figure 1B**). Simultaneously, at 14 days post-injury there was an increase of the miRNAs up-regulated relative to NO controls (fold-change >1.5), rising from 6% at 3 days post-injury to 18% at day 14 (**Figure 1B**). Interestingly, nearly 48% of the miRNAs detected at day 14 post-injury were expressed at similar levels to NO controls (unchanged), a percentage that greatly increased from day 3 to day 14 after injury (**Figure 1B**).

The time-dependent changes in miRNAs expression were further explored for those miRNAs with an absolute fold-change to NO controls >1.5, a threshold considered to be biologically significant [21]. Among the miRNAs with an upregulation >1.5 fold, compared with NO animals, only 2 were exclusively up-regulated at day 3, 10 at day 14, and 2 were maintained from day 3 to day 14 post-injury (**Figure 2A** and **Table S1**). Conversely, 28 miRNAs were down-regulated only at day 3, 17 were down-regulated at both timepoints after injury and only 5 were down-regulated exclusively at day 14 (**Figure 2B** and **Table S2**). Interestingly, 9 miRNAs down-regulated at day 3 were then up-regulated at day 14 post-injury (**Figure 2C** and **Table S3**), including miRNAs that were bellow detection at day 3 and up-regulated at day 14. On the other hand, only 1 miRNA was up-regulated at day 3 and then down-regulated at day 14 post-injury (**Figure 2D**).

Taken together, these results suggest a temporal regulation of miRNA expression after injury.

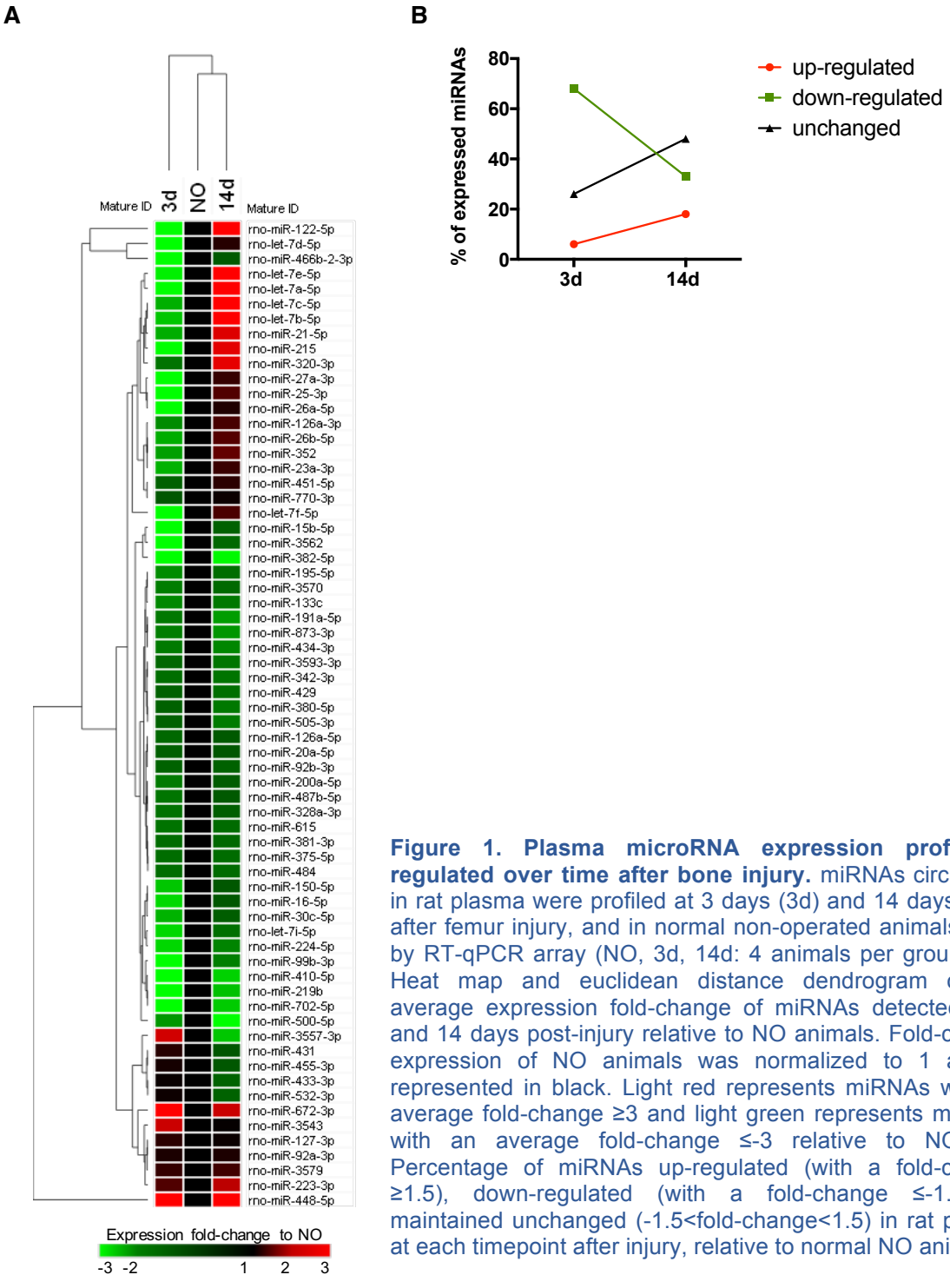


Figure 1. Plasma microRNA expression profile is regulated over time after bone injury. miRNAs circulating in rat plasma were profiled at 3 days (3d) and 14 days (14d) after femur injury, and in normal non-operated animals (NO) by RT-qPCR array (NO, 3d, 14d: 4 animals per group). (**A**) Heat map and euclidean distance dendrogram of the average expression fold-change of miRNAs detected at 3 and 14 days post-injury relative to NO animals. Fold-change expression of NO animals was normalized to 1 and is represented in black. Light red represents miRNAs with an average fold-change ≥3 and light green represents miRNAs with an average fold-change ≤-3 relative to NO. (**B**) Percentage of miRNAs up-regulated (with a fold-change ≥1.5), down-regulated (with a fold-change ≤-1.5) or maintained unchanged (-1.5<fold-change<1.5) in rat plasma at each timepoint after injury, relative to normal NO animals.

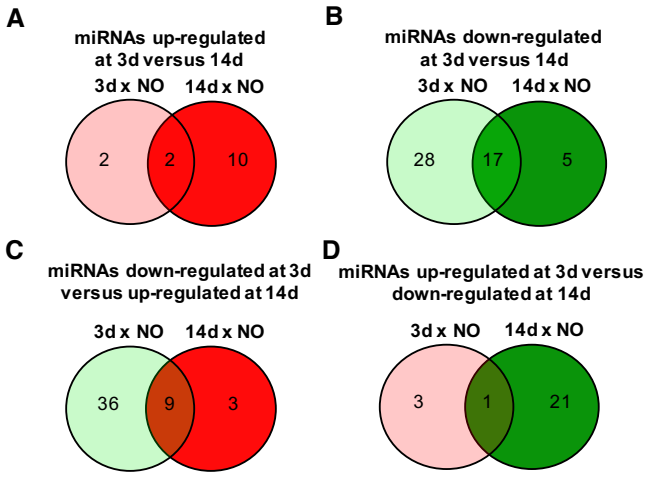


Figure 2. Progress of microRNA expression profile over time after injury. miRNA expression fold-changes at 3 days (3d) and 14 days (14d) after injury were calculated relative to non-operated (NO) control animals, and miRNAs up-regulated (fold-change ≥1.5) and down-regulated (fold-change ≤-1.5) were compared for both timepoints post-injury (NO, 3d and 14d: 4 animals per group). (**A**) Comparison of miRNAs up-regulated at 3 days post-injury (relative to NO) with miRNAs up-regulated at 14 days (relative to NO). (**B**) Comparison of miRNAs down-regulated at 3 days post-injury (relative to NO) with miRNAs down-regulated at 14 days (relative to NO). (**C**) Comparison of miRNAs down-regulated at 3 days post-injury (relative to NO) with miRNAs up-regulated at 14 days (relative to NO). (**D**) Comparison of miRNAs up-regulated at 3 days post-injury (relative to NO) with miRNAs down-regulated at 14 days (relative to NO). The number of miRNAs found in each category is indicated inside the circles and more information on the exact miRNAs is in Tables S1-S3.

3.3.2. Let-7 family of miRNAs is up-regulated at day 14 post-injury

To scrutinize in more detail the systemic regulation of plasma miRNA over time post-injury, we then focused our miRnome analysis on the comparison of day 14 versus day 3 post-injury (**Table 1**). The most up-regulated miRNA was miR-122, which was 88.89 times more expressed at day 14 than at day 3. Although this up-regulation was not significant (p=0.34), as miR-122 is often considered a biomarker of liver injury [31], we confirmed that this up-regulation was not related to liver lesions in this model. No histological alterations could be detected in the liver of injured animals (**Figure S2**). Amongst the miRNAs with the highest up-regulation and lowest False Discovery Rate (FDR) at day 14 were several members of the let-7 family, let-7a, let-7b, let-7c, let-7d, let-7e and let-7f; but, only let-7d and let-7a were statistically different (**Table 1**). Those results are illustrated in more detail in **Figure 3A**, with let-7a-5p and let-7d-5p being statistically significantly up-regulated at day 14 post-injury compared with day 3. Also, the prototypical oncogene miR-21-5p was significantly up-regulated, with a low FDR at day 14 compared to day 3 after injury (**Table 1** and **Figure 3A**). Importantly, these miRNAs have human homologs, and are not increased in sham operated animals between days 3 and 14 post-surgery (**Figure 3B**). On the other hand, only 3 miRNAs were markedly down-regulated (fold-change <-1.5) at day 14 post-injury

compared to day 3, namely miR-3557-3p, miR-3543 and miR-672-3p (**Table 1**), with miR-3557-3p being statistically significantly down-regulated. However, this miRNA was not considered for further analysis because it has complete homology with rno-miR-378a-5p, which was detected in our array at very low levels.

Table 1. Top 10 miRNAs most differently expressed in plasma of animals at 14 days post-injury compared to day 3. Left: plasma miRNAs up-regulated at 14 days relative to 3 days post-injury. **Right:** plasma miRNAs down-regulated at 14 days relative to 3 days post-injury. Data is represented as fold-change at day 14 relative to day 3 post-injury. p-value was calculated by Mann-Whitney test, and False Discovery Rate (FDR) correction for multiple comparisons is indicated for each miRNA (3d and 14d: 4 animals per group).

Up-regulated miRNAs				***Down-regulated miRNAs***			
miRNA name	Fold-change 14d x 3d	P-value	FDR	miRNA name	Fold-change 14d x 3d	P-value	FDR
rno-miR-122-5p	88.89	0.34	0.85	rno-miR-3557-3p	-5.70	0.03	0.35
rno-let-7d-5p	21.09	0.03	0.35	rno-miR-3543	-2.08	0.89	0.95
rno-let-7a-5p	11.70	0.03	0.35	rno-miR-672-3p	-2.05	0.69	0.95
rno-let-7e-5p	10.29	0.06	0.35	rno-miR-505-3p	-1.48	0.49	0.86
rno-miR-466b-2-3p	10.00	0.34	0.85	rno-miR-500-5p	-1.48	0.49	0.86
rno-let-7f-5p	8.59	0.06	0.35	rno-miR-380-5p	-1.41	0.49	0.86
rno-miR-215	7.69	0.34	0.85	rno-miR-433-3p	-1.39	0.49	0.86
rno-let-7b-5p	7.61	0.11	0.39	rno-miR-532-3p	-1.38	0.49	0.86
rno-let-7c-5p	7.20	0.11	0.39	rno-miR-429	-1.27	0.49	0.86
rno-miR-21-5p	5.72	0.03	0.35	rno-miR-3593-3p	-1.23	0.69	0.95

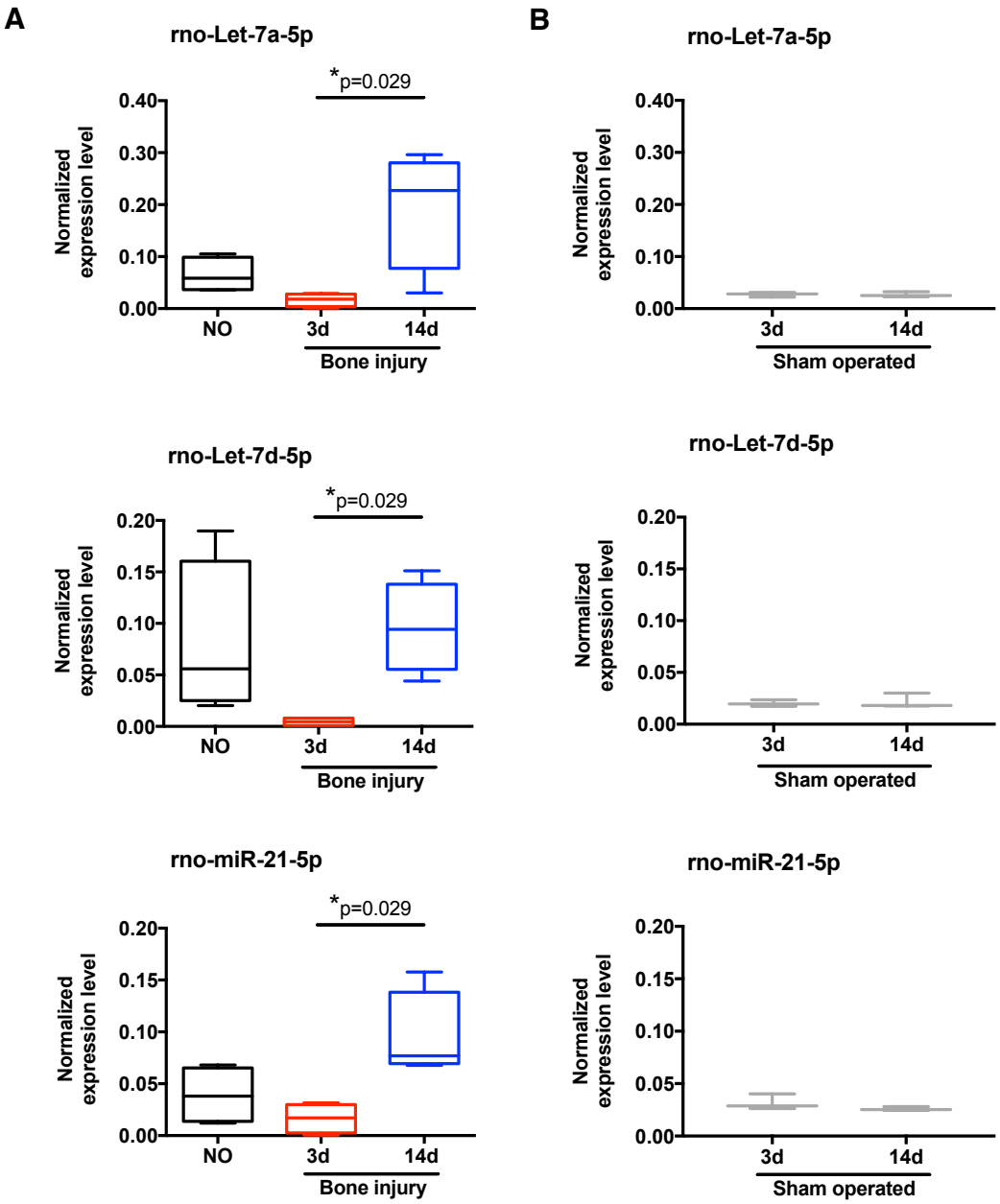


Figure 3. Expression of let-7 family members and miR-21 in plasma is altered by bone injury. (**A**) Detailed expression level ($2^{-\Delta Cq}$) variation of the miRNAs significantly up-regulated in plasma at day 14 after bone injury compared to day 3 (NO, 3d, 14d: 4 animals per group). (**B**) Expression levels of the same miRNAs in the plasma of sham operated animals at day 3 and day 14 after soft tissue surgery (3d, 14d: 3 animals per group). Box plots represent min-to-max distribution, and p-values calculated by Mann-Whitney test are indicated.

3.3.3. Detected miRNAs are predicted to control cellular processes important for bone regeneration

To uncover the biological processes regulated by the detected miRNAs, we then performed a bioinformatics analysis of the miRNAs circulating in the plasma of animals at 14 days post-injury compared with 3 days post-injury. According to the results obtained (**Table 2**), the changes in circulating miRnome from day 3 to 14 post-injury were predicted to control mainly cell differentiation (cellular development) and proliferation. miRNAs in plasma at day 14 post-injury were also predicted to regulate pathways of cell movement, apoptosis and cell survival, and cell cycle. A more detailed analysis of each biological process identified revealed that the pattern of circulating miRNAs at 14 days post-injury was associated with inhibition of tumour cell proliferation and cell cycle progression, suggesting an inactivation of cell proliferation pathways from day 3 to day 14 post-injury (**Figure 4A**).

Next, to functionally validate these predictions, we analysed the potential of plasma from animals at the different timepoints to promote proliferation of bone marrow-derived mesenchymal stem/stromal cells (MSC), which are important for bone regeneration [32]. Results are illustrated in **Figure 4B**, and quantified across different animals in **Figure 4C**. Plasma from animals at days 3 and 14 post-injury, but not from NO animals, promoted significant MSC proliferation when compared to negative control, and to similar levels as FBS positive control. These results do not agree completely with the bioinformatics prediction, as MSC proliferation in response to plasma from day 14 was not statistically different to that obtained with plasma from animals at day 3 (**Figure 4C**). We also analysed the number of cells in draining lymph nodes, and it increased as expected at initial stages of inflammation (day 3), reducing to levels close to or below control by day 14, with inflammation resolution. However, similar results were observed for sham and bone defect animal groups and so should not be related to the miRNA profile (data not shown).

Table 2. Biological pathways predicted to be regulated by the miRNAs circulating in plasma at 14 days post-injury relative to 3 days post-injury. Specific miRNAs predicted to be involved in each process, and p-value range for the association predicted, as calculated in Ingenuity pathway analysis, are indicated.

Biological functions regulated by miRNAs expressed at 14d versus 3d post-injury		
Molecular and Cellular Functions	**miRNAs assigned**	**P-value range**
Cellular development	rno-let-7d-5p, rno-miR-122-5p, rno-miR-126a-5p, rno-miR-15b-5p, rno-miR-20a-5p, rno-miR-215, rno-miR-429, rno-miR-21-5p, rno-miR-223-3p, rno-miR-224-5p, rno-miR-23a-3p, rno-miR-26a-5p, rno-miR-27a-3p, rno-miR-30c-5p, rno-miR-320-3p, rno-miR-451-5p	$4.55x10^{-2}$ to $1.08x10^{-5}$
Cellular growth and proliferation	rno-let-7d-5p, rno-miR-122-5p, rno-miR-126a-5p, rno-miR-15b-5p, rno-miR-20a-5p, rno-miR-21-5p, rno-miR-215, rno-miR-223-3p, rno-miR-224-5p, rno-miR-23a-3p, rno-miR-26a-5p, rno-miR-27a-3p, rno-miR-320-3p, rno-miR-429, rno-miR-451-5p	$4.55x10^{-2}$ to $1.08x10^{-5}$
Cellular movement	rno-let-7d-5p, rno-miR-122-5p, rno-miR-126a-5p, rno-miR-15b-5p, rno-miR-20a-5p, rno-miR-21-5p, rno-miR-224-5p, rno-miR-23a-3p, rno-miR-26a-5p, rno-miR-27a-3p, rno-miR-320-3p, rno-miR-429, rno-miR-451-5p	$4.49x10^{-2}$ to $6.20x10^{-7}$
Cell death and survival	rno-let-7d-5p, rno-miR-122-5p, rno-miR-15b-5p, rno-miR-20a-5p, rno-miR-21-5p, rno-miR-224-5p, rno-miR-30c-5p, rno-miR-320-3p, rno-miR-429, rno-miR-451-5p	$4.63x10^{-2}$ to $1.16x10^{-4}$
Cell cycle	rno-let-7d-5p, rno-miR-15b-5p, rno-miR-20a-5p, rno-miR-21-5p, rno-miR-23a-3p, rno-miR-25-3p, rno-miR-26a-5p, rno-miR-27a-3p, rno-miR-451-5p	$4.80x10^{-2}$ to $1.84x10^{-7}$

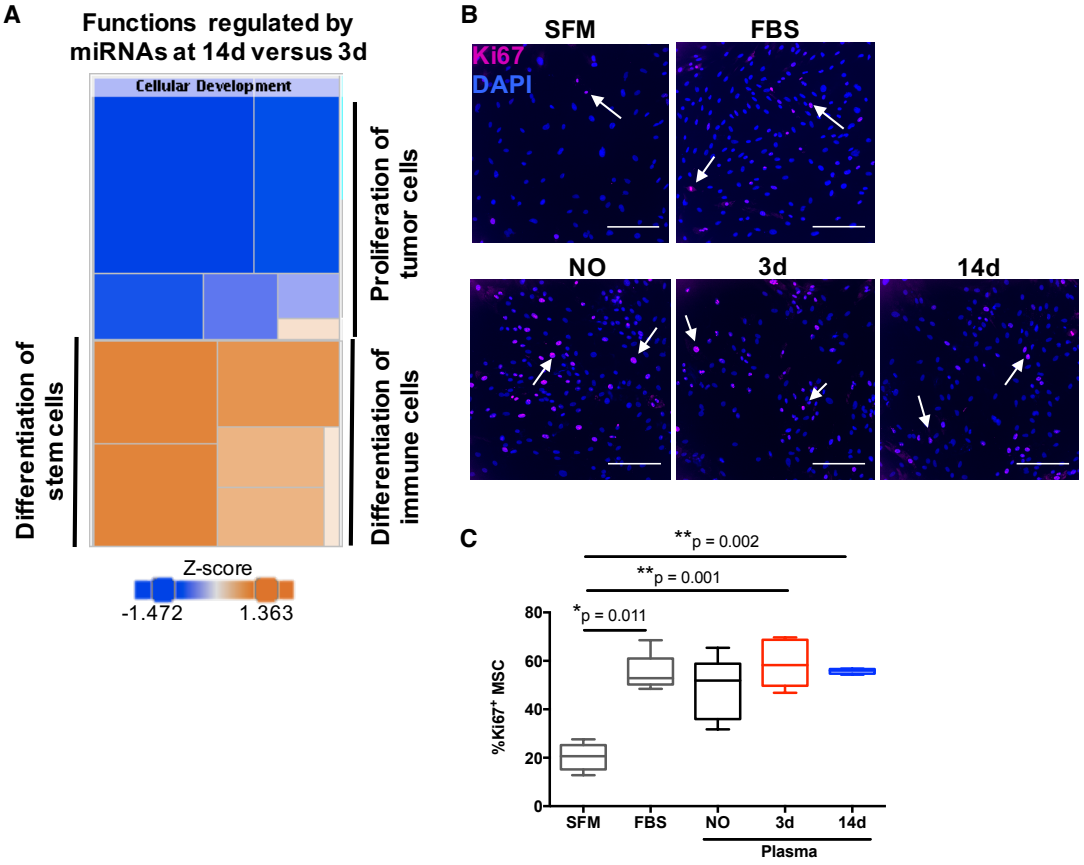


Figure 4. MiRnome circulating in plasma upon bone injury is predicted to control cell proliferation. (**A**) Diagram of the main biological processes associated by Ingenuity pathway analysis with the miRNA expression profile detected in plasma of animals at 14 days (14d) post-injury compared to day 3 (3d) (3d, 14d: 4 animals per group). The colour scheme represents the z-score for the activation status of each biological function, with dark blue representing inhibited functions and dark orange representing activated functions. The colour distribution in the diagram predicts an inhibition of cell proliferation processes at 14 days post-injury. (**B**) Representative micrographs of Ki67 immunostaining of rat bone marrow-derived mesenchymal stem/stromal cells (MSC) after 48 h of stimulation with plasma (diluted 1:3) from NO rats or animals at day 3 and day 14 after bone injury, or maintained in serum-free media (SFM; negative control) or media with FBS 10% v/v (FBS; positive control). Ki67 (AlexaFluor647) is depicted in magenta and nuclei (DAPI) in blue. White arrows indicate representative Ki67-positive nuclei. Scale bar: 200 µm. (**C**) Quantification of Ki67 immunostaining across multiple experiments, represented in the graph as the percentage of $Ki67^+$ MSC. Box plots represent min-to-max distribution (NO: 5 animals; 3d and 14d: 6 animals per group). P-values relative to SFM are indicated, as calculated by Kruskal-Wallis test, followed by uncorrected Dunn's multiple comparison test. None of the comparisons for the other conditions tested were statistically significant.

3.3.4. Detected miRNAs profile suggests changes in miRNA biogenesis pathways

At the molecular level, the bioinformatics analysis indicated that the expression profile of miRNAs over time after injury was caused by alterations in the activation status of the miRNA-related proteins Dicer and Argonaute 2 (Ago2), and of the ephrin receptor B6 (EphB6) (**Table 3**). These proteins were predicted to be inhibited at day 3 post-injury compared with NO controls, while Ago2 and EphB6 were predicted to become activated at day 14 relative to day 3 after injury. Furthermore, the miRnome profile at day 14 post-injury compared with day 3 could be overlapped into the networks of the signalling pathways of IGFR1, MAPK1/2, Smad2/3, Dicer and Drosha (**Figure 5**), which were predicted to be activated.

Table 3. Main canonical pathways predicted to regulate the miRNA expression profile found at day 14 compared to day 3 after bone injury. Status of activation and p-value range for the association predicted, as calculated by Ingenuity pathway analysis, are indicated.

Pathways regulating miRNAs expressed at 3d versus NO		
Causal networks	**Predicted status**	**P-value**
Ago2	Inhibited	$2.83x10^{-30}$
Dicer	Inhibited	$2.51x10^{-18}$
Ephrin receptor B6	Inhibited	$1.32x10^{-17}$
Pathways regulating miRNAs expressed at 14d versus NO		
Causal networks	**Predicted status**	**P-value**
Ago2	Not determined	$4.12x10^{-41}$
Dicer	Not determined	$2.81x10^{-22}$
Ephrin receptor B6	Not determined	$6.58x10^{-19}$
Pathways regulating miRNAs expressed at 14d versus 3d		
Causal networks	**Predicted status**	**P-value**
Ago2	Activated	$3.47x10^{-40}$
Dicer	Not determined	$1.06x10^{-21}$
Ephrin receptor B6	Activated	$1.67x10^{-20}$

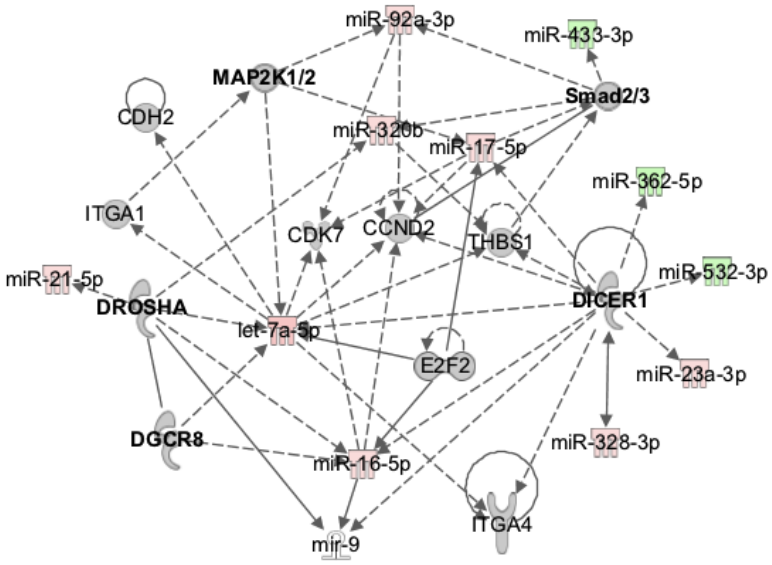


Figure 5. MiRnome circulating in plasma after injury is predicted to be controlled by miRNA processing enzymes and to affect different signalling pathways. Exemplificative diagram of a network of interaction of miRNAs detected in plasma at day 14 post-injury relative to day 3 (3d, 14d: 4 animals per group), with the canonical signalling pathways predicted to affect their expression (e.g., Dicer), and that they were predicted to regulate (e.g., Smad2/3) by Ingenuity pathway analysis.

In order to functionally explore these *in silico* predictions, we tested the expression profile of candidate miRNAs in splenocytes. These cells were chosen due to their involvement in the inflammatory response and reported contribution to the circulating miRNome [33]. Following the expression in plasma, let-7a, let-7d and miR-21 were significantly up-regulated in splenocytes at day 14 post-bone injury relative to 3 days (**Figure 6A**). In splenocytes from sham operated animals, the expression levels of these miRNAs were not different between days 3 and 14 post-surgery (**Figure 6B**). Next, candidate proteins were selected from the signalling pathways predicted to be altered by the bioinformatics analysis, and their protein levels were analysed by Western blot in cell lysates of splenocytes and PBMC from different animals. Results are illustrated in **Figure 7A, C**, and quantified across different animals in **Figure 7B, D**, respectively. When splenocytes of bone injury and sham operated animals were probed for the expression of these proteins (**Figure 7B**), we found that levels of Dicer and Ago2 were significantly higher at 14 days post-injury compared to 3 days and NO animals. Dicer levels were also higher at 14 days post-bone injury than in sham operated animals at the same timepoint, albeit not reaching statistical significance (p=0.066). EphB6 levels followed a similar trend and were significantly higher at 14 days post-bone injury when compared to sham operated and NO animals. On the other hand, Smad2/3 levels were significantly lower at 14 days after bone injury compared to 3 days and NO animals. In PBMC (**Figure 7D**), levels of Ago2 and EphB6 were significantly lower at day 3 and remained low at day 14 post-injury compared to NO animals. There was also a

reduction in the level of Dicer at 3 and 14 days compared to NO animals, albeit not statistically significant (p=0.062 and p=0.052, respectively).

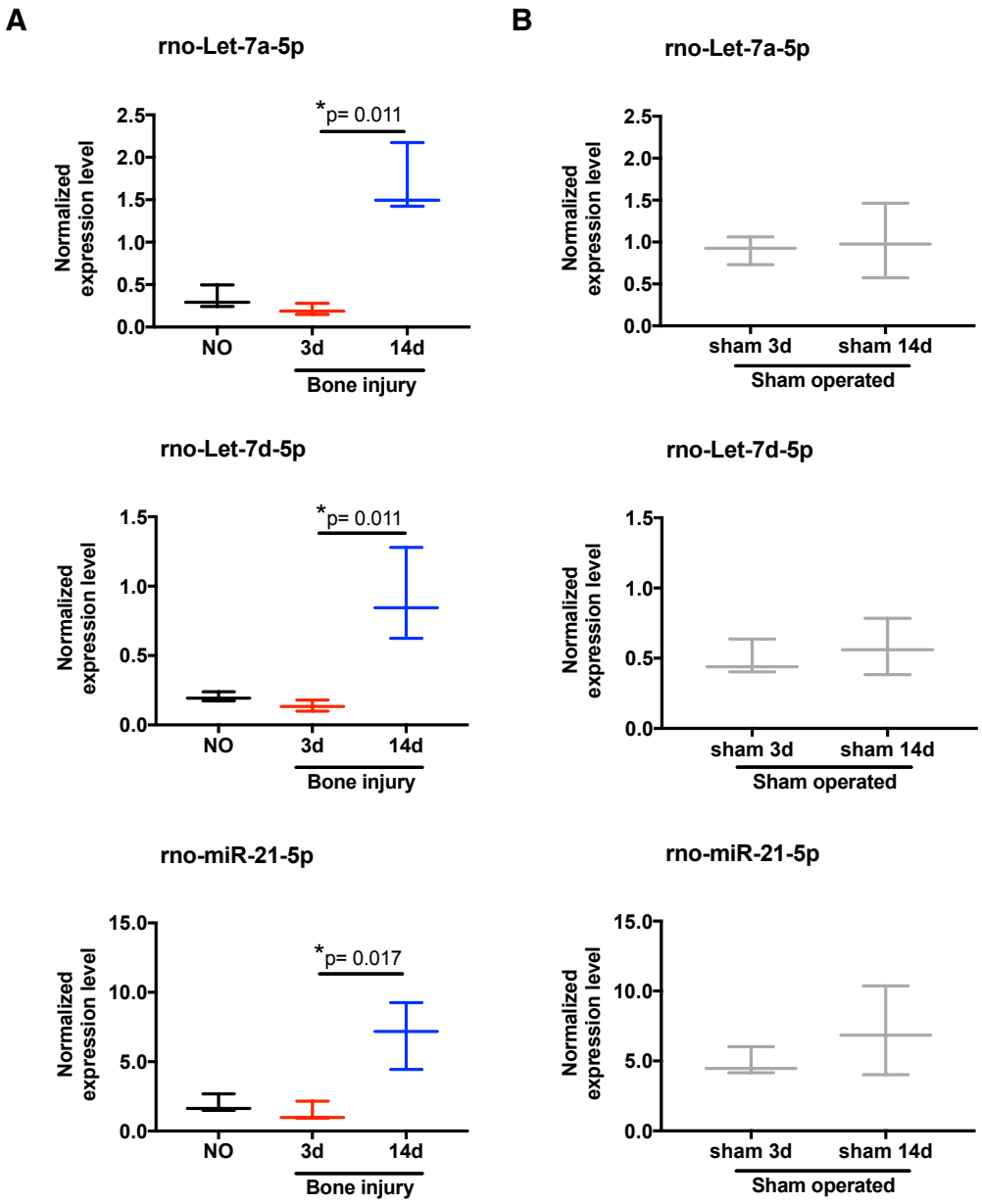


Figure 6. Temporal changes in miRNA expression in splenocytes. Normalized expression levels of rno-let-7a-5p, rno-let-7d-5p, and rno-miR-21-5p in splenocytes of animals from: (**A**) non-operated (NO), 3 days (3d) and 14 days (14d) after bone injury; and (**B**) 3 days and 14 days after sham surgeries. N = 3 animals per group. Box plots represent min-to-max distribution, and p-values are indicated, as determined by Kruskal-Wallis test followed by uncorrected Dunn's multiple comparison test (**A**), or Mann-Whitney test (**B**).

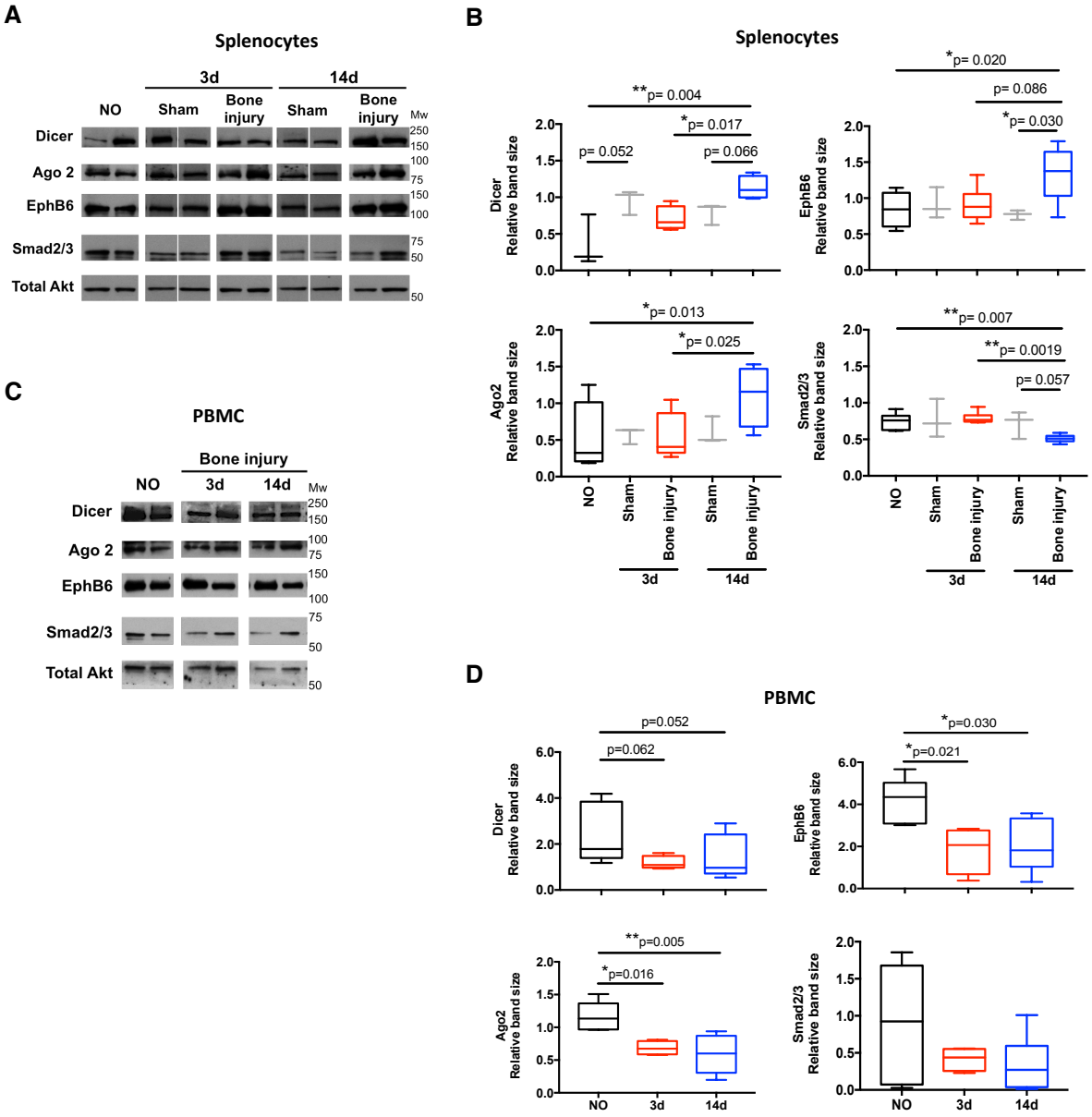


Figure 7. Protein levels of target proteins were altered in splenocytes and PBMC. Dicer (218 kDa), Ago2 (94 kDa), EphB6 (110 kDa), Smad2/3 (55-60 kDa) and total Akt (60 kDa) were analysed by Western blot: (**A**) Representative images in splenocytes isolated from non-operated (NO), 3 days (3d) and 14 days (14d) after sham or bone injury surgeries. Different lanes represent different animals. (**B**) Relative quantification of Western blot bands illustrated in (**A**) across different animals (NO, Sham 3d and 14d: 3 animals per group, and Bone injury 3d and 14d: 4 animals per group). (**C**) Representative images in PBMC of NO, 3 days and 14 days post-injury animals. Different lanes represent different animals. (**D**) Relative quantification of Western blot bands illustrated in (**C**) across different animals (NO: 5 animals; 3d: 4 animals; 14d: 6 animals). Total Akt protein bands were used as normalizer for (**B**) and (**D**). Mw: molecular weight. Box plots represent min-to-max distribution, and p-values are indicated, as determined by Kruskal-Wallis test followed by uncorrected Dunn's multiple comparison test.

3.4. Discussion

The response to bone injury is influenced by systemic factors, such as comorbidities or the co-existence of other injuries [5,34,35]. Also, our previous work shows that the inflammatory response to biomaterial implantation in bone impacts systemic immune cell proportions in blood, draining lymph nodes and spleen [3,4]. Moreover, the systemic response to injury may hold the key for biomarkers that can predict non-union and other bone repair complications [36]. This is particularly important considering that the criteria currently employed in the clinic to diagnose delayed or non-union fractures are often ambiguous and difficult to apply to all types of fractures [37]. Circulating miRNAs, measured in body fluids, are being greatly explored as biomarkers for the diagnosis and prognosis of several diseases [38], namely cancer, cardiovascular conditions, neurodegenerative diseases and infection [13]. Thus, the current work explores the changes in systemic miRNA profile upon bone injury. The response was analysed at day 3, the acute inflammatory phase in the bone defect animal model, and at day 14, the inflammation resolution phase [27]. Our findings show an initial overall down-regulation of miRNAs compared with NO controls. This is in agreement with the local response, as an extensive down-regulation of miRNA expression in mouse bone tissue after fracture has also been reported [21]. At day 14, miRNA expression in plasma started to recover, with several miRNAs returning to the levels found in NO animals, or even being up-regulated. Similarly, it was previously reported in a rat model of liver injury that a genome-wide down-regulation of miRNAs in the liver occurred at 24 h after the lesion [39], being related with the recovery of liver cell mass. A gradual increase in overall miRNA expression in the liver was then observed up to 72 h after injury, becoming closer to the basal expression pattern of control sham-operated animals. Moreover, this initial reduction of miRNA levels was accompanied by a decreased expression of Dicer1 and other enzymes of the miRNA-processing pathway. Interestingly, Dicer was recently implicated in the regulation of inflammatory responses in rheumatoid arthritis [40], being down-regulated in synovial fibroblasts from rheumatoid arthritis patients compared to osteoarthritis patients or healthy subjects. Furthermore, Dicer1 knockdown in healthy synovial fibroblasts increases IL-6 secretion, a key cytokine in the early stages of the bone repair/regeneration process. Recently, this enzyme was also reported to be up-regulated during osteogenic differentiation [41]. Importantly, our bioinformatics analysis predicted that the observed expression pattern was correlated with inhibition of Dicer and Ago2 at day 3 after injury, two key enzymes in the maturation and function of miRNAs [9]. In terms of protein expression pattern, in PBMC we found significant reductions in Ago2 and EphB6 at 3 and 14 days post-injury compared to NO animals, while the reduction for Dicer was not statistically significant. On the other hand, in spleen these reductions at 3 days were not

observed, but Dicer, Ago2 and EphB6 levels were significantly higher at 14 days compared to NO animals, and Dicer and Ago2 were also higher compared to 3 days. Altogether, these results show some resemblance, but do not completely overlap, with the pathways predicted to be activated and inhibited by the bioinformatics analysis of the miRNA profile observed in plasma.

In our study, we were able to identify several specific miRNAs circulating in plasma following bone injury that were also previously reported to be differently expressed in the plasma of human patients with bone-related conditions. Five miRNAs — miR-223-3p, miR-21-5p, miR-122-5p, miR-23a and miR-25-3p — were consistently up-regulated in plasma of injured animals at 14 days post-injury and have also been reported as up-regulated in the serum of osteoporotic fracture patients [24]. miR-122-5p and miR-21-5p were also found up-regulated in the serum of osteoporotic fracture patients by Panach *et al.* [25]. Kocijan *et al.* found an up-regulation of miR-320a in the serum of idiopathic osteoporosis patients with low impact traumatic fractures, and a down-regulation of miR-16-5p, miR-215-5p and let-7b-5p [26]. In our model, we found the same expression pattern for miR-320-3p (homologous to human miR-320a) and miR-16-5p at day 14 after injury. Moreover, miR-215-5p was not detected at day 3 post-injury, and, interestingly, it was up-regulated at day 14 compared to control animals. Similarly, let-7b-5p was down-regulated at day 3 post-injury, and up-regulated at day 14 post-injury.

From the miRNAs detected in plasma over time after bone injury, several members of the let-7 family were down-regulated at day 3 and specifically up-regulated at day 14 post-injury relative to NO control animals (as highlighted in bold in **Tables S1-S3**). This up-regulation of individual miRNAs belonging to the let-7 family is in agreement with the local expression pattern observed in murine union and non-union fractures over time after injury [21,22]. Thus, the current work demonstrates that the systemic response to bone injury also occurs at the miRNA level and reflects, at least in part, the local response that takes place upon bone injury. This is further supported by the up-regulation of several miRNAs (let-7a, let-7d and miR-21) in splenocytes in response to bone injury.

miRNAs of the let-7 family are classically considered as tumour suppressors, targeting mRNAs of genes such as RAS, cyclin D1/2, IGFR1 and AKT2 in different cancer models [42]. However, their role in tissue repair remains vastly uncharacterized. In the context of immune response, let-7 family members have been reported both as pro-inflammatory and anti-inflammatory miRNAs, depending on the cell type where they are being expressed and the inflammatory context. For instance, let-7c is reported to regulate murine bone marrow-derived macrophage polarization towards the M2 pro-regenerative phenotype [43]. On the other hand, let-7c is implicated in enhancing dendritic cell activation, with its overexpression in murine bone marrow-derived dendritic cells leading to an increase

in the secretion of IL-6 upon LPS stimulation [44]. This dual role of let-7 miRNAs is also extended to other biological processes that may impact later stages of bone tissue repair, such as cell proliferation. In general, let-7 miRNAs are considered inhibitors of cell proliferation and, in accordance with this, the bioinformatics analysis performed predicted an anti-proliferative role for the miRNA expression profile at day 14 versus day 3 post-injury. Previous studies have shown that let-7a inhibited proliferation of murine MSC [45]; but, in the work of Wang *et al.*, the overexpression of let-7c in human MSC did not significantly alter their proliferative capacity [46]. Additionally, different members of the let-7 family have been shown to regulate MSC differentiation. In the work of Wei *et al*., let-7c was demonstrated to promote MSC osteogenic differentiation [47]. On the other hand, let-7a, let-7b, let-7c, let-7d, let-7i, let-7f and let-7g were reported to be down-regulated in murine pre-osteoblasts stimulated to differentiate into osteocytes [48], further supporting the concept that miRNA expression regulation in time is needed for the successful regeneration of injured bone. Thus, it is possible that up-regulation of let-7 at 14 days post-injury is related with the initiation of MSC differentiation processes at the beginning of the repair phase, towards bone tissue regeneration [5]. From our results and those of others, reported in the literature, it is clear that the role of the let-7 family in bone tissue repair is complex and needs to be further studied. It is likely that different members of the let-7 family have different, but complementary, biological roles depending on the cell population where they are expressed, which overall can benefit tissue repair/regeneration at different stages of the process. Furthermore, our results may also be explained by the levels of expression of let-7 achieved in response to injury. Indeed, a recent study demonstrated that a tight regulation of the levels of the let-7 family members is necessary to allow regenerative processes to occur, maintaining the balance between promotion of regeneration and prevention of tumorigenesis [49].

From circulating miRNA expression profile, we found that miR-21, classically recognized as an oncomir, was up-regulated at day 14 after injury in comparison to day 3 (highlighted in bold in **Tables S1-S3**). However, miR-21 has also been reported as a mediator of angiogenesis and wound healing [50], and this expression pattern was also previously described in bone tissue of union and non-union fracture animal models [21,22]. Also, rat MSC overexpressing miR-21 induced better fracture healing, with a recent review suggesting that the pro-osteogenic role of miR-21 may be through the indirect regulation of *Col1a1*, and that it is induced by the TGF-β and BMP pathways [51]. Moreover, as mentioned above, a clinical study comparing circulating miRNAs in serum of patients with osteoporotic fractures with non-osteoporotic fracture patients revealed a significant up-regulation of miR-21 [24]; however, this study did not focus on the comparison with the plasma miRNA profile upon fracture in healthy subjects. Later, the work of Panach *et al.*

confirmed this result, showing a significant increase in miR-21 levels in serum of osteoporotic fracture patients compared with osteoarthritis patients [25]. Interestingly, miR-21 is one of the most described immunomodulatory miRNAs, having a major anti-inflammatory role. Expression of miR-21 can be triggered by pro-inflammatory stimuli, such as LPS and IL-6, and also by TGF-β, as demonstrated for murine bone marrow myeloid-derived suppressor cells [52]. In turn, the high levels of miR-21 initiate a negative feedback loop that controls inflammation and promotes its resolution. Indeed, transfection of a murine macrophage cell line with miR-21 decreased the levels of NF-κB and PDCD4, while increasing secretion of IL-10 [53]. Nonetheless, a study by Fabbri and colleagues showed that miR-21 can be recognized by Toll-like receptors and also pointed out a pro-inflammatory role for this miRNA in myeloid cells [54]. Proliferation is another biological process regulated by miR-21. Different studies have been suggesting that the effect of miR-21 on MSC proliferation may vary depending on cell origin, inhibiting proliferation of adipose tissue-derived MSC and of specific populations of amniotic fluid-derived MSC, but promoting proliferation of bone marrow-derived MSC [55,56]. Nevertheless, considering the simultaneous up-regulation of circulating let-7 and mR-21 that we found at day 14 post-injury, it is likely that the pro-proliferative action of miR-21 upon MSC is balanced by the inhibitory action of let-7, and thus the final outcome will depend on the strength of both signals in target cells. Interestingly, miR-21 has been identified both *in vitro* and *in vivo* as a strong promoter of osteogenesis [57,58], and could be involved in the initiation of MSC differentiation at day 14 post-injury.

In addition to these miRNAs, miR-122, found up-regulated at 14 days in the current study, has also been related to bone fractures, being highly up-regulated in the serum of patients with osteoporotic fractures [24,25]. Interestingly, the expression of miR-122 was reported to be dependent on inflammation in other injury models. In a rat model of chronic chemically-induced toxic hepatitis, higher levels of IL-6 and TNF-α were correlated with decreased levels of miR-122 in the affected liver tissue [59], which suggests that, in our study, the expected resolution of inflammation at day 14 after bone injury likely allowed miR-122 expression. Considering that miR-122 is a biomarker of liver injury and stress, it is unclear what causes its up-regulation in the plasma in our injury model (since no major alteration of liver tissue was detected) and in the serum of osteoporotic fracture patients, and further studies are needed in the field, namely exploring possible systemic metabolic changes caused by the dysfunctional bone in both situations [60]. On the other hand, miR-122 has been reported as a tumour suppressor miRNA in different tumour types, including osteosarcoma, inhibiting processes such as cell proliferation [61,62]. Thus, its overexpression in the plasma of injured animals at day 14 further supports an anti-proliferative role for the circulating miRNA expression profile over time after injury. In our previous works we demonstrated that miR-195 inhibits MSC proliferation, osteogenic

differentiation, and angiogenic activity [63]. Interestingly, in this work, the levels of miR-195 were decreased in plasma at day 14 post-injury, compared to NO animals (highlighted in **Table S2**), which is in accordance with the biological processes expected to occur at this stage of response to bone injury.

Considering the pleiotropic action of miRNAs, the ultimate biological effect they mediate in a target cell will depend on the balanced integration of signals induced by different miRNAs. This is only partially supported by our results on the effect of total plasma in MSC proliferation. Despite the prediction for a profile of miRNAs that reduced cell proliferation from day 3 to 14 post-injury, there was increased MSC proliferation in response to plasma from both time points. Of note, we cannot exclude the role of other mediators present in plasma on the observed effect on MSC proliferation. Other studies also demonstrated the capacity of plasma or serum to affect MSC proliferation. In the work by Kim *et al.*, serum from a rat stroke model was able to promote MSC proliferation via miRNA-20a up-regulation [64]. More interestingly, serum from bone trauma patients was also shown to enhance the proliferation of healthy bone marrow-derived MSC, although the timepoint after trauma for sample collection was not clear from this study, and comparison with the effects of stimulation with serum from healthy donors was not performed [65].

Our results also show that protein levels of Dicer and Ago2, predicted to be down-regulated at day 3 and to recover by day 14, were down-regulated, but remained low in PBMC, while no reduction was observed at 3 days in splenocytes, but there was an increase at day 14. Activation of T cells is reported to lead to a general miRNA depletion and the down-regulation of miRNA processing proteins like Ago2 [66], similar to what was observed in the plasma and blood cells of our animals, respectively. The EphB6 showed a similar pattern of expression, with down-regulation in PBMC at both time points and up-regulation in splenocytes at day 14. Interestingly, EphB6 was demonstrated to be important for eliciting an effective and strong T cell activation [67]. In addition, this receptor was previously implicated in the regulation of miRNA expression in breast carcinoma cells [68]. Together, our results support a systemic role for circulating mediators in plasma in the activation of different cell processes at target cells.

In conclusion, the current work provides a detailed profile of plasma miRNAs upon bone injury. The profile obtained and the similarities with the previously reported local miRNA expression indicates that the local response is mirrored at the systemic level and supports the future use of liquid biopsies for monitoring the regenerative process. The temporally regulated expression of the let-7 family, miR-21 and miR-122, supports their important role in the resolution of inflammation and start of the repair/regenerative stage, rendering them as attractive candidate biomarkers for such monitoring. Moreover, this work contributes to a

better understanding of healthy bone healing, which can be explored for the design of new therapies for bone repair and regeneration.

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Supplementary Information

Supplementary Figure S1

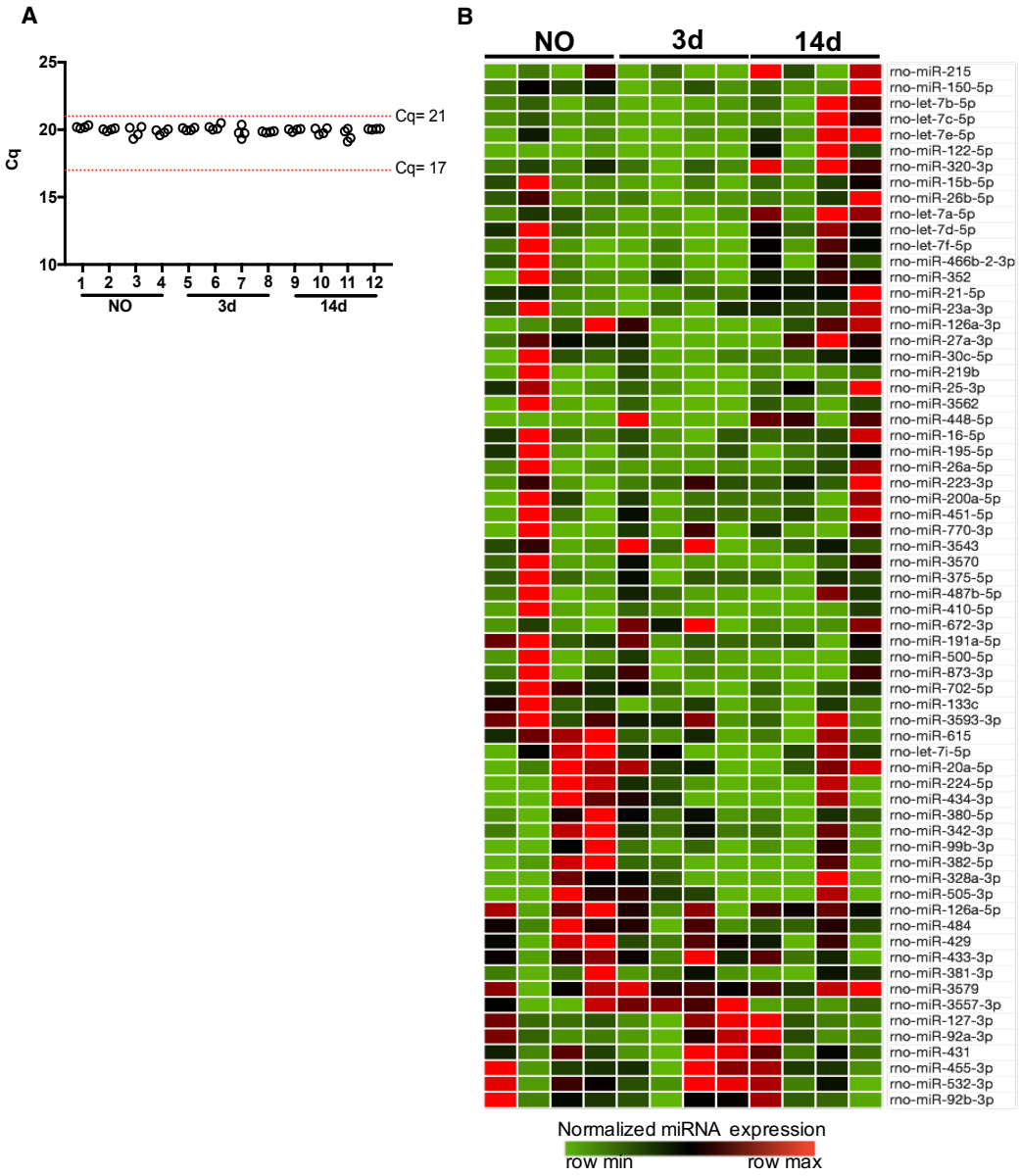

Figure S1 (Relative to Figure 1). miRNA normalized expression in plasma of non-operated rats, and animals at 3 and 14 days after bone injury. (**A**) Cq values of positive PCR controls included in the RT-qPCR arrays performed, for RNA from plasma of non-operated rats (NO), or at 3 (3d) and 14 days (14d) after femur injury. The RT-qPCR miScript® miRNA HC PCR arrays used in this study include positive PCR control wells for assessment of RT-qPCR reactions effectiveness, which takes into account the quality of the RNA used. If a reaction is effective and RNA is of high quality, the Cq values for these wells should range between 17<Cq<21, indicated by dashed red lines in the graph. Cq values are presented for all the samples included in this study, and each symbol represents a technical replicate. (**B**) Heat map of miRNA expression levels, normalized to endogenous and exogenous miRNA controls, for each animal included in the experimental groups (each column represents a different animal). In each row, light red represents the highest $2^{-\Delta Cq}$ value for that miRNA, and light green the lowest $2^{-\Delta Cq}$.

Supplementary Figure S2

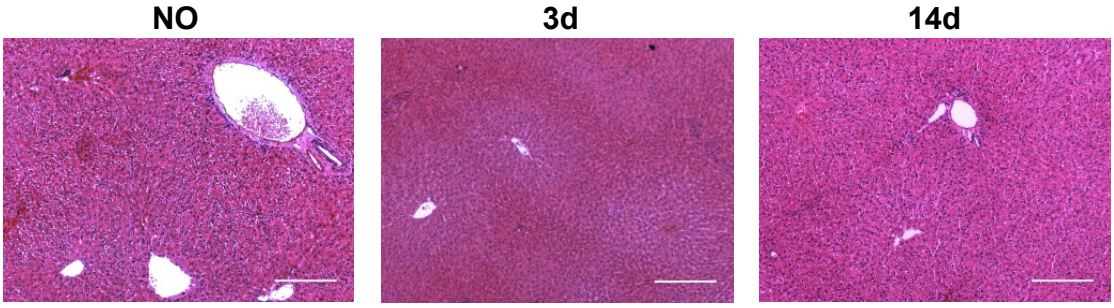

Figure S2 (Relative to Table 1). Histological analysis of rat liver does not reveal any tissue alteration upon bone injury. Representative micrographs of haematoxylin and eosin staining of liver sections from non-operated (NO) rats, and from rats at 3 days (3d) and 14 days (14d) after bone lesion (NO, 3d and 14d: 6 animals per group). Scale bar: 250 µm.

Table S1 (Relative to Figure 2). List of miRNAs up-regulated upon injury as determined by Venn diagram analysis. miRNA expression fold-change at 3 days (3d) and 14 days (14d) after injury was calculated relative to non-operated (NO) control animals, and miRNAs with a fold-change ≥1.5 were compared for both timepoints post-injury (NO, 3d and 14d: 4 animals per group). Specific miRNAs up-regulated only at day 3 (relative to NO), maintained up-regulated both at 3 days and 14 days (relative to NO), and up-regulated only at day 14 (relative to NO) are indicated. Relevant miRNAs, discussed in our analysis are highlighted in bold.

miRNAs up-regulated at 3d versus 14d		
Up-regulated at 3d only (relative to NO)	Up-regulated at 3d and 14d (relative to NO)	Up-regulated at 14d only (relative to NO)
rno-miR-3543	rno-miR-448-5p	**rno-let-7a-5p**
rno-miR-3557-3p	rno-miR-672-3p	**rno-let-7b-5p**
		rno-let-7c-5p
		rno-let-7e-5p
		rno-miR-122-5p
		rno-miR-21-5p
		rno-miR-215
		rno-miR-223-3p
		rno-miR-320-3p
		rno-miR-352

Table S2 (Relative to Figure 2). List of miRNAs down-regulated upon injury as determined by Venn diagram analysis. miRNA expression fold-change at 3 days (3d) and 14 days (14d) after injury was calculated relative to non-operated (NO) control animals, and miRNAs with a fold-change ≤-1.5 were compared for both timepoints post-injury (NO, 3d and 14d: 4 animals per group). Specific miRNAs down-regulated only at 3d (relative to NO), maintained down-regulated both at 3 days and 14 days (relative to NO), and down-regulated only at 14 days (relative to NO) are indicated. Relevant miRNAs, discussed in our analysis are highlighted in bold.

miRNAs down-regulated at 3d versus 14d		
Down-regulated at 3d only (relative to NO)	Down-regulated at 3d and 14d (relative to NO)	Down-regulated at 14d only (relative to NO)
rno-let-7a-5p	rno-let-7i-5p	rno-miR-3557-3p
rno-let-7b-5p	rno-miR-133c	rno-miR-3593-3p
rno-let-7c-5p	rno-miR-191a-5p	rno-miR-380-5p
rno-let-7d-5p	**rno-miR-195-5p**	rno-miR-429
rno-let-7e-5p	rno-miR-219b	rno-miR-505-3p
rno-let-7f-5p	rno-miR-224-5p	
rno-miR-122-5p	rno-miR-342-3p	
rno-miR-126a-3p	rno-miR-375-5p	
rno-miR-150-5p	rno-miR-382-5p	
rno-miR-15b-5p	rno-miR-410-5p	
rno-miR-16-5p	rno-miR-434-3p	
rno-miR-200a-5p	rno-miR-484	
rno-miR-21-5p	rno-miR-500-5p	
rno-miR-215	rno-miR-615	
rno-miR-23a-3p	rno-miR-702-5p	
rno-miR-25-3p	rno-miR-873-3p	
rno-miR-26a-5p	rno-miR-99b-3p	
rno-miR-26b-5p		
rno-miR-27a-3p		
rno-miR-30c-5p		
rno-miR-320-3p		
rno-miR-328a-3p		
rno-miR-352		
rno-miR-3562		
rno-miR-3570		
rno-miR-381-3p		
rno-miR-466b-2-3p		
rno-miR-487b-5p		

Table S3 (Relative to Figure 2). List of miRNAs with opposite expression from day 3 to day 14 after injury. miRNA expression fold-change at 3 days (3d) and 14 days (14d) after injury was calculated relative to non-operated (NO) control animals, and miRNAs up-regulated (fold-chane ≥1.5) and down-regulated (fold-change ≤-1.5) compared for both timepoints post-injury (NO, 3d and 14d: 4 animals per group). Specific miRNAs down-regulated only at 3 days (relative to NO), down-regulated at 3 days but then up-regulated at 14 days (relative to NO), and up-regulated only at 14 days (relative to NO) are indicated. Relevant miRNAs, discussed in our analysis are highlighted in bold.

miRNAs down-regulated at 3d versus up-regulated at 14d

miRNAs down-regulated at 3d (relative to NO)	miRNAs down-regulated at 3d and up-regulated at 14d (relative to NO)	miRNAs up-regulated at 14d (relative to NO)
rno-let-7d-5p	**rno-let-7a-5p**	rno-miR-223-3p
rno-let-7f-5p	**rno-let-7b-5p**	rno-miR-448-5p
rno-let-7i-5p	**rno-let-7c-5p**	rno-miR-672-3p
rno-miR-126a-3p	**rno-let-7e-5p**	
rno-miR-133c	rno-miR-122-5p	
rno-miR-150-5p	**rno-miR-21-5p**	
rno-miR-15b-5p	rno-miR-215	
rno-miR-16-5p	rno-miR-320-3p	
rno-miR-191a-5p	rno-miR-352	
rno-miR-195-5p		
rno-miR-200a-5p		
rno-miR-219b		
rno-miR-224-5p		
rno-miR-23a-3p		
rno-miR-25-3p		
rno-miR-26a-5p		
rno-miR-26b-5p		
rno-miR-27a-3p		
rno-miR-30c-5p		
rno-miR-328a-3p		
rno-miR-342-3p		
rno-miR-3562		
rno-miR-3570		
rno-miR-375-5p		
rno-miR-381-3p		
rno-miR-382-5p		
rno-miR-410-5p		
rno-miR-434-3p		
rno-miR-466b-2-3p		
rno-miR-484		
rno-miR-487b-5p		
rno-miR-500-5p		
rno-miR-615		
rno-miR-702-5p		
rno-miR-873-3p		
rno-miR-99b-3p		

III. General discussion and future perspectives

The contributions of immune cells and mediators for bone homeostatic metabolism and repair and regeneration are increasingly recognized [1]. However, many details of the crosstalk between these two systems remain unclear. For this doctoral thesis we focused on the crosstalk between DC, an immune cell population, and MSC, the bone osteoprogenitor cell population, hypothesizing that their communication could be mediated by EV. The work developed confirmed our hypothesis, and unravels some candidate molecular mediators.

Previous work from our team has demonstrated that blood-derived T cells, NK cells and macrophages are able to promote MSC recruitment *in vitro* [2]. Later, we extended those observations to monocyte-derived immature DC, demonstrating they are also able to promote MSC recruitment, via a paracrine mechanism of cell communication, and to an extent comparable to macrophages [3]. In this thesis we further explored the communication between DC and MSC. We focused our work on DC because their role in bone homeostasis and repair remains largely unexplored, in comparison with other immune cells populations, for which elements of the crosstalk with MSC and other bone cells were previously reported i*n vitro* and *in vivo* [1, 4]. Indeed, the knowledge on the influence of DC upon bone cells is limited and mainly restricted to osteoclasts, with a few studies suggesting that DC can differentiate into osteoclasts, particularly in a pro-inflammatory environment (reviewed in [5]). More recently, a work from Zhang *et al.* showed that conditioned media from murine DC grown in the presence of β-TCP significantly promoted osteogenesis of murine pre-osteoblasts *in vitro*, compared to conditioned media from DC cultured in standard conditions and β-TCP media extracts [6], suggesting a regulatory role of DC upon osteoblasts and osteogenesis.

There are several populations of DC, with origin both in myeloid and lymphoid progenitors, known for their role as professional APC, but with the different subsets performing more specialized functions [7]. DC can be naturally found in blood, secondary lymphoid tissues, and other tissues (e.g., skin, intestine), where they perform surveillance functions. DC can also be differentiated *in vitro* from blood monocytes or bone marrow hematopoietic progenitors [7]. Therefore, DC are versatile cells able to communicate and modulate the action of other cell populations belonging both to the innate and the adaptive immune system, linking both arms of this system, and thus playing a central role mediating immune responses, including in an injury context [8, 9]. In regard of their immunogenic potential, DC are being increasingly explored for anti-cancer therapies [10]. However, their tolerogenic potential remains underexplored, and renders DC particularly interesting for the development of new immunomodulatory therapies for bone trauma and diseases. In fact, the role of DC in bone remodelling and repair is still largely unknown, in comparison to other populations of immune cells. Future studies on the immunophenotyping of circulating DC in patients with impaired bone healing or suffering of bone diseases (e.g., osteoporosis,

osteoarthritis) could contribute to unravel further aspects of DC participation in bone homoeostasis and repair. A similar analysis of bone marrow or spleen-derived DC in animal models of impaired versus normal bone healing, as well as animal models of bone diseases, would further contribute to clarify the role of DC in bone remodelling and repair, allowing then more complex technical analysis, such as histological analysis of DC populations at bone injury sites to assess their spatial distribution and activation status. In addition, following an approach similar to that applied for other immune cell populations, depletion of specific subsets of DC *in vivo*, for instance in CRISPR-engineered knockout animals, could further highlight the function of DC in bone development and homoeostasis.

Remarkably, EV secreted by DC have been reported to play most functions assigned to their parent cells [11]. Furthermore, EV have been described to mediate *in vitro* the communication between immune cell populations found in bone marrow and cells that form the bone tissue [11]. This motivated our hypothesis that the capacity of immature DC to promote MSC recruitment *in vitro* [3] could be mediated by DC-EV. With the work developed in this thesis and described in section II.1, we demonstrated for the first time that DC-EV can promote *per se* the recruitment of MSC, in a mechanism likely dependent on their cargo. Indeed, DC-EV contained a plethora of proteins involved in the promotion of cell migration such as MMPs, osteopontin, and other chemokines, including SDF-1. So far, only EV from platelet lysates [12] and from a chronic lymphocytic cell line [13] had been described to have the same effect on MSC or stromal cells, *in vitro*. Thus, our findings greatly contribute to further uncover the action of immune cell-derived EV upon MSC.

Our early work also suggested that DC pre-conditioning in a pro-inflammatory environment impaired their capacity to promote MSC recruitment [3]. More recent work also implicated TLR4 pro-inflammatory signalling in murine $CD11c^+$ DC as deleterious for intramembranous bone healing [14]. Importantly, EV cargo, and ultimately their function, depends on the parent cell activation status, including their exposure to inflammatory environments [15]. This led us to our second hypothesis that bone injury would lead to changes in DC-EV, including their cargo, impacting on their capacity to promote MSC recruitment. In order to further explore this issue, a bone injury model of critical size bone defect in the femur of rats was used. This model of non-healing bone injury was used because non-healing fractures remain a medical challenge for which conventional treatments are unsatisfactory, as previously explained in Chapter I. By focusing our study in this model we were able to identify novel biological targets that might be used in the clinics for the treatment of non-healing bone lesions. Of note, most of the studies reported in the literature using a model of critical size bone defect in rats are based on the surgical induction of the defect in rat calvaria. However, a great percentage of the non-healing human bone lesions occur in long bones [16], that are different in morphology, biomechanical environment, and

mechanism of ossification (endochondral instead of intramembranous), which conditions the response to bone injury. Furthermore, this model does not require any additional stabilization/fixation of the lesion site, being more easily implemented without the interference of exogenous materials (like fixators), than alternative models commonly used, such as segmental bone defect models [17].

The bone injury response was analysed at two different timepoints, 3 and 14 days, representing the acute and resolution stages of the inflammatory phase of bone healing. The systemic nature of bone injury response, previously reported by us in response to biomaterials implantation [18, 19], was confirmed here by the changes in immune cell populations in spleen, draining lymph nodes and blood. Moreover, BM cells exposed to the injury microenvironment were isolated and differentiated *ex vivo* into DC and Mac for EV production. As described in section II.2, we showed for the first time that EV secreted by DC from precursors isolated at 3 days post-injury, and thus exposed to a microenvironment marked by the acute inflammatory response to bone injury, impaired MSC recruitment *in vitro* compared to DC-EV from NO animals, while EV secreted by DC from precursors isolated at 14 days post-injury, derived from the inflammation resolution microenvironment, promoted significant MSC recruitment. Our findings support the hypothesis that bone injury influences the precursors of immune cells populations at the injury site, and the vesicles that the differentiated cells secrete, further contributing to unravel the impact that immunomodulation has on bone repair, and reinforcing the concept that inflammation, and its timely resolution, are crucial for proper bone healing. Interestingly, our results also suggest that this MSC recruitment capacity of EV is preferential of DC-EV and for MSC, as Mac-EV did not influence significantly MSC recruitment, and DC-EV did not significantly impact the recruitment of fibroblasts. This is in line with several studies suggesting a certain degree of preferential tropism of EV from a specific parent cell, towards a specific target cell or tissue [20, 21], although this EV specificity is still a matter of intense research and controversy in the field. Moreover, in accordance with our previous work, this effect of EV from DC derived from different timepoints after bone injury, is likely dependent on the cargo they carry, which will be altered by exposure of DC precursors to the different microenvironments. Indeed, DC-EV derived from BM cells at the inflammation resolution phase contained several chemokines, and were highly depleted of inflammatory cytokines, such as TNF-α, and migration inhibitory proteins, such as TIMP-1, in comparison with DC-EV from the acute inflammatory phase. Despite the differences that we observed on the capacity of DC-EV to modulate MSC recruitment depending on BM cells pre-conditioning, an important limitation of this *ex vivo* approach is related with the fact that we cannot assure that cultured DC and Mac maintain the phenotype they would have had *in vivo*, particularly considering that their differentiation is performed in presence of cytokines previously suggested to impact DC

maturation and Mac polarization. In fact, IL-4 was reported to promote maturation (beyond differentiation) of rat BM-derived DC [22], whereas M-CSF was associated with an enhanced polarization of human and murine macrophages into the M2 phenotype [23]. Nonetheless, DC-EV from 14 days post-injury were able to significantly recruit MSC, and so their potential for therapeutic application, or of molecules in their cargo, are worth pursuing.

Considering our findings on the capacity of EV from DC populations to promote MSC recruitment, we questioned if they could also impact other aspects of MSC biology important for bone repair and regeneration, namely their (osteogenic and chondrogenic) differentiation, and proliferation. According to our results, human immature DC-derived EV did not significantly impact MSC proliferation or differentiation. Similar findings were observed for EV from DC and Mac derived at different timepoints after bone injury. In contrast, previous studies reported that EV derived from other immune cell populations, namely activated monocytes and platelet lysates, are able to promote MSC osteogenic differentiation [24, 12]. All together, this can lead us to speculate that in an *in vivo* bone injury setting, DC might contribute for bone repair by promoting MSC recruitment into the injury site, where EV from other cell origins might be able to modulate their differentiation, although this still remains to be demonstrated *in vivo*. On the other hand, it is possible that the role of MSC at bone injury sites might not only be restricted to their capacity to differentiate into the osteogenic (and chondrogenic) lineage, but also to their immunomodulatory capacity, an aspect of MSC biology that was not investigated in this thesis with/without stimulation with DC-EV. In fact, MSC were shown to inhibit T cell activation, regulate the function of T_{reg}, inhibit antibody secretion and activation of B cells, inhibit macrophage activation, and inhibit maturation, activation and antigen presentation capacity of DC, with some of these actions also being mediated by MSC-derived EV (reviewed in [25]). However, the potential impact of immature and activated DC on MSC activation and cytokine secretion profile remains unexplored. Conversely, the outcome of the immunomodulatory action of MSC greatly depends on the molecular signals driving their activation, with some authors even proposing an MSC activation profile into a pro-inflammatory M1 phenotype and a M2 anti-inflammatory phenotype [26]. Thus, it is likely that MSC stimulation with EV secreted by BM-DC derived from different microenvironments will also activate MSC differently, conditioning their immunomodulatory properties in a time-post-injury dependent manner, similarly to what happens to their capacity to promote MSC recruitment.

Taking into account the functional differences we observed for DC-EV depending on time after injury at which DC were derived, we hypothesized they would be due to changes in the cargo of the secreted EV. For the work developed in this thesis, we focused our analysis of the EV cargo on proteins with a role described in the regulation of cell migration, such as chemokines, cytokines and MMPs, in an attempt to correlate EV content with the effects

observed for DC-EV on MSC migration. Nonetheless, this analysis could have been extended to other mediators known to regulate MSC migration, as is the case of miRNAs. Moreover, several miRNAs have been described as regulators of MSC migration, with few miRNAs being described as promoters of MSC migration, as is the case of miR-10b [27], miR-9-5p [28] and miR-211 [29], while others are reported to inhibit it, for instance, miR-335 [30], miR-146a-5p [31] and miR-27b [32]. However, the limited amount of EV that could be recovered from primary DC cultures, prevented from doing all the analysis without sacrificing more animals.

Overall, the findings stemming from this thesis on DC-EV regulation of MSC recruitment, lead us to propose EV from immature or tolerogenic DC as new biological therapeutic agents to be further tested for improved bone repair and regeneration. Indeed, as described in section I.2, EV can become “off-the-cell” ready-made products, that carry a complex and complementary cargo likely more effective than bone therapies based on single molecule administration. DC can be obtained easily from peripheral blood monocytes of the patient, and are already being used in clinics, for anti-cancer immunotherapies [10]. Furthermore, EV cargo can be modified *in vitro* to include higher amounts of mediators known to contribute to bone repair [33]. Nonetheless, despite EV exploitation for therapeutical use, including in clinical trials, the widespread use of these vesicles as therapeutics is still far from reality. In fact, the methods currently available for EV isolation present some hurdles that need to be solved for their successful clinical application, namely: (i) reproducibility, a key issue that needs to be improved, to reduce variations in EV cargo content and EV yield from batch-to-batch; (ii) co-purification of other soluble mediators, that constitute a potential contamination of vesicle suspensions; (iii) scale up into a larger size compatible with the clinical demand, and at a reasonable cost; and (iv) official regulatory standards to assess EV safety and biological activity before patient administration, need to be set up [33].

However, it is important to note that a major limitation of this work is that our findings are based on *in vitro* and *ex vivo* models, set up to investigate the effect of DC-EV on different aspects of MSC biology. The contribution of DC for normal bone metabolism and bone repair and regeneration, as well as the role that DC-EV might play in those processes, dependent on MSC recruitment and beyond, remain to be demonstrated in appropriate *in vivo* models. Therefore, the next step to confirm the potential of EV would be the evaluation of their effects upon administration in *in vivo* models of bone injury. The primary outcome of DC-EV administration to be evaluated would be the capacity to promote MSC recruitment. This could be assessed either by measuring the mobilization of endogenous populations of MSC into circulation, and/or by the increase in the number of transplanted MSC at the lesion site. One approach could be using *in vivo* models of bone marrow reconstitution post-

irradiation, combined with labelled MSC transplantation, followed by *in vivo* imaging of long-term engrafted MSC upon induction of a bone injury. In more detail, recipient rats irradiated for bone marrow ablation would be transplanted with hematopoietic cells, together with purified MSC isolated from syngeneic rats ubiquitously expressing fluorescently tagged proteins, for example actin-GFP [34]. MSC injections would be performed intra-bone marrow, for instance in the femur, in order to increase the probability of cell engraftment [35]. Rats with reconstituted bone marrow would then be subjected to the critical size bone defect and treated with DC-EV. In a previous study, Muguruma *et al.*, following a similar approach of labelled hematopoietic stem cells and MSC (eGFP-positive) co-transplantation in mice, showed that at 10 weeks after transplantation, $eGFP^+$ cells could be found nearby the endosteum and associated with the vasculature, among other regions in the bone marrow [35]. Interestingly, $eGFP^+$ cells found near or within bone surface were positive for osteocalcin and some of them had a morphology resembling osteoblasts or osteocytes, suggesting the transplanted $eGFP^+$ MSC engrafted in the bone marrow at the long term and were able to differentiate into the osteogenic lineage [35]. Therefore, it is hypothesized that in the model here proposed, the administration of DC-EV in rats with long-term reconstituted bone marrow and subjected to the bone injury, would lead to the recruitment of labelled donor MSC, and their active participation in the bone injury response. These recruited labelled cells could be detected by *in vivo* imaging and histological analysis of bone sections. An important outcome of DC-EV administration to be evaluated would be the improvement of bone healing, which could be assessed independently of MSC detection, and using μCT, radiographic and histological methods, as well as molecular analysis of bone markers. This could be an indirect assessment of MSC fate upon recruitment into the bone injury site, although the distinction between the effects they may promote by their secretome, or by direct differentiation and integration in the new forming bone tissue would be technically challenging to distinguish, as there is no single marker specific for MSC. For this *in vivo* evaluation of DC-EV therapeutic potential, EV could be administered systemically by injection, or delivered locally at the bone lesion site, integrated in a delivery system. This last approach would be preferred, and likely lead to improved outcomes and less off-target effects. Thus, EV would need to be combined with a delivery system that can be implanted at sites of bone injury, where a controlled and localized release of DC-EV could promote MSC recruitment. To achieve this purpose, a biomaterial-based system for the controlled delivery of EV *in vivo* is already under development in our lab. If results obtained in the proposed *in vivo* studies confirm our hypothesis, exploratory clinical studies of DC-EV administration for the treatment of patients suffering from non-healing bone lesions may be envisaged in the long term.

Following our findings on the capacity of DC-EV to impact MSC recruitment, we intended to investigate if the content of circulating EV could reflect the local response to bone injury at the systemic level. However, the amount of EV, of appropriate quality, that were recovered from rats plasma by ultracentrifugation was limited, preventing us from profiling the molecular composition of these EV, namely regarding their miRNA content. As an alternative approach, since miRNAs may be secreted to the extracellular milieu either enclosed in EV or associated with protecting protein complexes [36], we sought to characterize the plasma miRnome directly, at the 3 and 14 days timepoints after bone injury.

In an era of intense search for improved systems of disease diagnosis and prognosis, biomarkers assume a central role, in particular those readily accessible in biofluids such as urine and blood. In this thesis, we demonstrate in an *in vivo* model of bone injury, that the circulating miRnome changes with time after injury, and as part of the systemic response to that injury. Moreover, we found that miR-21 and several members of the let-7 miRNA family were significantly up-regulated at the resolution phase of the inflammatory response to injury (14 days), compared to the acute inflammatory phase (3 days). Importantly, miR-21 is reported as a biomarker of inflammatory diseases, being induced by pro-inflammatory stimuli, and acting as a driver of inflammation resolution [37]. In fact, miR-21 targets the mRNA of genes such as PDCD4, PTEN and IL12p35, inducers of pro-inflammatory phenotypes [37]. Let-7c is also described to target the mRNA of the pro-inflammatory cytokine IL-6 [38]. On the other hand, miR-21 was recently reported as a promoter of MSC migration *in vitro* [39]. Moreover, miR-21 and let-7 are promoters of osteogenesis [40, 41], with miR-21 also promoting osteoclastogenesis [40]. This suggests that the miRNAs differently expressed along time after bone injury may play a concerted regulatory role upon different processes of the regenerative response. Furthermore, our findings support the fact that the profile of circulating miRNAs reflect, at least in part, the inflammatory response to bone injury and the progression of bone healing, providing the first evidence that plasma miRNAs may potentially be used as biomarkers of evolution of bone repair, as alternative or complementary, to the conventional methods used in the clinics. Assessment of bone healing is currently based on the radiographic analysis of bone union, which is reported as subjective, often inaccurate in the diagnosis, and very limited in the prognosis of impaired bone healing, such as delayed bone union and bone non-union [42]. Several serologic proteins have been proposed as biomarkers for bone healing monitoring, as is the case of TGF-β1 [43, 44], tartrate-resistant acid phosphatase (TRAP) 5b [45], C-terminal crosslinking telopeptide of type I collagen (CTX) [45], collagen III N-terminal propetide (PIIINP) [46], among others (reviewed in [47]). However, the limited number of patients included in the studies, and several confounding factors related to their genetic background and habits, as well as in the design of the studies, has been delaying the establishment of such proteins as

biomarkers for impaired bone healing [47], and consequently their analysis in clinical practice. On the other hand, miRNAs have been explored as biomarkers for diagnosis and prognosis of many different conditions, particularly cancer [48], but also injury of different organs [49, 50], infection [51] and disease, including bone-affecting diseases [52]. Thus, miRNAs circulating systemically along time after bone injury have the potential to be readily accessible indicators of proper bone healing. Furthermore, since they can be detected through precise quantitative techniques such as RT-qPCR, they might be more accurate indicators of the bone healing process than the methods currently used in the clinics.

Considering the limited knowledge on the differences between healing and non-healing bone lesions at the cellular and molecular level, it would also be interesting to further explore the effect of different lesions on the levels of plasma circulating mediators and in the modulation of the content of EV secreted by DC and other immune cells. Future studies exploring the composition and biological activity of EV from immune cells derived from the bone marrow of injured bones, in animal models of healing and non-healing bone defects, could reveal more specific targets for the development of improved therapies for non-healing bone injuries. The comparison of the circulating miRnome in different bone lesion models could not only uncover new miRNA targets for such therapies, but also reveal more specific biomarkers for the diagnosis, monitoring and prognosis of non-healing bone lesions. At this stage, the validation of such miRNA targets as biomarkers of bone healing could be undertaken in clinical studies dedicated to the molecular characterization of blood samples from patients with healing versus non-healing bone lesions.

Together, the work developed throughout this thesis confirmed our initial hypothesis, that EV can mediate the crosstalk between immune cells and bone cells, namely between DC and MSC, and led to the achievement of the main goals set. With this work we showed that bone injury triggers a local and a systemic inflammatory response, characterized by the secretion of mediators such as extracellular miRNAs and EV, that have potential to be used as new biomarkers and therapeutical drugs, opening new perspectives for the development of improved therapies for aggravated non-healing bone lesions, and chronic bone diseases. Nonetheless, the action of most immune cell types in modulating bone cells activity in homeostasis and disease, and the role that EV play in this cell crosstalk is still largely unknown. Future studies exploring the details of this cell communication will open new perspectives for the development of novel and more efficient approaches to treat bone injuries and diseases.

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