UNIVERSIDADE DO PORTO

Faculdade de Engenharia

Instituto de Ciências Biomédicas Abel Salazar

Instituto de Engenharia Biomédica

Mestrado Integrado em Bioengenharia

Biotecnologia Molecular

Dissertação

Steps to enhance bone regeneration:

The Role of Dendritic Cells on Mesenchymal Stem Cells Recruitment

By:

Andreia Machado da Silva

Porto, July 27th 2012

UNIVERSIDADE DO PORTO

Faculdade de Engenharia

Instituto de Ciências Biomédicas Abel Salazar

Instituto de Engenharia Biomédica

Mestrado Integrado em Bioengenharia

Biotecnologia Molecular

Dissertação

Steps to enhance bone regeneration:

The role of dendritic cells on mesenchymal stem cells recruitment

By:

Andreia Machado da Silva

Supervision:

Doutora Susana Santos

Porto, July 27th 2012

This thesis reports the work developed for the course *Dissertação* of the Integrated Master in Bioengineering – Molecular Biotechnology of the Faculty of Engineering and Institute of Biomedical Sciences Abel Salazar, University of Porto, and was submitted for evaluation to obtain the academic masters degree on July 16th 2012.

Evaluation panel:

Professor Doutor Alexandre Quintanilha
(Panel president)

Doutora Cristina Barrias
(Examiner)

Doutora Susana Santos
(Supervisor)

Evaluated and approved in public examination by the evaluation panel members on July 23rd 2012, at Institute of Biomedical Sciences Abel Salazar, University of Porto, Porto.

Acknowledgments

If I managed to conclude my course as well as this thesis it is all due to very important people who always stood by me and supported me. Namely:

Susana Santos, my supervisor during this thesis, always so helpful, dedicated and hard-working for my own good, even if I did not always corresponded to that. She is a true inspiration!

Professor Mário Barbosa, who kindly accepted me to work at INEB during my thesis and whose thoughts, ideas and knowledge make him a role model for my future.

Catarina Almeida, who kindly provide me the mesenchymal cells and always had an important question to me; Maria Oliveira, who always gave me a bit of her little time, helped me with zymographies and qPCR and contributed with important discussions about this work; Keila Fonseca, who nicely helped me with the results of my experiments; David Gomes, who taught me all that I know about histology.

All the colleagues from INEB, with a special remark to Ana Pinto and Marta Pinto, always so sweet about me, Filipa Lourenço, so caring through all those long work days, Joana Santos, with her good mood, Joana Silva, Rita Silva, Diana Leite and Aida Moreira.

Prof. Perpétua Pinto-do-Ó, Prof. Nuno Azevedo, Prof. Maria Helena Carvalho, Prof. Mónica Sousa and Prof. Maria João Saraiva, my teachers during the Integrated Master. They give a true meaning to the word Professor, reaching their students beyond the technical knowledge.

My Biotec fellows! We built this group together through the last 3 years and I will miss every single moment of it. You all had a role in what I am today. I was so lucky to found you!

Tatiana, Ana Isabel, Sandra, Rita and Ana Marta, who share with me their friendship.

My friend José Nuno Senra, who taught me the importance of disconnecting from the world outside.

My family, specially my parents, to whom I owe everything that I am and I reach, and my aunt Susana Machado who actually ended up doing this course along with me through very long work nights.

To all of them my deepest and sincere thank you!

Abstract

Bones and joints can be affected by physical damage, such as fractures, or diseases, like osteoporosis or osteoarthritis, which lead to bone tissue loss and degeneration. Millions of people are affected by these conditions every year and the strategies employed for their treatment are a burden for Health Systems worldwide, accounting for direct expenses in the order of billions of dollars. Hence, the development of enhanced strategies for bone regeneration is urgent and needed.

Bone healing commonly initiates with an inflammatory phase that sets up a chemoattractant environment, for multipotent mesenchymal stromal/stem cells (MSC) recruitment into the bone injury. These cells are known to differentiate into cells of the osteogenic lineage, and also to modulate the action of immune cells. Among them dendritic cells (DC) are particularly relevant since they bridge the innate and the adaptive immune systems. However, the influence of DC upon MSC and the role of this crosstalk in bone regeneration have not been widely studied so far. Hence the goal of this work was to investigate the capacity of DC to promote the recruitment of MSC, also investigating the influence of pro- and anti-inflammatory stimuli in this cross-talk.

In this study human monocyte-derived DC were used, together with primary human bone marrow-derived MSC. CD1a/CD83 expression profile and verification of ISCT criteria were followed to confirm DC and MSC identity, respectively, prior to their use in experiments. Matrigel-coated membranes were used in transwell invasion assays to study MSC recruitment by DC. TNF-α was used as the pro-inflammatory stimulus and resveratrol as the anti-inflammatory stimulus. Cell culture supernatants from transwell assays were used for MMP profiling and cytokine screening, by zymography and Luminex®-based multiplex assay, respectively. DC in the bottom compartment were used for RNA extraction and quantitative RT-PCR. Cell-cell contact approaches were also used to study MSC-DC interaction. Time-lapse video microscopy was performed, and NF-kB nuclear translocation was investigated by imaging flow cytometry (ImageStream^X) and confocal microscopy. DC and stimuli influence on MSC osteogenic differentiation were finally investigated, by ALP activity staining and biochemical assay.

Results demonstrate for the first time that DC significantly promote MSC invasion, for cell ratios of 1:10 and 1:20 (MSC:DC). Gelatine zymograms, together with qPCR, revealed a role of MMP-9, likely mainly produced by DC, in the promotion of MSC recruitment, but it does not seem to be the only key player in the process. IL-10, IL-8, IL-6 and TNF-α were found to be

secreted into the media, likely mostly by DC. Although IL-10 and TNF-α appear to be either consumed or their production inhibited by the presence of MSC, none of them was significantly up or down-regulated in DC-MSC crosstalk. The translation of the signals in DC-MSC communication at an intracellular level was studied monitoring the NF-kB signalling pathway. From the results obtained in imaging flow cytometry and confocal microscopy studies performed, this pathway does not appear to be activated as an immediate result of MSC-DC interaction. Finally, ALP activity changes indicate a potential role for DC in MSC osteogenic differentiation.

Taking together DC recruitment capacity towards MSC, demonstrated here, with MSC capacity to modulate DC, previously shown by others, the cross-talk between these cells is a promising target for the development of new strategies for enhanced bone regeneration.

Keywords: Mesenchymal stem cells; Dendritic cells; Osteoimmunology; Bone regeneration

Resumo

O sistema esquelético pode ser afetado por vários tipos de lesões, como por exemplo fraturas e doenças de diferente etiologia, como a osteoporose e a osteoartrite. Estas lesões caracterizam-se por uma perda de tecido ósseo e consequente degeneração do osso. Milhões de pessoas são afetadas todos os anos à escala global por lesões do sistema esquelético e as estratégias de tratamento atualmente aplicadas comportam custos económicos aos Sistemas de Saúde que ascendem aos milhares de milhão de dólares. Em consequência, é necessário desenvolver rapidamente estratégias novas e mais eficientes de regeneração óssea.

O processo de regeneração óssea tem início numa fase inflamatória, responsável pela criação de um microambiente quimiotático para as células mesenquimais multipotentes do estroma (ou células estaminais mesenquimais, MSC), que deste modo migram para a região da lesão óssea. Estas células são capazes de se diferenciar em células da linhagem osteoblástica, desempenhando também um papel modulatório sobre a ação das células do sistema imunitário. De entre as células do sistema imunitário, as células dendríticas (DC) são particularmente relevantes, uma vez que são das principais responsáveis pela comunicação entre o sistema imunitário inato e adaptativo. Todavia, a influência que as DC exercem sobre as MSC e as implicações desta inter-comunicação na regeneração óssea não foram até agora alvo de estudo pormenorizado. Por este motivo, esta dissertação tem como objetivo investigar a capacidade das DC na promoção do recrutamento das MSC e, em simultâneo, de que forma estímulos pró e anti-inflamatórios condicionam esta ação.

Para as experiências laboratoriais no âmbito deste trabalho, foram usadas DC diferenciadas a partir de monócitos primários de origem humana, bem como MSC isoladas a partir de medula óssea humana. Dada a natureza primária destas culturas celulares, a identidade das MSC e das DC foi verificada antes da realização dos trabalhos experimentais através da caracterização da expressão dos marcadores de superfície CD1a e CD83 e da concordância com critérios internacionais ISCT, respetivamente.

Para investigar o potencial das DC na promoção da migração das MSC, foram realizados ensaios de invasão através de membranas porosas sintéticas cobertas com um análogo da membrana basal, o *Matrigel*. Nestes ensaios um microambiente pró-inflamatório e anti-inflamatório foi recriado pela adição de TNF-α e de resveratrol, respetivamente. No final destas experiências, os meios de cultura foram recolhidos e testados e caracterizados para a presença de metaloproteases (MMP), por zimografia, e de citocinas, pelo sistema de ensaio múltiplo da Luminex®. As DC utilizadas nos ensaios de migração foram também recolhidas para

extração de RNA e avaliação da expressão génica por RT-PCR quantitativo. A interação entre as DC e as MSC foi também estudada ao nível da comunicação intercelular por contacto, através da monitorização da interação celular por vídeo-microscopia com lapso de tempo e, da translocação do fator NF-kB para o núcleo por citometria de fluxo com aquisição de imagem (ImageStream^X), bem como por microscopia confocal. Finalmente foi ainda investigada a influência de diferentes estímulos na potenciação da diferenciação osteogénica das MSC, através da deteção e quantificação da atividade da enzima fosfatase alcalina.

Os resultados obtidos demonstram pela primeira vez que as DC potenciam significativamente a invasão das MSC, quando o ensaio é realizado utilizando células na proporção 1:10 e 1:20 (MSC:DC). Os zimogramas de gelatina e o RT-PCR quantitativo mostram que a MMP-9, potencialmente produzida pelas DC, está implicada neste efeito invasivo, apesar de não ser o único fator determinante. As DC deverão ser também as principais secretoras das citocinas IL-10, IL-8, IL-6 e TNF-α detetadas nos meios de cultura analisados. No entanto, não foi detetada a sobre- ou sub-expressão de nenhuma delas na presença dos dois tipos celulares, apesar das citocinas IL-10 e TNF-α estarem provavelmente a ser consumidas ou a sua produção inibida pela presença das MSC. De acordo com os resultados obtidos para a translocação do fator NF-kB, esta via de sinalização não parece estar a ser ativada pelo contacto DC-MSC de uma forma imediata. Finalmente a atividade da fosfatase alcalina nas MSC muda por ação das DC, indicando um potencial papel das DC na potenciação da diferenciação osteogénica das MSC.

Os resultados aqui apresentados, em conjunto com a capacidade já anteriormente descrita das MSC para modular a atividade das DC, fazem desta interação DC-MSC um alvo promissor no desenvolvimento de novas estratégias terapêuticas que contribuam para uma melhoria da regeneração óssea.

Palavras-chave: Células estaminais mesenquimais; Células dendríticas; Osteoimunologia; Regeneração óssea

Index

List of figures

List of tables

List of abbreviations

BM	Bone marrow
BSA	Bovine serum albumin
CM	Conditioned medium
DAPI	4',6-diamidino-2-phenylindole stain
DC	Dendritic cells
dH_2O	Distilled water
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
ESCs	Embryonic stem cells
FITC	Fluorescein isothiocyanate
FBS	Foetal bovine serum
iDC	Immature dendritic cells
iPS	Induced pluripotent stem cells
LPS	Lipopolysaccharide
MMPs	Metalloproteinases
OPG	Osteoprotegerin
PLGA	Poly-lactic-co-glycolic acid
mDC	Mature dendritic cells
MSC	Multipotent mesenchymal stromal cells/mesenchymal stem cells
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline

PE	Phycoerythrin
RPMI 1640	Roswell Park Memorial Institute 1640
RSV	Resveratrol
RT	Room temperature
SERMs	Selective estrogen receptor modulators
SFM	Serum free medium
SLE	Systemic lupus erythematosus
TLR	Toll-like receptor
VCAM-1	Vascular cell adhesion molecule-1

Cytokines/Chemokines/Growth factors

BMP	Bone morphogenetic protein
FGF	Fibroblast growth factor
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte-macrophage colony stimulating factor
IGF	Insulin-like growth factor
M-CSF	Macrophage colony-stimulating factor
PDGF	Platelet-derived growth factor
RANK	Receptor activator of nuclear factor-κB
RANKL	Receptor activator of nuclear factor-κB ligand
TGF-β	Transforming growth factor-β

1. Introduction

1.1. Bone in health and disease

1.1.1. Bone development and homeostasis

As a characteristic of vertebrate organisms, human anatomy comprises an internal supportive skeleton made up of bones and cartilage. Human beings have approximately 206 bones in their bodies, which are responsible for allowing locomotion and load support, the protection of vital organs, lodging and homeostasis of the hematopoietic system and contributing to ion homeostasis, namely calcium and phosphorus.

Bones are formed during the embryonic development, being derived from the mesodermal germinative layer. The first bone-like structures appears in foetus around the fourth month of gestation, being a non-functional woven tissue of collagen fibres randomly arranged. This is the primary bone. Before birth, this tissue is replaced by a lamellar one, the secondary bone, composed of aligned collagen fibres that are already mechanically functional[1].

Independently of the type of bone to be formed, it occurs by one of the following two processes: intramembranous ossification or endochondral ossification.

In the intramembranous ossification (Figure 1A) mesenchymal stem cells (MSC) directly differentiate into bone cells[1]. In this pathway of ossification, MSC differentiate into osteoblasts, which in turn produce the bone tissue matrix. These osteoblasts become thus entrapped in the fibrous matrix, constituting osteocytes. All together these components form the trabecular bone, the spongy interior of bones that lodges the bone marrow. More osteoblasts surround these trabeculae, further depositing bone matrix and forming the cortical bone, which has a more compact structure[2]. On the other hand, the endochondral ossification (Figure 1B) is characterized by an initial step of formation of hyaline cartilage rudiments, which serve as models that are later replaced by woven bone[1]. In this ossification process, MSC differentiate into chondroblasts, which produce the cartilaginous rudiments shaped like the final bone form. The chondroblasts entrapped in the cartilage become chondrocytes. Lining these structure there are mesenchymal progenitor cells that differentiate into osteoblasts, concomitantly with the vascularisation of the cartilage. The osteoblasts are then responsible for the deposition of bone matrix, from the periphery to the centre of the cartilage. Along matrix formation, chondrocytes dye and, just like in intramembranous ossification, cortical and trabecular bone are formed[2].

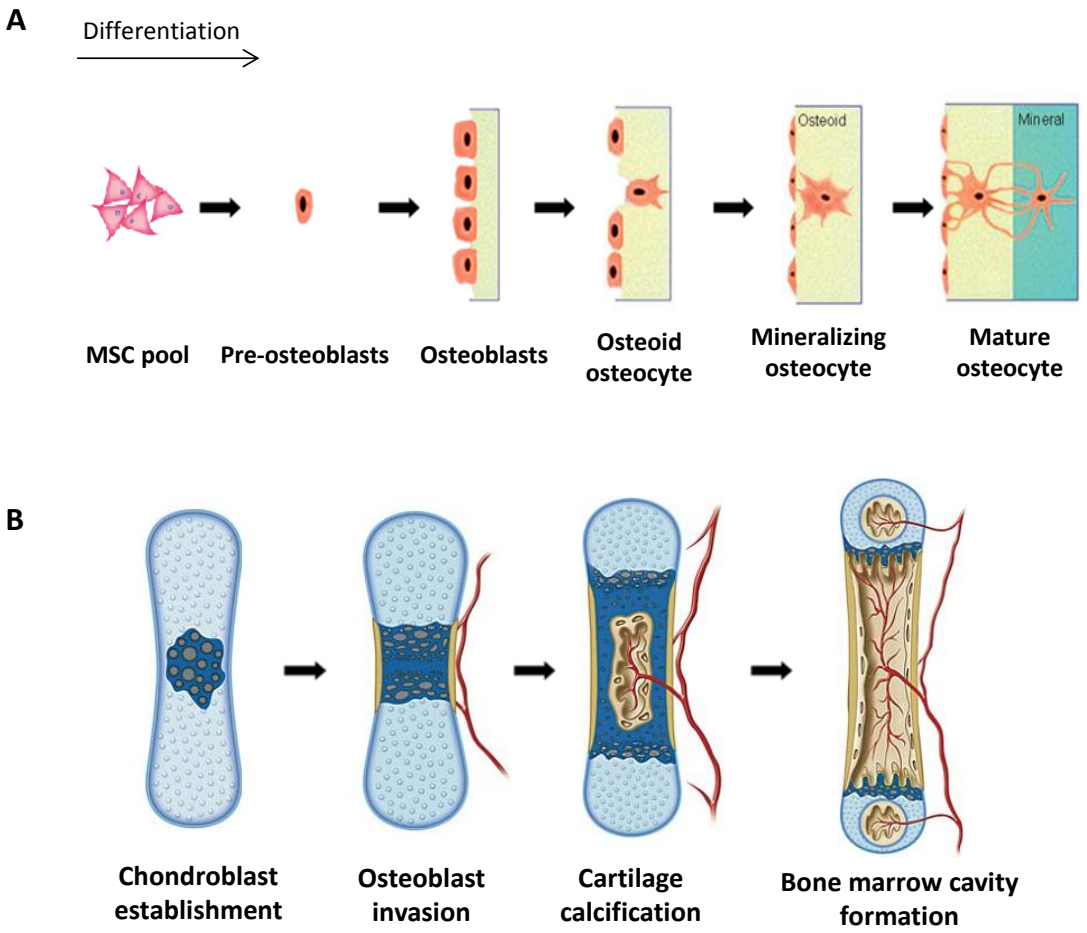


Figure 1. The ossification processes: (A) Key steps of intramembranous ossification. In the intramembranous ossification process, MSC from the stem cell pool of bone marrow start bone formation differentiating into the osteoblastic lineage. MSC go through several intermediate stages of differentiation till they become osteoblasts, which are responsible for the formation of an immature bone matrix, the osteoid. Osteoblasts further differentiate into osteocytes, which become entrapped in the mineralized bone matrix. Cells of the osteoblastic lineage at different stages of differentiation establish a network interconnected by canaliculi, forming the functional bone tissue (Adapted from Bonewald (2011)[3]). **(B) Key steps of endochondral ossification**. In endochondral ossification, MSC differentiate into chondroblasts, producing a cartilage matrix that will serve as a model for bone formation. A periostal region enriched in mesenchymal progenitor cells is then formed, providing the osteoblasts for the forming bone. These osteoblasts promote the calcification of the cartilagenous matrix and vascularisation takes place, along with chondroblast hypertrophy. In this way the bone marrow cavity is formed. In this process, trabeculae of calcified cartilage remains at the extremities of the newly formed marrow cavity (Adapted from Dijk *et al* (2011))[4].

After their complete development, bones are constituted by an extracellular matrix (ECM) mainly composed by collagen type I that is mineralized, being the most abundant salt hydroxyapatite. This matrix supports several types of cells. The most important for bone tissue composition are osteocytes, osteoblasts and osteoclasts[5].

Bone is constantly being remodelled at a number of different sites simultaneously. Osteoblasts, cells derived from bone marrow stromal/stem cells, are responsible for the replacement of bone tissue, a process that, in homeostatic conditions, lasts 3 to 4 months per site. On the other hand, osteoclasts originate in the hematopoietic lineage, and are

responsible for the resorption of bone tissue. This is a much faster process, lasting about 3 weeks per site of bone remodelling, in homeostatic conditions [6]. Thus, osteoblasts and osteoclasts are of particular importance in bone tissue, as their balanced action is responsible for bone remodelling/homeostasis throughout life.

Bone remodelling (Figure 2) can be described as proceeding through three principal phases: activation, resorption and formation[1]. During the activation step, osteoclast precursors are recruited to the remodelling site, where they maturate into osteoclasts. The recruitment mechanism is not well known, but several reports have been implicating different chemokines and metalloproteinases (MMPs) in the process[7,8]. Once in the remodelling site, osteoclasts are stimulated by osteoclastogenic signals, namely macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL)[9], initiating the resorption phase. Active osteoclasts adhere to the bone matrix, inducing the organization of resorption lacunae. This structure is sealed and inside an acidic environment is created, allowing the dissolution of the hydroxyapatite and, subsequently, the degradation of the organic components of the matrix by MMPs. Osteoclasts then go into apoptosis, and are removed from the area[10]. Finally, osteoblasts precursors are recruited to the site where resorption occurs. Once again, the mechanisms involved in cell mobilization are not known yet, but their maturation is mediated by several growth factors present at the site[11]. Here, osteoblasts proceed with intramembranous or endochondral ossification, repeating the mechanisms of embryonic bone formation. As a result of the process, the resorption site is refilled with new bone tissue of the same size as before.

1.1.2. Disruption of bone homeostasis

Sometimes the normal development and turnover of bones are affected by pathological conditions originating from very different events. The most important injuries that affect bone homeostasis are the following:

- **Fractures**

Fractures are breaks in the continuity of bone or cartilage that may be caused by traumatic events, such as falls and accidents, and also by bone affecting diseases that reduce bone mass, namely osteoporosis[12]. Thus, many of the diseases that affect bone have also as a drawback an increase in the risk of fracture.

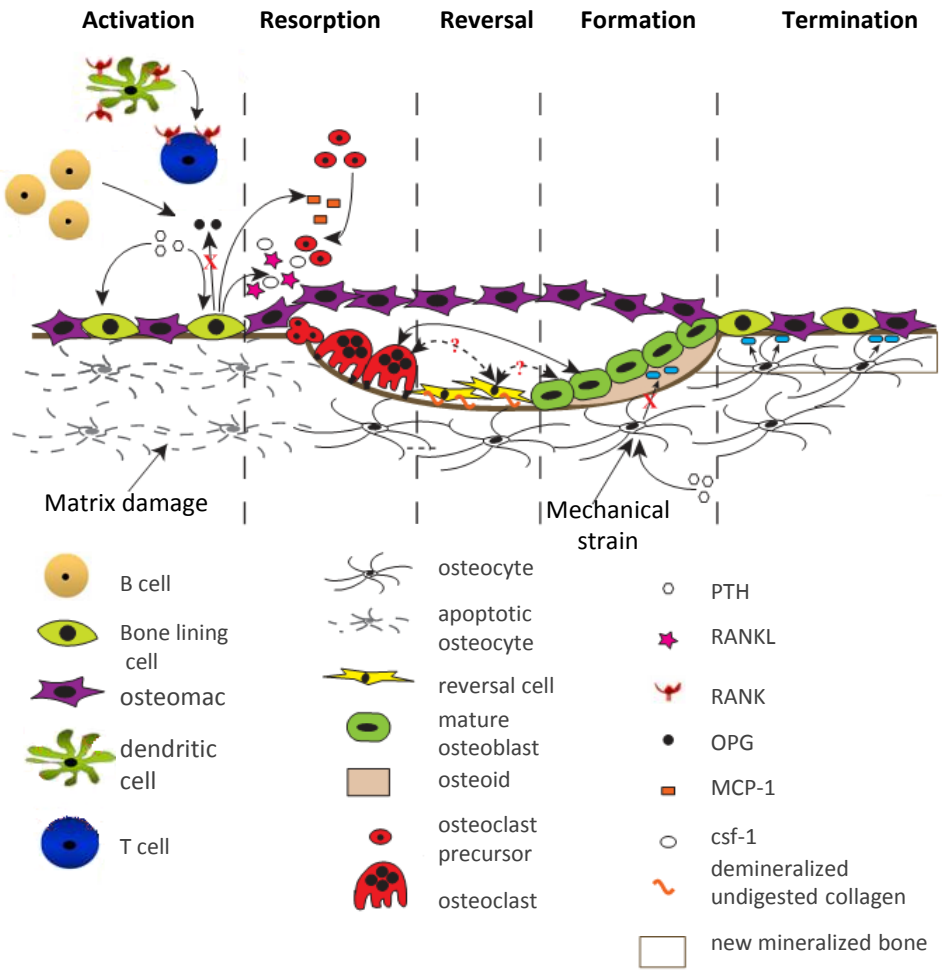


Figure 2. The bone remodelling process. Under homeostatic conditions, bone surface is covered with cells of the osteoblastic lineage in different differentiation stages. B cells in the bone marrow secrete OPG, suppressing osteoclastogenesis. Upon initiation of *Activation* phase, the endocrine-remodelling signal PTH (parathyroid hormone) binds its receptor on bone-lining pre-osteoblasts, activating them. Osteoblats recruit pre-osteoclasts to bone surface by MCP-1 secretion. Progression to the *Resorption* phase leads to an increase of the osteoclastogenic signals, such as colony stimulating factor (csf-1) and RANKL, which promotes pre-osteoclasts proliferation and differentiation into mature osteoclasts. These cells anchor to the bone matrix, originating resorption lacunae where the bone matrix is demineralised. During the R*eversal* phase, debris from resorption are removed. Meanwhile, activated osteoblasts are recruited to resorption sites and new bone starts to form. The *Termination* of bone formation occurs and homeostasis is thus reestablished (Adapted from Raggatt & Partridge (2010)[13]).

Fractures are the bone affecting injuries with the highest burden for society, since their occurrence is very common. For instance, in the year 2000 around 9 million osteoporotic fractures were estimated to have occurred worldwide[14]. Importantly, fracture treatment accounts for $12 to $19 billion of direct expenses, each year, in the United States alone[15]. In many cases, the treatment requires an extended follow-up, which makes the lifetime cost of the treatment of a single fracture event to rise till $81000[16].

Furthermore, fracture treatments usually restrict people's mobility, leading to indirect costs due to absence from work, increased psychological problems, among others[12]. In

addition, with population aging it is likely that more people are affected by bone diseases, potentiating fracture occurrence, as in osteoporosis[17].

- **Bone formation/resorption imbalances**

In this type of diseases, osteoclasts activity is not compensated by the activity of osteoblasts, leading to a rate of bone tissue resorption higher than bone tissue deposition. One of the most important examples of this type of diseases is osteoporosis, characterized by bones with a low mass and a deteriorated structure[12]. This disease is mostly frequent in women over the age of 40. Overall, in 2002, about 10 million individuals with an age over 50 was diagnosed with osteoporosis of the hip in the United States, but it is predicted that this number will rise, with one out of two Americans having or being at risk of developing this disease[12].

Another highly prevalent bone disease related with an imbalanced homeostasis of the bone tissue includes Paget's disease of bone, in which the overactivity of an increased number of osteoclasts is compensated by a rapid bone production that consequently assembles in a unstructured way[12].

- **Inflammatory diseases**

Rheumatoid arthritis is one of the most common inflammatory bone diseases, being characterized by an enhanced subchondral bone resorption, concomitantly with the destruction of articular cartilage. This effect is due to the accumulation of macrophages in the rheumatoid synovial membrane, which differentiate into osteoclasts, by the action of RANKL and pro-osteoclastogenic cytokines like IL-1 and IL-17 [6]. It is estimated that rheumatoid arthritis has a prevalence of 0.3% - 1% in the population of developed countries[18].

- **Malignancies**

Bones can be affected by tumours originating inside them, as well as by metastatic tumours from other anatomical structures[12], since they are one of the most common places for cancer spread[6]. In the former case, bone tissue becomes dysfunctional or is directly damaged by the tumour, while in the later case, the entering tumour cells need to establish, leading most of the times to an increase of osteoclast formation and activity[6]. The most common malignancies originating in the bone include multiple myeloma and osteosarcoma, whereas metastatic tumours are generally derived from breast and prostate cancers. In 2011,

it was estimated that more than 23000 new cases of multiple myeloma and bone and joint tumours were diagnosed in the American population, leading to approximately 12000 deaths[19].

- **Genetic disorders**

Genetically-determined bone diseases can affect bone homeostasis either by decreasing or increasing bone mass[12].

Osteogenesis imperfecta is one of the most prevalent bone diseases in the world, being characterized by a delayed bone growth and a low bone mass, which in turn leads to a high risk of fractures. It is caused by genetic mutations, most of which occurs in the collagen type I encoding genes COL1A1 and COL1A2[4], and the treatments available are yet very limited. In the USA, it was estimated that, in 2004, 20000 to 50000 people suffered from this disease[12].

Another common bone disease of genetic origin is osteopetrosis, in which osteoclasts are impaired and consequently so is bone resorption, leading to an excessive development of bone tissue[20].

Although medicine underwent great advances to treat bone injuries, the data here presented demonstrates that they are still a burden to national Health Systems, beyond the life constrictions that they cause on the people affected. Furthermore, it is probable that the figures presented here are largely underestimated, since many cases of bone affecting diseases are still underdiagnosed[12]. This greatly evidences the necessity of developing more efficient therapies for bone injuries.

1.2. Strategies for bone healing

According to the injury affecting bones, the desired outcome and the general clinical situation of patients suffering bone injuries, different treatments may be employed, to recover bone function (Figure 3).

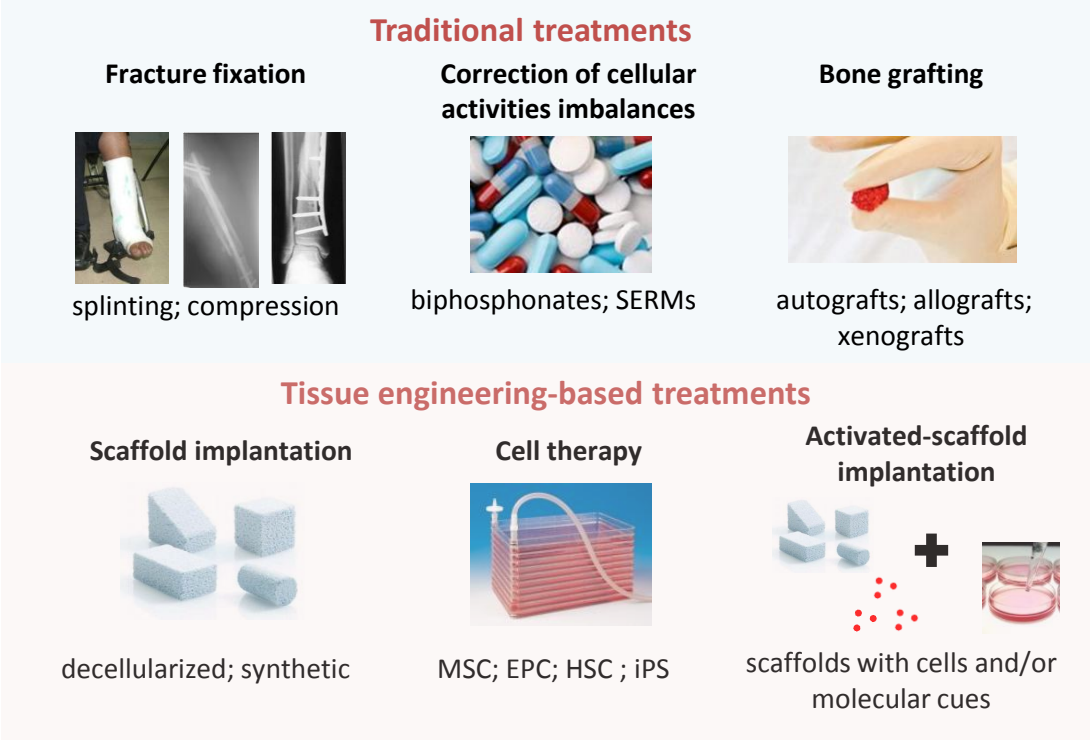


Figure 3. Strategies for bone regeneration. Bone injuries are still mostly treated by traditional methods. For bone fractures this consists on the stabilization of the lesion site, whereas bone metabolic diseases, namely those related with an increased osteoclastogenesis, are treated recurring to drug administration. The most severe cases of bone injury are treated by grafting. Since these strategies are not optimal for many cases of bone injuries, new treatments are under testing, namely the implantation of scaffolds in the injured zone, which can also be functionalized with molecular cues such as growth factors, and/or cells. Cell transplantation can also be used, with MSC being the most promising cell source.

In the case of a fracture, the realignment and reconnection of bone extremities are the main aims. This depends mainly on three conditions: the inflammatory response that sets up at the site of injury; the cellular events of ossification, described above, occurring at both ends of the bone; and also the mechanical stimuli that the bone is exposed to[21]. The treatments generally employed are focused on the modulation of these mechanical stimuli, consisting on the fixation of the fracture to stabilize and align the separated bone fragments. This stabilization can be done either externally, with no need of surgery, or internally, splinting and compression being the most used techniques for fracture fixation[21].

External splinting is usually achieved using plaster casts, braces or external fixators, generally allowing a higher interfragmentary movement[22]. In the case of internal splinting, intramedular nails, applied directly in the marrow of long bones[23], or internal fixators, placed on the bone surface[24], can be used, allowing a higher control of bone fixation. Using these two methods, bone regeneration commonly occurs by endochondral ossification. On the other hand, the compression technique requires the application of compression plates in the bones

that stimulate the intramembranous ossification[25]. In most of the cases, fracture treatments lead indeed to bone repair, however the process velocity is limited by the rate of bone remodelling imposed by osteoblasts and osteoclasts. In addition, for internal fixation, the devices used remain inside the patients even after bone healing.

In the cases of bone affecting injuries related with cellular activity imbalances, the most common therapies applied aim to inhibit the high bone resorption either reducing osteoclast generation or activity[6]. This is mainly accomplished by the prescription of drug-based therapies, targeting the signalling pathways of osteoclasts development and function. Among the drugs most used are N- containing biphosphonates. They are the most effective drugs applied nowadays for bone resorption inhibition, acting directly in the mevalonate pathway of cholesterol synthesis. This is turn affects the post-translational prenylation of GTP-binding proteins, such as Rho, Rab and Cdc42, determinant for osteoclast activity and survival[26]. Another therapy widely applied in women includes estrogens and selective estrogen receptor modulators (SERMs). It is probably based on the regulatory and/or suppressive role of estrogens in the production of osteoclastogenic cytokines[27] (see 1.1.2 - *Inflammatory diseases*), but the exact molecular mechanism has not been clarified yet. Moreover, secondary effects of estrogens over organs such as breast and uterus have been reported[6]. Along with estrogens, similar compounds known as SERMs were discovered to be selective for estrogens receptors, mimicking the role of estrogens themselves, with the advantage that some of them have a tissue specific action.

Although widely applied, the bone treatments described so far are not as effective as it is desirable, especially in pathological conditions such as non-union fractures, bone injuries derived from systemic inflammatory conditions and genetically determined diseases. In this context, new strategies to achieve the reconstitution of functional bones are being pursued. Depending on the injury conditions, the transplantation of bone grafts to the lesion site may be a suitable treatment. They were already used for the regeneration of bones from different anatomical places, such as mandible[28] and forearm[29], with satisfactory results. The efficacy of this approach is based on the fact that bone grafts have osteogenic activity, since they carry viable cells capable of generating osteoblasts, are osteoinductors, promoting the osteogenic activity of the tissue surrounding the lesion site, and are osteoconductors, allowing the growth of the host bone tissue into their structure[30]. Regarding their origin, bone grafts are usually recovered from the iliac crest, being then applied as an autologous or allogeneic graft. From the point of view of immunological rejection, autografts would be the best solution for a patient, so that it would not be recognized as a non-self tissue. However, in bone genetic diseases, for instance, this therapy would not retrieve any results. In this way allografts are

used, either from live or dead donors. As a consequence, there is an increased risk of immune rejection of the graft, as well as transmission of diseases from the donor[30]. It should also be mentioned that some bovine bone-derived products were already tried as periodontal xenografts in patients[31], but in this case there is also the risk of transmission of serious diseases to humans, for instance bovine spongiform encephalopathy. But, regardless of its origin, there is a very limited availability of bone for transplantation, either in number and graft size[30,32]. This prompted the search for alternative ways of bone regeneration, namely tissue engineering-based therapies.

Due to the importance of mechanical stability of bones during the repair process[33], as well in their normal function, new therapeutic strategies need to apply appropriate scaffolds to injury site[34], which can be also combined with cell transplantation and/or chemical factors delivery.

Scaffolds for bone repair can be either natural or synthetic. Natural scaffolds can be obtained by bone decellularization and demineralization, which originates a scaffold constituted by most of the organic ECM components of bone. These scaffolds may also retain growth factors, such as BMPs, which is the basis of their osteoinductive potential *in vivo*[35]. Their efficacy for bone repair has been successfully tested in studies with animal models[36] and also small clinical trials with patients[37]. At the same time, several different biomaterials are under research for the construction of synthetic bone grafts. Usually, different materials are chosen to construct the scaffold depending on the bone type that it should help repair. Indeed, for long bones, the ceramics hydroxyapatite- and β-tricalcium phosphate-based scaffolds are normally applied, whereas for cranial/facial bones gelatine, collagen and different synthetic polymers (e.g., poly-lactic-co-glycolic acid (PLGA)) are preferred[38]. Nowadays, several commercial products are already available for therapeutics, as summarized in (Table 1).

Furthermore, the osteogenic potential of scaffolds applied for bone regeneration can be enhanced through their biochemical and even biological activation. To accomplish this, the scaffolds can be embedded with osteoinductive chemical cues, such as BMPs (namely BMP-2 and BMP-7), platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), insulin-like growth factor (IGF) and fibroblast growth factor (FGF). Moreover, they can be implanted into the injury site along with cells. Nowadays, stem cells are recognized as one of the best cell source for transplantation in TE approaches due to their capacity of differentiation into the required cell type and of self-renewal, reconstituting tissues along the life time of patients.

According to the tissue intended to be regenerated, different sources of stem cell have to be chosen. Due to their pluripotency embryonic stem cells (ESC) could be chosen for

bone regeneration, but many ethical concerns restrict the generalized used of these cells[39]. Alternatively, induced pluripotent stem cells (iPS), which can be derived from autologous adult cells, could be used, but the phenotype of these stem cells does not totally resemble that of ESCs[40]. Further studies have yet to be performed before a wide application of iPS in therapeutics. At present, multipotent adult stem cells of the bone are the most appropriate cell source for bone regeneration, since in theory they should be capable of originating the tissue from where they were derived. The bone is a niche for three different types of stem cells: the hematopoietic stem cells, the endothelial stem cells and the mesenchymal stem cells[41], and all of them have been shown to contribute to the enhancement of bone regeneration. Besides promoting angiogenesis at the injury site, endothelial stem cells might stimulate osteoblasts through BMP-2 signalling[42]. Similarly, hematopoietic stem cells can influence MSC differentiation into the osteoblastic lineage through BMP-2 and BMP-6[43]. In this way, their role in bone regeneration is indirect, and MSC are revealed as the bone marrow-derived stem cells with the greater potential for bone reconstitution.

Table 1. Examples of commercially available scaffolds for bone regeneration. Adapted from Greenwald (2006).[44]

Product (Company)	Composition	Commercially available forms	Bone repair mechanism
CELLPLEX® (Wright Medical Technology)	Tricalcium phosphate	Various sized granules	Osteoconduction
OpteMx™ (Exactech)	HA/TCP biphasic combination	Granules, sticks, rounded wedges, wedges and cylinders in several sizes	Osteoconduction; osteogenesis and osteoinduction when combined with bone marrow aspirate
INFUSE™ Bone Graft (Medtronic Sofamor Danek)	rhBMP-2 protein on a collagen sponge	Freeze-dried powder and sponge in several sizes	Osteoinduction
BonePlast® (Biomet/EBI/Interpore Cross)	Calcium sulphate	Powder/fluid mixed with hardenable paste	Osteoconduction
ProOsteon® 500R (Biomet/EBI/Interpore Cross)	Coral HA composite	Granular or block	Osteoconduction

1.3. Multipotent mesenchymal stromal cells: their characteristics and their functions

Multipotent mesenchymal stromal cells (also commonly named mesenchymal stem cells) are undifferentiated cells, able to self-renew and also to differentiate into more committed cell types from the mesodermal layer[45,46]. Namely, MSC are able to differentiate into adipocytes, osteocytes and chondrocytes[46]. In addition, several reports have been describing the capacity of MSC to differentiate into cell types of other embryonic germ layers, such as neurons, which remains a very controversial issue[47]. The proliferation and the differentiation capacity of different MSC populations may vary, which may be related with the source from which they were isolated[46].

MSC were first identified by Friedenstein and colleagues in 1976[48] and since then they were mostly found in the bone marrow, synovial membrane and fluid, articular cartilage, peripheral blood, adipose tissue, placenta, umbilical cord (both blood and matrix), amniotic fluid and membrane, dental pulp and deciduous teeth (Figure 4).

Due to the lack of molecular signatures and features specific of MSC, collected cells have to be retrospectively analysed, in order to ensure their identity. In 2006, the International Society for Cellular Therapy proposed a set of minimal criteria that human cells should verify to be considered MSC (Figure 4). These are: (1) expression of CD105, CD73 and CD90 in ≥ 95% of the population and absence of the lineage markers CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR (≤ 2% of positive cells in the population), when a screening of the cell surface molecules with appropriate antibodies is performed; (2) the capability of cells to form cell colonies adherent to the plastic of tissue culture plates, when cells are maintained in standard culture conditions; and (3) the capability to differentiate into osteoblasts, chondroblasts and adipocytes, in functional tests, where cells are cultured in appropriated stimulating culture media.

The physiological functions of these cells are mainly related with the normal turnover and maintenance of adult mesenchymal tissues and regulation of hematopoietic stem cells homeostasis, in the bone marrow niche[49]. They are also ascribed as immunomodulatory players, influencing the recruitment and activity of immune cells in different situations[49,50]. In addition they are thought to be able to migrate across whole body into sites of injury, although controversy on this still remains[51].

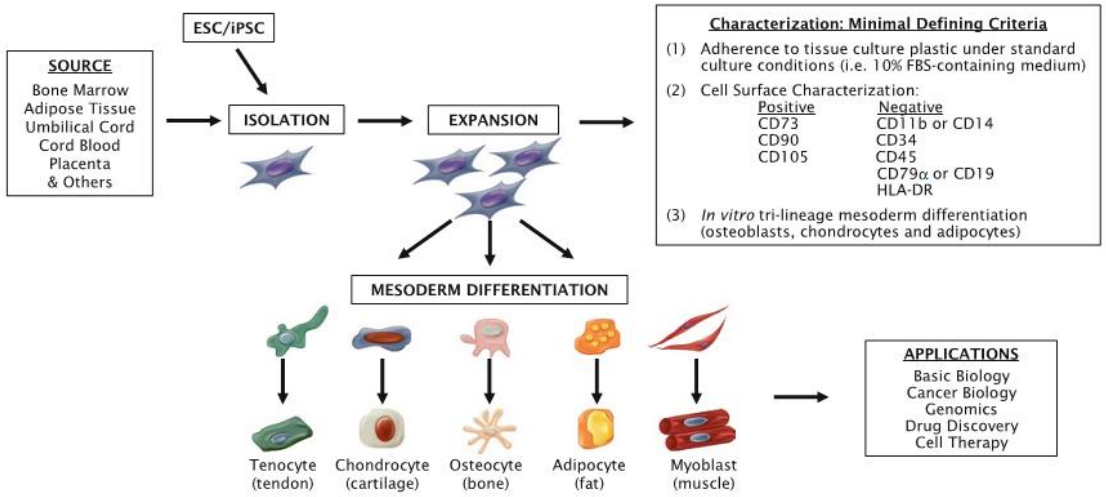


Figure 4. Characteristics of multipotent mesenchymal stromal cells. MSC isolated from different sources are being used for studies of potential clinical application. Their capacity to differentiate into different cell types of the mesodermal lineage, combined with their immune properties, makes them candidates for tissue engineering applications. Due to the ambiguity of their identification, ISCT defined criteria based on molecular expression and differentiation capacity to consider a cell population as MSC (Adapted from Vemuri *et al* (2011)[52]).

1.3.1. Multipotent mesenchymal stromal cells as immunomodulatory agents

MSC are implicated in the modulation of the immune system, through different mechanisms, generally imposing an immunosuppressive effect (Figure 5). In what concerns innate immunity, MSC can control one of the first lines of immune defence, i.e. activation of the complement system, by secreting regulatory proteins of that system[53]. MSC suppress the proliferation and the functions of natural killer cells, changing their expression pattern of surface receptors[54]. In addition, i*n vitro* studies show that MSC suppress immature dendritic cells (iDC) formation, inhibiting the differentiation of monocytes into this lineage[55]. At later stages of DC development, MSC were also shown to suppress DC maturation and to prevent the $CD4^+$ T cell proliferation mediated by antigen-activated DC[56]. In this line, MSC can directly affect T cells inhibiting their proliferation and diminishing the levels of cytokines they produce[57]. In parallel, MSC directly promote the proliferation of regulatory T cells, namely the $CD4^+CD25^{high}FOXP3^+$ subset[58], which have a physiological immunosuppressive role[59]. In the adaptive immune system, MSC also affect B cells. They inhibit B cell proliferation by arresting them on the cell cycle checkpoint G0/G1, their capacity to produce immunoglobulins and chemotaxis[60]. These immunomodulatory properties of MSC are mediated by the plethora of

cytokines, chemokines and growth factors, among other factors, they produce and secret, being thus considered as cell-factories[61].

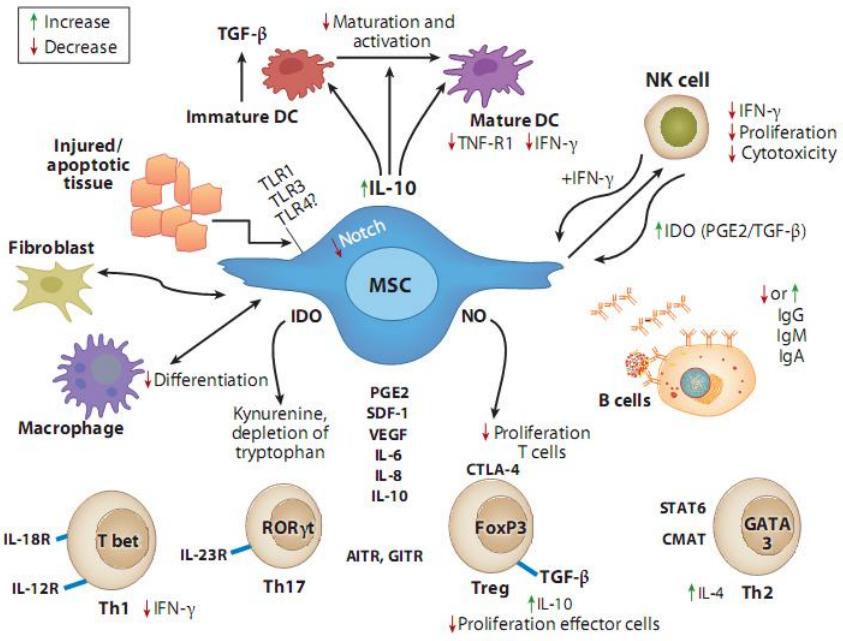


Figure 5. Overview of the immunomodulatory role of MSC on cells of the immune system. Depicted are the effects of MSC exert upon immune cells, as well as the mediators of their interaction (Singer & Caplan (2011)) [50].

At this point, it should be highlighted these are the effects of MSC upon immune cells commonly observed, but their immunological behaviour strongly depends on the environmental factors that stimulate them. As an example, Waterman *et al.* described a phenotypic change in human MSC depending on the activation of different Toll-like receptors (TLR) on their surface by external stimulus: the stimulation of TLR3 with poly(I:C) induced an immunossupressive phenotype in MSC, whereas the stimulation of TLR4 with LPS lead to a pro-inflammatory behaviour by these stem cells[62]. Hence, MSC properties appear to depend on the environment they are in.

The immunomodulatory function of MSC benefits from their immunoprivileged state, since they have low levels of MHC class I antigens, do not constitutively express MHC class II antigens and lack the co-stimulatory molecules B7, CD40 and CD40L (co-stimulatory receptors for T cell activation)[63]. In consequence, the immunological characteristics of MSC are extremely important for their clinical application, since the probability of being recognized by an unrelated host and rejected is lower than for other adult stem cells. This has been specially explored in several clinical trials where human allogeneic MSC have been used to treat patients with graft-versus-host disease or autoimmune diseases[64].

1.3.2. Migratory capacity of multipotent mesenchymal stromal cells in health and disease

In 1997, Fernandez *et al.* reported the isolation of cells with a phenotype resembling MSC from the peripheral blood of breast cancer patients treated with granulocyte-colony stimulating factor (G-CSF) or granulocyte-macrophage colony stimulating factor (GM-CSF)[65]. Later, Mansilla *et al.* detected MSC-like cells in blood samples from acute burn patients[66]. These findings suggest that MSC may be mobilized upon injury, but results are inconsistent between different cases[67]. On the other hand, this MSC property has been better described for injuries causing an inflammatory reaction, where MSC homing to the lesion site might be related to their immunomodulatory properties.

Here homing is defined as the process whereby migrating MSC are arrested in the blood vessels of a tissue, extravasate to that tissue and produce an effect[51]. This migratory process of MSC resembles leukocyte migration and thus can be defined as proceeding in 5 steps (Figure 6):

1) crossing of tissue ECM till blood vessels are reached;
2) crossing of vascular basement membrane into blood vessels lumen;
3) travelling in the blood stream and/or rolling in blood vessels wall to the injury place;
4,5) crossing of basement membrane and ECM.

During phase 1 of migration, MSC remodel the ECM, in order to make space through the glycosaminoglycan network to reach blood vessels basal membrane. During phase 2, basal membrane of endothelium is crossed by ECM remodelling, allowing MSC to extravasate into the blood stream. In phase 3 of migration, MSC may be dragged by blood stream until they establish shear resistant adhesive interactions with vascular endothelial cells. The successive establishment and disruption of these adhesive connections guides MSC along blood vessel wall in a rolling movement and as a response to a chemotactic gradient. In the vicinity of the injury locations, these chemokines lead to integrin activation, promoting a stronger adhesion with the endothelial cells that results in MSC extravasation back to a tissue[68]. Here MSC again remodels ECM to penetrate to the interior of the tissue (phase 4 and 5 of migration), where they exert their action[68].

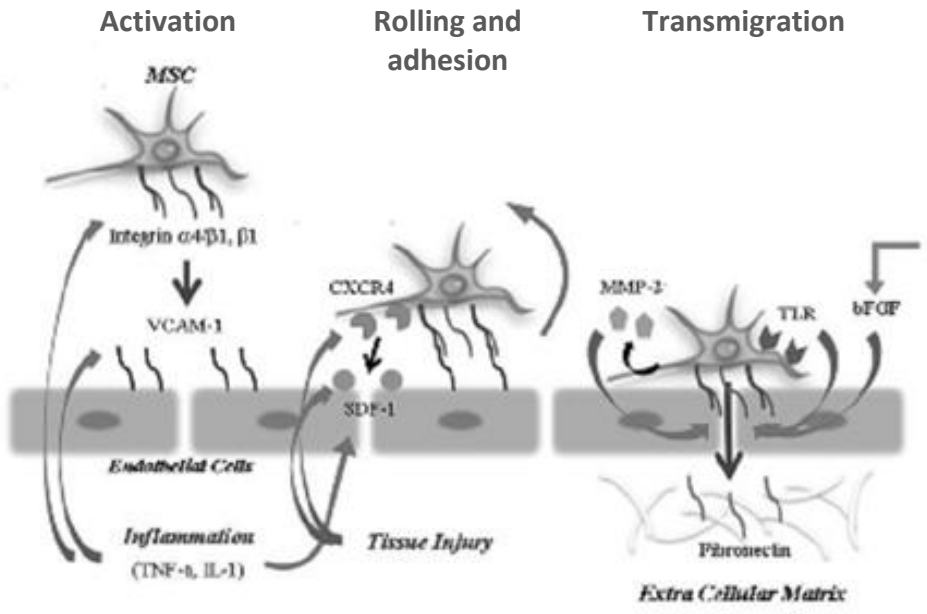


Figure 6. MSC extravasation in their homing process. During the *activation* step, local inflammation induce endothelial cells to express adhesion molecules that are linked by integrins on MSC activated by inflammatory signals. Thigh *adhesion* of MSC on the vessel lining cells allows their *rolling* promoted by chemoattractant signals. MSC are then able to extravasate into the tissues, remodelling ECM through MMPs to reach the injured site. (Reproduced from Yagi *et al* (2010)[68]).

These steps in MSC migration are mediated by molecules with different effects which ultimately facilitate cell mobilization. In this way, integrins, cell surface receptors involved in cell adhesion, are intervenient in the interaction of MSC with ECM and endothelial cells. One of the most important is the α4β1 integrin. It is expressed by MSC[69] and binds ECM components, such as fibronectin[70], and the vascular cell adhesion molecule-1 (VCAM-1)[71]. A novel ligand for the P-selectin adhesion molecule expressed in endothelial cells was discovered to be involved in MSC rolling[72]. MMPs, such as MMP-2, are also involved in the interaction with ECM and endothelial basement membrane, opening space for cell mobilization by breaking glycosaminoglycans present in their network[73].

Besides the players on MSC homing described so far, chemokines and cytokines are probably the most important ones, creating the chemotactic gradient that promotes MSC mobilization. One of the most described systems involved in the process is the CXCR4/SDF-1 axis. Although present at low levels on the MSC surface, CXCR4 is produced in large quantities by these cells, translocating to cell membrane when challenged by other chemokines[74]. In addition, MSC express a set of CC and CXC chemokine receptors[75] that makes them responsive to other chemokines, such as MCP-1 and MIP-1α. Regarding the cytokine effect on MSC mobilization, TNF-α is one of the most implicated in the process, exhibiting a chemotactic role

towards MSC[76]. Growth factors, for instance G-CSF, PDGF and IGF, are also strong MSC chemotrancts[77].

Chemokines and cytokines promoting MSC mobilization and homing can be produced by different cell types in the injured tissues, and immune cells are important contributors to the pool of chemotactic factors. Interestingly, previous work revealed the capacity of natural killer cells, T cells and macrophages to recruit MSC through a Matrigel-coated insert membrane, an effect particularly evident for macrophages[78]. Later on, Anton *et al* showed that conditioned medium from macrophages was able to recruit MSC in a transwell assay with inserts of uncoated membrane, and that this medium was enriched in chemokines/cytokines, namely CCL5, CCL12 and IL-8, reinforcing the importance of cytokines/chemokines in MSC recruitment[79].

1.3.3. MSC in therapies for bone repair/regeneration

The existence of MSC in bone and their capacity to originate osteoblasts makes them a suitable cell source for bone regeneration through direct differentiation. On the other hand, their immumodulatory actions may be an additional or an alternative pathway through which they contribute to bone repair in certain disease contexts, modulating the chronic inflammatory environment often associated with osteoarticular diseases. Furthermore, their potential to home to injury sites allows the development of therapeutic approaches encompassing their systemic infusion.

MSC transplantation alone has been reported to be beneficial in the treatment of different bone diseases and traumas, both in studies with animal models and clinical trials. As an example, in 2005, Le Blanc *et al.* reported the transplantation *in utero* of foetal liver-derived allogeneic MSC into a 32 week human foetus with severe onset of osteogenesis imperfecta. At the 9 month of age, histological analysis revealed structured bone trabeculae and during the first 2 years of life the psychomotor development of the child was normal[80]. In a more integrative approach, the efficacy of strategies of bone repair/regeneration combining MSC and scaffolds were also described in *in vivo* studies using animal models of bone fractures, from small rodents[81,82], to dogs[83] and sheep[84]. In these studies, the concurrent implantation of MSC in a scaffold increased local bone production and contributed to the reconstitution of bone tridimensional structure. In addition, the benefits of this approach were also demonstrated with a limited number of patients with critical size defects originated by fractures[85].

A few studies are also being performed to genetically modify MSC in order to improve their potential to treat bone injuries. This can be accomplished inducing the overexpression of pro-osteogenic factors, such as BMP-2, TGF-β and FGF, in MSC. As shown by Zachos *et al*., the transplantation of genetically modified MSC expressing BMP-2 lead to a quicker healing of a distal femoral articular osteotomy in nude rats when compared with the transplantation of non-modified cells[86]. Despite the promising results obtained with genetically modified MSC in different animal models, their application in human clinical trials is scarce.

A wide application of MSC will only be achieved after appropriate prospective clinical trials are carried out. During these trials some safety issues concerning MSC transplantation have to be tested, namely their tumorigenic capacity, since murine MSC were shown to induce sarcoma both *in vivo* and *in vitro*[87].

1.4. Osteoimmunology: the interplay between immune cells and mesenchymal stem cells in bone remodelling

MSC and immune cells are in close proximity and interaction throughout their life span, sharing the bone marrow niche. Here, MSC strongly influence immune cells formation and development[88]. On the other hand, immune cells also influence MSC phenotype and behaviour[89]. Bone biology is thus strongly influenced by the interplay between skeletal and immune systems (Figure 5, 7).

Several reports have been implicating T lymphocytes both in bone formation and resorption, as follows. Activated T lymphocytes were seen to be capable of inducing *in vitro* the osteoclastogenesis of human peripheral blood stem cells and monocytes cultures[90]. Accordingly, phenylketonuria patients suffering enhanced bone osteoclastogenesis showed an increase number of circulating activated T cells, as well as osteoclast precursors[91]. On the other hand, an established murine model of endotoxin-induced bone resorption, implicated T lymphocytes in the suppression of osteoclast formation, through the production of IFN-γ, balancing their production of the osteoclastogenic RANKL[92]. Furthermore, conditioned medium from activated T cells was shown to influence MSC osteogenic differentiation, increasing the expression of RUNX2 and osteocalcin mRNA and the activity of alkaline phosphatase[93].

B lymphocytes were also shown to be involved in bone turnover, mainly influencing osteoclastogenesis. Manabe *et al.* showed that this role can be accomplished either by the secretion of the stimulator of osteoclast differentiation ODF/RANKL by cells of the B lymphoid lineage, or by their direct differentiation into osteoclasts[94]. In contrast, mature B cells can also

be involved in osteoclastogenesis suppression, since they are responsible for the production of most of the bone marrow osteoprotegerin (OPG), a decoy receptor of RANKL[95].

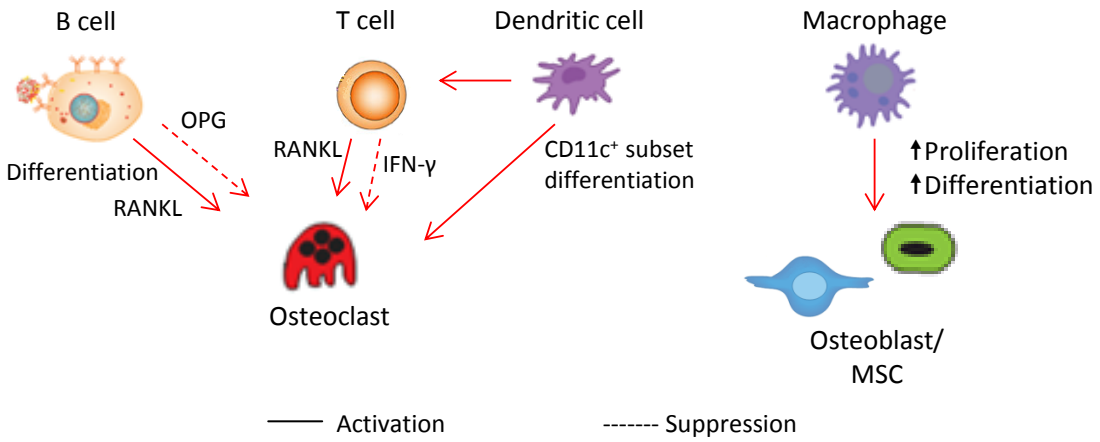


Figure 7. Immune cells influence on the major effector cells of bone remodelling. Immune cells influence bone remodelling modulating osteoclast/osteoblast pool in bone through processes of direct differentiation or molecular stimulation. The activity of osteoclasts/osteoblasts and their progenitors is also influenced by the interaction with immune cells.

Similarly to many other tissues, bone has a population of resident macrophages, the osteomacs. This macrophage population was co-isolated with osteoblasts from mice bone explants and i*n vitro* studies showed their involvement in osteoblast driven mineralization[96]. Moreover, the influence of macrophages on bone formation was recently extended to MSC. It was reported that human derived monocytes/macrophages were able to increase hMSC proliferation, also influencing their early differentiation into the osteogenic pathway. Indeed, at 7 days of culture under the influence of monocytes/macrophages, the ALP activity was increased in hMSC and osteocalcin and osteopontin were overexpressed. These effects were probably enhanced by monocytes/macrophage-derived BMP-2[97].

Natural killer cells are important in the promotion of the differentiation of monocytes to osteoclasts, at least when co-cultured *in vitro*, probably through the action of RANKL and M-CSF[98].

The close relationship between immune cells/molecular mediators and MSC is further evidenced in pathologic conditions such as fractures. When a fracture occurs, bone regeneration is coordinated along three phases: inflammatory, repair and remodelling phases, whereby an inflammatory response is set up due to tissue damage, MSC are recruited to the injured place for ossification and the transient bone matrix is replaced by functional bone tissue, respectively. This inflammatory phase was shown to be necessary for proper fracture

healing, since, for example, the administration of cyclooxigenase-2 inhibitors, an anti-inflammatory drug, was related with impaired fracture healing[99].

1.4.1. Immune system and MSC cross-talk: the particular case of dendritic cells

Dendritic cells are considered the professional antigen presenting cells of the immune system, and contrarily to other immune cell populations, their influence on bone biology is not well described. It is thought that they are probably not key contributors to bone homeostasis, since DC depleted animals show no skeletal abnormalities[100]. However they can have a more important role on bone remodelling under inflammatory conditions, as supported by their presence in synovial tissues of rheumatoid arthritis patients[101], and by the existence of DC with altered biological features in the rat model of spondyloarthropathies[102].

DC can have a lymphoid or myeloid origin, existing in an immature state in the peripheral tissues that they monitor, until they are activated. Upon a contact with an antigen DC: (i) start maturation (mDC), (ii) process the antigens they internalized, loading epitopes into MHC class I and II molecules (iii) migrate to secondary lymphoid organs and (iv) present antigens to T cells[103]. In this way they represent a strong link between the innate and the adaptive immune systems, and thus are good candidates to affect bone homeostasis/remodelling either directly, by producing soluble mediators or indirectly through their action upon T cells (Figure 7).

This is supported by studies concerning DC behaviour when in contact with biomaterials used for bone regeneration. DC were found in PLGA scaffolds implanted subcutaneously in mice, and *in vitro* experiments showed that DC primed with PLGA films enhanced T cell proliferation in mixed lymphocyte reactions[104]. But DC can also play a role on osteoclastogenesis in a direct way. Indeed, immature $CD11c^+$ DC were shown to differentiate into functional osteoclasts in response to M-CSF, RANKL, TNF-α or human rheumatoid synovial fluid[105] (Figure 7). These cells were also found in the eroded bone surfaces of arthritic joints in rheumatoid arthritis mouse models[106]. On the other hand, studies on systemic lupus erythematosus (SLE) pathologies where nonerosive arthritis of the joints occurred, suggested an indirect role of DC on osteoclastogenesis inhibition. Patients with SLE show elevated levels of IFN-α, a cytokine mainly expressed by plasmacytoid DC that stimulates the differentiation/maturation of myeloid DC. An increased stimulation of DC maturation decreases the pool of myelomonocyte precursors in the organism, which also originates oesteoclasts. In this way, plasmacytoid DC indirectly controlled osteoclastogenesis[107]. Taking

together these data, it seems that different subsets of DC influence bone repair/remodelling in different ways, acting in a self-regulated communication network.

Despite the knowledge about the role of DC in osteoclastogenesis, very little is known about their interaction with osteoblasts and mesenchymal stem cells.

1.5. Objectives

In the context of the discussed above about MSC and DC, about their interaction and their participation in skeletal and immune systems biology, this work aimed to contribute to our knowledge on the potential influence exerted by DC upon MSC, and its possible implications for bone regeneration.

In vitro work was performed at a functional and molecular level, both in physiological, pro-inflammatory and anti-inflammatory environments.

To accomplish our goal four specific objectives were delineated:

1. **Characterize the capacity of DC to recruit MSC.**

   The extent of MSC mobilization by DC was evaluated in transwell assays, where the molecular effectors promoting the event, namely cytokines and MMPs were screened/identified.

2. **Investigate the potential of DC to interact with MSC, inducing alterations in their behaviour, namely their motility.**

   Co-cultures of the two cell types were monitored using time-lapse video microscopy.

3. **Correlate the activation of specific signalling pathways, both in DC and MSC, with the interaction of DC-MSC.**

   NF-kB signalling pathway activation was investigated by imaging flow cytometry and confocal laser scanning microscopy.

4. **Uncover the potential of DC to promote osteogenic differentiation of MSC.**

   MSC osteogenic differentiation with conditioned medium from DC and other stimuli was evaluated.

2. Methods and materials

2.1. Multipotent mesenchymal stromal cell culture and characterization

MSC used in this work were kindly provided by Catarina Almeida (INEB), who previously isolated them from the BM of patients undergoing total hip arthroplasty, as described[78]. The patients were less than 50 years old and did not suffer from any known inflammatory disease.

The cells were provided in passage 2 and then expanded until passage 4. MSC growth medium used was Dulbecco's modified Eagle's medium (low glucose with Glutamax) (DMEM; Invitrogen), supplemented with 10% foetal bovine serum (FBS; Invitrogen) and 1% penicillin/streptomycin (Invitrogen). FBS used was previously tested and selected for MSC growth in the laboratory and was heat inactivated prior use at 57 °C, for 30 min. Routinely, MSC detachment was performed rinsing the culture twice with phosphate buffered saline (PBS) 1x and incubating with 0.05% trypsin (Invitrogen), at 37 °C, for 10 min. Cells were grown in 150 cm^2 tissue culture flasks (BD Falcon), incubating at 37 °C in 5% CO_2. MSC at passage 4 were stored in freezing medium (MSC growth medium with 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich)), in liquid nitrogen, until use.

Prior to each experiment, frozen aliquots of MSC were thawed and cultured in MSC growth medium until 70-80% confluence. All the assays were performed using MSC between passages 5 and 9.

2.1.1. Multipotent mesenchymal stromal cell characterization according to the ISCT criteria

Cell phenotyping by flow cytometry:

For each marker under analysis, $1x10^5$ MSC were washed with PBS 1x and incubated in 50 µL PBS 1x/2% FBS/0.01% azide (FACS buffer) with the appropriate antibody, for 30 min, on ice. Monoclonal anti-human antibodies were used for cell labelling as follows: fluorescein isothiocyanate (FITC)-labelled CD34 (at 2 µL in 50 µL) and phycoerythrin (PE)-labelled CD105 (at 2 µL in 50 µL), CD73 (at 5 µL in 50 µL), CD90 (at 5 µL in 50 µL), CD14 (at 2 µL in 50 µL), CD19 (at 2 µL in 50 µL), CD45 (at 2 µL in 50 µL) and HLA-DR (at 2 µL in 50 µL). Control samples were prepared as above using as antibodies FITC-labelled anti-mouse IgG1, PE-labelled IgG1 and PE-labelled IgG2a (all at 2 µL in 50 µL). All antibodies were purchased from ImmunoTools, except CD90, which was from BD Biosciences. After labelling, cells were washed 3 times in FACS buffer and ressuspended in 2% paraformaldehyde (Sigma-Aldrich) for fixation. Cells were analyzed in

a Flow Cytometer FACSCalibur (Becton Dicksinson), acquiring a total of 10 000 events. Data was analysed with FlowJo.

Osteogenic differentiation:

$15x10^3$ cells were plated for 7, 14, 21 and 28 days in DMEM (low glucose, with Glutamax; Invitrogen) supplemented with 10% FBS (PAA Laboratories) and 1% penicillin/streptomycin (Invitrogen), further supplemented with 100 nM dexametasone, 10 mM β-glycerophosphate (Sigma-Aldrich), 50 μM ascorbic acid (Fluka). Medium was replaced twice per week. MSC differentiation was evaluated by alkaline phosphatase activity staining at all time points. In this way, MSC were fixed with 4% paraformaldehyde for 15 min, at RT (room temperature), and washed with distilled water (dH_2O). Then, were incubated at RT with Fast Violet B salt (Sigma-Aldrich) solution 250 μg/mL containing Naphtol AS-MX phosphate, for 45 min, and washed twice with dH_2O. Differentiation evaluation was complemented detecting mineralization by Von Kossa staining, at time points 21 and 28 days. MSC were fixed with 4% paraformaldehyde for 15 min, at RT, and washed with dH_2O. Then were incubated in silver nitrate 2.5%(w/t) (Sigma-Aldrich) for 30 min under UV light, followed by incubation in sodium thiosulphate 5%(w/t) (Sigma-Aldrich) for 3 min. Cells were finally washed with dH_2O. In both cases samples were stored in PBS 1x until analysis under an inverted microscope (Zeiss Axiovert 200). Images were recorded using AxioVision Release 4.8.2 software.

Chondrogenic differentiation:

$2x10^5$ cells were ressuspended in DMEM (low glucose with Glutamax with 1% penicillin/streptomycin) supplemented with glucose to a final concentration of 4.5 g/L, 50 μg/mL ascorbic acid (Fluka), 40 μg/mL L-proline (Sigma-Aldrich), 100 μg/mL sodium pyruvate and 100 μg/mL ITS (insulin-transferring-selenium) (BD Biosciences), without or with chondrogenic differentiation factors (0.1 μM dexamethasone and 10 ng/mL TGF-β (Immunotools)). Cell suspensions were transferred to 15 mL Falcon conical tubes, spun at 450 g for 10min at RT and incubated at 37ºC in 5%CO_2 for 14 and 28 days. Medium was replaced twice per week. At each time point, samples were recovered, fixed with 4% paraformaldehyde for 20 min at RT, washed with PBS 1x, and stored in PBS 1x until further processing. Cell pellets were transferred to histology cassettes, dehydrated with a series of ethanol in a paraffin tissue processor (Microm STP 120-2) and embedded in paraffin blocks. Sections of 5 μm were then cut, transferred to laminas, rehydrated in xylol and a series of ethanol and stained with

toluidine blue 1% (Fluka) in sodium borate 1% (Sigma-Aldrich), for 5 min. Laminas were rinsed, prepared with mounting media and stored at 4°C until analysis under the inverted microscope.

Adipogenic differentiation:

$6x10^3$ MSC were plated in a 24-well plate until reaching confluence (7 days) with DMEM (low glucose with Glutamax; Invitrogen) supplemented with 10% FBS (PAA Laboratories) and 1% penicillin/streptomycin (Invitrogen), further supplemented with dexamethasone 100 µM (Sigma-Aldrich), 3-isobutyl-1-methylxanthine 500 µM (Sigma-Aldrich) in 500 mM KOH (Merck), insulin 10 µg/mL (Sigma-Aldrich) in 20 mM HCl (Sigma-Aldrich), indomethacin 100 µM (Sigma-Aldrich) (adipogenic medium). After 2-3 days of culture, adipogenic medium was replaced by insulin medium (MSC medium supplemented only with 10 µg/mL insulin). After 1-2 days, medium was replaced by adipogenic medium and, after 3 days again by insulin medium. Cells were treated a third time with adipogenic medium and then maintained in insulin medium for 1 week. After that MSC were fixed with 4% paraformaldheyde for 20 min, washed with PBS 1x and incubated with isopropanol 60% for 5 min. Cells were then stained with Oil Red O 1.8 mg/mL for 5 min and after counterstained with hematoxylin for 1 min. Cells were washed with dH_2O and stored in PBS 1x, until analysis under the inverted microscope. All procedures were performed at RT.

2.2. Dendritic cell culture and characterization

Dendritic cells were differentiated from monocytes isolated from human buffy coats (Figure 8). The buffy coats were kindly donated by Instituto Português do Sangue (Porto) and Hospital de São João (Porto) and were prepared from blood of healthy blood donors. Buffy coats were centrifuged at 1200xg (without brake), for 20 min, at RT. The interface corresponding to the peripheral blood mononuclear cells (PBMC) was recovered and monocytes were enriched by negative selection, incubating with RosetteSep™ Human Monocyte Enrichment Cocktail (StemCell™) in a ratio of 15:1, for 20 min at RT, under gentle shaking. The tetrameric antibody complexes of the reagent link unwanted cells to red blood cells. The suspension was then diluted in PBS 1x/FBS 2% in a ratio of 1:2 and after gently overlaid on the top of 1 volume of Ficoll-Histopaque™ (Sigma-Aldrich). The complexes were removed by density gradient centrifugation at 1200xg, for 20 min (without brake) at RT. The monocyte-enriched interface was recovered and washed 2-3 times with PBS 1X by centrifuging at 700 rpm for 17 min. Monocytes were cultured in 6-well plates, plating 2 million cells in each well in Roswell Park Memorial Institute 1640 medium (RPMI-1640) (low glucose with

Glutamax; Invitrogen) supplemented with 10% heat inactivated FBS (Lonza) previously tested in the laboratory, 1% penicillin/streptomycin (Invitrogen) and 50ng/ml of each of the differentiation factors IL-4 and GM-CSF (both from ImmunoTools), as previously described[108]. Cells were then incubated at 37 °C in 5% CO_2 and allowed to differentiate into DC, at least 7 days, prior to use. For each experiment freshly differentiated DC were recovered from the culture wells by gentle pipetting, following centrifuging at 300xg and ressuspending in the appropriate volume of culture medium. Cells were always used between day 7 and day 9 of differentiation.

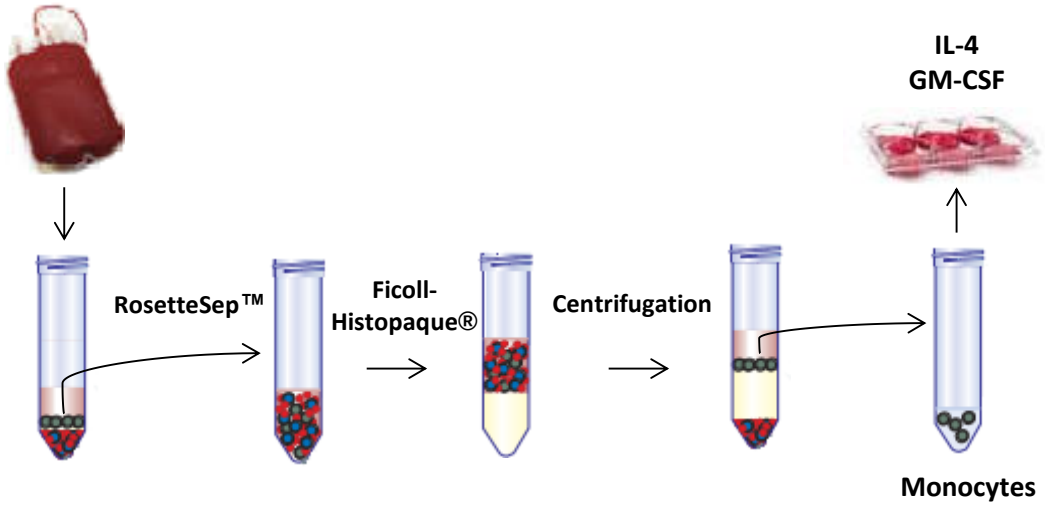


Figure 8. Diagram of the process for the recovery of monocytes, from human buffy coats. The buffy coats are centrifuged, originating an interface (tube 1, in grey) where peripheral blood mononuclear cells (PBMC) are concentrated. Cells in this mix bearing CD2, CD3, CD8, CD19, CD56, CD66b, CD123 and glycophorin A are linked by RosetteSep™ to red blood cells. The complexes formed are removed by a density gradient centrifugation with Ficoll-Histopaque®. The interface obtained, enriched in monocytes, is cultured in medium supplemented with DC differentiating factors. After 7 days of culture, DC are fully differentiated (Adapted from StemCell Technologies[109]).

DC phenotype and activation status was always confirmed by visual inspection under the light microscope and for some experiments, by flow cytometry, after DC stimulation with lipopolysaccharide (LPS) 50 ng/mL or TNF-α 100 ng/mL 24 h before assay.

Flow cytometry:

DC were gently recovered from the culture wells, washed with PBS 1x and incubated in 50 µL of FACS buffer with the appropriate antibody, for 20 min, on ice. The monoclonal anti-human antibodies used were: PE-labelled CD1a (Immunotools) and FITC-labelled CD83 (AbDSerotec), all at 2 µL in 50 µL of FACS buffer. Control samples were prepared as above for

non-stained DC and for single stained DC with antibodies anti-human FITC-labelled IgG1 and PE-labelled IgG1 (ImmunoTools). Data acquisition and analysis was performed as described for MSC.

2.3. Multipotent mesenchymal stromal cells recruitment: transwell invasion/recruitment assays

The capacity of DC to recruit MSC was evaluated in transwell assays. Boyden chambers with membranes of 8 µm pore size and coated with Matrigel were used (BD Biosciences) as inserts for the 24-well plates, as exemplified in Figure 9. In all experiments, the bottom compartment (BC) contained the stimuli for MSC recruitment in 750 µL of MSC medium, with or without FBS. The top compartment (TC) contained $4x10^4$ MSC in 500 µL MSC medium with or without FBS.

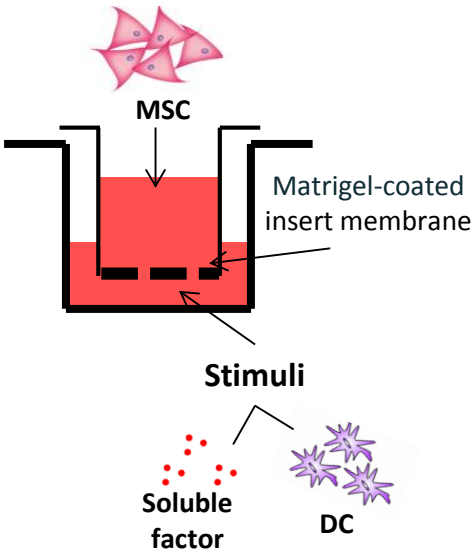


Figure 9. Scheme of the apparatus used for transwell assays. DC and/or TNF-α/RSV in DMEM (with or without FBS) are placed in the wells of a 24-well plate (bottom compartment - BC), being the stimuli for MSC invasion across the Matrigel-coated membrane of the Boyden chamber (top compartment - TC).

DC were recovered as described above, plated in BC ensuring the appropriate MSC:DC ratio and either left unstimulated or stimulated with 100ng/ml of TNF-α and/or 10µM of resveratrol (trans-3, 5, 4'-trihydroxystilbene, RSV) 4-5 h, prior to TNF-α stimulation, as indicated. Assays were performed 24h after TNF-α stimulation (DC at day 7 of differentiation). Controls were made using serum free medium (SFM; MSC medium without FBS), MSC medium supplemented with 30% FBS and MSC medium supplemented with stimulation factors in the

appropriate assays. Where indicated the MMP inhibitors gallardin (Calbiochem) or doxycycline (Fluka) were added both to the BC and TC to a final concentration of 10 µM and 15 µg/mL, respectively. Experiments were run in culture medium without serum and, since gallardin is prepared in DMSO, a control sample was included with 10% DMSO in TC and BC.

MSC were allowed to invade the membranes for 24h. After, culture media were removed, the membranes of the inserts were washed with PBS 1x and fixed for 20 min with 4% paraformaldehyde. Insert membranes were washed again and stored in PBS 1x at 4 °C until further use. Then, the membrane side facing the interior of the insert was thoroughly scrubed with a cotton swab and washed with PBS 1x. The membrane was then carefully removed, placed on a microscope slide, previously prepared with mounting media containing DAPI (4',6-diamidino-2-phenylindole), and covered with a cover-slip. MSC nuclei were stained with the DAPI and could be counted in the inverted fluorescence microscope as above.

2.4. Metalloproteinases activity detection

The contribution of MMP for MSC recruitment was assayed by the detection of their activity in gelatine and β-casein zymographies.

Culture media from recruitment experiments, or from DC cultured alone in serum free medium in the same conditions as in the transwell assays, were collected from top and bottom compartments together or separate, and labelled TC and BC, respectively. Samples were then centrifuged at 1200 rpm for 10 min, at 4 °C to remove cells and debris. Supernatants were stored at -20 °C until use.

For experiments conducted in serum free culture, proteins in the supernatants were precipitated before use. In this way, 1 volume of sample was mixed with 6 volumes of acetone at -20 °C and kept at -20 °C overnight. Tubes were then centrifuged at 14000, for 5 min, at 4 °C and the supernatant was carefully discarded. Acetone remnants were evaporated letting the tubes open at RT. Protein pellets were finally dissolved in 30 µL of ultrapure water, at RT, under gentle agitation. Protein content of samples was quantified using Bio-Rad protein assay according to the manufacturer's instructions.

Unless stated otherwise, samples were mixed with sample buffer (SDS 0.1% plus 0.04% sucrose in Tris buffer 0.25 M, pH 6.8) and 12 µg of protein (directly from culture medium), or 2.25 µg of protein (from precipitated protein pellets) were loaded into the gels. Stacking gels were 5% acrylamide (Bio-Rad) and resolving gels were 10% acrylamide gels, containing 0.1% of gelatine (Sigma-Aldrich). Precision Plus Protein™ Dual Color Standards (Bio-Rad) were used as protein standards. Electrophoresis was run at 60 V until samples exit the stacking gel, and then

at 80 V until the 25 KDa band of the protein marker reach the end of the gel. Gels were then washed twice in 2% Triton X-100 (Sigma-Aldrich) for 15 min, washed with dH_2O and incubated in MMP substrate buffer ($CaCl_2$ 10 mM in Tris buffer 50 mM, pH 7.5) for 16-18h, at 37 °C. After, gels were washed with dH_2O, incubated with Comassie Brilliant Blue solution for 20 min and washed again with dH_2O until clear bands were seen in a blue background. Finally, gels were scanned in a GS800 calibrated densitometer (Bio-Rad) and band size quantified using Quantity One 1-D Analysis Software v 4.6 (Bio-Rad) or ImageJ software.

2.5. RNA extraction and gene expression evaluation

DC used for transwell assays and in control experiments were recovered for mRNA extraction. After centrifugation of the culture media from BC, cell pellets and the adherent DC in the culture plate used for the transwell were processed using the PureLink™ RNA Mini Kit (Invitrogen) according to the manufacturer's protocol, but the 300 µL lysis buffer recommended were added to the culture plate to lyse the adhered cells and then the lysate was transferred to the tubes containing the cell pellets obtained after centrifugation of culture supernatants. Lysis was completed by passing the suspension 5 times in a 18-gauge needle, as recommended by the manufacturer. The mRNA extracted was then stored at -80°C until use.

The expression of MMP-9 gene in the transwell assays was accessed by qPCR using RNA recovered from DC used in the assay. In this way RNA samples were thawed on ice and RNA content and purity were quantified by UV spectrophotometry, using a NanoDrop® spectrophotometer. Then, 300 ng of RNA were diluted in RNase-free water and mixed with Random Primers (Invitrogen) to a final volume of 12 µL for cDNA synthesis. The mix was incubated at 70 °C for 10 min and after maintained at 4°C, in order to anneal the primers with the RNA template. The mix was complemented with 0.75 µL RNase-free water, 1 µL dNTPs, 2 µL DTT, 0.2 µL RNAsin and 0.2 µL reverse transcriptase in 4 µL reaction buffer and cDNA was synthesized incubating at 37 °C for 1 h. All reagents were from Invitrogen, except RNAsin, from Promega.

qPCR reactions were prepared diluting 0.5 µL of cDNA in 4 µL of RNAse-free water plus 5 µL of TaqMan reaction buffer, following the addition of 1.6 µL of MMP-9 (Hs00234579_m1 from Applied Biosystems) or GAPDH (Part Number: 4352934E from Applied Biosystems) human probe. qPCR was then run on an Applied Biosystems GeneAmp Prism 7700 System for 40 temperature cycles as follows: 20 s at 50 °C, 10 min at 95 °C followed by more 15 s at 95 °C and finally 1 min at 60 °C. Data was analyzed using the $2^{-\Delta ct}$ method.

2.6. Cytokine detection

Aliquots of culture media from transwell invasion assays were used for cytokine screening with a multiplex cytokine assay (Luminex®). Culture media samples were mixed with coded MagPlex® magnetic microspheres (Luminex®) linked to antibodies anti-human IL-1b, IL-6, IL-8, IL-12p70, TNF-α or IL-10 according to the manufacturer's protocol. Fluorescence was read and the coded beads identified using the MAGPIX® system (Luminex®). Cytokine concentration was determined comparing sample fluorescence, against a standard curve.

2.7. Time-Lapse Video Microscopy of multipotent mesenchymal stromal cells and dendritic cells

Co-cultures were prepared plating $5x10^3$ MSC on 24-well plates in MSC medium 24h before DC addition. DC were carefully collected as before and added to MSC cultures in a ratio 1:10 in fresh MSC medium. Controls were made plating MSC and DC alone. The plate with cell cultures was then placed in the inverted microscope with an incubation system (Zeiss Axiovert 200), setting temperature to 37 °C and CO_2 concentration to 5%. Cell positions were defined and phase contrast photographs were recorded every 5 min for 12 h (records started 2-3h after MSC-DC co-culture). The videos obtained were then analyzed with MTrackJ (ImageJ) for single cell tracking, in order to determine the distance travelled by the cells and their velocity.

2.8. Detection of NF-kB nuclear translocation by imaging flow cytometry

DC-MSC at 1:5, 1:10 and 1:20 ratios were placed together in tubes for reciprocal stimulation. Cells were then carefully collected 0, 15, 30 and 60 min, washed and incubated with monoclonal anti-human PE-labelled CD45 antibody (at 5 µL in 50 µL). Cells were fixed with 4% paraformaldehyde for 10 min, at RT and washed. NF-kB was then immunostained incubating cells in 0.1% Triton X-100 in PBS 1x/FBS 2% with monoclonal anti-human p65 NF-kB subunit primary antibody in a ratio 1:5000 for 20 min. Cells were washed again and incubated with anti-mouse FITC-labelled IgG secondary antibody in PBS 1x/2% FBS for 20 min. After washing, nuclei were stained for 5 min with DRAQ5 20 µM (Biostatus). Controls were made mixing inactivated and activated DC with no antibody staining and NF-kB, secondary antibody alone or DRAQ5 single stains. Samples were analyzed on imaging flow cytometer ImageStream^X (Amnis), acquiring at least 10000 events. The data obtained was analysed using the nuclear localization wizard of the IDEAS software (Amnis).

2.9. Detection of NF-kB translocation by confocal microscopy

Cover-slips were placed on the bottom of 24-weel plates and $6x10^3$ MSC were cultured on top, in MSC medium. DC were added to MSC cultures at 10:1 ratio, and TNF-α was added to a final concentration of 100 ng/mL. Stimulations occurred for 30 min, 1h, 12 h and 24 h. At the end of each time point, culture medium was recovered from the wells, cells were washed with PBS 1x and fixed with 4% paraformaldehyde for 20 min, at 37 °C. Cover-slips were then transferred to a petri dish and NF-kB immunostain was performed as described for co-cultures in suspension, except for nuclei stain. Cover-slips were mounted onto microscope slides using mounting media containing DAPI. Samples were stored at 4 °C until visualization by confocal laser scanning microscope (Leica SP2 AOBS SE). Images recorded were analysed in ImageJ software.

2.10. Osteogenic differentiation of MSC in response to different stimuli

Cultures of unstimulated DC or DC stimulated with TNF-α, RSV or both were prepared as described above. After 24 and 72h of TNF-α stimulation, culture media (conditioned media (CM)) was recovered from the wells, centrifuged at 1200 rpm for 10 min, at 4 °C, and stored at -20 °C until use. $5x10^3$ MSC were cultured for 2 days in MSC medium for expansion and then culture medium was replaced by CM, or MSC medium containing RSV at 10 µM or 50 µM. Controls were made with MSC cultured in MSC medium and in osteogenic medium as prepared in 2.1. Cells were maintained in culture for 7 days, replacing culture medium once. After this time the degree of osteogenic differentiation of MSC was evaluated by ALP activity staining, as described in 2.1.

2.11. ALP activity quantification by biochemical assay

MSC were cultured in medium alone or containing RSV at 10 µM or 50 µM for 7 days before culture media was removed from the wells, cells were washed with PBS 1x and the culture plate was frozen at -20°C. Later, plates were defrosted on ice and cells were incubated with 1% Triton X-100 for 30 min, at 4 °C and under shaking at 200 rpm, followed by sonication for 2 min on ice, in order to lyse cells. Meanwhile, ALP reaction media were prepared with p-nitrophenyl phosphate in sodium bicarbonate buffer supplemented with $MgCl_2$. Cell extracts in each well were homogenized and 20 µL were added to this reaction media, incubating at 37 °C for 1h. During the reaction, active ALP catalyzes the hydrolysis of p-nitrophenyl phosphate into p-nitrophenol, a chromogenic product. The hydrolysis was stopped adding NaOH 20 mM to the

reaction media. Absorbance was then read at 405 nm and ALP activity was determined comparing against a standard curve of p-nitrophenol standard solutions. Total protein in the cell lysates was also quantified, using Bio-Rad protein assay as above.

2.12. Statistical analysis

Statistical analysis of the results obtained was performed using GraphPad Prism 5.02 (GraphPad Software, Inc.). The data obtained was first tested for normality using D'Agostino and Pearson omnibus normality test. As data did not pass the normality test, it was compared using the non-parametric test of Kruskal-Wallis, followed by Dunns post test comparing of user defined sets of data. Tests were run for a significance level α of 0.05 and data statistically significant was identified in plots with the symbol *

3. Results

In this work human primary MSC and DC were used. Primary MSC require a phenotypic and functional characterization prior to use, in order to demonstrate that these cells fulfil the criteria to be considered true MSC. Dendritic cells, on the other hand, were differentiated from primary human monocytes, freshly isolated for every experiment, but a periodic characterization on cell phenotype is essential.

3.1. Phenotypic characterization of MSC and DC

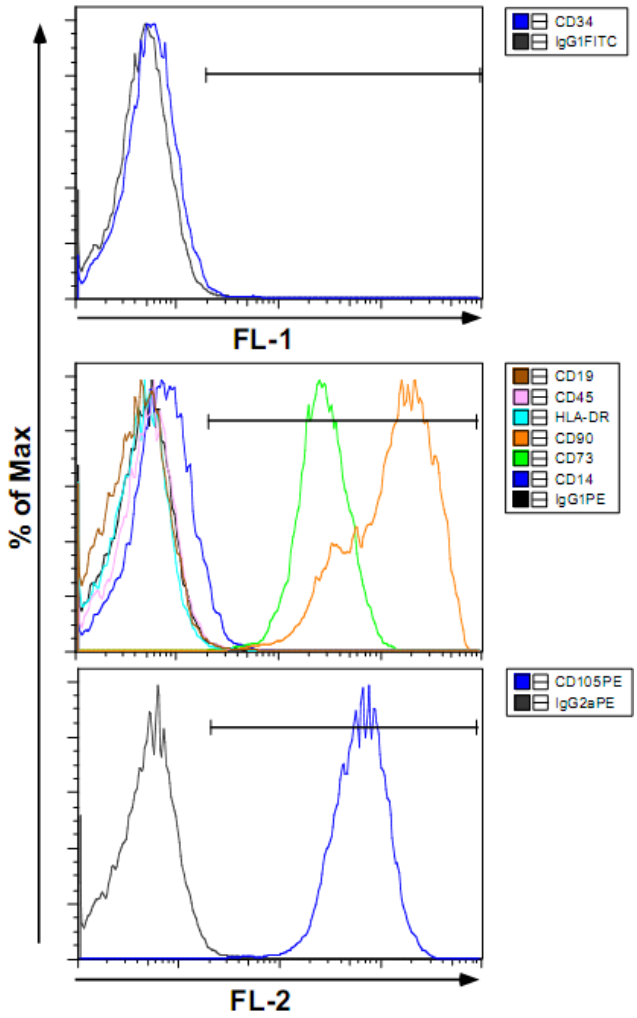
MSC were characterized according to the internationally defined ISCT criteria, which involves combined characterization of cell surface markers and differentiation potential[110].

The characterization of phenotypic markers on MSC surface by flow cytometry revealed that more than 95% of cell population used in this work are $CD105^+$, $CD73^+$ and $CD90^+$ (Figure 10). Simultaneously, less than 2% of these cells express the markers CD45, CD19, CD34 and HLA-DR (Figure 10). Although ~5% of the cell population in test express CD14, this is likely due to the particular clone of CD14 antibody used.

Next we wanted to determine MSC differentiation capacity, so the cells were cultured in media containing osteogenic, chondrogenic or adipogenic chemical stimuli.

The osteogenic differentiation capacity of MSC was tested monitoring the activity of ALP during 28 days of culture, and complemented with Von Kossa Staining for the visualization of mineralization foci.

As illustrated in Figure 11, ALP activity staining (red) gradually increased along time, in osteogenic medium, and was already considerable after 7 days of osteogenic stimulation. As expected, ALP enzymatic activity is higher under osteogenic conditions than for MSC cultured in basal conditions (negative control). Nevertheless, ALP activity staining also increases under basal conditions from the first week of cell culture, although to a lesser extent than observed in osteogenic conditions.



Sample	% of Positive
CD105	99.7
CD73	99.9
CD90	100

Sample	% of Positive
CD19	0.88
CD14	5.04
CD45	0.84
HLA-DR	0.84
CD34	1.47

Controls	% of Positive
PE-IgG1	1.04
PE-IgG2a	0.94
FITC-IgG1	1

Figure 10. Phenotypic characterization of the MSC population. MSC were immunostained for the surface markers defined in ISCT criteria for MSC identification and then analyzed by flow cytometry. The percentage of positive cells for a surface marker was calculated according to the gate defined in the plots of the isotype controls.

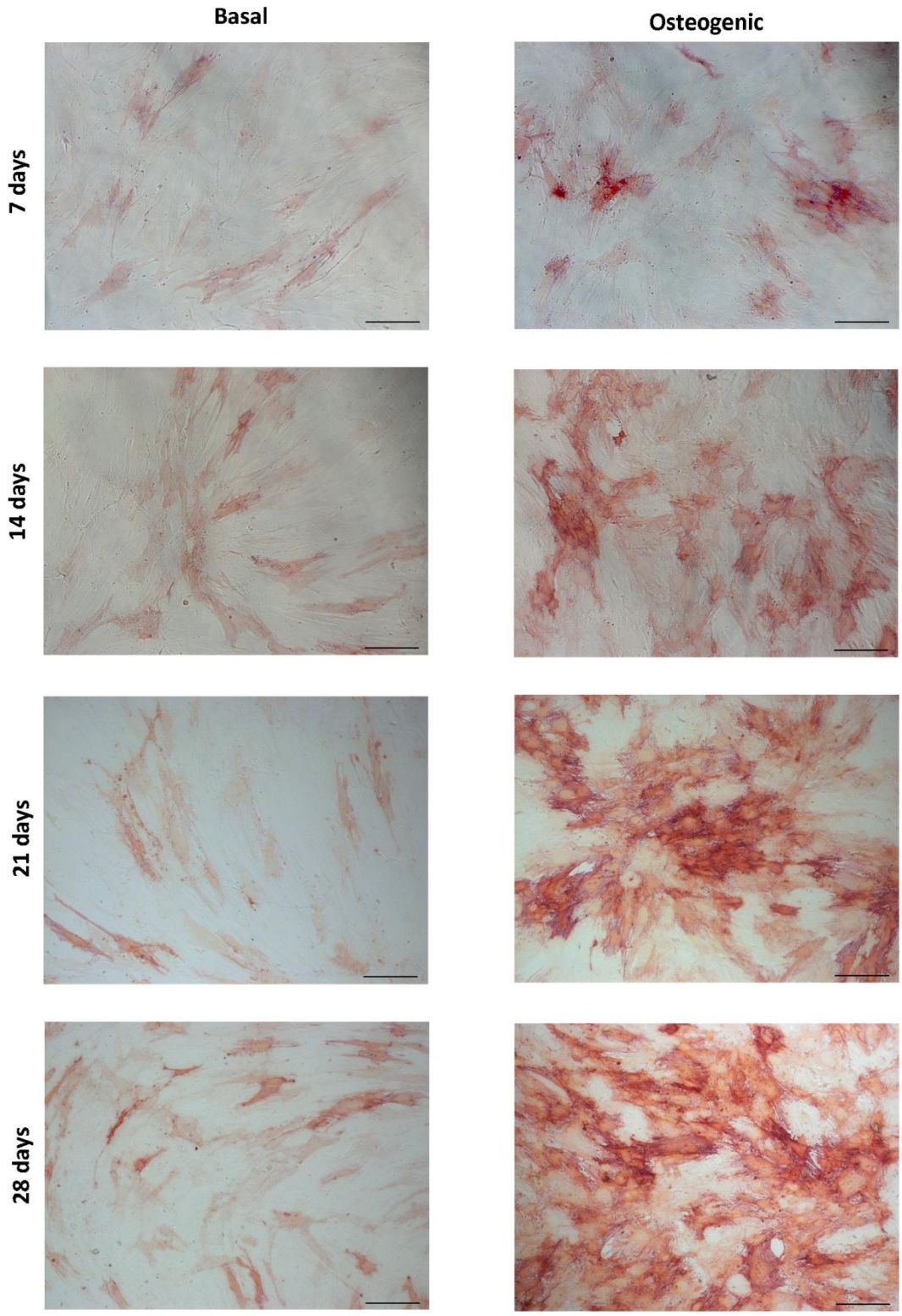


Figure 11. Osteogenic differentiation capacity of MSC as assessed by ALP activity staining. MSC were cultured at 7, 14, 21 and 28 days in osteogenic medium or in control basal conditions. The osteogenic differentiation capacity of MSC was assayed by ALP activity staining with Fast Violet B salt solution containing phosphate compounds. ALP activity is revealed by the red staining. Scale bars: 200 µm.

In agreement with these results, mineralization occurred in MSC cultured in osteogenic conditions, as assayed by Von Kossa staining (Figure 12). Comparing with ALP activity detection, mineralization occurred, as expected, at a later time point. After 28 days of culture, dark brownish spots are visible. In contrast to the observations for ALP activity, mineralization was not detected in MSC cultured in basal conditions during the course of the experiment.

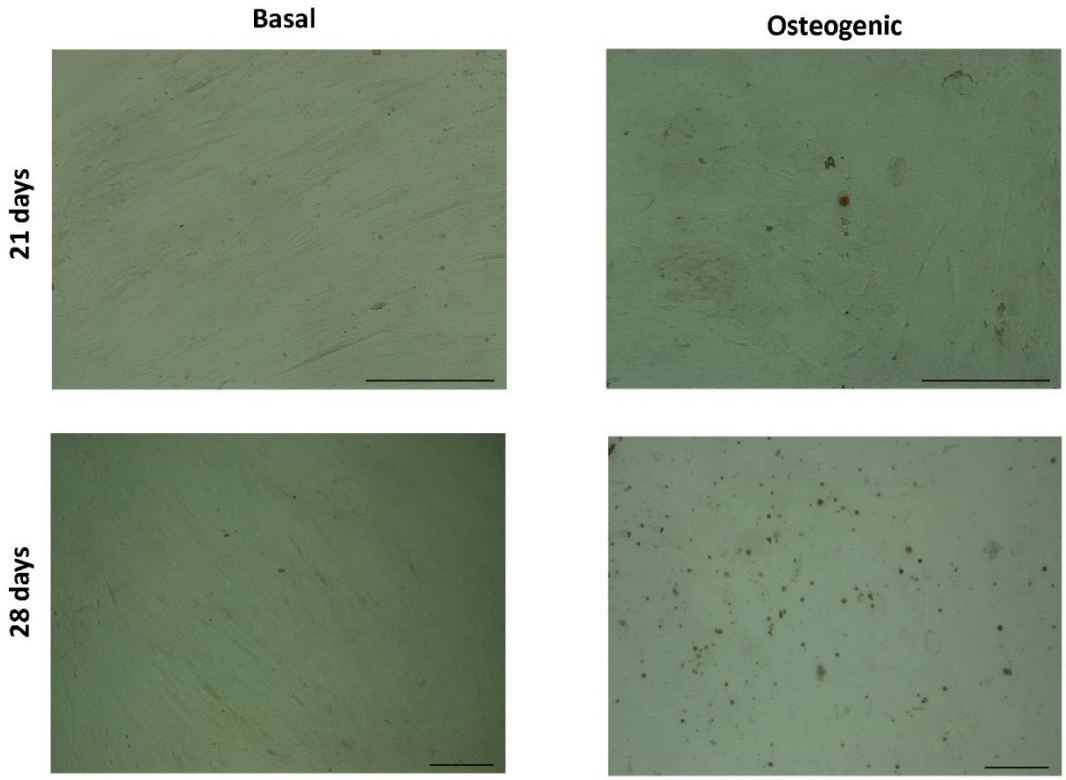


Figure 12. Osteogenic differentiation capacity of MSC as assessed by Von Kossa staining, for mineralization. MSC were cultured in osteogenic or basal medium for 28 days. The mineralization capacity of MSC was assayed by Von Kossa staining. Mineralization nodules are formed in osteogenic conditions, as shown by the dark spots, indicating a capacity to incorporate phosphate into the matrix producing mineralized nodules. Scale bars: 200 µm.

MSC also revealed a potential to differentiate into the chondrogenic lineage. As illustrated in Figure 13, a change in colour from blue to purple was noticeable in MSC pellets cultured for 28 days in chondrogenic medium and stained with toluidine blue. The colour of MSC pellets cultured in basal conditions also changed, but much more slightly towards purple. Also, the size of pellets cultured in chondrogenic conditions was larger than those cultured in basal conditions.

When cultured in adipogenic medium for 7 days, MSC formed fat deposits to a large extent, as shown by Oil Red O staining (Figure 14). This was not seen in MSC cultured under basal conditions.

Taken together, these results show that the cells used are in fact MSC, as defined by the ISCT criteria.

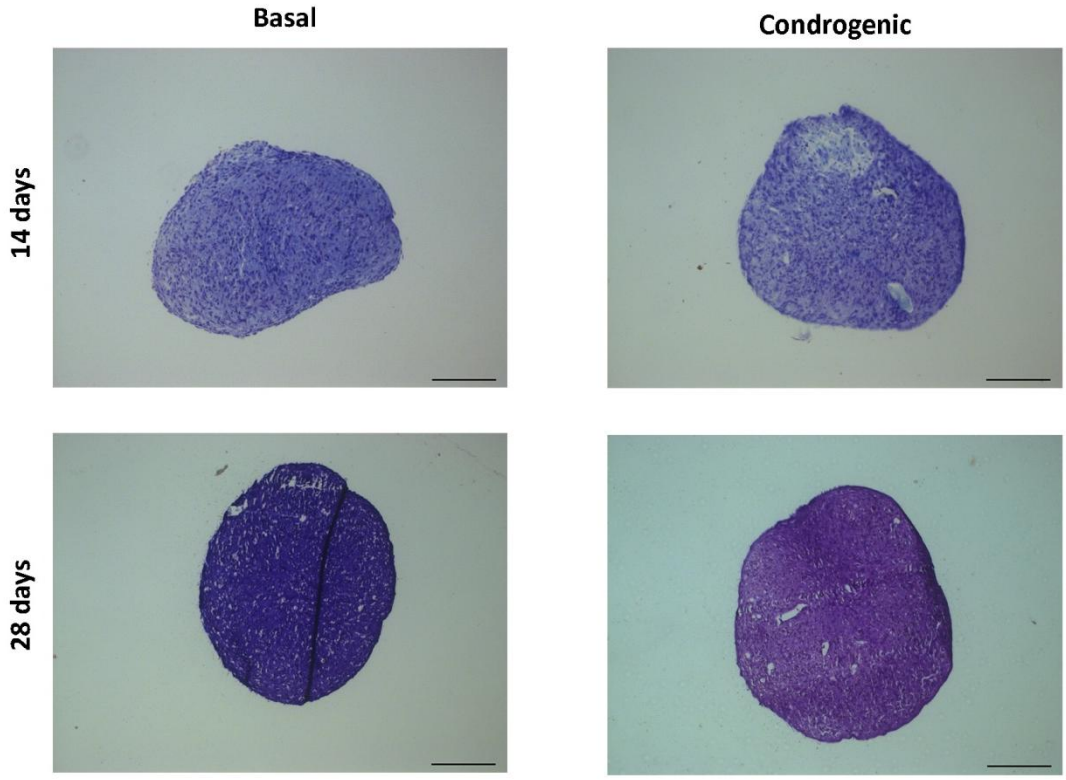


Figure 13. Chondrogenic differentiation capacity of MSC. Hypoxic MSC pellets were maintained in chondrogenic or basal media, before being histologically processed and stained with toluidine blue. The increased production of glycosaminoglycans along culture time, was revealed by the purple colour, particularly at day 28. MSC were thus capable to differentiate into the chondrogenic cell lineage. Scale bars: 200 µm.

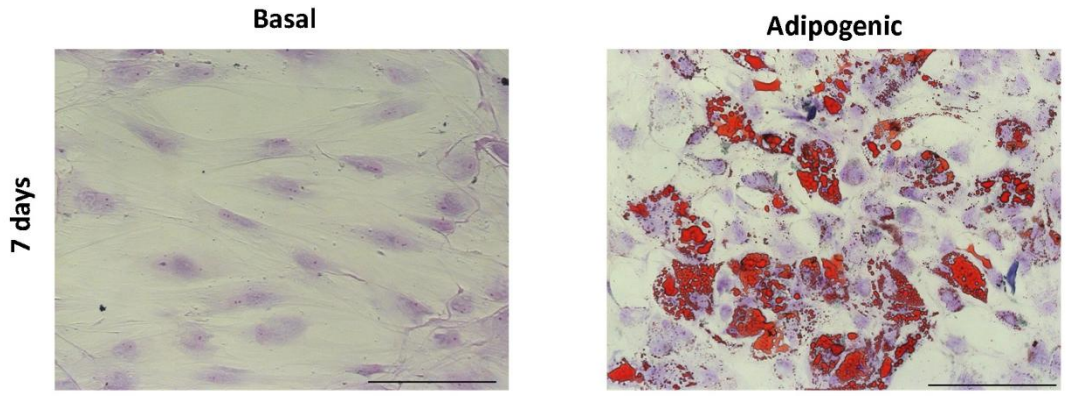


Figure 14. Adipogenic differentiation capacity of MSC. MSC were cultured in adipogenic or basal medium, before staining with Oil Red O. Fat deposits are visible in red, and cell nuclei were stained purple, with hematoxylin. Scale bars: 200 µm.

Finally, the differentiation and responsive capacity of DC used in this work was also verified. Since these cells were differentiated from monocytes populations isolated from different blood donors, periodic analysis of DC phenotype were performed. The cell population expressed the lineage marker CD1a, independently of any previous stimulation (Figure 15). On the other hand, the expression of CD83 was increased in DC samples stimulated with TNF-α or LPS, comparing to non-stimulated cells (Figure 15). Isotype and single staining controls are included in Annex 1.

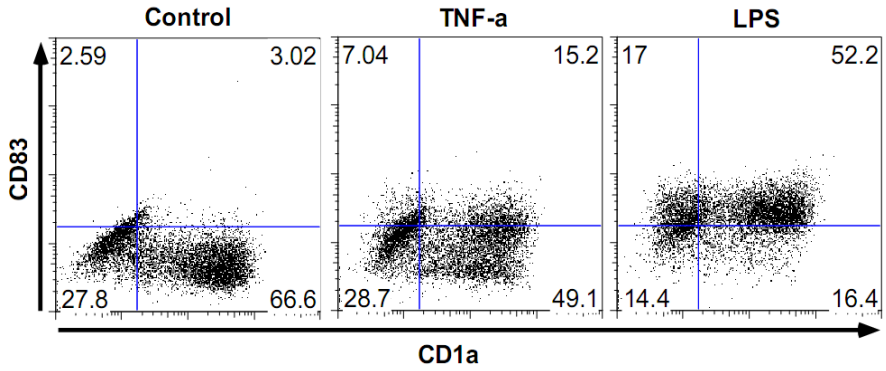


Figure 15. Phenotypic characterization of DC. Monocyte-derived DC, differentiated for 7 days and either left unstimulated or stimulated with TNF-α or LPS were immunostained for the surface markers CD1a (lineage marker) and CD83 (activation marker), and analysed by flow cytometry. All samples show good expression of the lineage marker, while the activation marker increased with cell stimulation, particularly with LPS.

3.2. The influence exerted by DC on MSC invasion capacity

3.2.1. DC promote MSC recruitment *in vitro*

Boyden chambers with Matrigel-coated membranes (Figure 9) were used to investigate the potential of DC to stimulate MSC recruitment. An example of invading MSC with the nuclei stained blue is illustrated in Figure 16A. The presence of DC in different proportions in the BC of transwell assays influenced MSC invasion. Indeed, increasing numbers of DC lead to a higher number of MSC crossing insert membranes outwards (Figure 16B). The invasion capacity of MSC when DC were present in the ratio 1:10 and 1:20 was statistically significantly higher, when compared with the negative control, where in the BC was only SFM. According to the results obtained, the MSC:DC proportion of 1:10 was selected to perform next assays.

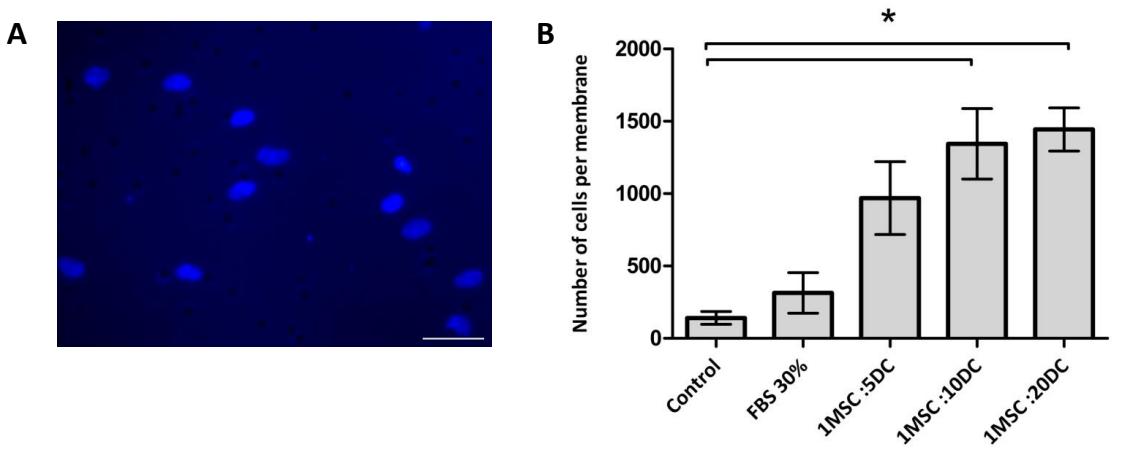


Figure 16. DC stimulate MSC invasion capacity. Serum free media (control), media supplemented with 30% FBS (FBS 30%) or the number of DC appropriate for the indicated ratios (in normal culture media with 10% FBS) were placed in the bottom compartments (BC). A Boyden chamber with Matrigel-coated membranes with 8 µm pores was placed in the well, and MSC seeded on top. **(A)** Representative image of an insert membrane with invading MSC stained in blue (DAPI). Scale bar: 50 µm. **(B)** Quantification of the results obtained for 3 independent experiments. (n=3; *P-value < 0.05 in Kruskal-Wallis, followed by Dunns multiple comparisons test).

3.2.2. Inflammatory conditions do not influence significantly MSC recruitment by DC

Next we wanted to evaluate the potential influence of a pro- or anti-inflammatory environment on the capacity of DC to recruit MSC. Thus DC were stimulated with the pro-inflammatory cytokine TNF-α or the anti-inflammatory compound resveratrol (RSV) 24h prior to transwell assays being set up. Both stimuli were also applied to the same sample, RSV first and TNF-α after, in an attempt to inhibit the pro-inflammatory stimulation. The results obtained are illustrated in Figure 17A and show that DC stimulations affect only slightly their capacity to recruit MSC. The numbers of invading MSC are not significantly different than the ones obtained when unstimulated DC are used. This assay was conducted using MSC at different passages. By plotting the number of invading MSC it was found that MSC capacity to invade the membrane diminishes with increasing passage numbers (compare red with black symbols).

Furthermore, as illustrated in Figure 17B, TNF-α or RSV molecular stimuli alone cannot promote MSC membrane invasion to an extent comparable to the presence of DC.

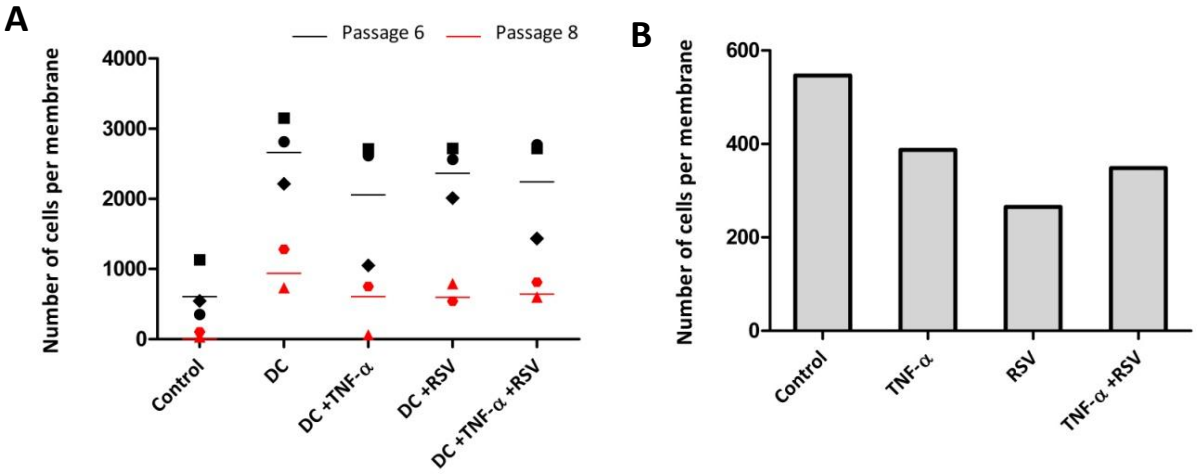


Figure 17. Influence of DC stimulation in their capacity to promote MSC recruitment. (A) Transwell assays were performed with DC stimulated 24h prior to assay set up with the pro-inflammatory cytokine TNF-α, the anti-inflammatory compound resveratrol or both (RSV first and then TNF-α). Each symbol represents a different donor from which DC were derived. Traces represent the mean for each condition. Passage 6 and passage 8 refer to the MSC passage used. **(B)** Control conditions were also assayed using the stimuli alone in the BC. In both cases the assay was performed in SFM.

3.2.3. MMP and cytokines are secreted during MSC recruitment by DC

As MMPs have been implicated in *in vitro* assays for MSC extravasation before[73], their role in MSC Matrigel invasion in the presence of DC was studied. The presence of MMPs in the culture supernatant recovered at the end of transwell assays was analyzed by gelatine zymography, which allows the detection of MMP-9 and MMP-2. According to the molecular weight described in the literature for MMPs[111] – pro-MMP-9: 92 KDa; MMP-9: 83 KDa; pro-MMP-2: 72 KDa; MMP-2: 64 KDa –, it is likely that proforms of MMP-2 and MMP-9 are detected in zymography gels (Figure 18A). Results also show that the amount of pro-MMP-9 increases with increasing DC numbers.

For invasion assays, MSC are cultured on top of a transwell, creating two compartments, so we next decided to analyse the supernatant of each compartment separately. The band patterns obtained for top (TC) and bottom (BC) compartments are different, as shown in Figure 18B. The increase in pro-MMP-9 form is much more evident in BC, while in TC another band, likely pro-MMP-2, increases with increasing DC. Moreover, in agreement with the invasion results the profile of MMPs observed when stimulated DC were used does not appear to change significantly (Figure 18C). MMPs activity in control samples of stimulated DC are depicted in Annex 3. In order to further clarify if MMPs could be mediating the attractant effect of DC on MSC, invasion assays were performed in the presence of MMPs inhibitors.

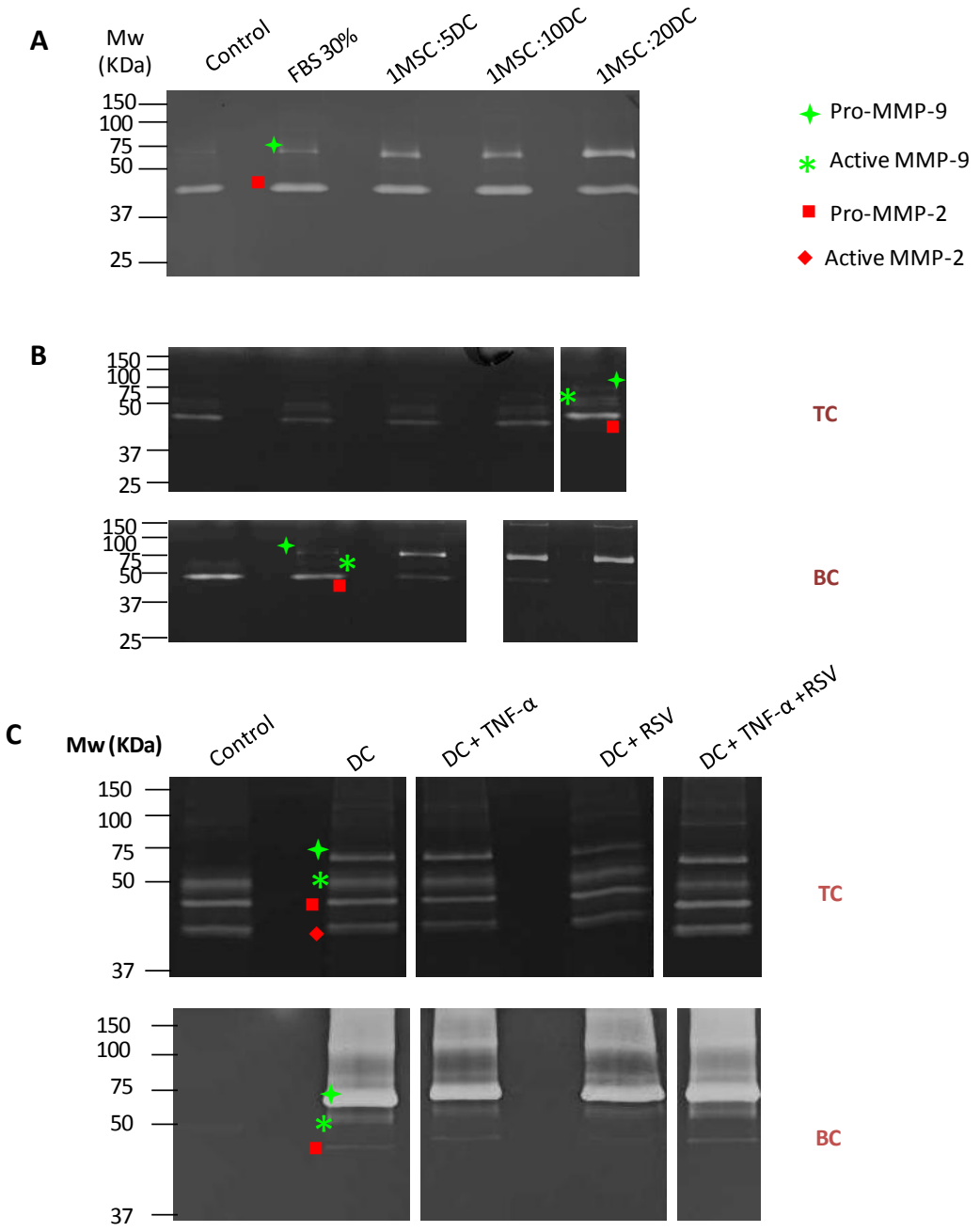


Figure 18. Profile of secreted MMP during invasion assays. (A) Culture medium collected from the transwell assays were tested for MMP content loading 12 µg of protein in gelatine-containing acrylamide gels. Gelatine degradation revealed the presence of pro-forms of MMP-2 and MMP-9, based on their known molecular weights. **(B)** Supernatant was collected separately from the top (TC) and bottom (BC) compartments of transwell invasion assays and tested for MMP activity, loading 12 µg of protein in gelatine-containing acrylamide gels. **(C)** Zymograms of protein samples precipitated from the supernatants recovered from TC and BC of MSC invasion assays performed in serum free medium. DC were stimulated, as indicated, 24h prior to assay set up with the pro-inflammatory cytokine TNF-α, the anti-inflammatory compound resveratrol or both (RSV fist and then TNF-α). After the invasion assay, supernatant was collected from the TC and BC, proteins were precipitated and quantified before 2.25 µg of protein were analyzed by zymography. In control conditions BC has only SFM. Gelatine degradation revealed the presence of pro and active forms of MMP-9, as well as the pro form of MMP2, in patterns differing with the compartment considered. The symbols + and ✱ probably correspond to the pro-form and the active form of MMP-9, respectively. In the same way, the symbols ■ and ◆probably correspond to the pro-form and the active form of MMP-2, respectively.

Results illustrated in Figure 19A, show that the presence of doxycycline led to a reduction in the number of invading MSC, while gallardin does not seem to influence MSC invasion. Interestingly, the intensity of pro- and active MMP-9 bands on zymograms of culture medium recovered from samples with doxycycline appear reduced, in relation to conditions with DC alone in the BC, while MMP-2 seems to disappear. Interestingly, in presence of gallardin, in the BC, the active form of MMP-9 is no longer and there is a slight reduction in the pro-MMP-2 band intensity (Figure 19B).

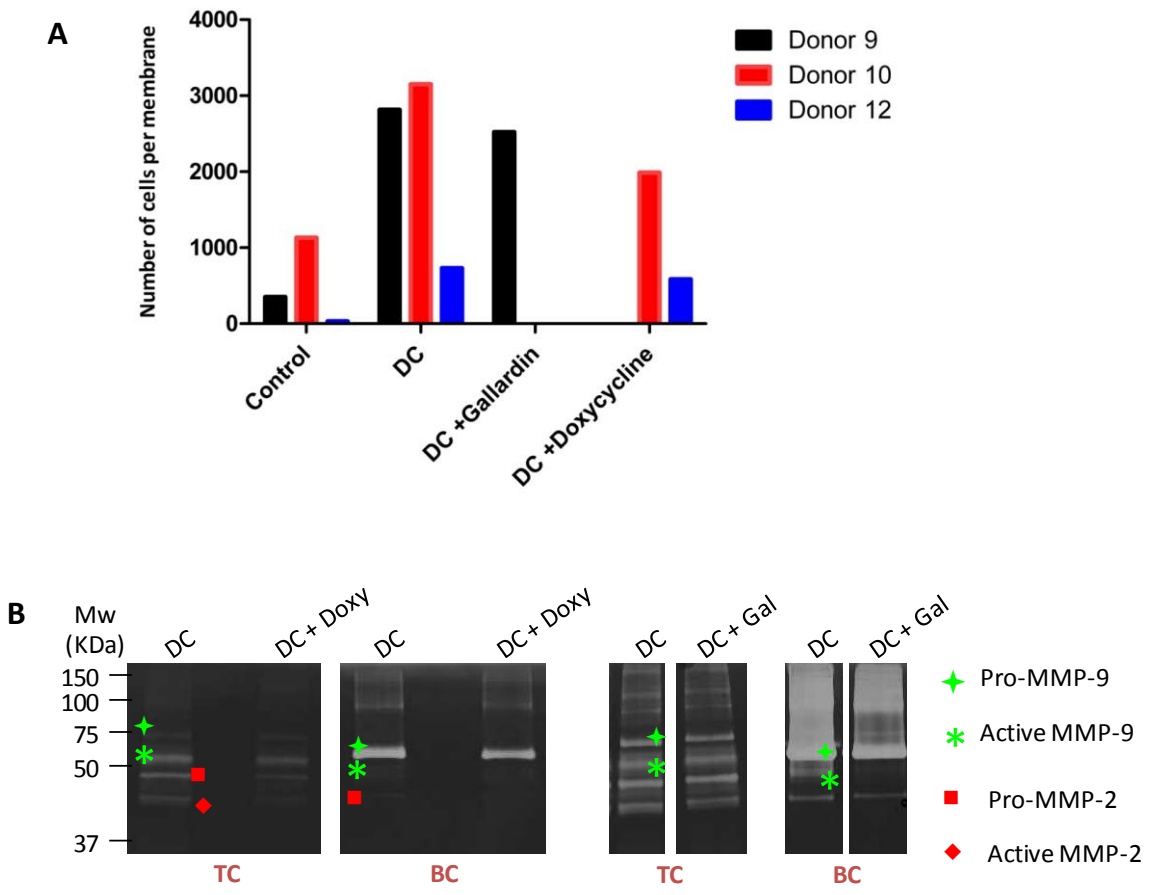


Figure 19. Effects of MMP inhibitors on MSC invasion and MMP activity. DC alone, or in the presence of MMP inhibitors gallardin or doxycycline were placed in the bottom compartment of transwell assays. Matrigel-coated transwells were placed in the wells and MSC were cultured on them. **(A)** Number of invading MSC for different DC donors in the different conditions (donor 9 was used for the gallardin experiment and donors 10 and 12 for the doxycycline experiments). **(B)** Zymograms of protein samples precipitated from the supernatants recovered from TC and BC of MSC invasion assays described in A. After the invasion assay, supernatant was collected from the TC and BC, proteins were precipitated and quantified before 2.25 µg of protein were analyzed by zymography. Gelatin degradation revealed the presence of pro and active forms of MMP-9, as well as the pro form of MMP2, in patterns differing with the compartment considered. The symbols + and ✱ probably correspond to the pro-form and the active form of MMP-9, respectively. In the same way, the symbols ■ and ◆probably correspond to the pro-form and the active form of MMP-2, respectively.

Adding to these proteomic studies, changes at the transcriptional level of MMP-9 in the different experimental conditions was also investigated, in the DC that were in BC of the transwell assays. Comparing the conditions where DC were previously stimulated with the condition unstimulated DC (Figure 20), expression of MMP-9 is increased, which does not correlate with the similar degree of MSC invasion observed. This effect is particularly high when DC are stimulated with both RSV and TNF-α, which may be acting synergistically, in contrast to what was expected. The smallest increase in MMP-9 expression occurs for DC stimulated with RSV or doxycycline, which may be related with the inhibitory roles described here for these two compounds. On the other hand, comparing the expression levels in DC used in the transwell assays and DC cultured alone a decrease in MMP-9 expression is observed.

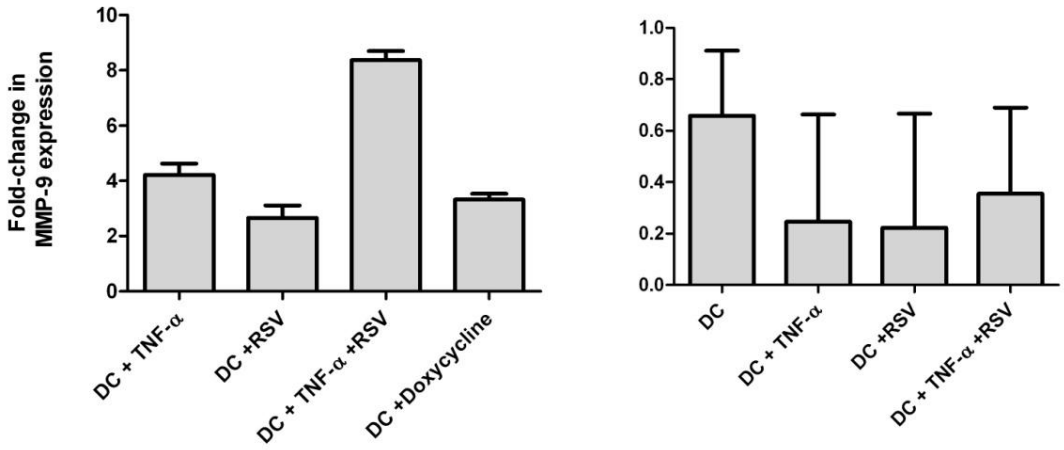


Figure 20. MMP-9 gene expression profile in DC used in transwell assays. At the end of transwell assays, DC were recovered and total mRNA extracted. This was then used to perform a qPCR in order to quantify MMP-9 expression. **(A)** Fold-change in MMP-9 expression in the different conditions where DC were stimulated relatively to the condition where DC were unstimulated**. (B)** Fold-change in MMP-9 expression occurred in DC when cultured in the presence of MSC in the transwell assays relatively to control conditions they are cultured alone. In both cases, mRNA expression was normalized to GAPDH mRNA expression. No statistical significant differences were found in both cases.

It is likely that DC also mediate MSC recruitment through the production of chemotactic factors, such as chemokines and cytokines. So, we used the culture supernatants from the invasion assays to perform a small screening of cytokines/chemokines present.

The presence of the cytokines IL-10, IL-6, TNF-α, IL-1b and IL-12p70, as well as the chemokine IL-8, was tested. Of the cytokines tested IL-1b levels were below the detection limit for all conditions assayed. Interestingly, MSC were found to produce high levels only of IL-6, while the other cytokines/chemokines appear to be mainly produced by DC (Figure 21).

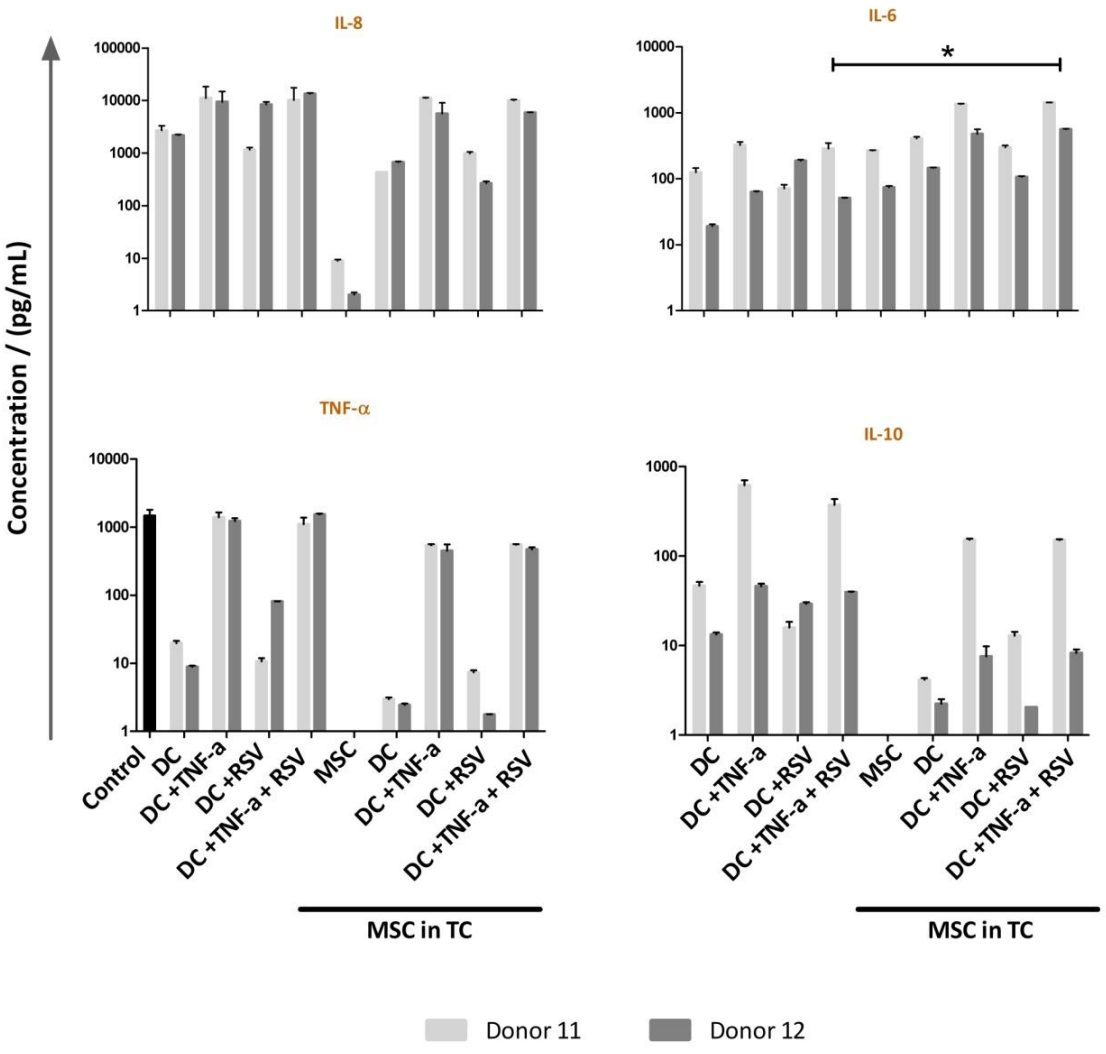


Figure 21. Cytokine secretion profile in supernatants of invasion assays. Supernatants from the invasion assays were used to perform a Luminex®-based cytokine arrays. In the plot for TNF-α, control condition corresponds to DMEM supplemented with 100 ng/mL TNF-α (the amount added to samples stimulated with TNF-α). Differences in cytokine/chemokine secretion were analyzed using the Kruskal-Wallis test, followed by Dunns multiple comparisons test, statistically significance is marked by * ($p<0.05$). TNF-a stands for TNF-α.

DC alone produce high levels of cytokines, as assessed by the quantifications of DC single cultures. The quantifications for cytokines/chemokines for cultures where MSC and DC are present do not differ significantly from the amounts found for DC cultured alone. Nonetheless, it is interesting to notice that the levels of IL-8 and IL-10 when both cells are present tend to be lower that for DC alone under the same stimulation (except IL-8 in conditions with TNF-a). Also, the levels of IL-6 show the opposite effect, i.e. they are higher in

assays where the two cells are present, than for DC alone, and are even significantly higher in the conditions with RSV and TNF-α for when the two cells are together, in relation to DC alone. Since some of the stimulation conditions under test imply the addition of TNF-α to culture media, the quantification of TNF-α levels had to be taken into account the amount added. Nonetheless, when DC and MSC are present, and in the absence of added TNF-α, a lower level was found than for DC alone. The same is true also for the condition in presence of RSV.

3.3. Cell motility: DC and MSC synchronize when cultured together

Given the influence of DC on MSC recruitment, we next wanted to investigate the interaction of these two cell types when cultured together. Cultures of DC and MSC alone and in co-culture were monitored by time-lapse video-microscopy. Observations of the co-culture along time show that DC interact with MSC, attaching to their extremities in such a way that follow their movement. Also, DC spread along and move on top of MSC, frequently switching position, on top to underneath them and vice-versa (Figure 22A). Moreover, quantification of distance travelled and mean velocity (Figure 22B,C) shows that when cultured alone DC move significantly more and faster than MSC. However, when cultured together this difference is much attenuated, MSC and DC start travelling more similar distances at more similar velocities. This leads to MSC significantly increasing their mean velocity (Figure 22B,C).

3.4. MSC-DC interaction: NF-kB nuclear translocation was not detected

The interaction of DC with MSC causes effects that can be mediated at the molecular level by different signalling pathways. In this work the activation of the NF-kB signalling pathway upon DC-MSC interaction was studied.

In the preliminary tests performed to validate the technique, imaging flow cytometry allowed to detect the translocation of NF-kB to DC nucleus upon their stimulation with TNF-α for 15 min, as shown by the co-localization of green labelled-NF-kB in the in the red stained nuclei (Annex 4, Figure 23A). This co-localization was detected in 61.94 % of the cell population in test, indicating the activation of the NF-kB signalling pathway (Figure 23B). In contrast, in the absence of TNF-α stimulation NF-kB is located in DC cytoplasm, as evidenced by the separation of green and red colours in the majority of stained cells. In addition, NF-kB was detected as translocated in only 0.76% of the population under analysis.

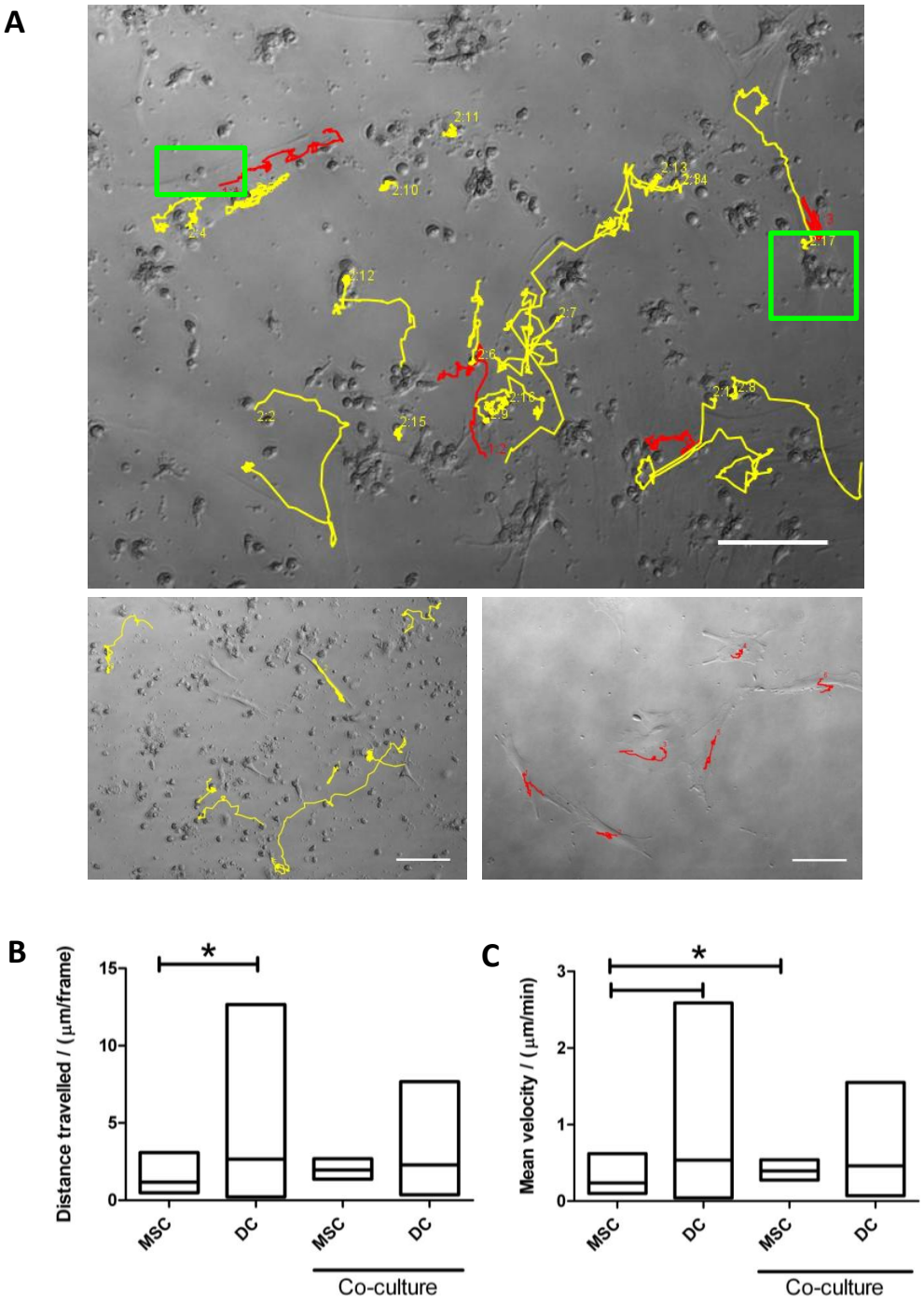


Figure 22. MSC and DC interaction when in co-culture and cultured separately. (A) MSC and DC in co-culture were monitored by time-lapse video microscopy for 12h. The videos obtained were then analyzed in MTrackJ performing single cell tracking in order to determine the pathways travelled by the cells. Central panel: MSC-DC co-culture. Bottom left panel: DC single culture; Bottom right panel: MSC single culture. Red lines represent MSC pathways and yellow lines DC pathways. Green boxes identify locations where DC are in tight interaction with MSC. Scale bar: 100µm. **(B)** Quantification of the distance travelled by each single cell followed. It was calculated dividing the length of the complete pathway by the number of frames during which the cell was followed. **(C**) Mean velocity of each cell followed was calculated as the mean of cell velocities determined by the software for each frame of the video. The horizontal line in the bars of the plot is the mean value for that distribution. Statistical analysis was performed using the Kruskal-Wallis test, followed by Dunns multiple comparisons test; statistical significance is marked by * ($p<0.05$).

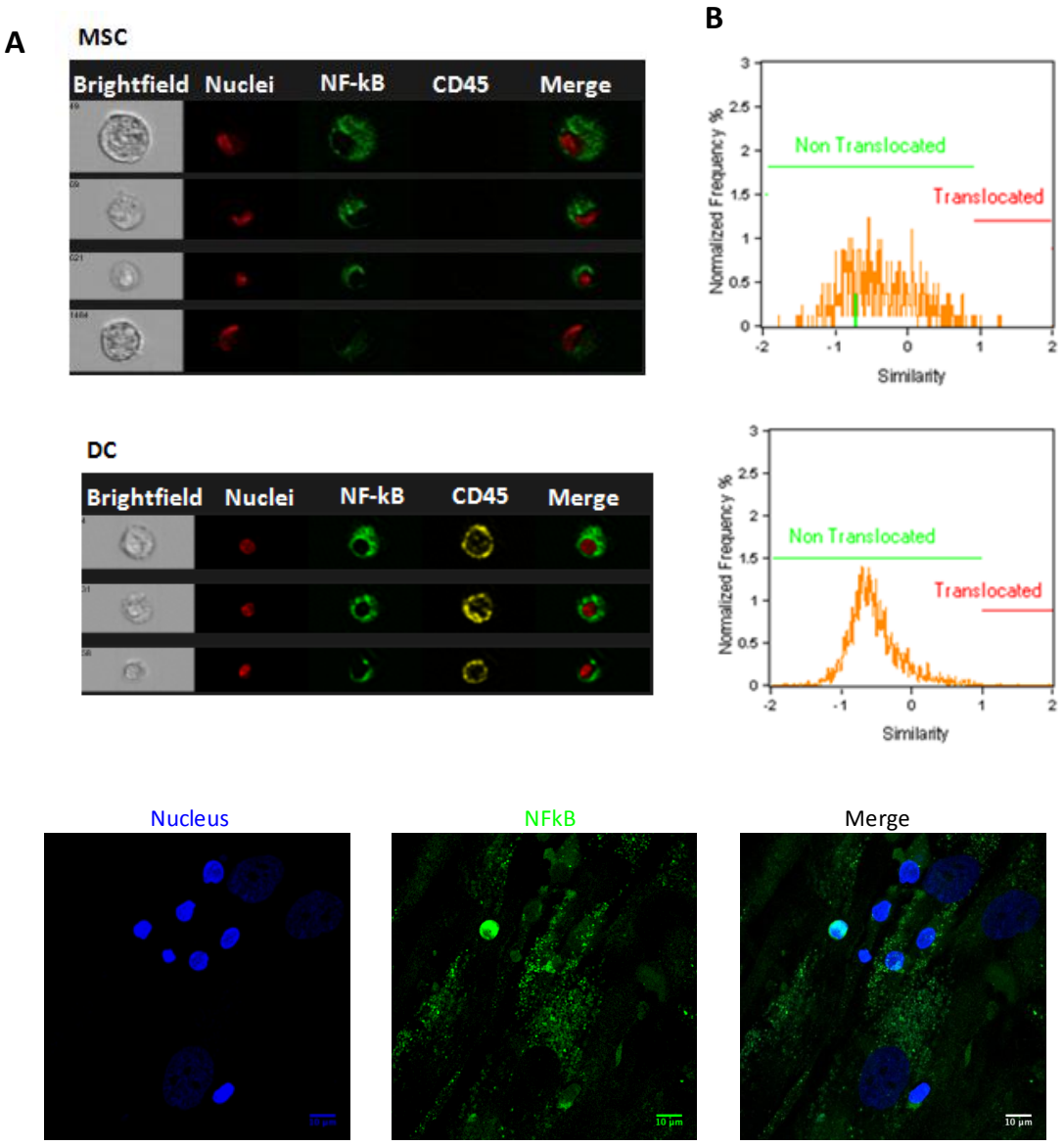


Figure 23. NF-kB signalling pathway is not activated following MSC-DC interaction. (A) MSC and DC cultured together for 30 min were immunostained for NF-kB and analysed by imaging flow cytometry. NF-kB translocation was not detected, as proved by cell images and very low similarity scores for the cell populations. Left panel: Brightfield and fluorescence snapshots of stained MSC and DC obtained on ImageStreamX. Right panel: Plots of the similarity score of colour distribution in MSC and DC after their co-culturing. **(B)** Analysis of NF-kB translocation after MSC and DC co-culture by confocal laser scanning microscopy. Merged images show that NF-kB translocated to cell nucleus only occasionally in DC, indicating the activation of the NF-kB signalling pathway, but the same was not seen for MSC.

Culturing together MSC and DC, translocation of NF-kB into cell nucleus could not be detected in either of the two cell populations. This can be verified by the separation of the green and red colour in upon merge of cells snapshots acquired during the assay (Figure 23A). Furthermore, the evaluation of the similarity in colour distribution in the cell population did

not reveal high scores for any of the two populations, which is indicative of NF-kB localization in the cell cytoplasm. CD45 was used as a marker for DC as it is a hematopoietic lineage marker that is not expressed by MSC.

In order to confirm these results, assays of adherent MSC interacting with DC along different times were performed and NF-kB nuclear translocation characterized by confocal microscopy (Figure 23C). In concordance to the results obtained by imaging flow cytometry, the majority of cell population did not presented a co-localization of NF-kB and nuclear staining. Nevertheless, NF-kB translocation could be found in some DC, as supported by co-localization of DAPI and NF-kB stain (Figure 23C).

3.5. MSC osteogenic differentiation under different stimuli

Finally, we wanted to investigate if besides influencing MSC recruitment DC had the capacity to influence MSC osteogenic differentiation.

3.5.1. Conditioned media from DC cultures induce MSC osteogenic differentiation

MSC were cultured for 7 days in media collected from DC cultures (conditioned medium, CM), stimulated with pro-inflammatory and anti-inflammatory cues, before ALP activity staining. As illustrated in Figure 24, CM from DC cultures that underwent stimulation with RSV (conditions DC + TNF-α and DC + TNF-α + RSV) lead to slightly higher ALP activity, as indicated by a stronger red staining. CM from unstimulated DC also appeared to increase ALP activity, relative to basal conditions. As expected, MSC cultured in osteogenic medium showed higher levels of ALP activity, and MSC cultured under basal conditions also show some ALP activity. In a preliminary study, MSC were also cultured together with DC for 7 days and submitted to the same pro-inflammatory and anti-inflammatory stimuli. Analysis of cellular ALP by flow cytometry did not reveal any differences in MSC osteogenic differentiation relatively to basal conditions (results not shown), but the assay has to be optimized and repeated in order to confirm results. Results of ALP detection in MSC cultured in the stimuli alone are depicted in Annex 5.

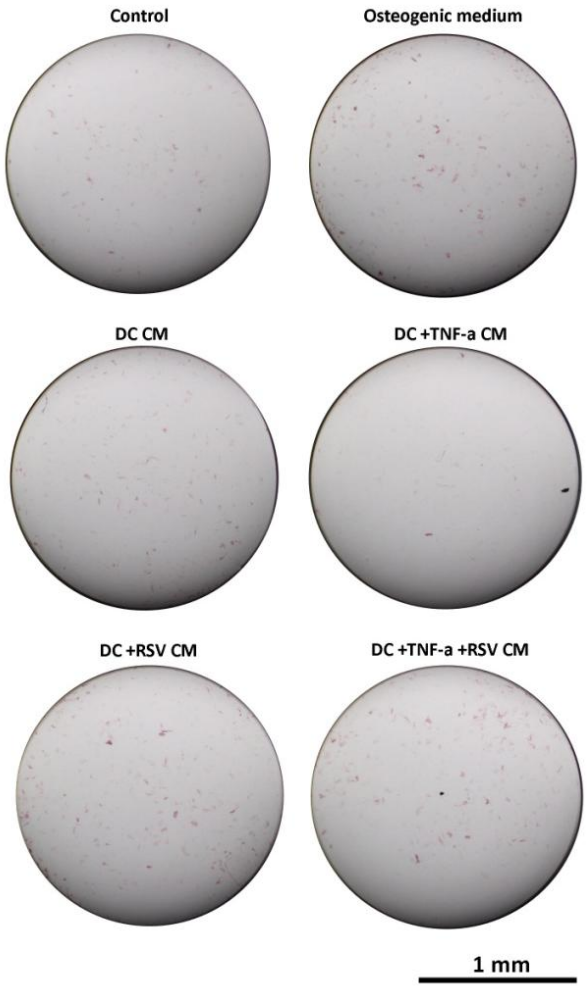


Figure 24. Osteogenic differentiation of MSC when stimulated with CM derived from DC cultures. Culture medium collected from DC cultures stimulated or not with pro-inflammatory and anti-inflammatory cues was used to culture MSC. Osteogenic differentiation of MSC was tested after 7 days in culture by ALP activity staining. For positive control MSC were cultured in osteogenic medium, whereas culture in basal conditions was the negative control of the experiment.

3.5.2. Resveratrol induces human BM-derived MSC osteogenic differentiation

In order to evaluate the contribution of the RSV stimulus to the results observed above, MSC differentiation into the osteogenic lineage in presence of different concentrations of RSV was evaluated by ALP activity, after 7 days of culture. As shown in Figure 25A, ALP activity was not significantly different from the activity detected in cultures of MSC in basal conditions. Interestingly, RSV at a concentration of 10 µM appears more prone to induce MSC differentiation than 50 µM. As expected, positive results were seen for the positive control of MSC cultured in osteogenic medium (see 2.1.1.). In basal conditions, MSC also showed some ALP activity, as seen before (see 3.1). ALP activity quantification was also performed for MSC

cultured in the presence of RSV (Figure 25B), but no significant stimulation by RSV was observed.

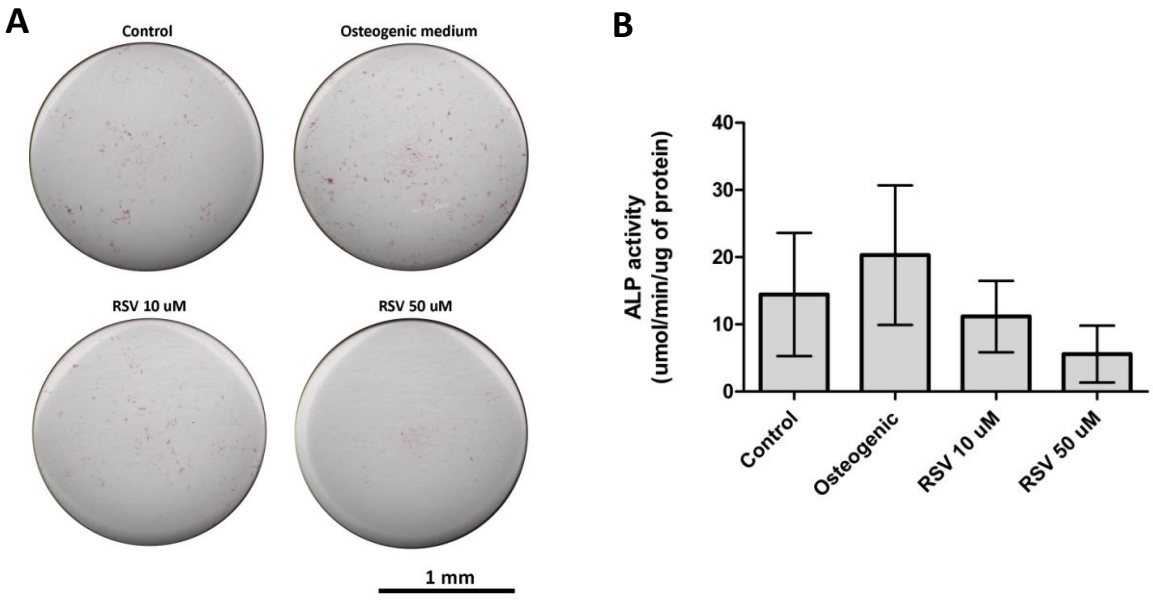


Figure 25. Osteogenic differentiation of MSC cultured in the presence of resveratrol. Culture medium supplemented with RSV at different concentrations was used to culture MSC for 7 days. **(A)** ALP activity red staining indicates MSC osteogenic differentiation. **(B)** ALP activity was then quantified through the conversion of p-nitrophenyl phosphate into p-nitrophenol. Results are expressed in function of the total protein in cell lysates. In the plot, umol and uM stand for µmol and µM, respectively.

4. Discussion

The work described here was developed using primary cultures of cells of human origin, thus avoiding the interspecies differences in both MSC and DC populations. The main problem faced is also its main advantage, the biological variability associated with different human individuals. In this context, the results obtained and discussed below should be interpreted taking into account this intrinsic variability and that for some results statistical significance would require further repeating of experiments, to increase the number of individuals analysed.

4.1. MSC characterization and DC phenotype

It is difficult to prospectively isolate MSC based on their phenotype, so the procedures for their obtaining relay on the progressive purification from a mix of different cell populations[49]. Thus, the isolated cells have to be analysed for specific features that identify them as MSC. According to the ISCT criteria for MSC identification[110], more than 95% of the cell population in analysis should express the markers CD105, CD73 and CD90, while no more than 2% of the population should express lineage markers characteristic of other cell types. Indeed, CD19 is a lineage marker for B cells, capable to adhere to MSC in culture, CD14 is highly expressed in monocytes and macrophages, CD45 is a hematopoietic marker, CD34 is expressed in early hematopoietic progenitors and endothelial cells and HLA-DR is only expressed in MSC after their stimulation and activation[110]. Except for the surface marker CD14, the minimum positive and negative percentages were verified for the population. However, the percentage of population that is $CD14^+$ can still be considered low. It is possible that the antibody used in the immunostain has some background staining that is non-specific. This is further corroborated by the fact that, if there were 5% of monocyte/macrophages in the cultures then they would have to be also positive for CD45, which is a hematopoietic marker, and so expressed by monocytes/macrophages. Since there are only 0.84% of $CD45^+$ cells the staining for CD14 is most likely non-specific. The main goal of the phenotypic testing is to exclude the presence of other cells in the population being used, which are present in the BM in close relationship with MSC.

Besides the phenotypic characterization of the cells, they also have to be able to differentiate into the osteogenic, chondrogenic and adipogenic lineages, under appropriate stimulation. From the assays to test the osteogenic differentiation it is concluded that MSC were capable of differentiating into the osteoblastic lineage. Indeed, ALP activity increased visibly with time in MSC cultures, under osteogenic stimulation conditions. This correlates with

the results later obtained by Von Kossa staining, which detected phosphate deposited under osteogenic conditions. Along differentiation *in vitro*, MSC produce a collagen matrix that is mineralized after the second to third week of culture[112]. The ALP produced during the first stages of MSC differentiation hydrolyse β-glycerophosphate contained in the culture medium into phosphate, that is incorporated in the collagen matrix[112]. This temporal aspect of osteogenic differentiation was seen in these MSC cultures, since Von Kossa staining retrieved results only at day 28 after cell culture initiation.

MSC were also shown to differentiate into the condrogenic lineage, as revealed by the purple colour observed in toluidine staining. Along the time course of chondrogenic differentiation, cells produce glucosaminoglycans, components of the ECM, in large amounts, namely collagen type II and aggrecan, which can be linked by toluidine blue[113]. Toluidine stains cell nuclei (links to nucleic acids) in blue, but when linked to glucosaminoglycans it acquires a purple colour, allowing the visualization of chondrogenic differentiation. From the results obtained for culture in basal conditions, this MSC population also show a slight tendency to naturally differentiate into the chondrogenic lineage, and hypoxia induced inside the cell pellet could have stimulated that capacity. Nonetheless, in chondrogenic conditions the differentiation is much more evident and the pellets were also bigger in size.

Finally, MSC were also shown to be able to differentiate into the adipogenic lineage, as shown by the incorporation of the fat-soluble dye oil Red O in fat deposits.

Taking all these results together, it was considered that the BM-derived cell population tested was in fact MSC, and the cells were used in subsequent experiments. During this study cells were always used between passages 4 and 9. Although the karyotype stability was not analysed along this time, previous works[45] have shown that bone marrow derived-hMSC maintained a normal karyotipe and telomerase activity until passage 12 and thus cell integrity/identity should have not been compromised.

DC used in this work were derived from $CD14^+$ monocytes by cell culture for 7 days in the presence of the differentiation stimulating factors GM-CSF and IL-4[114,115]. This method to obtain DC is well established, but cell cultures should be monitored in order to guarantee that differentiation is occurring as expected, and that cells obtained are responsive. In this work this was achieved by periodically analysing the expression profile of cell surface markers in selected DC populations. Peripheral blood monocyte-derived dendritic cells can be characterized as $CD1a^+$, a marker that is commonly expressed in antigen-presenting cells and that has been widely used as human DC marker[115–117]. From the analysis of flow cytometry results, the majority of the cell population was $CD1a^+$, indicating DC differentiation. Besides testing for DC differentiation, DC maturation by the pro-inflammatory stimuli TNF-α and LPS[115]

was also tested by staining the cells for the activation marker $CD83^{+}$. When unstimulated, (control) DC are $CD83^{-/low}$ but the pro-inflammatory stimulation causes a shift of the cell population towards a $CD83^{+}$ phenotype. Although cell surface expression of CD83 is not very high it is in agreement with previous reports[115]. Moreover, LPS, a common danger signal for immune cells, causes a stronger maturation response in DC, when compared with TNF-α. These results have been obtained in the lab, for several different donors (Santos, S. (INEB); personal communication), and are also in agreement with previous reports[115]. The authors hypothesized that this different maturation behaviour could be related with the existence of DC subsets in different degrees of differentiation in the population under analysis[115].

4.2. Implications of DC in MSC recruitment: effects and mediators

The culture of MSC in the presence of DC in transwell assays showed that DC positively influence the invasion capacity of the former. Statistical significance, relative to spontaneous MSC migration (control), was obtained for 1:10 and 1:20 MSC:DC ratios and thus increasing numbers of DC promote the invasion of higher number of MSC through the Matrigel-coated membranes. Previous works in the laboratory also showed that NK cells, and specially macrophages were able to promote MSC invasion through Matrigel-coated membranes in transwell assays[78]. In these studies, a ratio of MSC to NK cell of 1:5 was not able to promote significant MSC invasion neither. However, when NK cells were present in a proportion of 15:1 (NK:MSC) significant recruitment was found, with an average of 500 MSC invading outwards through the coated membrane[78]. In our study the MSC:DC ratio 1:10 led to an average of 1300 invading cells, which is more than twice the average for NK cells at a lower ratio. Thus, DC seem to have more capacity to promote MSC recruitment than NK cells. When the same assay performed in the presence of macrophages at 1:5 ratio (MSC:macrophage), a chemotactic index (number of migrated MSC per insert divided by the number of MSC migrated in the negative control) between 7 and 13 was observed [78]. In this work, the chemotactic index of MSC in the presence of DC at 1:5 ratio was between 4 and 10, and at 1:10 was between 6 and 12. Thus, it seems that the DC effect on MSC is similar to that of macrophages, who were also recently implicated in MSC osteogenic differentiation[97].

Through this assay it was possible to simulate MSC extravasion through blood vessels, as the Matrigel coating the membranes has a composition that mimics that of the basement membrane[118]. Thus, it is tempting to speculate that DC may be implicated in one of the steps of the endogenous MSC mobilization process (see 1.3.2.). Nevertheless, once MSC reach the homing site, they likely have to cross into the tissue, occupying a place in its 3D structure

composed by cells entrapped in the ECM, which MSC will have to remodel[119]. This microenvironment that MSC are exposed *in vivo* was not mimicked in this assay and hence it would be an useful issue to inspect. The effect of DC on MSC recruitment when both cells are cultured in 3D conditions could then be studied using a collagen invasion assay or a 3D radial migration assay, as describe before[120].

Osteoarticular diseases are often linked to an inflammatory state, as in the case of different types of arthritis. Consequently, immune cells present a different phenotype from resting states, changing their expression profile of cytokines, chemokines, MMPs, adhesion molecules, among others. This motivated the study of the effect of DC on MSC recruitment when DC are in presence of the pro-inflammatory cytokine TNF-α (Figure 17), which has a prominent role in these diseases[121], with several anti-TNF-α drugs being used in the clinic[122]. Nonetheless, anti-TNF-α therapies have worrying side effects as they reduce the capacity to fight opportunistic infections[122]. Moreover, in a rat model of spondyloarthropathies DC have been shown to be abnormal and likely linked to disease development[102,123]. So, it was also important to understand if exposure of DC to an anti-inflammatory compound, could change their effect upon MSC, when in an inflammatory environment. In this work resveratrol (trans-3, 5, 4'-trihydroxystilbene) was used as the anti-inflammatory stimuli. Resveratrol is a polyphenolic phytoestrogen capable of binding estrogen receptor-α and -β, having anti-inflammatory effects, anticancer activity and inducing cardiovascular protection, being implicated as well in the modulation of bone metabolism[124]. According to the results obtained in the transwell assays performed, exposure of DC to pro-inflammatory and anti-inflammatory stimuli did not induce statistically significant changes in MSC invasion, even though DC phenotype changed upon stimulation (assayed through CD83 activation marker, by flow cytometry, and by S. Santos, personal communication). Nevertheless, taking into account the apparent slight changes in the pattern of MSC invasion capacity, it seems that the presence of high amounts of TNF-α may affect negatively the capacity of DC to recruit MSC, and the presence of RSV in cultures may slightly inhibit that effect. Although, these results are not significant and will need to be repeated to be able to draw more definitive conclusions. This reduction in MSC recruitment was also observed when the molecular stimuli where added to BC in the absence of DC.

The effect of DC on MSC recruitment is likely to be mediated by different soluble factors, such as chemokines, cytokines, MMPs, among others. Comparing the assays where DC and stimuli were used simultaneously and assays using only the molecular stimuli, the number of invading MSC is ~ 5 times higher in the former. This may be explained by the balance between molecular mediators consumption by MSC and production by DC. The continuous

production of stimulants by DC replaces the factors being consumed, which allows the establishment of a gradient of chemotactic factors along time. This strongly supports the importance of the establishment of a chemotactic gradient for MSC recruitment[125,126].

MMPs are a subset of metaloproteinases, with 23 distinct forms in humans. They function as endopeptidases, being usually secreted in an inactive form where a prodomain shields the catalytic domain of the enzyme[111]. This prodomain can be removed by several mechanisms, being its proteolytic cleavage by other proteases the most important one[127]. Once activated, MMPs are the main responsible for the turnover of ECM, cleaving their components[111]. Consequently, given the described role of MMPs in cell migration [73,111], the expression and activity of MMP-2 and MMP-9 was investigated. Although the cell source of MMPs cannot be surely assigned from this assay, it is more likely that the MMPs detected in the culture medium of a compartment are produce by the cells cultured there. Overall, when DC are used for MSC recruitment a clear increase in pro-MMP-9 is detected. This is particularly clear in serum free-samples from BC (Figure 18C). It is likely that DC themselves change their pattern of MMP expression, in response to stimuli present in the medium. Those MMPs may then be used to degrade the Matrigel-coating of the membrane, helping MSC to invade. The activity of MMP-2 in the TC does not seem to change for the different conditions tested, but it seems to be reduced in the BC, both in samples from assays performed in the presence or absence of serum. When performed in β-casein gels (Annex 3), the band profile obtained follows the profile observed in gelatine gels and thus MMP-9 should be the major MMP mediating the MSC invasion process. Moreover, the influence of MMP on MSC invasion was corroborated by a slight decrease in the number of invading MSC, when doxycycline, an antibiotic that inhibits MMPs activity and/or expression[128], was added to culture media during the transwell assay, accompanied by a decrease in MMP-9 band intensity.

A true assessment of MMP contribution to MSC invasion in the different conditions would be favoured by quantification of the bands in the gel. This can be done using different software programmes, such as Quantity-One or ImageJ. These two start the data analysis correcting gel images for background effects. In our samples, we experienced problems with different areas in the gel having different backgrounds and so leading to aberrations in correction and unreliable band density quantification. A new attempt at quantifying the bands obtained will be performed in a near future.

Regarding the assay itself, analysis of samples of culture media not supplemented with serum allows a much more detailed and sensitive screening of MMP activity. This may be due to the presence of less serum contaminating proteins correlating with higher amounts of MMPs being resolved in the gel, which may be relevant for the detection of the active forms of

these enzymes. This has also positive implications to the recruitment assays themselves, as the presence of serum may mask the effect of other factors on MSC.

According to the results of qPCR for MMP-9 expression in DC, their stimulation promotes a higher production of MMP-9, which diffuses in the medium and theoretically would increase MSC invasion. However, zymograms show an increase of the density of a band corresponding to the inactive form of MMP-9 in BC. In this way, although production of MMP-9 increases in the system, its activation does not. Hence the key factor controlling MSC invasion does not seem to be MMPs production per se, but could be related to their activation, which is influenced by other factors, such as other proteases and protease inhibitors, like the tissue inhibitors of metalloproteinases, TIMPs[129].

From the qPCR results comparing MMP-9 expression in DC cultured in the presence of MSC and cultured alone, an influence of MSC upon DC seems evident, with MSC probably exerting immunomodulatory role upon DC[57]. In an injury context *in vivo*, the inhibition of MMP-9 expression by DC would constraint their motility, so diminishing their probability of encountering other immune cells and consequently inhibiting their contribution to an inflammatory state.

A small prospective screening for the presence of cytokines and a chemokine, in the culture medium of transwell assays, revealed the presence of IL-6, TNF-α, IL-10 and IL-8. Previous reports have implicated TNF-α[130] and IL-8[131] in the promotion of MSC migration *in vitro.* However, in our experiments this effect was not detected, since only slight changes on MSC invasive capacity occurred in the different conditions where DC were stimulated. This could be due to the fact that the authors were looking only to MSC migration and not invasion, which implied the disruption of the basement membrane. Our results show no significant changes in the levels of the cytokines tested. This may be due to the fact that only 2 experiments were quantified and more would need to be done to have more conclusive results. Nonetheless, it is interesting to note that for some cytokines, like IL-10 levels in cultures of DC alone are higher than in the corresponding condition when we had MSC presence, which suggests that MSC may be consuming the cytokines produced by DC or inhibiting their secretion. In order to test this qRT-PCR could be performed on DC to evaluate potential changes in gene expression, or intracellular cytokine stainings could be performed to evaluate differences in cytokine production at the protein level.

As MSC recruitment was enhanced when DC were present, next we wanted to investigate the effect of co-culturing the two cell populations on their motility. Video inspection showed clearly that DC and MSC were interacting, with DC moving along and around MSC. Quantifying the results from time-lapse video microscopy showed that DC and

MSC synchronize their speed of movement, when in co-culture. DC slow down, while MSC move significantly faster when in co-culture, further indicating that DC and MSC are able to establish cell contacts that may persist in time. This potentially implicates that communication between MSC and DC may also involve cell-contact dependent pathways. This is in agreement with a previous report in the literature showing Notch activation upon DC-MSC contact [132].This may have implications as DC are known migratory cells and so could potentially interact with MSC in different locations.

We first hypothesised that DC and MSC would communicate through secreted factors, including cytokines. As cytokines are known to activate the NF-kB signalling pathway[133], we looked into p65 NF-kB subunit translocation into the cell nucleus, a result of NF-kB activation[134]. Contrary to what was expected, NF-kB was not found to be translocated to the cell nucleus, in the conditions assayed. This may be due to the time-points used in our assays, so optimization of the experimental protocol and a more detailed time-course should be performed. Also, promoting DC-MSC cell contact would likely improve the chances of detecting signalling pathway activation. Another practical difficulty had to do with data acquisition in the ImageStream^X device and data analysis in IDEAS software. MSC have an auto-fluorescence higher than DC, and we had much less MSC when compared to DC, which together significantly impaired our data analysis. This aspect would also require optimization of acquisition settings. Finally, it is important to mention that this experimental approach is worth optimizing, as we could quantify the translocation of NF-kB to the nucleus, in DC in response to TNF-α and along time in thousands of cells (Annex 4).

4.3. Modulation of MSC osteogenic differentiation

Besides contributing to MSC recruitment, DC were also shown in this work to influence MDC differentiation capacity into the osteogenic lineage, supporting the idea that DC may be directly implicated in the modulation of MSC behaviour in places of bone injury.

During this work, a study was published reporting that RSV stimulates osteogenic differentiation of MSC[135]. Nevertheless, so far it was not demonstrated for human bone marrow-derived MSC. From the assays performed in this study it appears that small doses of RSV may enhance MSC osteogenic differentiation. However, these results are very preliminary and require confirmation.

5. Conclusions and future perspectives

With this work we demonstrated for the first time that DC are capable of influencing MSC behaviour, namely their motility and invasion capacity. This was demonstrated by an increase in the number of invading cells in transwell assays and by the synchronization of DC and MSC motility in co-culture. Unfortunately, a clear demonstration of the influence of pro-inflammatory or anti-inflammatory environments in DC-MSC crosstalk was not possible so far, but further experiments may help compensate the great variability among DC populations, allowing more conclusive results.

Attempts were made in order to unravel the molecular factors mediating DC-MSC crosstalk and the effects produced on motility. From the cytokines and chemokines studied, no one showed to be determinant in DC-MSC communication. On the other hand, MMP-9 was shown to be implicated in MSC invading capacity, but from our results it is not likely the only element conditioning the effects observed. Since TIMPs are good candidates as modulators of MMP activity, studies to detect their expression and activity should be performed. A reverse zymography could be used for an initial screening of these molecules, in the protein samples recovered from the transwell assays. In addition, it was not possible to demonstrate the role of NF-kB signalling pathway in the intracellular translation of MSC-DC communication. An optimization of the assay is needed, but other relevant signalling pathways should be screened, such as the NOTCH pathway. Also, other soluble mediators should be investigated, such as those contained in exosomes. Exosomes are small vesicles produced by many types of cells, including MSC and DC and present also in body fluids such as plasma and urine[136]. Exosomes have been shown to contain many regulatory factors, like cytokines and MMPs, but also microRNAs[136], which are small non-coding RNA molecules that are capable to modulate cell behaviour.

In addition to their role in MSC recruitment, results indicate that DC may influence MSC differentiation into the osteogenic lineage. If verified by more extensive studies, this effect would make DC optimal modulators of bone regeneration, promoting MSC recruitment to injury sites and modulating their differentiation into functional osteoblastic bone cells. In addition, MSC could exert a immunomodulatory role upon DC to regulate their activity, with both cells acting in a balanced way to achieve enhanced bone regeneration.

Considering what was achieved during this work, its major contribution to the scientific knowledge is the establishment of an opportunity to shed light and focus attention into the overlooked crosstalk between DC and MSC, creating a basis for the development of therapeutic strategies that take advantage of it to improve bone injuries treatments.

6. References

1. Teti, A. Bone development: overview of bone cells and signaling. *Current Osteoporosis Reports* **9**, 264-273 (2011).

2. Seeley, R., Stephens, T.D. & Tate, P. Sistema esquelético: Ossos e tecido ósseo. *Anatomia & fisiologia* 173-204 (2005).

3. Bonewald, L.F. The amazing osteocyte. *Journal of bone and mineral research* **26**, 229-38 (2011).

4. van Dijk, F.S. *et al.* Osteogenesis imperfecta: a review with clinical examples. *Molecular Syndromology* **2**, 1-20 (2011).

5. Fattore, A., Teti, A. & Rucci, N. Bone cells and the mechanisms of bone remodelling. *Frontiers in Bioscience* **E4**, 2301-2321 (2012).

6. Rodan, G.A. & Martin, T.J. Therapeutic approaches to bone diseases. *Science* **289**, 1508-1514 (2000).

7. Yu, X., Huang, Y., Collin-Osdoby, P. & Osdoby, P. Stromal cell-derived factor-1 (SDF-1) recruits osteoclast precursors by inducing chemotaxis, matrix metalloproteinase-9 (MMP-9) activity, and collagen transmigration. *Journal of Bone and Mineral Research* **18**, 1404-1418 (2003).

8. Yu, X., Huang, Y., Collin-Osdoby, P. & Osdoby, P. CCR1 chemokines promote the chemotactic recruitment, RANKL development, and motility of osteoclasts and are induced by inflammatory cytokines in osteoblasts. *Journal of Bone and Mineral Research* **19**, 2065-2077 (2004).

9. Braun, T. & Zwerina, J. Positive regulators of osteoclastogenesis and bone resorption in rheumatoid arthritis. *Arthritis Research & therapy* **13**, 235 (2011).

10. Fattore, A., Teti, A. & Rucci, N. Osteoclast receptors and signalling. *Archives of Biochemistry and Biophysics* **473**, 147-160 (2008).

11. Henriksen, K., Neutzsky-Wulff, A.V., Bonewald, L.F. & Karsdal, M.A. Local communication on and within bone controls bone remodeling. *Bone* **44**, 1026-1033 (2009).

12. U. S. Department of Health and Human Sciences *Bone health and osteoporosis: a report of the surgeon general*. 40-217 (Rockville, 2004).

13. Raggatt, L.J. & Partridge, N.C. Cellular and molecular mechanisms of bone remodeling. *The Journal of biological chemistry* **285**, 25103-8 (2010).

14. Cole, Z.A., Dennison, E.M. & Cooper, C. The impact of methods for estimating bone health and the global burden of bone disease. *Salud Pública de México* **51**, S38-45 (2009).

15. Tosteson, A. Economic impact of fractures. *osteoporosis in men: The effects of gender on skeletal health* 15-27 (1999).

16. Braithwaite, R.S., Col, N.F. & Wong, J.B. Estimating hip fracture morbidity, mortality and costs. *Journal of the American Geriatrics Society* **51**, 364-370 (2003).

17. Oden, A. *et al.* Lifetime risk of hip fractures is underestimated. *Osteoporosis International* **8**, 599-603 (1998).

18. Woolf, A.D. & Pfleger, B. Burden of major musculoskeletal conditions. *Bulletin of the World Health Organization* **81**, 646-656 (2003).

19. American Cancer Society *Cancer facts and figures 2011*. 1-55 (2011).

20. Vernejoul, M.C. & Bénichou, O. Human osteopetrosis and other sclerosing disorders: recent genetic developments. *Calcified Tissue International* **69**, 1-6 (2001).

21. Claes, L., Recknagel, S. & Ignatius, A. Fracture healing under healthy and inflammatory conditions. *Nature reviews. Rheumatology* (2012).doi:10.1038/nrrheum.2012.1

22. Claes, L.E. & Ito, K. Biomechanics of fracture fixation and fracture healing. *Basic orthopaedic biomechanics and mechano-biology* 563-584 (2005).

23. Duan, X., Li, T., Mohammed, A.Q. & Xiang, Z. Reamed intramedullary nailing versus unreamed intramedullary nailing for shaft fracture of femur: a systematic literature review. *Archives of Orthopaedic and Trauma Surgery* **131**, 1445-1452 (2011).

24. Perren, S.M. Evolution of the internal fixation of long bone fractures. *The Journal of Bone & Joint Surgery* **84-B**, 1093-1110 (2002).

25. Uhthoff, H.K., Goto, S. & Cerckel, P.H. Influence of stable fixation on trabecular bone healing: a morphologic assessment in dogs. *Journal of Orthopaedic Research* **5**, 14-22 (1897).

26. Bergstrom, J.D., Bostedor, R.G., Masarachia, P.J., Reszka, A.A. & Rodan, G. Alendronate is a specific, nanomolar inhibitor of farnesyl diphosphate synthase. *Archives of Biochemistry and Biophysics* **373**, 231-241 (2000).

27. D'Amelio, P. *et al.* Estrogen deficiency increases osteoclastogenesis up-regulating T cells activity: a key mechanism in osteoporosis. *Bone* **43**, 92-100 (2008).

28. Mimura, H. & Fukuyo, S. Orthodontic reconstruction with autotransplantation and bone grafting after a traffic accident. *American Journal of Orthodontics and Dentofacial Orthopedics* **141**, S119-S129 (2012).

29. Van Isacker, T. *et al.* Forearm reconstruction with bone allograft following tumor excision: a series of 10 patients with a mean follow-up of 10 years. *Orthopaedics & Traumatology: Surgery & Research* **97**, 793-799 (2011).

30. Shegarfi, H. & Reikeras, O. Bone transplantation and immune response. *Journal of Orthopaedic Surgery* **17**, 206-211 (2009).

31. Yilmaz, S., Kabadayi, C., Ipci, S.D., Cakar, G. & Kuru, B. Treatment of intrabony periodontal defects with platelet-rich plasma versus platelet-poor plasma combined

with a bovine-derived xenograft: a controlled clinical trial. *Journal of Periodontology* **82**, 837-844 (2011).

32. Peres, J.A. & Lamano, T. Strategies for stimulation of new bone formation: a critical review. *Brazilian Dental Journal* **22**, 443-448 (2011).

33. Claes, L.E. & Heigele, C.A. Magnitudes of local stress and strain along bony surfaces predict the course and type of fracture healing. *Journal of Biomechanics* **32**, 255-266 (1999).

34. Babis, G.C. & Soucacos, P.N. Bone scaffolds: the role of mechanical stability and instrumentation. *Injury* **36**, S38-S44 (2005).

35. De Long, W.G. *et al.* Bone grafts and bone graft substitutes in orthopaedic trauma surgery. A critical analysis. *The Journal of Bone and Joint Surgery* **89**, 649-658 (2007).

36. Einhorn, T.A., Lane, J.M., Burstein, A.H., Kopman, C.R. & Vigorita, V.J. The healing of segmental bone defects induced by demineralized bone matrix. A radiographic and biomechanical study. *Journal of Bone and Joint Surgery* **66**, 274-279 (1984).

37. Michelson, J.D. & Curl, L.A. Use of demineralized bone matrix in hindfoot arthrodesis. *Clinical Orthopaedics and Related Research* **325**, 203-208 (1996).

38. Arthur, A., Zannettino, A. & Gronthos, S. The therapeutic applications of multipotential mesenchymal/stromal stem cells in skeletal tissue repair. *Journal of Cellular Physiology* **218**, 237-45 (2009).

39. Kastenberg, Z.J. & Odorico, J.S. Alternative sources of pluripotency: science, ethics, and stem cells. *Transplantation Reviews* **22**, 215-222 (2008).

40. Robinton, D.A. & Daley, G.Q. The promise of induced pluripotent stem cells in research and therapy. *Nature* **481**, 295-305 (2012).

41. Allen, T.D. & Dexter, T.M. Long term bone marrow cultures: an ultrastructural review. *Scanning Electron Microscopy* **4**, 1851-1866 (1983).

42. Zaidi, N. & Nixon, A.J. Stem cell therapy in bone repair and regeneration. *Annals of the New York Academy of Sciences* **1117**, 62-72 (2007).

43. Jung, Y. *et al.* Hematopoietic stem cells regulate mesenchymal stromal cell induction into osteoblasts thereby participating in the formation of the stem cell niche. *Stem cells* **26**, 2042-2051 (2008).

44. Greenwald, A.S. *et al. Bone-graft substitutes: facts, fictions & applications*. (Chicago, 2006).

45. Pittenger, M.F. Multilineage Potential of Adult Human Mesenchymal Stem Cells. *Science* **284**, 143-147 (1999).

46. Kern, S., Eichler, H., Stoeve, J., Kluter, H. & Bieback, K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood or adipose tissue. *Stem Cells* **24**, 1294-1301 (2006).

47. Hunt, C.J. Cryopreservation of human stem cells for clinical application: a review. *Transfusion Medicine and Hemotherapy* **38**, 107-123 (2011).

48. Friedenstein, A.J., Gorskaja, J.F. & Kulagina, N.N. Fibroblast precursors in normal and irradiated mouse hematopoietic organs. *Experimental Hematology* **4**, 267-274 (1976).

49. Shi, C. Recent progress toward understanding the physiological function of bone marrow mesenchymal stem cells. *Immunology* **136**, 133-138 (2012).

50. Singer, N.G. & Caplan, A.I. Mesenchymal stem cells: mechanisms of inflammation. *Annual Review of Pathology - Mechanisms of Disease* **6**, 457-478 (2011).

51. Karp, J.M. & Leng Teo, G.S. Mesenchymal stem cell homing: the devil is in the details. *Cell stem cell* **4**, 206-16 (2009).

52. Vemuri, M., Chase, L. & Rao, M. *Mesenchymal stem cells and applications*. (Humana Press Inc.: 2011).

53. Tu, Z., Li, Q., Bu, H. & Lin, F. Mesenchymal stem cells inhibit complement activation by secreting factor H. *Stem Cells and Development* **19**, 1803-1809 (2010).

54. Sotiropoulou, P.A., Perez, S.A., Gritzapis, A.D., Baxevanis, C.N. & Papamichail, M. Interactions between human mesenchymal stem cells and the natural killer cells. *Stem Cells* **24**, 74-85 (2006).

55. Nauta, A.J., Kruisselbrink, A.B., Lurvink, E., Willemze, R. & Fibbe, W.E. Mesenchymal stem cells inhibit generation and function of both CD34+-derived and monocyte-derived dendritic cells. *Journal of Immunology* **177**, 2080-2087 (2006).

56. Zhang, W. *et al.* Effects of mesnchymal stem cells on differentiation, maturation, and function of human monocyte-derived dendritic cells. *Stem Cells and Development* **13**, 263-271 (2004).

57. Groh, M.E., Maitra, B., Szekely, E. & Koç, O.N. Human mesenchymal stem cells require monocyte-mediated activation to suppress alloreactive T cells. *Experimental Hematology* **33**, 928-934 (2005).

58. Selmani, Z. *et al.* Human leukocyte antigen-G5 secretion by human mesenchymal stem cells is required to suppress T lymphocyte and natural killer function and to induce CD4+CD25highFOXP3+ regulatory T cells. *Stem Cells* **26**, 212-222 (2008).

59. Vignali, D.A., Collison, L.W. & Workman, C.J. How regulatory T cells work. *Nature reviews. Immunology* **8**, 523-532 (2008).

60. Corcione, A. *et al.* Human mesenchymal stem cells modulate B-cell functions. *Blood* **107**, 367-372 (2006).

61. Meirelles, L., Fontes, A.M., Covas, D.T. & Caplan, A.I. Mechanisms involved in the therapeutic properties of mesenchymal stem cells. *Cytokine & Growth Factor Reviews* **20**, 419-427 (2009).

62. Waterman, R.S., Tomchuck, S.L., Henkle, S.L. & Betancourt, A.M. A new mesenchymal stem cell (MSC) paradigm: polarization into a pro-inflammatory MSC1 or an Immunosuppressive MSC2 phenotype. *PloS one* **5**, e10088 (2010).

63. Gebler, A., Zabel, O. & Seliger, B. The immunomodulatory capacity of mesenchymal stem cells. *Trends in Molecular Medicine* **18**, 128-134 (2012).

64. Ghannam, S., Bouffi, C., Djouad, F., Jorgensen, C. & Noël, D. Immunosuppression by mesenchymal stem cells: mechanisms and clinical applications. *Stem cell research & therapy* **1**, 1-7 (2010).

65. Fernandez, M. *et al.* Detection of stromal cells in peripheral blood progenitor cell collections from breast cancer patients. *Transplantation* **20**, 265-271 (1997).

66. Mansilla, E. *et al.* Blood stream cells phenotypically identical to human mesenchymal bone marrow stem cells circulate in large amounts under the influence of acute large skin damage: new evidence for their use in regenerative medicine. *Transplantation Proceedings* **38**, 967-969 (2006).

67. Lazarus, H.M., Haynesworth, S.E., Gerson, S.L. & Caplan, A.I. Human bone marrow-derived mesenchymal (stromal) progenitor cells (MPCs) cannot be recovered from peripheral blood progenitor cell collections. *Journal of Hematotherapy* **6**, 447-455 (1997).

68. Yagi, H. *et al.* Mesenchymal stem cells: Mechanisms of immunomodulation and homing. *Cell Transplantation* **19**, 667-679 (2010).

69. Ugarte, D.A. *et al.* Differential expression of stem cell mobilization-associated molecules on multi-lineage cells from adipose tissue and bone marrow. *Immunology Letters* **89**, 267-270 (2003).

70. Valenick, L.V., Hsia, H.C. & Schwarzbauer, J.E. Fibronectin fragmentation promotes alpha4beta1 integrin-mediated contraction of a fibrin-fibronectin provisional matrix. *Experimental Cell Research* **309**, 48-55 (2005).

71. Garmy-Susini, B. *et al.* Integrin alpha4beta1-VCAM-1-mediated adhesion between endothelial and mural cells is required for blood vessel maturation. *Journal of Clinical Investigation* **115**, 1542-1551 (2005).

72. Ruster, B. *et al.* Mesenchymal stem cells display coordinated rolling and adhesion behavior on endothelial cells. *Blood* **108**, 3938-3944 (2006).

73. De Becker, A. *et al.* Migration of culture-expanded human mesenchymal stem cells through bone marrow endothelium is regulated by matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-3. *Haematologica* **92**, 440-449 (2007).

74. Wynn, R.F. *et al.* A small proportion of mesenchymal stem cells strongly expresses functionally active CXCR4 receptor capable of promoting migration to bone marrow. *Blood* **104**, 2643-2645 (2004).

75. Honczarenko, M. *et al.* Human bone marrow stromal cells express a distinct set of biologically functional chemokine receptors. *Stem Cells* **24**, 1030-1041 (2006).

76. Zhang, A. *et al.* Mechanism of TNF-α-induced migration and hepatocyte growth factor production in human mesenchymal stem cells. *Journal of Cellular Biochemistry* **111**, 469-475 (2010).

77. Ponte, A.L. *et al.* The in vitro migration capacity of human bone marrow mesenchymal stem cells: comparison of chemokine and growth factor chemotactic activities. *Stem cells* **25**, 1737-45 (2007).

78. Almeida, C.R., Vasconcelos, D.P., Gonçalves, R.M. & Barbosa, M.A.- Enhanced mesenchymal stromal cell recruitment via natural killer cells by incorporation of inflammatory signals in biomaterials. *Journal of the Royal Society, Interface* **9**, 261-271 (2012).

79. Anton, K., Banerjee, D. & Glod, J. Macrophage-associated mesenchymal stem cells assume an activated, migratory, pro-inflammatory phenotype with increased IL-6 and CXCL10 secretion. *PlosOne* **7**, e35036 (2012).

80. Le Blanc, K. *et al.* Fetal mesenchymal stem-cell engraftment in bone after in utero transplantation in a patient with severe osteogenesis imperfecta. *Transplantation* **79**, 1607-1614 (2005).

81. Hamajima, S., Hayashi, T., Sato, Y., Sasaki, K. & Kawai, T. Osteogenesis after transplantation of bone marrow-derived mesenchymal stem cells using polyvinylidene chloride film as a scaffold. *Dental Materials Journal* **30**, 707-716 (2011).

82. Endres, S. *et al.* Angiogenesis and healing with non-shrinking, fast degradeable PLGA/CaP scaffolds in critical-sized defects in the rabbit femur with or without osteogenically induced mesenchymal stem cells. *Clinical Hemorheology and microcirculation* **48**, 29-40 (2011).

83. Bruder, S., Kraus, K., Goldberg, V. & Kadiyala, S. The effect of implants loaded with autologous mesenchymal stem cells on the healing of canine segmental bone defects. *The Journal of Bone & Joint Surgery* **80**, 985-996 (1998).

84. Field, J.R. *et al.* The efficacy of allogeneic mesenchymal precursor cells for the repair of an ovine tibial segmental defect. *Veterinary and Comparative Orthopaedics and Traumatology* **24**, 113-121 (2011).

85. Marcacci, M. *et al.* Stem cells associated with macroporous bioceramics for long bone repair: 6- to 7-year outcome of a pilot clinical study. *Tissue Engineering* **13**, 947-955 (2007).

86. Zachos, T., Diggs, A., Weisbrode, S., Bartlett, J. & Bertone, A. Mesenchymal stem cell-mediated gene delivery of bone morphogenetic protein-2 in an articular fracture model. *Molecular Therapy* **15**, 1543-1550 (2007).

87. Tolar, J. *et al.* Sarcoma derived from cultured mesenchymal stem cells. *Stem Cells2* **25**, 371-379 (7AD).

88. Mercier, F.E., Ragu, C. & Scadden, D.T. The bone marrow at the crossroads of blood and immunity. *Nature reviews. Immunology* **12**, 49-60 (2012).

89. Sfeir, C., Ho, L., Doll, B.A., Azari, K. & Hollinger, J.O. Fracture Repair. *Bone Regeneration and Repair: Biology and Clinical Applications* 21-44 (2005).

90. Weitzmann, M.N. *et al.* T cell activation induces human osteoclast formation via receptor activator of nuclear factor kappaB ligand-dependent and -independent mechanisms. *Journal of Bone and Mineral Research* **16**, 328-337 (2001).

91. Roato, I. *et al.* Bone impairment in phenylketonuria is characterized by circulating osteoclast precursors and activated T cell increase. *PloS One* **5**, 1-9 (2010).

92. Takayanagi, H. *et al.* T-cell-mediated regulation of osteoclastogenesis by signalling cross-talk between RANKL and IFN-gamma. *Nature* **408**, 600-605 (2000).

93. Rifas, L., Arackal, S. & Weitzmann, M.N. Inflammatory T cells rapidly induce differentiation of human bone marrow stromal cells into mature osteoblasts. *Journal of cellular biochemistry* **88**, 650-9 (2003).

94. Manabe, N. *et al.* Connection between B lymphocyte and osteoclast differentiation pathways. *The Journal of Immunology* **167**, 2625-2631 (2001).

95. Li, Y. *et al.* B cells and T cells are critical for the preservation of bone homeostasis and attainment of peak bone mass in vivo. *Blood* **109**, 3839-3848 (2007).

96. Chang, M.K. *et al.* Osteal tissue macrophages are intercalated throughout human and mouse bone lining tissues and regulate osteoblast function in vitro an in vivo. *The Journal of Immunology* **181**, 1232-1244 (2008).

97. Pirraco, R.P., Reis, R.L. & Marques, A.P. Effect of monocytes/macrophages on the early osteogenic differentiation of hBMSCs. *Journal of Tissue Engineering and Regenerative Medicine* **DOI:10.100**, (2012).

98. Söderström, K. *et al.* Natural killer cells trigger osteoclastogenesis and bone destruction in arthritis. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 13028-13033 (2010).

99. Boursinos, L.A., Karachalios, T., Poultsides, L. & Malizos, K.N. Do steroids, conventional non-steroidal anti-inflammatory drugs and selective Cox-2 inhibitors adversely affect fracture healing? *Journal of Musculoskeletal & Neuronal Interactions* **9**, 44-52 (2009).

100. Alnaeeli, M. & Teng, Y.T. Dendritic cells: a new player in osteoimmunology. *Current Molecular Medicine* **9**, 1875-5666 (2009).

101. Thomas, R. *et al.* Dendritic cells and the pathogenesis of rheumatoid arthritis. *Journal of Leukocyte Biology* **66**, 286-292 (1999).

102. Dhaenens, M. *et al.* Dendritic cells from spondylarthritis-prone HLA-B27-transgenic rats display altered cytoskeletal dynamics, class II major histocompatibility complex expression, and viability. *Arthritis and Rheumatism* **60**, 2622-2632 (2009).

103. Griffin, M.D., Ritter, T. & Mahon, B.P. Immunological Aspects of Allogeneic Mesenchymal Stem Cell Therapies. *Human Gene Therapy* **21**, 1641-1655 (2010).

104. Babensee, J.E. Interaction of dendritic cells with biomaterials. *Seminars in Immunology* **20**, 101-108 (2008).

105. Speziani, C. *et al.* Murine dendritic cell transdifferentiation into osteoclasts is differentially regulated by innate and adaptive cytokines. *European Journal of Immunology* **37**, 747-757 (2007).

106. Grassi, F. *et al.* Oxidative stress causes bone loss in estrogen-deficient mice through enhanced bone marrow dendritic cell activation. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 15087-15092 (2007).

107. Mensah, K.A. *et al.* Nonerosive arthritis in lupus is mediated by IFN-α stimulated monocyte differentiation that is nonpermissive of osteoclastogenesis. *Arthritis Rheumatoid* **62**, 1127-1137 (2011).

108. Pickl, W.F. *et al.* Molecular and functional characteristics of dendritic cells generated from highly purified CD14+ peripheral blood monocytes. *The Journal of Immunology* **157**, 3850-3859 (1996).

109. STEMCELL tecnhologies Humane Monocyte Enrichment Cocktail. at <http://www.stemcell.com/~/media/Technical Resources/2/0/28435_rosetteSep.ashx>

110. Dominici, M. *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* **8**, 315-317 (2006).

111. Klein, T. & Bischoff, R. Physiology and pathophysiology of matrix metalloproteases. *Amino acids* **41**, 271-90 (2011).

112. Hoemann, C.D., El-Gabalawy, H. & McKee, M.D. In vitro osteogenesis assays: influence of the primary cell source on alkaline phosphatase activity and mineralization. *Pathologie Biologie* **57**, 318-323 (2009).

113. Mesenchymal stem cell assays and applications. *Mesenchymal stem cell assays and applications* (2011).

114. Sallusto, F. & Lanzavecchia, A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *The Journal of Experimental Medecine* **179**, 1109-1118 (1994).

115. Palucka, K. a, Taquet, N., Sanchez-Chapuis, F. & Gluckman, J.C. Dendritic cells as the terminal stage of monocyte differentiation. *Journal of Immunology* **160**, 4587-4595 (1998).

116. Brigl, M. & Brenner, M.B. CD1: antigen presentation and T cell function. *Annual Review of Imunology* **22**, 817-890 (2004).

117. Kou, P.M. & Babensee, J.E. Macrophage and dendritic cell phenotypic diversity in the context of biomaterials. *Journal of Biomedical Materials Research Part A* **96**, 239-260 (2011).

118. Kleinman, H.K. & Martin, G.R. Matrigel: Basement membrane matrix with biological activity. *Seminars in Cancer Biology* **15**, 378-386 (2005).

119. Saleh, F.A., Whyte, M. & Genever, P.G. Effects of endothelial cells on human mesenchymal stem cells activity in a three-dimensional in vitro model. *European Cells and Materials* **22**, 242-257 (2011).

120. Decaestecker, C., Debeir, O., Van Ham, P. & Kiss, R. Can anti-migratory drugs be screened in vitro? A review of 2D and 3D assays for the quantitative analysis of cell migration. *Medicinal Research Reviews* **27**, 149-176 (2007).

121. de Filippis, L.G. *et al.* Rheumatic symptoms in patients with human immunodeficiency virus are related to levels of tumor necrosis factor-alpha but not to viral load. *International Journal of Tissue Reactions* **27**, 9-13 (2005).

122. Thalayasingam, N. & Isaacs, J.D. Anti-TNF therapy. *Best Practice and Research. Clinical Rheumatology* **25**, 549-567 (2011).

123. Glatigny, S. *et al.* Proinflammatory Th17 cells are expanded and induced by dendritic cells in spondylarthritis-prone HLA–B27–transgenic rats. *Arthritis and Rheumatism* **64**, 110-120 (2012).

124. Gehm, B.D., McAndrews, J.M., Chien, P. & Jameson, J.L. Resveratrol, a polyphenolic compound found in grapes and wine, is an agonist for the estrogen receptor. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 14138-14143 (1997).

125. Sordi, V. *et al.* Bone marrow mesenchymal stem cells express a restricted set of functionally active chemokine receptors capable of promoting migration to pancreatic islets. *Blood* **106**, 419-427 (2005).

126. Singh, M., Berkland, C. & Detamore, M.S. Strategies and applications for incorporating physical and chemical signal gradients in tissue engineering. *Tissue Engineering. Part B. Reviews* **14**, 341-366 (2008).

127. Nagase, H. Activation mechanisms of matrix metalloproteinases. *Bioilogical Chemistry* **378**, 151-160 (1997).

128. Tsai, L.-K., Leng, Y., Wang, Z., Leeds, P. & Chuang, D.-M. The mood stabilizers valproic acid and lithium enhance mesenchymal stem cell migration via distinct mechanisms. *Neuropsychopharmacology* **35**, 2225-2237 (2010).

129. Visse, R. & Nagase, H. Matrix Metalloproteinases and Tissue Inhibitors of Metalloproteinases. *Circulation Research* **92**, 827-839 (2003).

130. Fu, X. *et al.* Migration of bone marrow-derived mesenchymal stem cells induced by tumor necrosis factor-alpha and its possible role in wound healing. *Wound Repair and Regeneration* **17**, 185-191 (2009).

131. Kim, S.M. *et al.* CXC chemokine receptor 1 enhances the ability of human umbilical cord blood-derived mesenchymal stem cells to migrate toward gliomas. *Biochemical and Biophysical Research Communications* **407**, 741-746 (2011).

132. Li, Y. *et al.* Human mesenchymal stem cells license adult CD34+ hemopoietic progenitor cells to differentiate into regulatory dendritic cells through activation of the Notch pathway. *Journal of Immunology* **180**, 1598-1608 (2008).

133. Hayden, M.S. & Ghosh, S. NF-κB in immunobiology. *Cell research* **21**, 223-244 (2011).

134. Brasier, A.R. The NF-κB Regulatory Network. *cardiovascular Toxicology* **6**, 111-130 (2006).

135. Shakibaei, M. *et al.* Resveratrol mediated modulation of Sirt-1/Runx2 promotes osteogenic differentiation of mesenchymal stem cells: potential role of Runx2 deacetylation. *PloS one* **7**, e35712 (2012).

136. Pant, S., Hilton, H. & Burczynski, M.E. The multifaceted exosome: biogenesis, role in normal and aberrant cellular function, and frontiers for pharmacological and biomarker opportunities. *Biochemical pharmacology* **83**, 1484-1494 (2012).

7. Annexes

Phenotypic characterization of DC

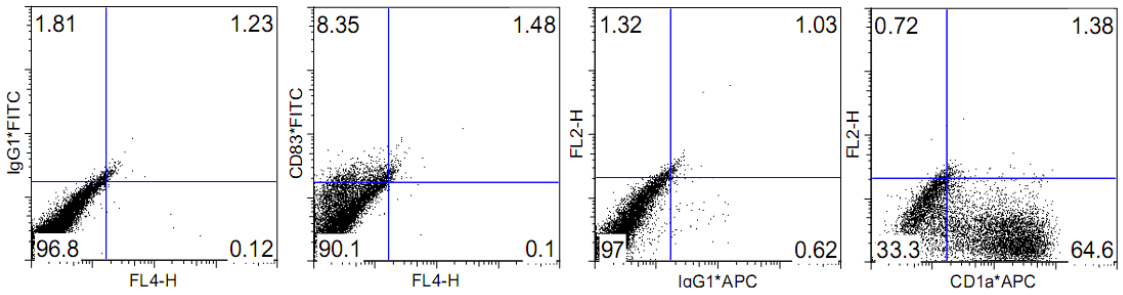


Figure 26. Control samples from the assay for DC phenotype characterization. Samples were prepared as described and single stains for CD1a, CD83 and appropriate isotypes were performed.

Metalloproteinases activity on supernatants from DC cultures

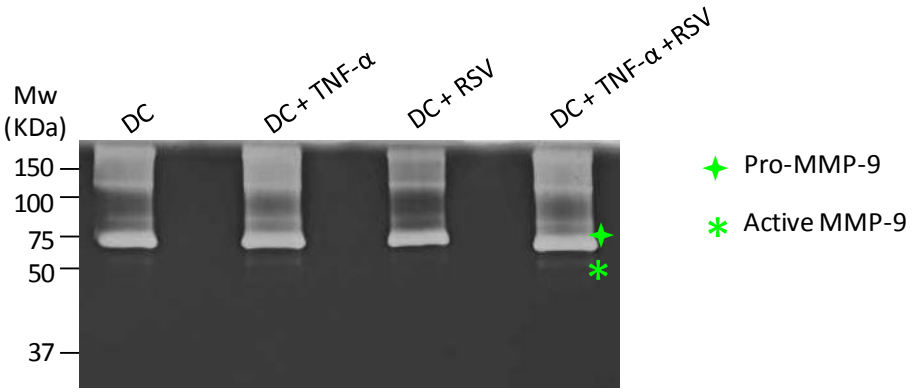


Figure 27. Zymograms of protein samples precipitated from supernatants of DC cultured alone. DC were cultured in the same conditions as for transwell assays, being subjected to the same stimuli. Culture medium was recovered and total protein precipitated and quantified as described. Samples were prepared and loaded on gelatine gels, which were then processed as described before.

β-casein zymograms of supernatants from transwell assays

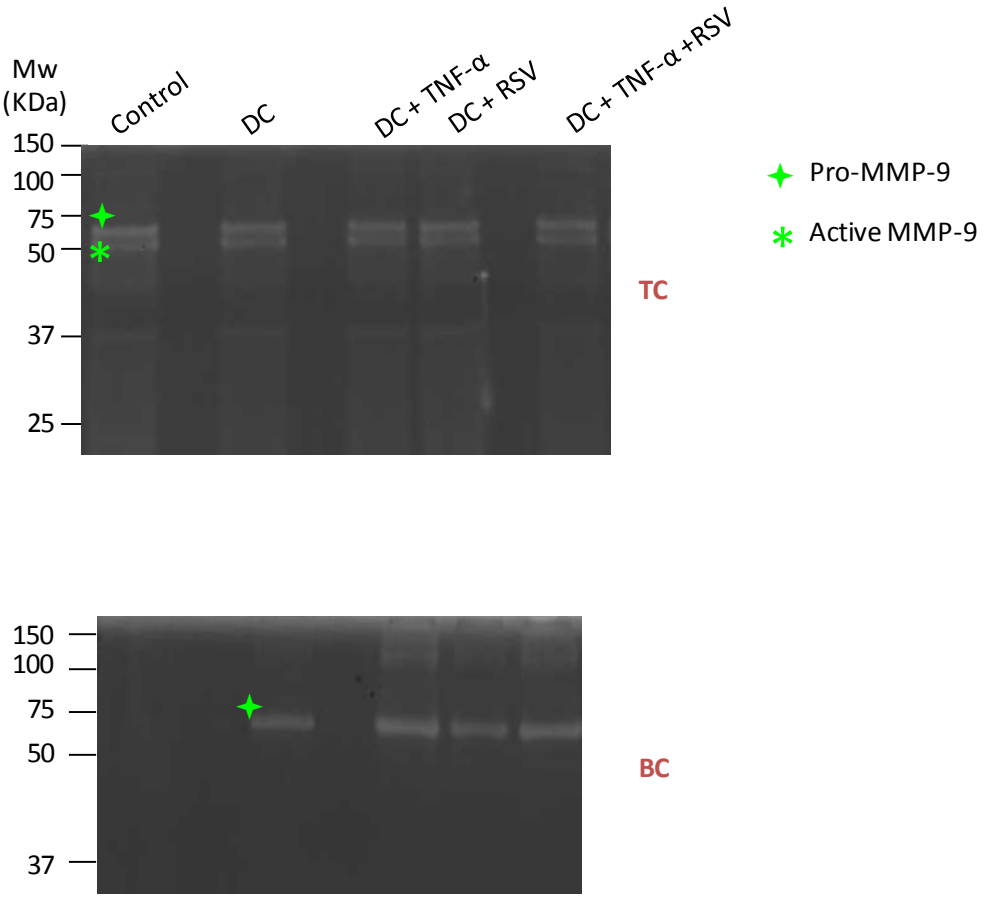


Figure 28. β-casein zymograms of protein samples precipitated from the culture medium of transwell assays. Culture medium from experiments where DC were stimulated with TNF-α, RSV or both, was recovered from TC and BC. Total protein was precipitated and quantified as described. Samples were prepared as for gelatine zymographies and loaded into gels of the same composition as before, except for gelatine that was replaced by β-casein (Sigma-Aldrich) at the same concentration. Gels were run and treated as before and MMP activity was detected by incubating in MMP buffer with the following composition: NaCl 200 mM, CaCl2 5 mM, Triton X-100 1% in Tris buffer 50 mM, pH 7.4. Control conditions refers to BC with SFM.

Validation of ImageStream^X analysis of NF-kB nuclear translocation

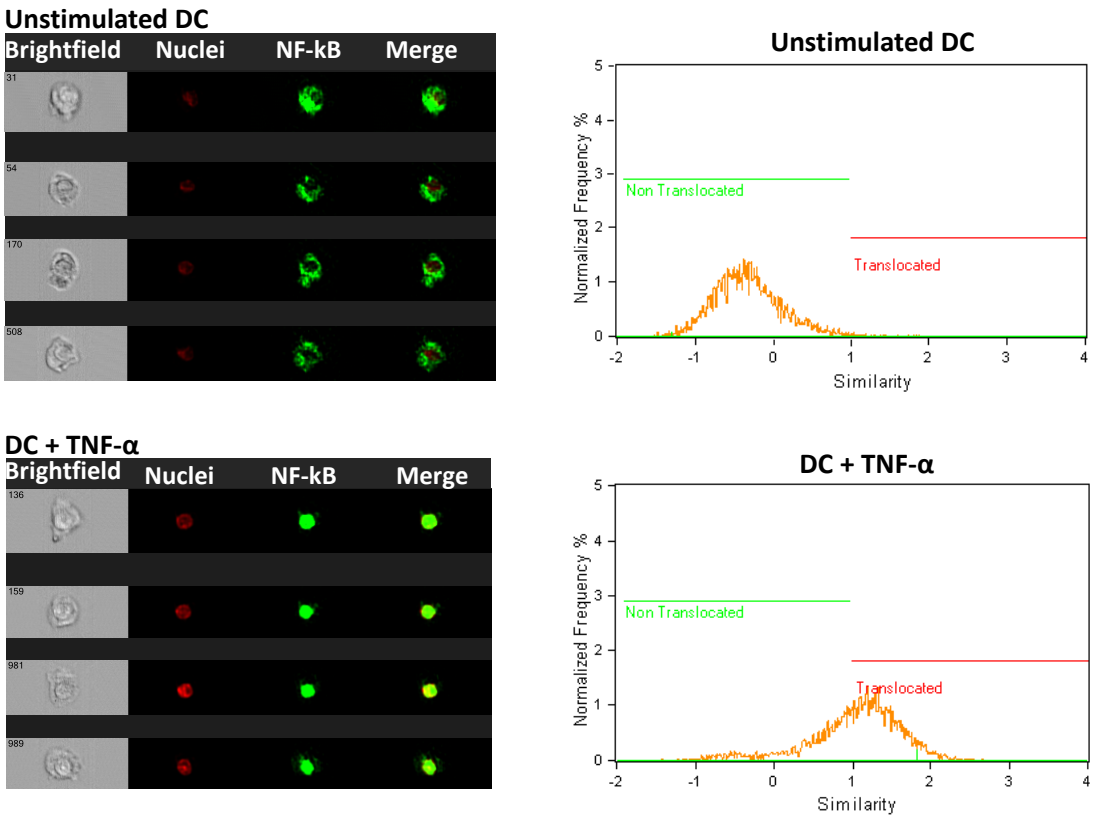


Figure 29. Assessment of NF-kB signalling pathway activation in DC by imaging flow cytometry. DC were stimulated with TNF-α 100 ng/mL for 0, 15, 30 and 60 min and then immunostained for NF-kB. Briefly, fixed cells were incubated in 0.1% Triton X-100 in PBS 1x/FBS 2% with monoclonal anti-human p65 NF-kB subunit primary antibody in a ratio 1:5000 for 20 min. DC were then incubated with anti-mouse FITC-labelled IgG secondary antibody in PBS 1x/2% FBS for 20 min. Cell nuclei were stained for 5 min with DRAQ5 20 µM. Controls were made mixing inactivated and activated DC with no antibody staining and NF-kB, secondary antibody alone or DRAQ5 single stains. Samples were then analyzed on ImageStream^X, acquiring at least 10000 events. The data obtained was analysed using the nuclear localization wizard of the IDEAS software.

Left panel: Brightfield and fluorescence snapshots obtained for each event that is detect by the ImageStream^X. DC stimulated for 30 min are depicted. Merging the images obtained in the IDEAS software it is possible to detect NF-kB translocation to cell nucleus by co-localization with the DRAQ5-stained red nucleus. **Right panel:** To determine the population percentage where NF-kB was activated, the similarity score for red and green colour distribution in the cell area was calculated using the IDEAS software. More than 50% of the analysed population was activated for NF-kB signalling when stimulated with TNF-α for 30 min.

ALP detection of MSC subjected to different stimuli

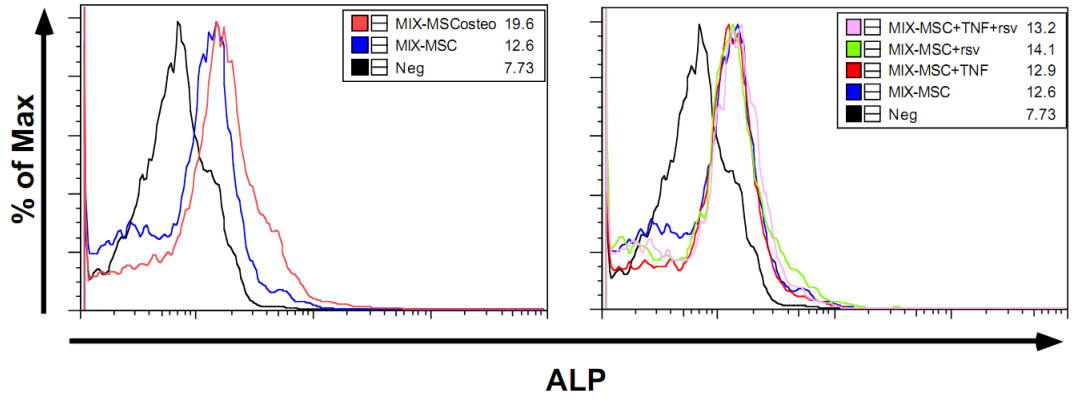


Figure 30. Effect of pro-inflammatory and anti-inflammatory stimuli on MSC osteogenic differentiation. MSC were cultured in MSC medium supplemented with TNF-α 100 ng/mL, RSV 10 µM or both for 7 days. MSC cultured in MSC medium and osteogenic medium (see section 2.1.1.) were used as controls. At the end of the assay, MSC were trypsinized for 15 min and immunostained for flow cytometry analysis. Briefly, MSC were incubated for 30 min, at 4°C, in FACS buffer containing monoclonal anti-human antibodies as follows: FITC-labelled CD45 and APC-labelled ALP (both at 2µL in 50 µL). Controls were made with no stain and single stains for CD45, ALP and the appropriate IgG isotypes. After, cells were washed and analysed in a Flow Cytometer FACSCalibur, acquiring ~10000 events.

This work was financed by FEDER funds through the Programa Operacional Factores de Competitividade – COMPETE and by Portuguese funds through FCT – Fundação para a Ciência e a Tecnologia in the framework of the project PEst-C/SAU/LA0002/2011.