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Decoding the role of inflammatory transcripts and microRNAs
in macrophage polarization for bone repair

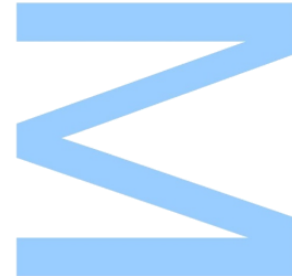
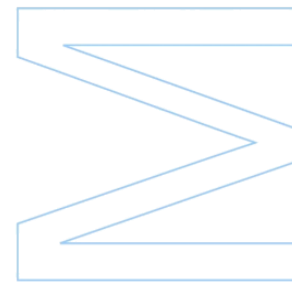
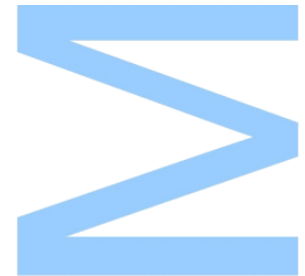
Maria Rodrigues Lopes



Decoding the role of inflammatory transcripts and microRNAs in macrophage polarization for bone repair

Maria Rodrigues Lopes

Dissertação de Mestrado apresentada à
Faculdade de Ciências da Universidade do Porto, Instituto de
Ciências Biomédicas Abel Salazar em
Bioquímica
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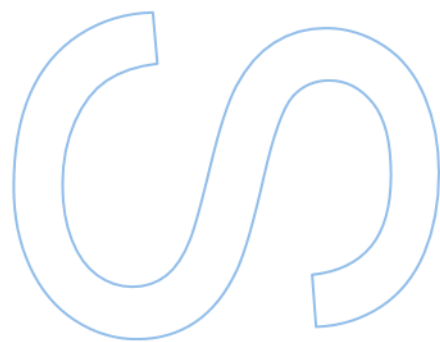
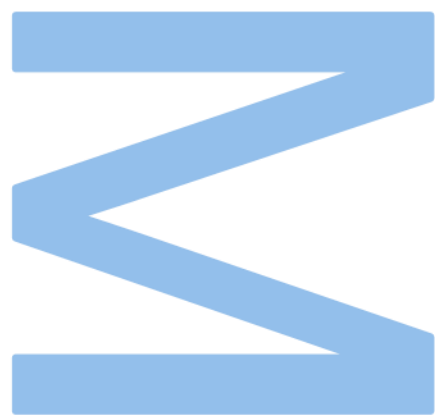
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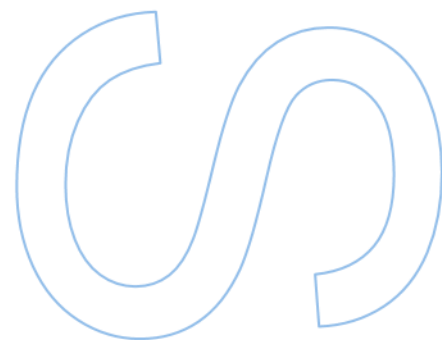
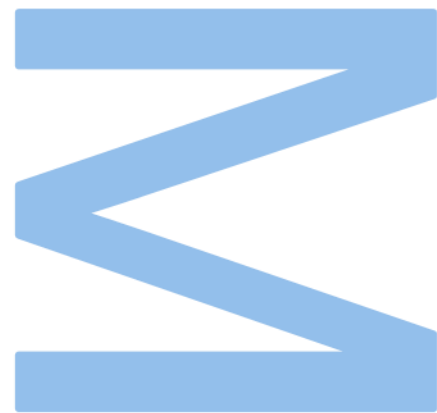


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Não sou nada
Nunca serei nada
Não posso querer ser nada
À parte isso, tenho em mim todos os
sonhos do mundo

In Tabacaria
Álvaro de Campos, 1928

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Resumo

As fraturas ósseas são um problema global de saúde pública. A regeneração óssea após uma fratura é um processo biológico e biomecânico regulado, compreendendo três fases: a fase inflamatória, a fase de reparação e a fase de remodelação. As células inflamatórias, como os macrófagos, migram para a fratura durante a fase inflamatória e têm um papel crucial no processo de regeneração óssea. Assim, a modulação do fenótipo dos macrófagos para promover a regeneração óssea é uma perspectiva terapêutica estimulante. Os microRNAs (miRNAs) são uma classe de pequenos RNAs não-codificantes (sncRNAs) que atuam como reguladores pós-transcricionais da expressão génica e que influenciam funções celulares chave, incluindo a polarização de macrófagos. Assim, os miRNAs podem modular a resposta inflamatória, alterando os níveis de expressão de citocinas, dos recetores de superfície e de outras moléculas relacionadas com a inflamação, o que pode ter consequências sobre o fenótipo dos macrófagos.

O objetivo desta tese é explorar o papel de mediadores inflamatórios, incluindo miRNAs, em macrófagos estimulados pelo tumor necrosis factor alpha (TNF- α) e abordar o impacto do seu secretoma no recrutamento de células estaminais mesenquimais da medula óssea (BM-MSCs).

Para atingir este objetivo, os macrófagos diferenciados de monócitos primários foram estimulados com lipopolissacarídeo (LPS) ou TNF- α e interleucina (IL)-4, para induzir diferentes respostas. Os níveis de expressão de TNF- α , IL-1 β e TGF- β foram avaliadas por PCR quantitativo em tempo real (RT-qPCR), após estimulação dos macrófagos. Além disso, os níveis de citocinas inflamatórias foram testados nos meios condicionados de macrófagos estimulados com TNF- α , utilizando a tecnologia de laser de multiplexação. A expressão de miR-16, miR-195, miR-342 e let-7d em macrófagos estimulados com TNF- α , LPS ou IL-4 foi avaliada por RT-qPCR. A capacidade de migração das BM-MSCs induzida pelo meio condicionado de macrófagos tratados com TNF- α foi testada usando ensaios transwell. Finalmente, uma estratégia de transfeção utilizando fosfato de cálcio foi testada para a entrega do miR-195 a macrófagos.

Os resultados demonstram que a estimulação de macrófagos com TNF- α levou a um aumento da expressão de TNF- α e IL-1 β após 6h. O TNF- α induziu os macrófagos a secretarem maiores concentrações de IFN- γ , IL-1 β , IL-10, IL-12p40 e IL-13, quando comparado com o grupo de controlo (macrófagos não estimulados). Embora não

estatisticamente significativo, observámos também um aumento da expressão miR-342 em macrófagos tratados com TNF- α , quando comparados com o grupo de controlo. Por outro lado, a expressão miR-195 está significativamente diminuída em macrófagos tratados com TNF- α em comparação com o grupo de controlo. Os ensaios de transwell revelam que o secretoma dos macrófagos estimulados com TNF- α não altera a migração das MSCs. Finalmente, os resultados da transfeção mostram que o protocolo selecionado é eficiente para a entrega de miRNAs, levando à sobre-expressão do miR-195 nos macrófagos transfetados.

Em conclusão, os resultados desta tese apoiam a hipótese de que os miRNAs medeiam o perfil inflamatório dos macrófagos. Esta tese contribui também para a avaliação dos sistemas de entrega de miRNAs com o objetivo de promover a regeneração óssea, através da modulação da inflamação.

Palavras-chave: RNA não codificante, microRNAs, inflamação, células estaminais mesenquimais

Abstract

Bone fractures are a global public health issue. Bone healing after an injury is a regulated biological and biomechanical process, comprising three phases: the inflammatory phase, the repair phase, and the remodeling phase. Inflammatory cells, like macrophages, migrate to the fracture during the inflammatory phase and have a crucial role in the process of bone repair. As such, modulating macrophage phenotype to promote bone regeneration is an exciting therapeutic prospect. MicroRNAs (miRNAs) are a class of small noncoding RNAs (sncRNAs) that act as important post-transcriptional regulators of gene expression and influence key cellular functions, including macrophage polarization. Thus, miRNAs can modulate the inflammatory response, by changing expression levels of cytokines, surface receptors and other inflammation-related molecules, which may have consequences on the macrophage phenotype.

The goal of this thesis is to explore the role of inflammatory mediators, including miRNAs, in tumor necrosis factor-alpha (TNF- α) stimulated macrophages and to address the impact of their secretome on the recruitment of bone marrow mesenchymal stem cells (BM-MSC).

To achieve our goal, primary monocyte-derived macrophages were stimulated with lipopolysaccharide (LPS) or TNF- α and interleukin (IL)-4, to induce different responses. Expression levels of TNF- α , IL-1 β and TGF- β were assessed by reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) following macrophage stimulation. Specific inflammatory cytokines in the conditioned media of TNF- α -stimulated macrophages were tested using multiplexing laser bead technology. RT-qPCR was used to evaluate the expression profiles of miR-16, miR-195, miR-342 and let-7d in TNF- α -, LPS- and IL-stimulated macrophages. The migration capacity of mesenchymal stem/stromal cells (MSCs) induced by the conditioned media of TNF- α -treated macrophages was tested using transwell assays. Finally, a transfection strategy based on the calcium phosphate method was tested for the delivery of miR-195.

The results demonstrate that stimulation of macrophages with TNF- α for 6h increased the expression of TNF- α and IL-1 β . The results also show that TNF- α induced macrophages secrete higher levels of Interferon (IFN)- γ , IL-1 β , IL-10, IL-12p40 and IL-13, when compared with the control group (non-stimulated macrophages). Although not statistically significant, we also observed increased miR-342 expression in TNF- α treated macrophages, when compared with the control group. On the other hand, miR-195 expression in macrophages is decreased by TNF- α treatment. Transwell assays reveal

that the secretome of TNF- α -stimulated macrophages does not change MSC migration. Finally, transfection results show that the selected protocol is efficient for miRNA delivery, leading to miR-195 overexpression in transfected macrophages.

In conclusion, the results from this thesis indicate that miRNAs regulate the inflammatory profile of macrophages. This thesis also contributes towards the improvement of miRNA delivery systems aiming to promote bone regeneration, through modulation of inflammation.

Keywords: non-coding RNA, inflammation, mesenchymal stem cells, microRNA delivery

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Abbreviations

A

AAV - Adeno-associated virus
AGO - Argonaute
AMAC-1 - Alternative macrophage activation-associated CC chemokine-1
AT-MSCs - Adipose tissue MSCs

B

BC - Buffy coats
BM-MSCs - Bone marrow mesenchymal stem cells
BMPs - Bone morphogenetic proteins

C

CB-MSCs - Cord blood MSCs
CCL2 - C-C motif chemokine ligand 2
CO₂ - Carbon dioxide
Conc - Concentration

D

DCP - Decapping protein
DGCR8 - DiGeorge syndrome critical region 8
DMEM - Dulbecco's modified eagle's medium

E

ECM - Extracellular matrix
ELISA - Enzyme-linked immunosorbent assay
EMT - Epithelial-to-mesenchymal transition

F

FBS - Fetal bovine serum
FC - Fold-change
FN - Fibronectin

G

GAG - Glycosaminoglycans
GAMs - Gene-activated matrices
GC - Glucocorticoids

H

h - Hour
HA - Hydroxyapatite
hMSC - Human mesenchymal stem cell

I

IC - Immune complexes
IFN - Interferon
IL - Interleukin
IL-1RA - IL-1 receptor antagonist

L

LPS - Lipopolysaccharide

M

MCP-1 - Monocyte chemotactic protein-1
MHC - Major histocompatibility complex
Min - Minute

MIP-1 α/β - Macrophage inflammatory protein-1 alpha and beta

miRNAs - MicroRNAs

MMPs - Metalloproteinases

mRNA - Messenger RNA

MSCs - Mesenchymal stem cells

N

NF- κ B - Nuclear factor- κ B

O

OCN - Osteocalcin

ON - Osteonectin

OPG - Osteoprotegerin

P

P/S - Penicillin/streptomycin

PAMPs - Pathogen associated molecular patterns

PBMCs - Peripheral Blood Mononuclear Cells

PBS - Phosphate-buffered Saline

PEG - Polyethyleneglycol

PEI - Polyethyleneimine

PFA - Paraformaldehyde

PMNs - Polymorphonuclear neutrophils

Pol II - RNA Polymerase II

pre-miRNA - miRNA precursor

pri-miRNAs - Primary miRNAs

R

RANKL - Receptor activator of NF- κ B ligand

RBCs - Red blood cells

RISC - RNA-induced silencing complex

rRNA - Ribosomal RNA

RT - Room temperature

RT-qPCR - Reverse transcription quantitative real-time polymerase chain reaction

S

SCR - Scramble

sncRNAs - Small non-coding RNAs

sRNA - Small RNA

T

TGF - Transforming growth factor

Th - T helper

TLR - Toll-like receptor

TNF - Tumor Necrosis Factor

U

UC-MSCs - Umbilical cord MSCs

V

VEGF - Vascular endothelial growth factor

VLP - Virus-like particle

W

WJ-MSCs - Wharton's Jelly MSCs

Y

YLD - Years lived with disability

1. Introduction

In orthopaedics and related medical fields, an understanding of the mechanisms underlying bone repair and regeneration are required for the treatment of traumatic injuries (fractures and non-unions) [1]. Research on bone repair/regeneration has been centered on osteoblasts and osteoclasts. It has been demonstrated the complex interactions between mesenchymal stem cell to osteoblast lineage cells, and the monocyte to macrophage-osteoclast lineage. However, inflammation has a critical role in the first stages of bone healing, following a fracture. It is now commonly accepted by the scientific community that the communication between inflammatory cells and bone cells is critical for bone formation, repair, and remodeling [2].

1.1 Bone tissue

The skeletal system comprises bone, cartilage, ligament, and other connective tissues. This system is responsible for sustaining the physical structure and it facilitates mobility, protects internal organs such as the heart and lungs, stores and releases fat and minerals, and provides support to blood cells [3]. Adipose tissue is located in yellow bone marrow, and the triglycerides stored in its adipocytes can be released to supply energy to other body tissues [4]. The red bone marrow is where blood cells such as leukocytes, erythrocytes, and platelets are formed (a process known as hematopoiesis) [5, 6]. The adult human skeleton comprises more than 200 bones that vary in size, structure, and composition. All these bones have the common feature that they are not composed of a homogenous material [7]. Typically, bones consist of an exterior layer of compact bone, also identified as cortical bone, and a more porous interior, the trabecular bone. Bone marrow is present within the highly vascularized trabecular bone and in the larger cavities of long bones [8].

Osseous tissue is a type of connective tissue that has a low cell count and high amounts of extracellular matrix (ECM). The matrix is formed by an inorganic and an organic phase [9]. The inorganic portion of the matrix is primarily made up of hydroxyapatite (HA), a mineral formed by calcium and phosphate that gives bones their strength and hardness. The organic portion is mainly composed of type I collagen, and noncollagenous proteins such as osteocalcin (OCN), fibronectin (FN) and osteonectin (ON), and glycosaminoglycans (GAG). Calcium, phosphorus, magnesium and sodium are stored in the bone matrix and released in minute amounts into the bloodstream when needed, promoting mineral homeostasis [10].

1.1.1 Bone cells

Bone tissue contains four types of cells: osteoblasts, osteocytes, osteogenic/osteoprogenitor cells, and osteoclasts (Figure 1) [11].

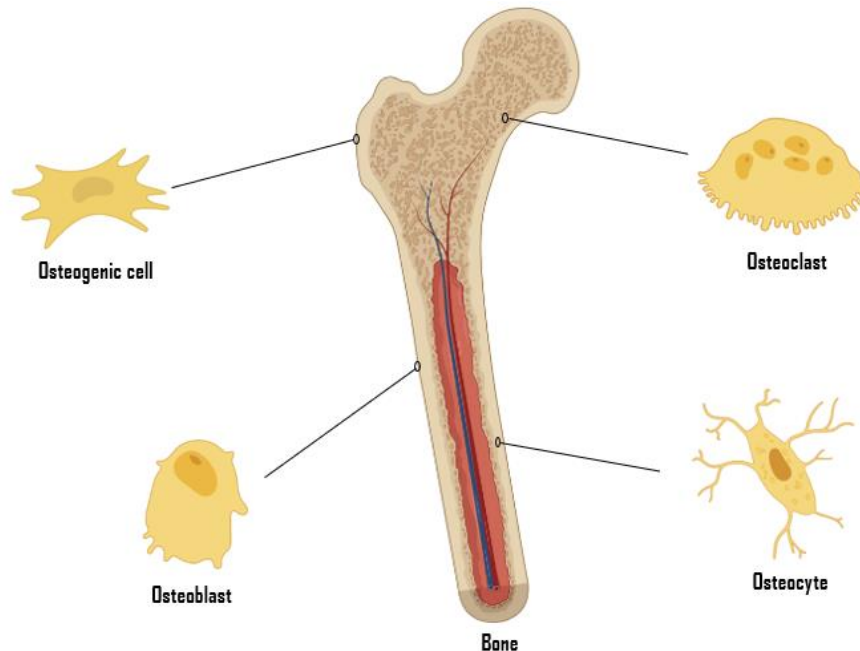


Figure 1. Four distinct types of bone cells: Bone tissue contains four different cell types. Osteoprogenitor cells are immature and differentiate into osteoblasts. When osteoblasts become imprisoned within the calcified matrix, they undergo a structural and functional transformation into osteocytes. Osteoclasts are differentiated from monocytes and contribute to bone resorption.

Osteoblasts are bone-forming cells that derive from mesenchymal stem/stromal cells (MSCs) and are responsible for ECM deposition and mineralization [12]. When confined in bone ECM, osteoblasts can mature into osteocytes [13], which are the most common type of bone cells and the primary cells of adult bone. Osteocytes, like osteoblasts, do not undergo mitosis [14]. Osteoprogenitor cells are the only undifferentiated bone cells and have high mitotic activity. After differentiating, they grow into osteoblasts. Due to the dynamic nature of bone, new tissue is constantly created as old, injured, or useless bone is dissolved for repair or calcium release. The cells responsible for bone resorption are the osteoclasts. Old bone is constantly being reabsorbed by these multinucleated cells, while osteoblasts constantly form new bone. The constant equilibrium between osteoclasts and osteoblasts activity causes the continuous remodeling of bone [15, 16].

1.1.2 Bone structure

Figure 2 presents a simplified representation of the cortical and trabecular bone structures that are found in the human body. In comparison to trabecular bone, cortical bone is more compact and rigid. Cortical bone is organized into osteons, subunits of the osteon system. Every osteon is formed around a Haversian canal, a passageway containing nerves and blood vessels. The osteon comprises multiple layers of compact bone, known as lamella, that are organized concentrically around the Haversian canal. Osteocytes sandwiched in between the lamella, in their own individual lacuna, and communicate with one another via cytoplasmic protrusions that run through channels known as canaliculi. The canaliculus is an essential component of the mechanosensing system, responsible for communication between osteocytes and osteoblasts [7]. Trabecular bone is composed by a disorganized network of fine tissue spicules or trabeculae that are interconnected in irregular patterns. In contrast to the osteon, each of these trabecula is formed by osteocyte-lined lamellae; however, this structure does not contain a Haversian canal and has a more irregular structure [17].

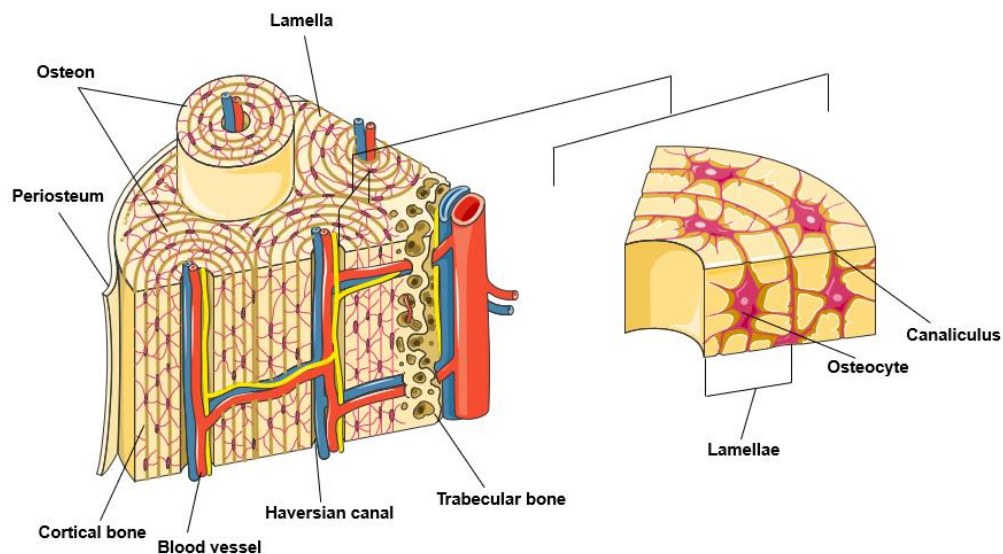


Figure 2. Bone structure.

In addition to this division of bone types, the bone extracellular matrix can also be divided into two different types according to the pattern in which collagen fibers are deposited. Woven bone is characterized by a random organization of collagen fibers, resulting in bone tissue with limited mechanical strength. Osteoblasts are responsible for the production of extracellular matrix, which is then succeeded by lamellar bone as the subsequent type of tissue. In contrast to the woven bone matrix, the collagen fibers in the lamellar extracellular matrix have a high degree of parallel alignment, which results

in the formation of collagen sheets and bone tissue that has a high level of mechanical strength [17].

1.1.3 Bone fractures

Poor diet, inactivity, bone septic illness, tumors, and musculoskeletal diseases such as osteoporosis, rheumatoid arthritis, osteoarthritis, low back pain, and limb trauma all contribute to bone abnormalities. Bone fractures are a global public health problem with considerable economic cost. In 2019, there were an estimated 178 million new fractures worldwide, 445 million prevalent fractures, and 25,8 million cases of years lived with disability (YLDs) [18]. In Portugal, the economic burden of new and prior fractures was €1.0 billion in 2019 [19, 20]. Fractures can result in lost time at work, lower productivity, disability, diminished quality of life, health loss, and increased healthcare expenditures, imposing a considerable financial burden on people, families, societies, and healthcare systems. The incidence and prevalence of bone fractures, as well as YLDs, have all increased in recent years due to population growth and aging. In addition, men are more likely than women to suffer a bone fracture, although this pattern reverses beyond the age of 50, when the female-to-male ratio of significant osteoporotic fractures increases. Fractures are more frequent with advancing age in both sexes [18, 21, 22].

Delayed union or non-union tibial fractures are common orthopedic problems that typically require numerous surgical procedures or bone regeneration replacements (such as bone grafting), resulting in a prolonged treatment period. Fractures that do not heal, as well as their magnitude and severity at certain anatomical locations, contribute to the growth in YLDs and place a higher strain on healthcare systems [18]. Thus, new techniques for reducing the incidence and burden of bone fractures must be implemented to improve the population's well-being.

1.2 Bone injury and regeneration

1.2.1 Bone healing

Bone healing after injury is a complex biological and biomechanical process that can be divided into two types: primary (direct) and secondary (indirect) bone healing [23, 24]. Primary bone healing is a rare occurrence during the healing of a fracture, and it takes place under extremely firm fixation with no displacement of bony fragments [23, 24]. Secondary bone healing, on the other hand, is the most prevalent kind of fracture healing. In general, secondary bone healing is divided into three phases: the inflammatory phase, the repair phase, and the remodeling phase [25].

In secondary bone healing, the inflammatory reaction begins immediately after the bone fracture. Blood vessels rupture inside and around the fracture site as a result of the trauma, resulting in a hematoma. The inflammatory cascade is initiated by inflammatory cells that release inflammatory cytokines such as interleukin (IL)-1, IL-6 and tumor necrosis factor- α (TNF- α), C-C Motif Chemokine Ligand 2 (CCL2), and others which peaks only 24 hours post-fracture [26]. Polymorphonuclear neutrophils (PMNs) are recruited first, followed by monocytes/macrophages infiltrating the fracture site [27]. The process evolves into the resolution of inflammation, which is a complex and well-regulated activity, as PMNs and macrophages clear the area of dead cells. The agents that trigger the inflammatory response and the production of pro-inflammatory mediators are reduced, and immune cells are gradually removed from the tissue during this phase [28]. To induce osteogenesis and angiogenesis, recruited inflammatory cells and bone marrow mesenchymal stem cells (BM-MSCs) engage an important intercellular communication or crosstalk via proinflammatory and anti-inflammatory cytokines, such as transforming growth factor (TGF), bone morphogenetic proteins (BMPs), and growth factors [29]. This process may also result in the production of a reparative granuloma, which will serve as a template for the subsequent formation of callus. The initial inflammatory reaction peaks between 24 and 48 hours after the fracture and then fade away after roughly a week [30].

During the repair phase, callus is formed, and the collagen matrix is laid down. Callus production is assured by two processes: intramembranous ossification and endochondral ossification [31]. In intramembranous ossification, MSCs differentiate into osteoprogenitor cells, which then proliferate and differentiate into osteoblasts, which produce woven bone. In endochondral ossification (Figure 3), BM-MSCs become chondrocytes and secrete a cartilage matrix that serves as a cartilaginous template. Chondrocytes then undergo hypertrophic differentiation and mineralization of the surrounding matrix, resulting in the formation of cartilaginous callus. Finally, apoptosis occurs in hypertrophic chondrocytes, culminating in vascular invasion and osteoblast migration. Afterwards, the cartilage matrix transforms into the bone matrix [31].

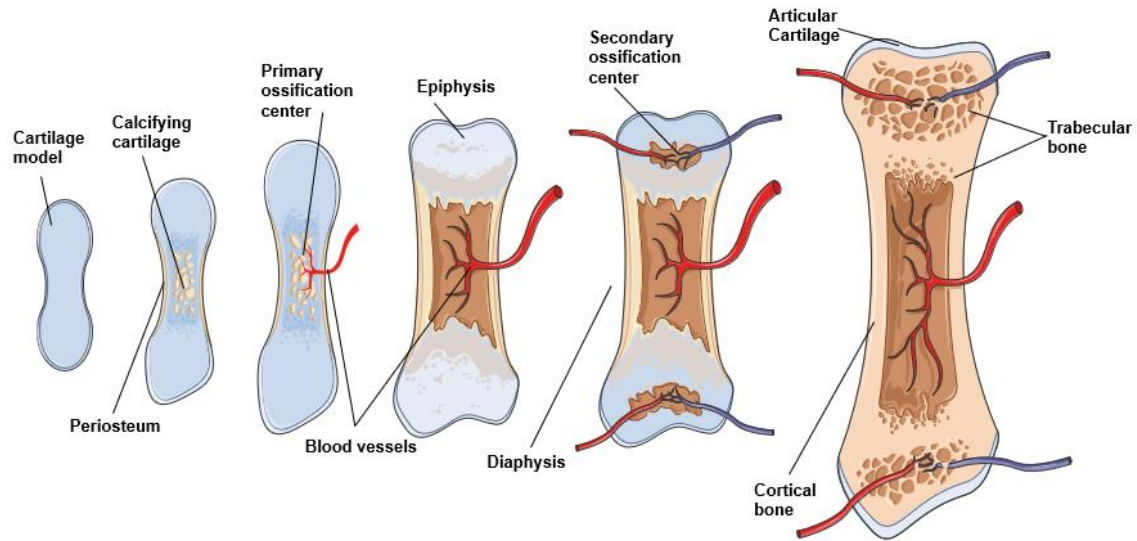


Figure 3. Endochondral bone formation. Progressive evolution of endochondral bone development during embryogenesis.

IL-1 and IL-6 are proinflammatory cytokines that are lacking during this period [32]. TNF- α is reduced in the early stages of healing but increases in the latter stages [33], promoting chondrocyte death, mineralized cartilage resorption, and vascularization during the endochondral ossification process [33]. Low levels of this cytokine also promote osteoblast growth, whereas, at high levels, it inhibits these activities [33]. Furthermore, osteal macrophages (OsteoMacs) which are a special subtype of macrophage residing in bony tissues, are abundant in bone production locations, and in the healing and remodeling phases. These cells are linked to the maturation of bone tissues [34, 35].

In the remodeling phase, the structure, form, and mechanical characteristics of bone are restored. During this final stage, the balance between osteoblastic and osteoclastic activity, which results in lamellar bone formation and bone resorption, is critical [31, 32] (Figure 4).

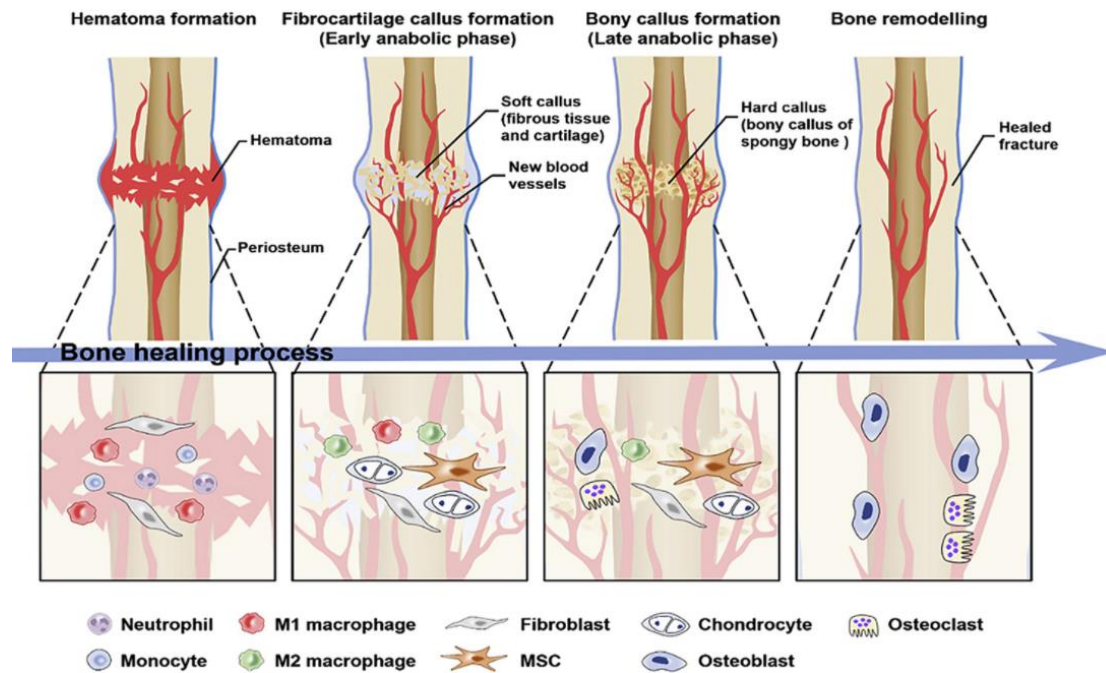


Figure 4. Bone healing process. The biological process of bone healing can be divided into three stages (inflammation, repair, and remodeling) (Adapted from [36]).

1.3 Inflammation

Inflammation plays a key role in the process of bone healing. Inflammation is the tissue's protective response to a noxious stimulus, and it leads to the removal of harmful stimuli as well as the start of the healing process [37, 38]. In most cases, the acute inflammatory reaction that is triggered by such stimuli comes to an end at a specific point, which is characterized by resolution and repair of the damaged tissue. On the other hand, there are situations in which a pathological dysregulation of the inflammatory process can result in a protracted and chronic inflammation that is instead characterized by permanent tissue damage, fibrosis, and/or scarring [39].

1.3.1 Inflammatory cells

When tissue-resident macrophages and mast cells are activated, pro-inflammatory cytokines such as $\text{TNF-}\alpha$, IL-1, and IL-6 are released, as are leukocyte-recruiting chemokines such as monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1 alpha and beta (MIP-1 α/β), and IL-8 [40]. Mast cells release histamines, which act on vascular endothelial cells to increase blood vessel permeability [41]. Neutrophils are activated at the site of inflammation either by direct contact with

pathogens or by proinflammatory cytokines released by the affected tissue's cells [41, 42]. Recruited monocytes/macrophages play different roles in the inflammatory process, depending on the signals present in the injured tissue. Monocytes and macrophages can control the course of inflammation by responding in a pro-inflammatory, anti-inflammatory, or repair-oriented manner [43]. Monocytes release proinflammatory cytokines in response to stimuli such as lipopolysaccharide (LPS), interferon-gamma (IFN- γ) and TNF- α in order to increase the cell-mediated immune response and recruit more cells to the site [43]. TNF- α is a strong pro-inflammatory agent that controls many different parts of macrophages function. It is quickly released after injury, infection, or exposure to LPS, and it is one of the most common early mediators in inflamed tissue [44]. TNF- α coordinates the production of a pro-inflammatory cytokine cascade, having a crucial role in the initial inflammatory response [45]. If this initial inflammatory response fails to eradicate the unpleasant stimuli, the inflammatory process persists and gains chronic features, such as continual low-grade tissue damage, neovascularization, and fibrosis [46]. IL-4 and/or IL-13 stimulation induces the repair monocyte/macrophage phenotype, which is characterized by enhanced expression of the mannose receptor, major histocompatibility complex (MHC) class II, alternative macrophage activation-associated CC chemokine-1 (AMAC-1) and MCP-1. This subpopulation of monocytes also produces anti-inflammatory cytokines like IL-1 receptor antagonist (IL-1RA) and tissue formation-stimulating GFs like TGF- β [47].

1.4 Macrophages

Macrophages are one of the most important type of cells in the inflammatory process [48]. Macrophages patrol the body for infection and can be found in a range of tissues [49]. As a result, tissue macrophages are frequently the first cells to encounter pathogens [50]. Macrophages are highly heterogeneous cells that can perform a variety of functions depending on their tissue location and environmental signals, including pathogen and debris phagocytosis, bacterial killing, inflammation regulation via pro and anti-inflammatory cytokines production, and wound repair [51]. The phenotype of macrophages changes as wound healing progresses [52].

1.4.1 Macrophage origins and development

Macrophages are mobilized during an infection as a result of chemokine production at the infection site. In general, macrophages are classified as either circulating macrophages or tissue resident macrophages [53]. Circulating macrophages are generated from bone marrow monocytes and circulate through the bloodstream. These

macrophages can get recruited to different tissues and are commonly linked to blood recycling and response to lesions [53]. Tissue-resident macrophages are derived from neonatal development and primarily home to the organ from which they originated. Tissue-resident macrophages can be found in nearly every organ, including the brain, liver, heart, spleen, skin, and lungs [53]. Each macrophage is specialized to fulfil duties relevant to the organ in which it dwells, and they are often slightly different from one another. Tissue-resident macrophages, by definition, respond to challenges or stimuli that occur in their native organ. Macrophages are able to respond to a variety of situations due to their widespread distribution throughout the body [53].

1.4.2 Macrophage functions in immunity and homeostasis

Macrophages are involved in every stage of the response to an infection. Inflammatory macrophages are phagocytic and antimicrobial early in infection, producing chemokines, proinflammatory cytokines, and reactive oxygen/nitrogen species, as well as presenting antigen to effector CD4⁺ T cells [54-56]. Macrophages are immunosuppressive during the resolution of inflammation [54, 55]. They are also responsible for clearing debris from the infection site, including dead or dying neutrophils [57-59]. Macrophages also play an important role in wound healing and regeneration, secreting a variety of growth factors and soluble mediators that promote fibroblast differentiation and extracellular matrix synthesis [60]. Macrophages also play a role in removing apoptotic cells from various tissues [61].

Macrophage phenotypes are regulated in response to a range of signals. These include not only microbial markers like LPS, flagellin, and others [62-65], but also endogenous signals from cytokines such as IFN- γ or IL-4 [51], or host defense peptides [66]. These signals can lead to alterations in epigenetic and transcriptional programs. The phenotype of macrophages can be determined not only by the pathogen detected but also by the extracellular environment. Macrophage polarization refers to the variation in the phenotype of activated macrophages as a response to these environmental inputs [67].

1.4.3 Macrophage phenotypes

Macrophage plasticity is accompanied by considerable changes in gene expression at the transcriptional level, including different sets of lipid mediators, G-protein-coupled receptors, and chemokines [68]. Macrophage activation involves alterations in metabolic responses in addition to known immunological pathways, and some of these metabolic activities provide additional immunomodulatory effects [69-71].

The M1 inflammatory type, induced by LPS and T helper type 1 (Th1) cytokines like IFN- γ , and the M2 wound healing and/or anti-inflammatory type, induced by T helper type 2 (Th2) cytokines like IL-4, IL-13, and IL-10 [51], are the two most common types of macrophage polarization states (Figure 5). Within the M1 and M2 categories, however, activation is diverse, with different polarizing stimuli eliciting various subsets of markers and functional responses [72]. As a result, a more sophisticated categorization based on the precise signals employed to induce activation has been proposed [72]. After initial polarization, macrophages can still be switched from one state to another, as seen in M2 macrophages treated with IFN- γ [73]. *In vivo*, macrophages can adopt states with markers and activities that are similar to distinct polarization spectrum points [72]. Tissue macrophages normally exist in an M2-like state, performing repair tasks, but can transition to an M1-like state in response to infection, demonstrating plasticity [74].

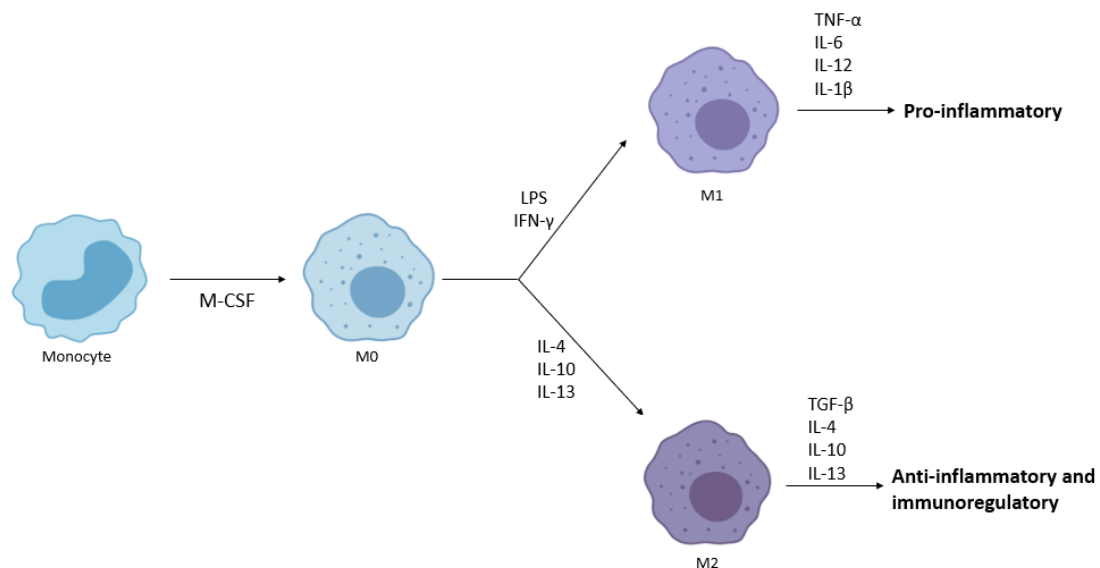


Figure 5. Macrophages phenotypes.

In the M1 stage, macrophages are largely responsible for clearing early infections and contributing to host inflammation [67]. IFN- γ increases macrophage phagocytosis and phagosomes, and M1(IFN+LPS) produces reactive oxygen species [73] as well as inflammatory cytokines such TNF- α , IL-1, and IL-12, as well as IL-6 [51, 72]. M1 macrophages can induce cell death and tissue damage as a result of this activation [54]. M2 macrophages have a variety of roles, including protection against extracellular parasites, wound healing and tissue repair, and immune response regulation, including inflammation [51, 73, 75, 76].

Pro-inflammatory M1 macrophages are replaced by wound healing and anti-inflammatory M2 macrophages during the resolution phase. Surprisingly, this can

happen even when M2-inducing cytokines are absent [54, 72]. It is unclear whether this represents a timed switch from a M1 to a M2 phenotype, or a migration of a different population of M2 macrophages such as tissue macrophages [54].

M2 macrophages have been studied in more depth than M1 macrophages. Activation by IL-4 or IL-13, IL-10, TGF- β , immune complexes (IC) with or without toll-like receptor (TLR) agonists, or glucocorticoids (GC) are some of the notable variants [51, 72]. Sub-classifications such as M2a, M2b, are frequently used to refer to these, while this terminology is not currently recommended. To characterize these variations, a number of receptors, cytokines, chemokines, and other indicators have been proposed [72]. However, there are gaps in this categorization, and there is debate about whether arginase is expressed in M2b macrophages [51, 77]. Humans and mice can have different markers. While arginase is a critical hallmark of the M2 state in mice, human macrophages do not express arginase [76], and whether IL-10 is an M2 marker in mice remains unknown [51, 78]. Just as the immune response requires coordination between M1-driven inflammation and M2-driven resolution, inflammation resolution necessitates a delicate balance between wound healing and M2 cell immunosuppressive actions. M2 macrophages can promote fibrosis during wound healing, in part by producing pro-fibrotic cytokine TGF and extracellular matrix degrading matrix metalloproteinases (MMPs). Immune suppressive M2 macrophages, on the other hand, are antifibrotic, in part because they express a distinct set of MMPs and inhibit myofibroblasts, which are implicated in the repair response [54, 60].

Macrophage dysregulation can cause major health problems because of the critical functions that macrophages play in immune responses and homeostasis. The number of illnesses induced by or partly ascribed to impairments in macrophage function reflects this. Cancer [79], infection [80], diabetes [81], and atherosclerosis [82] are all diseases that can result from altered macrophage functions. Surprisingly, these diseases are usually caused by macrophage overactivation or the presentation of traits that actively contribute to the disease environment, rather than a lack of macrophage activity [83]. As a result of this tendency, the capacity to control macrophage function, for example through genetic silencing, becomes increasingly appealing, as it is expected that changing aberrant macrophage phenotype can lead to therapeutic benefits.

1.5 Mesenchymal stem/stromal cells (MSCs)

Friedenstein and co-workers demonstrated in 1966 that heterotopic transplantation of bone marrow stroma could generate bone, fat cells, and cartilage [84]. In the 1990s, MSC emerged as an easily isolated progenitor cell with multipotency and the ability to

self-renew [85, 86]. Since then, MSC multipotency has been restricted to three lineages: osteoblast, adipocyte, and chondrocyte[87]. MSCs are a type of cell that can produce fully differentiated tissues within its lineage *in vivo*, demonstrating its multipotency, as well as reconstruct itself *in vivo* and give rise to cells with the same phenotypic and potency, demonstrating self-renewal [88]. The International Society for Cellular Therapy has established a set of criteria for designating multipotent MSCs. These cells must adhere to plastic, express specific surface antigens such as CD105, CD73, and CD90, and be devoid of CD45, CD34, CD14 or CD11b, CD79 α or CD19, and human leucocyte antigen (HLA) class II. Furthermore, the cells must be capable of *in vitro* differentiation into osteoblasts, adipocytes, and chondrocytes [89]. In addition CD29, CD44, CD146, and CD166 are used to identify MSC populations [90].

1.5.1 MSC sources

Since MSCs were first isolated from bone marrow, this tissue has served as the basis for research in this field. On the other hand, MSCs have been discovered in adipose tissue, connective tissue of umbilical cord, and cord blood. Although the MSC-populations isolated from these distinct sources are comparable in many ways, they may differ in terms of potential and phenotype. When it comes to trilineage multipotency, MSCs isolated from diverse sources have variable potential. Adipose tissue MSCs (AT-MSCs) are more prone to adipogenic differentiation than the other varieties, but Wharton's jelly-MSCs (WJ-MSCs) and Cord blood-MSCs (CB-MSCs) have been shown to be more osteogenic than BM-MSCs [91-93]. AT-MSCs have a lower potential for osteogenesis and chondrogenesis than BM-MSCs, while CB-MSCs have a lower adipogenic potential than both AT-MSCs and BM-MSCs [91, 94].

1.5.2 MSC and inflammatory stimuli

The inflammatory milieu created by the immune response to an infection or in inflammatory illnesses such as rheumatoid arthritis is harmful to bone and has a deleterious impact on bone producing cells and their progenitors, the MSCs. Simultaneously, MSCs have been shown to impact on both the adaptive and innate immune systems, for example, by inhibiting the expression of pro-inflammatory cytokines [95]. MSC immunomodulatory capabilities are well documented, making these cells an intriguing tool in cell-based therapy for autoimmune and inflammation. However, the link between MSC immunomodulatory capabilities and their regeneration capacity is only now being discovered.

TLRs are one probable link between these two traits. TLRs are expressed on innate immune system cells and may recognize pathogen associated molecular patterns (PAMPs) [96]. Aside from recognizing molecules associated with invading pathogens, these receptors can also detect host-specific molecules crucial for "self-recognition" and have been linked to the etiology of autoimmune and chronic inflammatory diseases [97]. Several TLRs, which have been suggested as important proteins in immunomodulation, are expressed on MSCs [98]. Furthermore, despite research yielding contradictory results, new data demonstrate that activation of distinct TLRs on MSCs can influence their differentiation *in vitro* [99].

1.6 Inflammatory signals: communication with bone cells

The spatial and temporal control of proinflammatory signals and components are essential for optimal bone remodeling (Figure 6) [100]. TNF- α , IL-1, IL-6, IL-11, and IL-17 are examples of cytokines that can stimulate bone resorption [101, 102]. They do this by either increasing the differentiation and activity of osteoclasts or decreasing the differentiation and function of osteoblasts, as well as decreasing the production of collagen and bone. On the other hand, anti-inflammatory cytokines such as IL-10 and IL-13 have the opposite impact [103].

Contradictory effects of TNF- α on osteogenesis and bone formation highlight the need for stringent regulation of cytokine release. Depending on concentration, cell type, and exposure duration, TNF- α may either inhibit or stimulate osteogenesis [104]. TNF- α has an essential role in intramembranous and endochondral bone formation, including MSC recruitment, chondrocyte apoptosis activation, and osteoclast recruitment [105, 106]. In contrast, high, persistent TNF- α levels are systemically damaging to tissues and can produce symptoms including chronic inflammation, decreased bone volume, and decreased bone mechanical strength [107].

Both TNF- α and IL-1 have comparable effects on bone healing. IL-1 promotes fracture healing by promoting the development of the primary cartilaginous callus and angiogenesis at the site of injury [108]. Nevertheless, the absence of IL-1 has little impact on fracture healing [109].

Normal bone healing requires controlled IL-6 expression, which increases angiogenesis, the production of growth factors such as vascular endothelial growth factor (VEGF), and the differentiation of osteoblasts and osteoclasts [104, 110]. In the first phases of fracture healing, the lack of IL-6 inhibits mineralization and remodeling of the fracture callus [100, 111]. In contrast, unusually elevated blood levels of IL-6 following a bone fracture are related with reduced function of the lower extremities in patients [112].

Macrophage-produced cytokines and chemokines are also capable of modulating osteoclast activity. TNF- α and IL-1 promote osteoclastogenesis both directly and indirectly by raising receptor activator of NF- κ B ligand (RANKL) expression in osteoblasts and fibroblasts and lowering osteoprotegerin (OPG) expression [113-115]. TNF- α attracts osteoclasts by promoting the apoptosis of osteocytes [116]. During the early stages of bone repair, inflammatory cells are the primary source of inflammatory signals [108]. However, the ability of bone cells to secrete pro-inflammatory cytokines can explain why bone regeneration is not always inhibited by the absence of inflammatory cells [117].

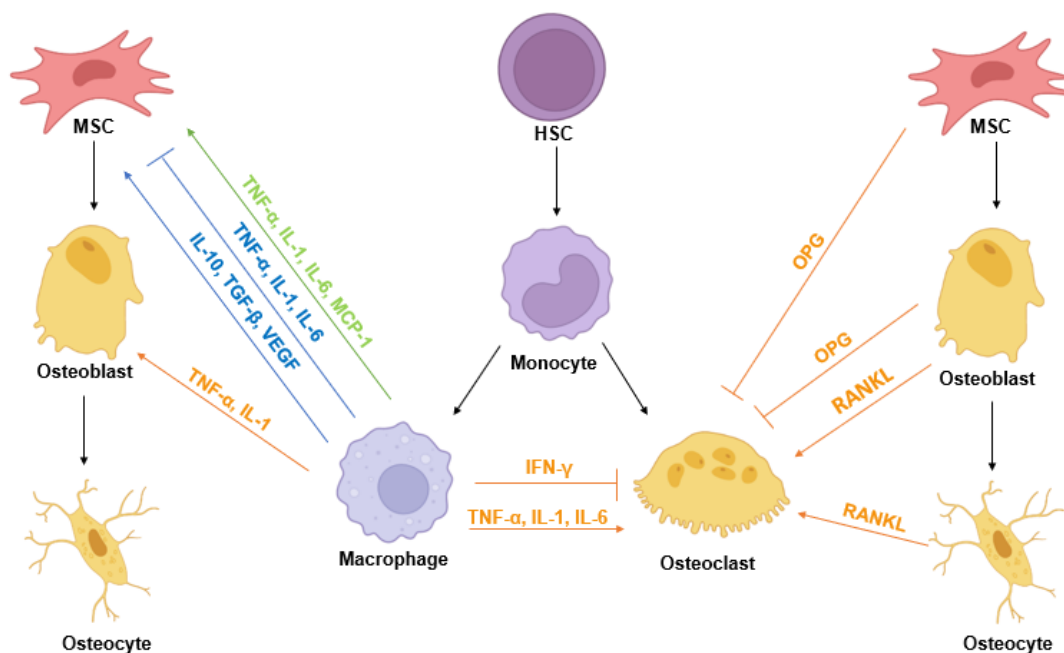


Figure 6. Crosstalk between bone precursor cells and inflammatory cells. Orange lines and arrows show differentiation inhibition and promotion. Blue lines/arrows show proliferation inhibition/promotion. Green arrows represent migratory stimulation.

1.7 MicroRNA

MicroRNAs (miRNAs) are a class of small non-coding RNAs (sncRNAs) with approximately 22 nucleotides in length. These conserved ribonucleic acids (RNAs) play a crucial role as regulators of gene expression, mainly by promoting post-transcriptional repression of their targets [118]. It is estimated that each miRNA can bind to several messenger RNAs (mRNAs), in a tissue-specific manner[119-121]. Thus, miRNAs are important in the regulation of many biological processes, and their deregulation can affect processes such as cell differentiation and proliferation, apoptosis, and

angiogenesis, which can lead to the emergence of neurological diseases, heart diseases and cancer [122].

1.7.1 miRNA biogenesis pathway

The canonical biogenesis pathway (Figure 7) is the dominant pathway by which miRNAs are processed. This pathway starts in the nucleus by the transcription of the miRNA genes by RNA Polymerase II (Pol II) which originates a long RNA molecule, the primary miRNA (pri-miRNA) [118, 123, 124]. This structure is then processed by the Microprocessor, which is a heterotrimeric complex constituted by two molecules of the RNA binding protein DiGeorge Syndrome Critical Region 8 (DGCR8) and by one molecule of the ribonuclease III enzyme, Drosha. DGCR8 recognizes an N6-methyladenylated motif within the pri-miRNA, while Drosha cleaves the pri-miRNA resulting in a miRNA precursor (pre-miRNA), that displays a hairpin-like secondary structure of approximately 60 nucleotides [118, 125, 126].

These pre-miRNAs are then exported from the nucleus to the cytoplasm, by the action of Exportin 5 and RAS-related Nuclear protein (RAN)-GTP, where the loop region of the pre-miRNA molecules is cleaved into a mature miRNA duplex by DICER, an RNase III enzyme [124]. The resulting miRNA duplex is constituted by one miRNA mature strand and by one passenger strand, also called miRNA*. This duplex is loaded onto Argonaute (AGO) protein that will select the miRNA strand based on the thermodynamic stability at the 5' ends of the miRNA duplex or a 5' U at nucleotide position 1. Generally, the strand with lower 5' stability or 5' uracil is deemed the guide strand. Then the selected strand along with other proteins including TNRC6 forms the miRNA-Induced Silencing Complex (miRISC) [118, 123, 127]. Whereas this complex targets the mRNAs for regulation, promoting its degradation, cleavage, or preventing its translation due to complementarity with the target. On the other hand, the other miRNA strand is degraded [128].

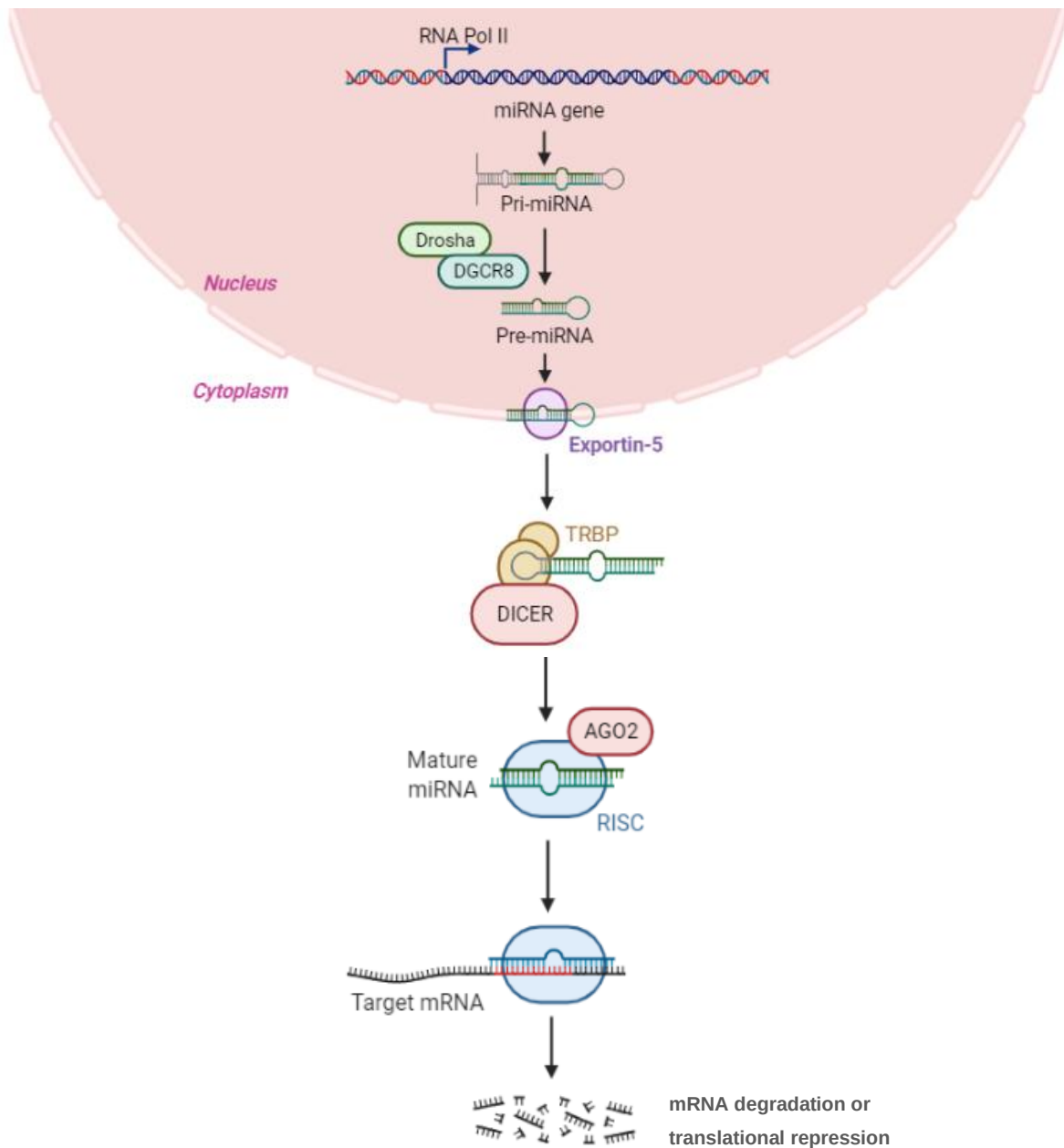


Figure 7. miRNA biogenesis pathway. Briefly, RNA polymerase II transcribes primary miRNA transcripts (pri-miRNA). Following transcription, the Microprocessor complex, which includes the RNAse III enzyme Drosha and the double-stranded RNA binding protein DGCR8, processes the pri-miRNA, resulting in the hairpin precursor miRNA (pre-miRNA). The pre-miRNA is subsequently exported to the cytoplasm by Exportin-5, where it is further processed into a miRNA duplex by Dicer in collaboration with TRBP. The mature strand of this miRNA duplex is loaded into the miRNA induced Silencing Complex (miRISC).

1.7.2 MicroRNA targets

MiRNAs act as guides for the miRISC complex, promoting its binding with the target mRNA and thus directing the target gene's post-transcriptional repression via translation repression, target mRNA degradation, or both. As a result, identifying miRNA targets is

critical to comprehending miRNA function [123, 129]. The identification of the targets is challenging and poses a difficulty in the field due to the small size of the miRNA and the fact that just a partial matching between the miRNA and the target mRNA is usually required [123].

In animals, the common interaction between miRNAs and their targets is partial base-pairing, which frequently involves matching between sites of the target mRNA's three primary untranslated regions (3'UTR) and the seed region of the miRNA 5'-end [118, 123]. Only nucleotides 2-5 are available for pairing with mRNA at the start of the target recognition, but later a conformational change permits nucleotides 2-8 and 13-16 to be exposed [130]. The existence of an adenine in the nucleotide 1 is preferred due to the interaction with the Argonaute protein [118]. Furthermore, ribosomes might interfere with miRNA targeting [131].

There are also non-canonical binding sites where the seed sequence and target mRNA are not perfectly matched. There is substantial compensatory base pairing in the 3' region of the miRNA in these situations. Apart from the seed region, the presence of these pairing areas may help to reduce regulatory redundancy in the action of miRNAs from the same family. MiRNA families are made up of miRNAs with the same seed sequence [118].

1.7.3 MicroRNA mechanisms for the regulation of gene expression

There are two complementary modes for miRNAs to repress their target transcripts, involving target mRNA degradation and translational repression [118, 132]. Several studies have shown that miRNAs primarily destabilize their mRNA targets, as lower levels of mRNA were followed by with decreased protein levels in almost all documented miRNA interactions with their targets [133, 134]. In plants, if the target and miRNA have nearly perfect complementarity, mRNA is degraded by slicing the target transcript through endonucleolytic cleavage driven by a catalytically active Argonaute [135, 136]. In mammals, most targets are only partially complementary to the miRNA, and the cellular 5'-to-3' mRNA decay pathway, which consists of three main steps outlined below, is used to degrade the miRNA. The miRISC recruits and directs the 5'-to-3' mRNA decay machinery to the target through interactions between the decay pathway complexes and the GW182 protein scaffold family [137-139].

There are three basic phases in the mRNA degradation pathway. The action of the cytoplasmic deadenylase complexes, initially catalysed by the poly(A) nuclease 2 and 3 (PAN2-PAN3) and later by CCR4-NOT, deadenylates mRNA [140]. After, the decapping protein 2 (DCP2) and other decapping cofactors, including DCP1, decap the

deadenylated mRNA [141]. Finally, the cytoplasmic nuclease 5'-to'3' exoribonuclease 1 (XRN1) degrades the deadenylated and decapped mRNA [141, 142]. The steps of the mRNA decay cascade are interconnected, and all of the complexes involved interact with one another - deadenylation complexes engage with decapping factors, and decapping factors, in turn, interact with XRN1 to recruit it to the decapped mRNA [121, 141]. Although mRNA degradation is the most prominent effect of miRNA-mediated silencing, translational repression mediated by the miRISC complex can still occur, either alone or in combination with mRNA degradation [134], because miRISC has been shown to repress translation in the absence of mRNA degradation [143]. The method by which miRNAs suppress translation is still a source of debate. However, evidence suggests that miRNAs block cap-dependent translation at the initiation stage by interfering with the eukaryotic initiation factor 4F (eIF4F) complex, which recruits and scaffolds the pre-initiation complex [144-146].

1.7.4 miRNAs nomenclature

With the development of next-generation sequencing techniques, such as high-throughput expression profiling, the identification of new miRNAs has become much easier. As a result, databases such as the “microRNA Registry” (miRBase) were needed to ensure that newly discovered miRNAs were properly annotated and registered to avoid redundancies and misannotations. MiRBase assigned new names to novel miRNA families and miRNAs belonging to known miRNA families in a systematic way under specific standards to avoid misannotations and overlapping miRNA names [147]. Around 2 812 mature miRNAs transcripts are currently deposited in miRBase (<http://www.mirbase.org/>) for the human genome. Thus, the currently used nomenclature is outlined in Table 1(adapted from [147]).

Table 1. Nomenclature of microRNAs.

Nomenclature format	Description
miR-Y or miRY	The use of a dash sign distinguishes animal miRNAs from plant miRNAs. e.g. miR-195 (animals) and miR195 (plants)
mir or miR MIR or miR	Lowercase and italic characters are used to name animal pre-miRNAs (e.g. mir-195). The capitalization of the “mir” prefix and the lack of the dash sign distinguish

	the names of plant pre-miRNAs (e.g. MIR195).
hsa-miR-Y, mmu-miR-Y, rno-miR-Y, ...	A three-letter prefix, species designators, followed by a dash, and the same identifier number format are used to name identical miRNA from different species. hsa - Homo sapiens; mmu – Mus musculus (mice); rno – Rattus norvegicus (rat).
X-miR-Ya, X-miR-Yb, X-miR-Yc, ...	The addition of a lettered suffix allows close related mature miRNA sequences that belong to the same family and may arise from separate precursors, but may not have the same target or function, to be distinguished within a specie. e.g. mmu-miR-195a and mmu-miR-195b
miR-Y-5p or miR-Y-3p	The prefixes -5p and -3p are used to designate which arm of the pre-miRNA hairpin the miRNA sequence originates from, regardless of its abundance. e.g. miR-195-5p and miR-195-3p
X-miR-Y-1, X-miR-Y-2, ...	An additional number is added after the dash symbol when different loci produce similar mature miRNA sequences in certain species and members of the same family. e.g. hsa-miR-19b-1 and hsa-miR-19b-2.

1.7.5 Delivery systems for miRNAs

The interest in miRNA-based therapeutics has increased dramatically over the last few decades, owing to the wide range of biological anomalies that have been discovered [148, 149]. There have been several therapeutic techniques established to either reduce or restore the expression of disease-associated miRNAs. It has been shown that miRNA mimics can be employed to restore the expression and function of miRNAs in cases where their expression has been lowered as a result of the disease [149-152]. The anti-miRNAs (antagomirs), on the other hand, are being used to inhibit the activity of elevated

miRNAs that are associated with disease [152-154]. However, the distribution of miRNA mimics or antagomirs to target tissues in a safe and efficient manner continues to be a substantial barrier for miRNA-based therapeutics. The sensitivity of miRNA delivery to destruction by nucleases, immunotoxicity, the quick clearance of miRNA from the bloodstream, and limited tissue permeability are all significant drawbacks of miRNA distribution [155-158].

Genetically modified viruses can efficiently transmit desired oligonucleotides into a variety of tissue types while maintaining high levels of gene expression for extended periods of time. The past few decades have seen the development of a range of viral delivery vehicles that can be tailored to deliver specific transgenes, treatment regimens, and cell types to specific recipients. Some of the most commonly utilized virus-based vectors for miRNA or anti-mRNA distribution are retroviral, lentiviral, adenoviral and adeno-associated virus (AAV) vectors, as well as bacteriophage-based virus-like particle (VLP) vectors [159, 160]. Apart from being extremely efficient miRNA delivery systems, viral-based miRNA delivery systems have a high immunogenicity, toxicity, and size constraint. To circumvent these obstacles, non-viral miRNA delivery approaches have been developed to be less damaging and biocompatible. Non-viral delivery approaches ensure that miRNA or miRNA-expressing vectors enter the cell successfully and are not prone to nuclease degradation. Chemical methods of non-viral miRNA distribution have been developed using lipid, polymer, inorganic, and extracellular vesicle carriers [161].

1.8 Bone tissue engineering

Bone engineering is a promising approach for healing bone defects caused by trauma or illness since it is less invasive, safer, and more effective than standard procedures such as autogenous bone grafts [162]. Given the drawbacks of bone grafts, identification of key molecules (such as growth factors, cytokines, and morphogens) involved in bone development and fracture healing, lead to the introduction and rapid expansion of biomimetic materials in the field of bone tissue engineering[163]. Tissue engineering techniques for bone regeneration include recombinant protein therapy to supply osteogenic cytokines and growth factors, delivery of nucleic acids (DNA, mRNA) encoding growth factors that stimulate bone formation, and transplantation of osteogenic cells to bone defect locations [164].

1.8.1 Gene therapy

Gene therapy is regarded as an efficient method for delivering growth factors in a sustained manner while avoiding the drawbacks of large protein dosages. Gene delivery

is less expensive than bolus protein administration and can be precisely regulated. Moreover, delivery of numerous genes simultaneously is conceivable, and customization is extremely simple [165]. Successful bone healing/repair involves the coordinated action of four crucial factors: (1) osteoconduction, (2) osteoinduction, (3) osteogenic signal-responsive cells, and (4) vascular supply. Bone tissue engineering tries to provide these components to drive *in vitro* or *in vivo* bone regrowth [166]. Gene therapy requires three essential material components: pDNA encoding the desired therapeutic protein, a vector to allow cellular uptake of the pDNA, and *in situ* or *ex vivo* target tissue or cells that produce the intended protein upon transfection [164].

1.8.2. Gene delivery vectors

There are two types of gene delivery vectors: viral vectors and nonviral vectors. Retrovirus/lentivirus, adenovirus, AAV, baculovirus, and other viral vectors are in use, whereas plasmid is the most prevalent nonviral vector. Using genetically modified cells, the gene vector may be delivered *in vivo* or *ex vivo*. Genes may be delivered systemically or locally in any case [167-170].

Although viral gene therapy has been shown to be effective in animal research, perceived safety issues continue to offer problems in performing human clinical trials, and hence clinical use of viral gene therapy for non-lethal diseases is typically unwanted. Despite having lower transfection efficiency than viral vectors, non-viral vectors are less dangerous and may be therapeutically translated for possible bone regeneration applications [164]. A vector that mediates effective gene therapy should ideally have a number of critical properties that allow for therapeutic levels of transgene expression over an acceptable period of time. Transgenes must be delivered to target cell nuclei in a way that promotes effective transfection while minimizing cytotoxicity and safety issues. Furthermore, it should preferably be cell-specific and have a regulated protein expression timeline [171].

1.8.3 Gene-activated matrices (GAMs)

Gene-activated matrices (GAMs) are inert scaffold systems that incorporate viral or non-viral gene delivery vectors that have been exploited in wound healing and tissue engineering applications [172]. GAMs are temporary three-dimensional templates that direct the production of new functional tissue and act as a guide for the growth of new functional tissue. This allows them to more effectively encapsulate and keep the gene within the sponge matrices for longer periods of time, resulting in increased matrix deposition and blood vessel creation in the developing bone tissue [173].

1.8.3.1 GAMs and tissue engineering

GAMs should ideally be constructed of biodegradable and biocompatible polymeric materials that bioresorbable after *in vivo* implantation into osseous deformities. When the invading reparative cells carrying the transgene attach to the scaffold matrix, they offer structural support for osteogenesis or bone remodeling to take place. Three-dimensional GAMs are often very porous in nature and can be created with a variety of desirable geometries that can regenerate functional bone tissue while keeping the original size and shape of the bone tissue being replaced [174].

GAMs should be osteoconductive, mechanically compatible, and able to integrate with native bone during healing. The matrix must generate and maintain appropriate space and a suitable environment *in vivo* to permit tissue development while managing the size and shape of space-filling regenerating tissue [164]. It must be structurally and mechanically stable, homogeneously porous, and have an optimum pore size for cell adhesion and growth. The scaffold matrices employed in guided bone regeneration must have the right physical and mechanical properties to promote cell survival and proliferation. Mechanical properties and degradability of scaffolds impact their ability to create and manipulate bone tissue. The scaffold must preserve its structural integrity after being implanted in the patient for progressive bone-formation [175]. The biocompatible scaffold must dissolve (by releasing non-toxic breakdown products) at a pace that allows new bone tissue to be produced, progressively replacing the biocompatible scaffold. Progenitor cells from the surrounding tissue should be able to migrate and connect to the scaffold, allowing the scaffold to function properly. As another consideration, the pore size must allow maximum cell entry and movement while being small enough to provide a high specific surface area for cell attachment and matrix deposition [176]. To transport metabolites and nutrients effectively, scaffolds must have an adequate pore volume fraction (high porosity, frequently >90%) and a linked pore network. The scaffold's pore size, shape, and porosity determine total specific surface area and ligand delivery to cells [177].

1.8.3.2 Biocompatibility of GAMs

Non-viral GAMs for tissue engineering are more cost-effective and perhaps than recombinant protein or viral therapy for inducing sustained expression of recombinant growth factors by transfected infiltrating wound healing cells. Implanted GAMs stimulate gene expression and protein synthesis, improving osteogenesis and bone regeneration. Localized gene therapy may minimize or reduce systemic toxicity from dosage dumping, which can occur with protein therapies [178]. *In vivo*, GAMs may produce a long-term

healing effect due to continuous transgene expression. The gene transfer from GAMs causes some cells to evolve into desired, chosen cell types over time, avoiding the need for repeated gene transfers. The porous scaffold protects gene vectors from physiological degradation and slows clearing from the wound site, leading to long-term transgene availability to GAM cells [179]. Non-viral gene delivery systems based on GAMs have the greatest potential to overcome key barriers to clinical translation when compared to viral gene delivery systems, provided that the safety and efficacy of these materials can be demonstrated in appropriate animal and human studies in the future [164]. Among the many applications of GAM technology in bone tissue engineering, one of the most versatile is its ability to impact sustained localized gene delivery and extended expression of tissue inductive growth factors. The polymeric scaffold design of GAMs can govern tissue development by regulating the release of growth factors and ensuring that appropriate amounts of growth factors are available at the site of injury [164, 166].

2. Objectives

2.1 Objectives

MiRNAs are currently being studied as potential therapeutic targets, in a wide range of human diseases. The fact that miRNAs control almost every biological mechanism and regulate a variety of distinct targets contributes to the expanding interest in this field of research. Currently, modulation of miRNA levels had been explored as an approach for bone regeneration therapies.

The main goal of this study is to explore the role of inflammatory mediators, including microRNAs, in TNF- α -stimulated macrophages and to address the impact of their secretome on the recruitment of mesenchymal stem cells. Specifically, we intended to:

- Assess the expression of inflammatory genes in TNF- α -stimulated macrophages;
- Evaluate the effect of the secretome derived from TNF- α -treated macrophages in MSC migration;
- Evaluate the effect of TNF- α treatment on macrophages miRNA expression;
- Test effectiveness of a miRNA delivery protocol to macrophages.

3. Materials and Methods

3.1 Human primary monocyte isolation

Human primary monocytes were isolated from buffy coats (BCs) from healthy blood donors (Figure 8) kindly provided by Serviço de Imunohemoterapia from Hospital Universitário São João, Porto. Firstly, BC was transferred to 50mL falcon and centrifugated at 1200 g, 30 minutes (min), at room temperature (RT), with slow acceleration and without brake, for blood components separation. After centrifugation, three distinct phases were observed: an upper phase (enriched in plasma), a middle phase (ring of Peripheral Blood Mononuclear Cells - PBMCs) and a lower phase (enriched in red blood cells - RBCs). PBMCs layer was collected into a 15mL falcon and incubated with RosetteSep human monocyte enrichment isolation kit (67 μ L per 1 mL of sample) during 20 min in a horizontal shaker at RT. The mixture was diluted on a proportion of 1:1 in 2% fetal bovine serum (FBS) in phosphate buffered saline (PBS), gently layered over Histopaque and centrifuged at 1200 g, 30 min, at RT, without acceleration or brake. The enriched monocyte layer was collected and washed with 50 mL of PBS 1x at least three times for platelet depletion (centrifugation at 1300 g for 6 min).

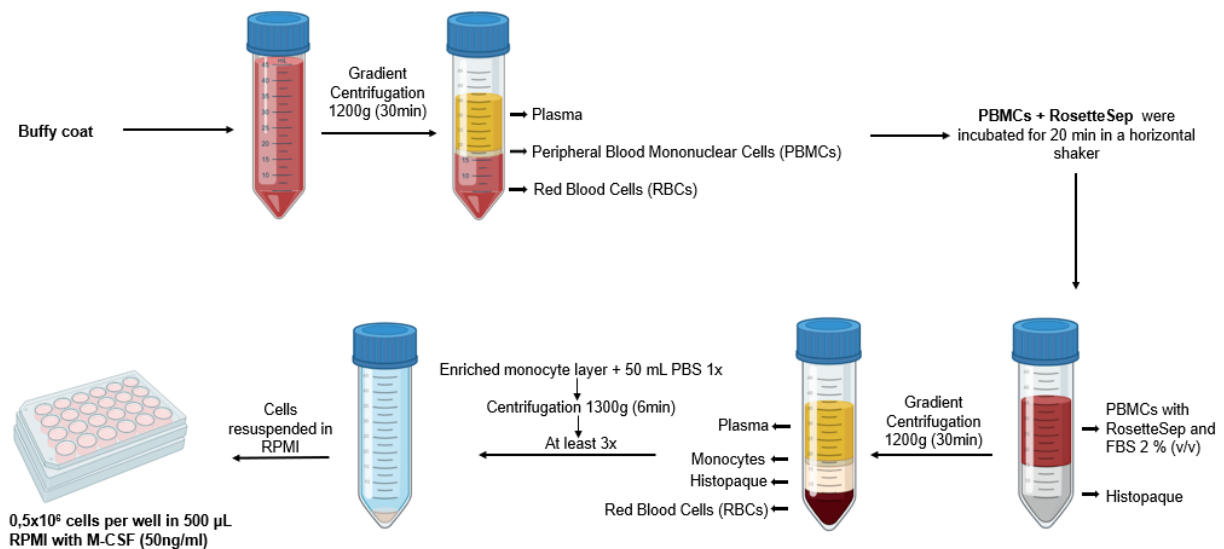


Figure 8. Monocyte isolation from human buffy coats. After the first centrifugation three different layers are formed: the lower layer with Red Blood Cells (RBCs), the middle layer with Peripheral Blood Mononuclear Cells (PBMCs) and the upper layer with the plasma. Then, PBMCs and RBCs are incubated with RosetteSep Human Monocyte Enrichment cocktail that is designed to separate monocytes from other blood cells, by negative selection. It binds cell surface antigens on human hematopoietic cells, except monocytes, crosslinking unwanted cells with RBCs in immunorosettes that pellet along with the free RBCs when centrifuged over a density medium such as Histopaque. Following centrifugation, monocytes were collected, washed, and cultured *in vitro*.

Recovered monocytes were plated at a density of 0.5×10^6 cells/well on top of sterile 13 mm glass coverslips in 24-well plates and cultured in a complete culture medium (RPMI) supplemented with 10% FBS and 1% penicillin-streptomycin (P/S) at 37°C and 5% carbon dioxide (CO₂). Primary human monocytes were allowed to differentiate into macrophages. On day 7, polarization of macrophages was induced. Cells were treated with 100 ng/mL LPS or 20 ng/mL TNF- α to stimulate pro-inflammatory M1 macrophages or with 20 ng/mL IL-4 to induce anti-inflammatory M2 macrophages and incubated for 6 or 72 hours (h) (Figure 9).

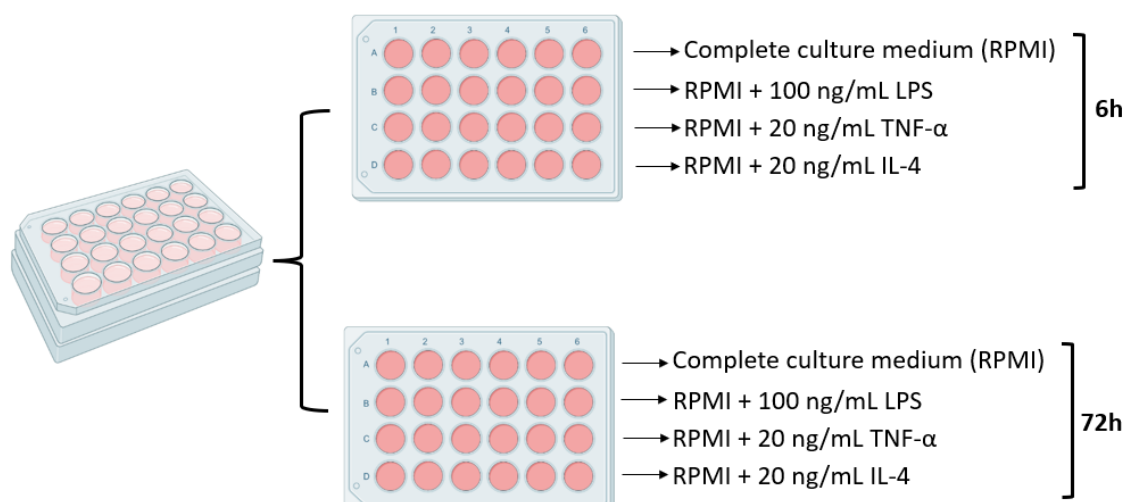


Figure 9. Stimulation of macrophages polarization. Cells were treated with 100 ng/mL lipopolysaccharide (LPS) or 20 ng/mL tumor necrosis factor alpha (TNF- α) to stimulate pro-inflammatory phenotype or with 10 ng/mL interleukin-4 (IL-4) to induce anti-inflammatory phenotype and incubated for 6 or 72 hours.

3.2 RNA isolation

The RNA was extracted from cells using TRIzol Reagent, according to the manufacturer's instructions. Briefly, after cell disruption with TRIzol, 0.2 mL of chloroform was added, and the tubes were vigorously inverted for 15 seconds (s), following incubation of 5 min at RT. Centrifugation was performed (12 000 g, for 15 min, at 4 °C) to obtain the separation of components into three phases: an upper transparent aqueous phase (containing RNA), a white interphase (containing DNA) and a lower red organic phase (containing protein). The upper phase, containing the RNA, was transferred to a clean RNase-free microtube without touching the interface. The RNA was then precipitated with 0.5 mL of isopropanol, and the microtubes were mixed gently and incubated for 10 min at RT. The samples were centrifuged for 10 min at 12 000 g and 4 °C. The supernatant was discarded, and the pellet was washed with 0.75 mL of ethanol

75 % (v/v). Then, centrifugation of 10 000 g was performed for 5 min at 4 °C, the supernatant was discarded, and the pellet was allowed to dry. Finally, the RNA pellet was resuspended in RNase-free water (Figure 10). RNA concentration was determined using a NanoDrop by measuring the absorbance at 260 nm. The RNA samples were then stored at -80 °C until further use.

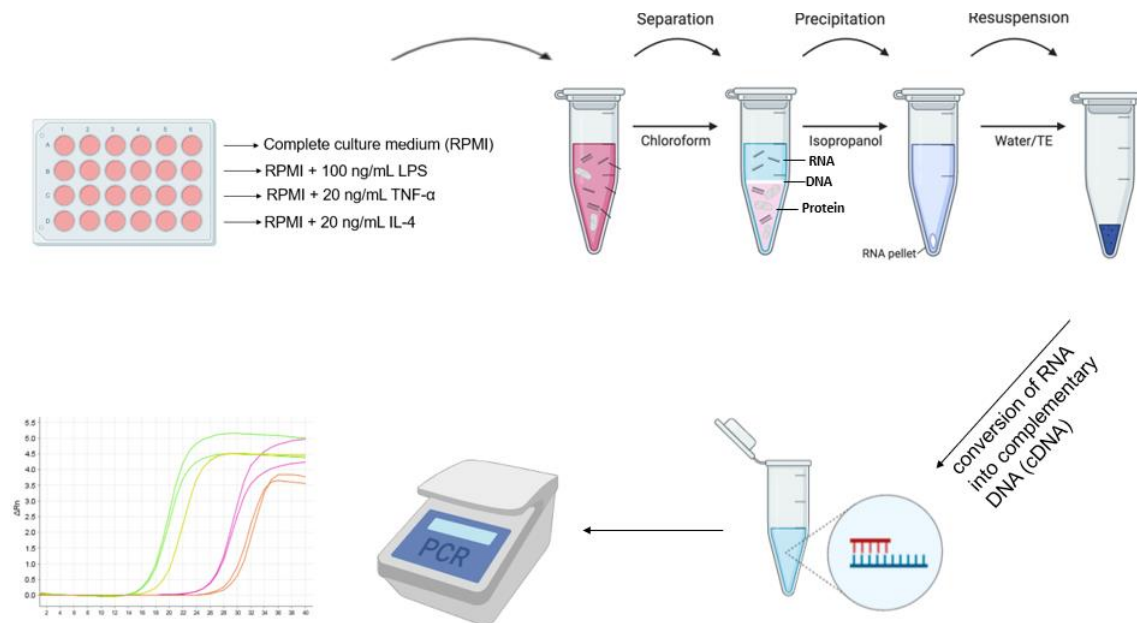


Figure 10. RNA isolation and Polymerase chain reaction (PCR).

3.3 Real-time quantitative polymerase chain reaction (RT-qPCR)

3.3.1 mRNA expression levels

RNA samples were first treated with TURBO DNA-free kit for gene expression analysis. Briefly, DNase and buffer were added to the samples and incubated at 37 °C for 30 min. DNase inhibitor was added to the samples that were vortexed and centrifuged at 10 000 g for 1 min. The supernatant containing the RNA was collected. Random Hexamers and dNTPs were mixed with the RNA and incubated at 65 °C for 5 min in a thermal cycler. Then, Buffer 5x, 0.1 M DTT and Superscript III were added, and the reaction was incubated under the following conditions: 10 min at 25 °C, 60 min at 50 °C and 15 min at 70 °C. Finally, the cDNA samples were diluted in 80 µL of nuclease-free water and stored at -20 °C until further use.

qRT reaction was performed using SYBR Green Mastermix. cDNA was added to the reaction mix containing SYBR Green dye, nuclease-free water, and the pair of primers (reverse and forward) corresponding to the gene of interest. The reaction was performed with 10 µL of final volume according to the following conditions: 3 min at 95 °C and 40

cycles of 30 s at 95 °C, 30 s at 58 °C and 30 s at 72 °C. β -actin was used as reference gene.

3.3.2 miRNA expression levels

miRNA expression levels were analyzed in primary macrophages using TaqMan MicroRNA Reverse Transcription Kit and gene-specific stem-loop Reverse Transcription primers. To synthesize cDNA, RNA was mixed with 10x RT Buffer, dNTP Mix, RNase inhibitor, miR-16 primer, miR-195 primer, miR-342 primer, let-7d primer, U6 primer and MultiScribe Reverse Transcriptase in RNase free microtubes. The samples were then vortexed, centrifuged and incubated in a thermal cycler under the following conditions: 16 °C for 30 min, followed by 30 min at 42 °C and 5 min at 85 °C. Finally, cDNA was diluted with 50 μ L of nuclease-free water and stored at -20°C until further use.

For the real-time PCR reaction, 5.5 μ L of reaction mix containing SsoAdvanced™ Universal Probes Supermix and miR-16, or miR-195, or miR-342 or let-7d or small nuclear RNA U6 TaqMan probe was mixed with 4.5 μ L of each cDNA sample in each well of the 96-well PCR plate, in a total of 10 μ L per well. The RT-qPCR reaction was performed under the following conditions: 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Small nuclear RNA U6 was used as a housekeeping gene.

3.4 Enzyme-linked immunosorbent assay (ELISA)

Quantification of IL-1 β secreted by macrophages was assessed by Enzyme-linked Immunosorbent Assay (ELISA) using the ELISA MAX™ Deluxe Set Human IL-1 β (BioLegend) (Figure 11). The ELISA MAX™ Deluxe Set Human IL-1 β is a Sandwich ELISA.

At first, a human IL-1 β specific mouse monoclonal antibody was coated on a 96-well plate. Standards and the samples (conditioned medias of control macrophages and conditioned media of macrophages stimulated with TNF- α) were added to the wells, and IL-1 β was bound to the immobilized capture antibody. Next, a biotinylated goat polyclonal anti-human IL-1 β detection antibody was added, producing an antibody-antigen-antibody “sandwich”. Avidin-horseradish peroxidase was subsequently added, followed by TMB Substrate Solution, producing a blue color in proportion to the concentration of IL-1 β present in the sample. Finally, the Stop Solution changed the reaction color from blue to yellow, and the microwell absorbance was read at 450 nm with a microplate reader. Cytokine concentrations (pg/mL) were determined using a standard calibration curve.

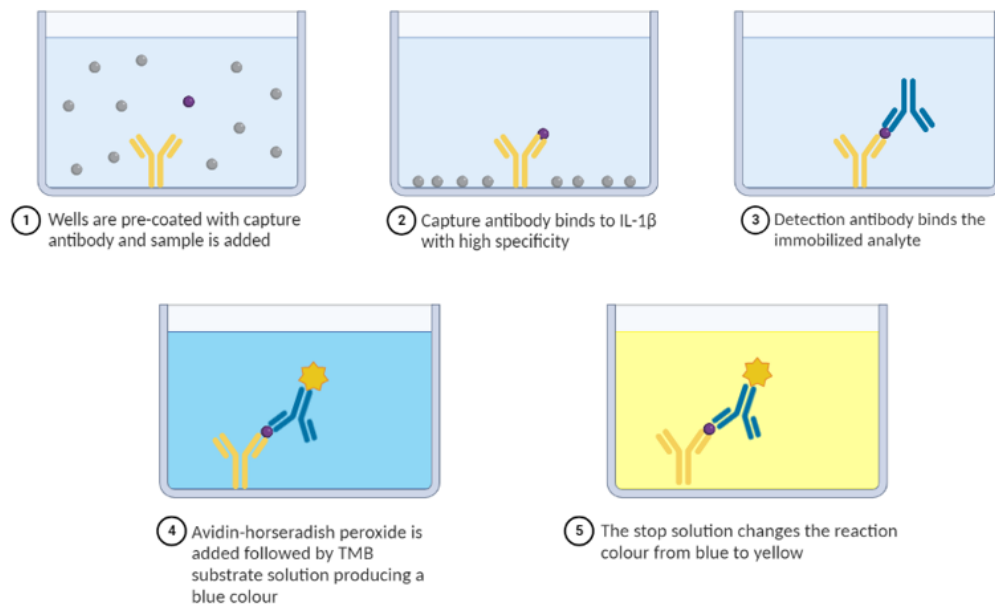


Figure 11. Schematic representation of ELISA assay.

3.5 Migration assays

Transwell assays assessed the TNF- α effect on the capacity of macrophages to recruit MSCs (Figure 12). Firstly, monocytes were isolated and differentiated into macrophages, as described in section 3.1. Then macrophages were stimulated with 20 ng/mL TNF- α for 6h in 24-well plates, and the conditioned media was collected.

Primary human mesenchymal stem/stromal cells (hMSC) were isolated by our group from discarded bone tissues of patients that underwent total hip arthroplasty or anterior cruciate ligament injury at Centro Hospitalar de São João (Porto, Portugal) and signed informed consent for tissue use in research. Patients were aged less than 60 years old. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10 % (v/v) FBS and 1 % (v/v) P/S up to eight passages.

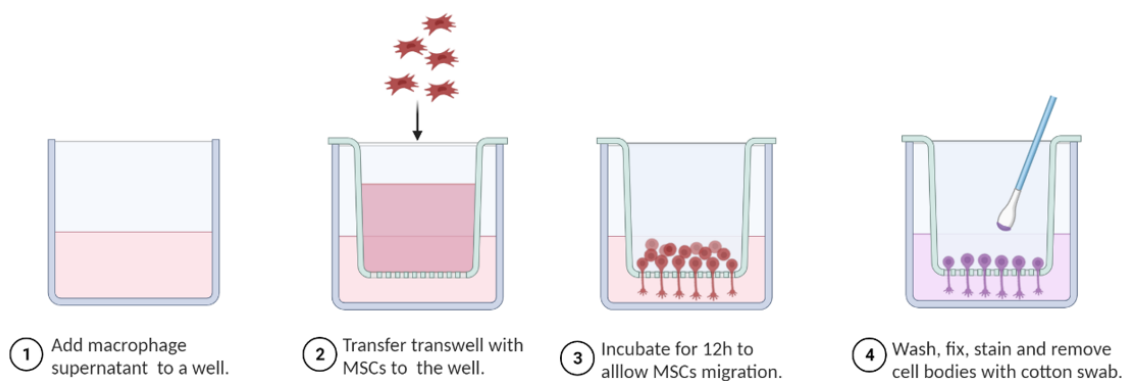


Figure 12. Migration assay protocol.

When a minimum of 75% confluence was achieved, MSCs were trypsinized and counted. 0.06×10^6 MSCs in FBS-free media were plated per transwell insert with PET membrane of 8 μm pore size, previously rehydrated with FBS-free media (top compartment of the transwell). Inserts were placed in contact with the conditioned media of macrophages, and MSCs were allowed to migrate through the membrane for 12h. FBS-free medium, medium with 10% FBS and medium with 20ng/mL TNF- α were used as controls for MSCs migration (Figure 13). Then, culture media was removed, and cells were fixed with 4% paraformaldehyde (PFA) for 20 min at RT. After 20 min, PFA was removed, and the cells were stained with Crystal Violet for 20 min at RT. Insert membranes were washed three times with PBS, and the inner side of the membrane was scrubbed with a cotton swab to remove non-migrated cells. Finally, % of cell migration in microscope images was evaluated using MATLAB software.

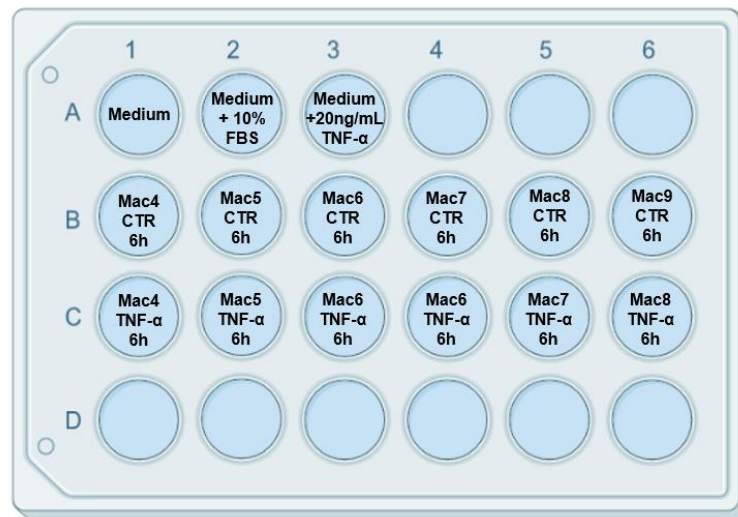


Figure 13. Schematic representation of the conditioned medias used to evaluate MSC migration.

3.6 miRNA transfection

Macrophages were transfected with hsa-miR-195 mimic or miRNA Precursor Negative Control (Scramble - SCR). First, human primary monocytes were isolated and cultured for 3 days. On the transfection day, an equal volume of a phosphate solution (12 mM) containing 0.017% (V/V) Darvan 821-A dispersant reagent was added to a solution of calcium chloride (20 mM). Then the mixture was filtered using a 0.2 μm filter. Calcium phosphate (CaP) particles were added into a solution containing CaCl_2 , TE buffer, and either the SCR or the hsa-miR-195 mimic that had been prepared at a final concentration of 20nM per well. Lastly, macrophages were incubated with the CaP-miRNA solution for ten minutes before the growth media was added. After 24h, the medium was switched

to one containing 20 ng/mL TNF- α (Figure 14). Then, 72h after transfection, the RNA from the cells was isolated and gene expression quantified by RT-PCR.

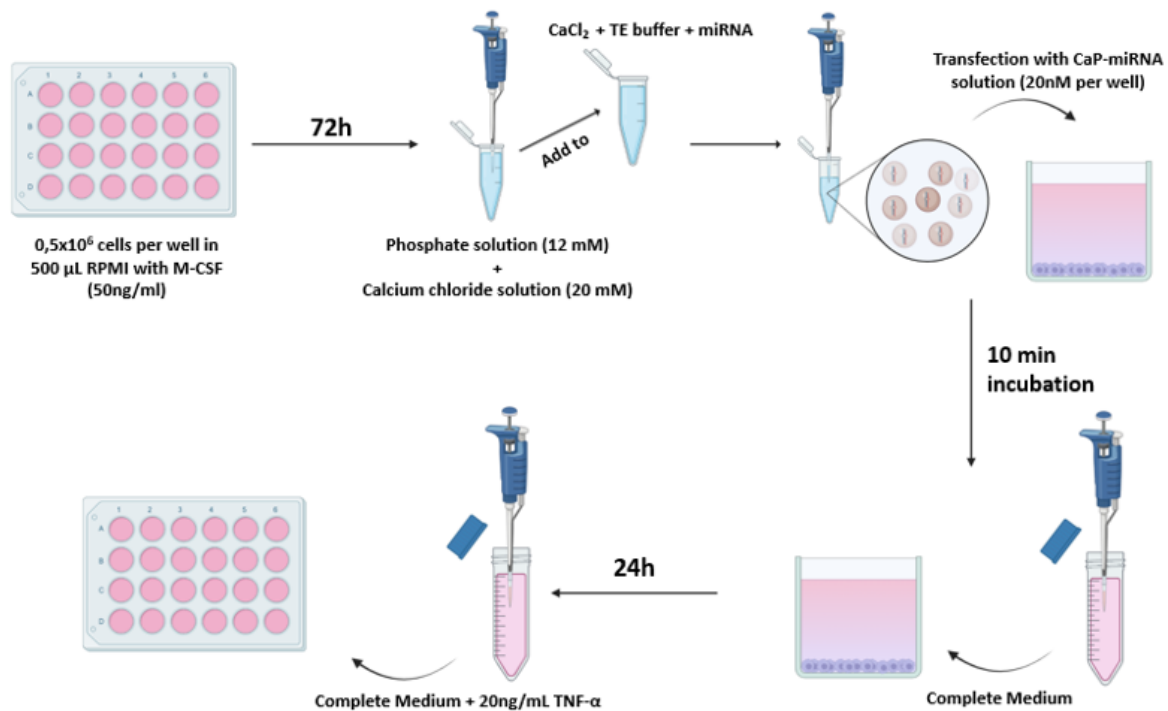


Figure 14. MicroRNA transfection protocol. First, monocytes were isolated from buffy coats of healthy blood donor, and after 72 hours were transfected with a CaP-miRNA solution. After 24 hours of transfection the medium is changed by one containing 20ng/mL TNF- α . Finally, the RNA of macrophages was extracted, and the expression of miRNAs was analyzed by RT-qPCR.

3.7 Statistical analysis

Statistical data analysis was performed using GraphPad Prism 9 (GraphPad Software, Inc.). To evaluate if the data followed a Gaussian distribution, the D'Agostino and Pearson omnibus normality tests were performed. If the raw data passed the normality test, a t-Student test (2 groups) or one-way ANOVA (more than two groups) were used to compare samples. If data did not follow a normal distribution, the non-parametric Mann-Whitney (2 groups) or Kruskal-Wallis test, followed by Dunn's multiple comparisons test (more two groups), was used to evaluate significant differences between samples. Statistical significance was considered when $p < 0.05$ (* - $p < 0.05$; ** - $p < 0.01$).

4. Results

4.1 TNF- α stimulation increases expression of pro-inflammatory markers in macrophages

To determine macrophage polarization, the expression levels of pro-inflammatory (TNF- α and IL-1 β) and anti-inflammatory (TGF- β) genes was evaluated after 6 and 72h of macrophages stimulation with LPS, TNF- α or IL-4. LPS was used as positive control to assess pro-inflammatory genes expression in the early time point.

Results show that TNF- α levels increase in TNF- α -treated macrophages from 4 out of 5 donors when compared with control group after 6h of stimulation. In contrast after 72h, TNF- α remains unchanged. The results also show that TNF- α is significantly downregulated after 6h of polarization of macrophages with IL-4, compared with control (FC=0.70 $\pm$ 0.17, p=0.04) (Figure 15). After 72h, the TNF- α expression levels show a tendency to be decreased in 4 out 5 donors in IL-4 treated macrophages, compared with control. Analysis of the expression of an additional pro-inflammatory gene, IL-1 β , shows that after 6h of macrophage stimulation with TNF- α , there is an increase (although not statistically significant) in IL-1 β expression relative to the control, in all 5 donors. The expression of this gene also increases after 72h. Moreover, treatment with IL-4, decreases the expression of IL-1 β compared to the control group after 6h and 72h (Figure 15).

Next, we analyzed the anti-inflammatory gene TGF- β . No significant differences are found for the expression of this gene when macrophages are stimulated with TNF- α or IL-4, except for the decrease following 6h of LPS stimulation (FC=0.56 $\pm$ 0.21, p=0.02), (Figure 15).

Together, these findings suggest that macrophage stimulation with TNF- α led to an increased expression of TNF- α and IL-1 β .

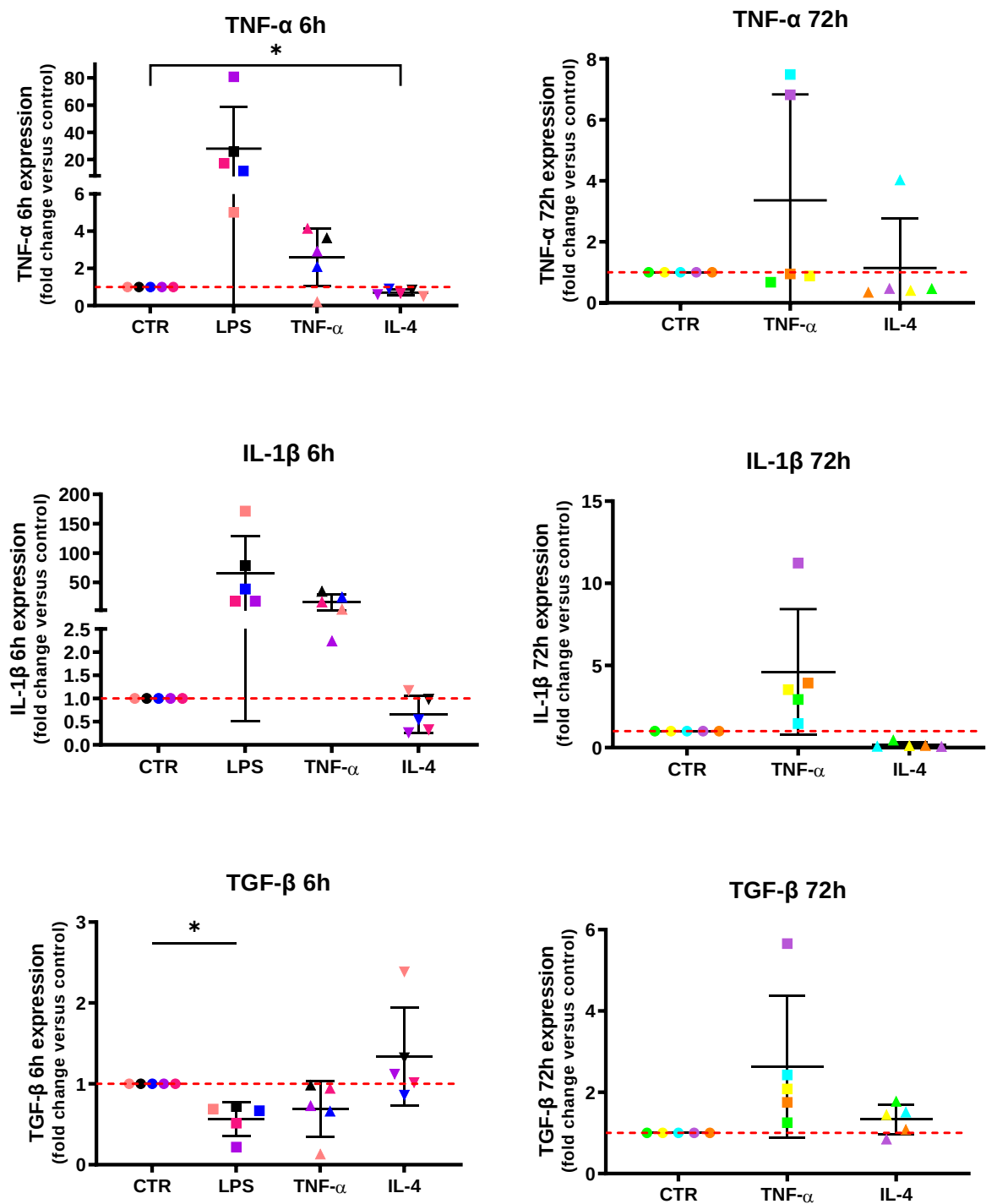


Figure 15. Expression of inflammatory genes. Human primary monocytes were differentiated into macrophages for 7 days and then stimulated for 6h or 72h with LPS, or TNF-α, or IL-4. Gene expression was determined by RT-qPCR.

4.2 TNF- α -induced macrophages secrete IFN- γ , IL-1 β , IL-10, IL-12p40 and IL-13

IL-1 β secretion was analyzed by ELISA. In non-stimulated macrophages (M0), IL-1 β protein levels could not be detected in the secretome. On the other hand, in 5 out of 6 donors, IL-1 β protein levels were detected in TNF- α stimulated macrophages, as shown in Table 2.

Table 2. TNF- α treated macrophages secrete IL-1 β . Human primary monocytes were differentiated into macrophages for 7 days and then stimulated for 6h with TNF- α . Levels of IL-1 β were quantified by ELISA in macrophages conditioned media. Nd (Non-detected).

	IL-1 β _{CTR} (pg/mL)	IL-1 β _{TNF-α} (pg/mL)
Donor 1	Nd	Nd
Donor 2	Nd	3.32
Donor 3	Nd	4.60
Donor 4	Nd	2.13
Donor 5	Nd	3.05
Donor 6	Nd	1.73

To expand the panel of cytokines analyzed, the Human Cytokine Proinflammatory Focused 15-Plex Discovery Assay (Eve technologies) was used, specifically levels of GM-CSF, IFN- γ , IL-1, IL-1RA, IL-2, IL-4, IL-5, IL 6, IL-8, IL-10, IL-12(p40), IL-12(p70), IL-13, MCP-1, and TNF- α were quantified. In order to accomplish this, supernatants from control macrophages (no stimulation) and supernatants from macrophages that had been activated by TNF- α for 6h were tested, in three distinct donors.

Results show that the concentration of IFN- γ (Conc=3.24 $\pm$ 0.81), IL-10 (Conc=669.1 $\pm$ 353.2), IL-12(p40) (Conc=6.74 $\pm$ 1.07) and IL-13 (Conc=4.51 $\pm$ 0.83) increase in the supernatants of macrophages that had been stimulated with TNF- α (Figure 16), compared with the supernatants from control macrophages.

Results also show that the concentration of TNF- α is increased in the supernatants of macrophages that had been stimulated with TNF- α (Annex 1- Supplemental Figure 1). However, we cannot exclude the hypothesis that this increase is caused by the recombinant protein added to the cells rather than increase in secretion.

Results show that GM-CSF was not detected in the conditioned media tested. Cytokines IL-2, IL-4, IL-5 and IL-12p70 were present in the conditioned media derived from TNF- α activated macrophages and from control macrophages but in minimal concentrations (extrapolated data, sample's signals were outside of the standard curve). The results also revealed that IL-1, IL-1Ra, IL-6, and IL-8 were present in all the tested conditioned media. However, their levels do not follow the same pattern for the 3 donors. In the case of MCP-1, the concentration values are above of the detection limit, indicating the possibility of saturation (Annex 1- Supplemental Figure 1). Consequently, a dilution should be required to distinguish sample trends.

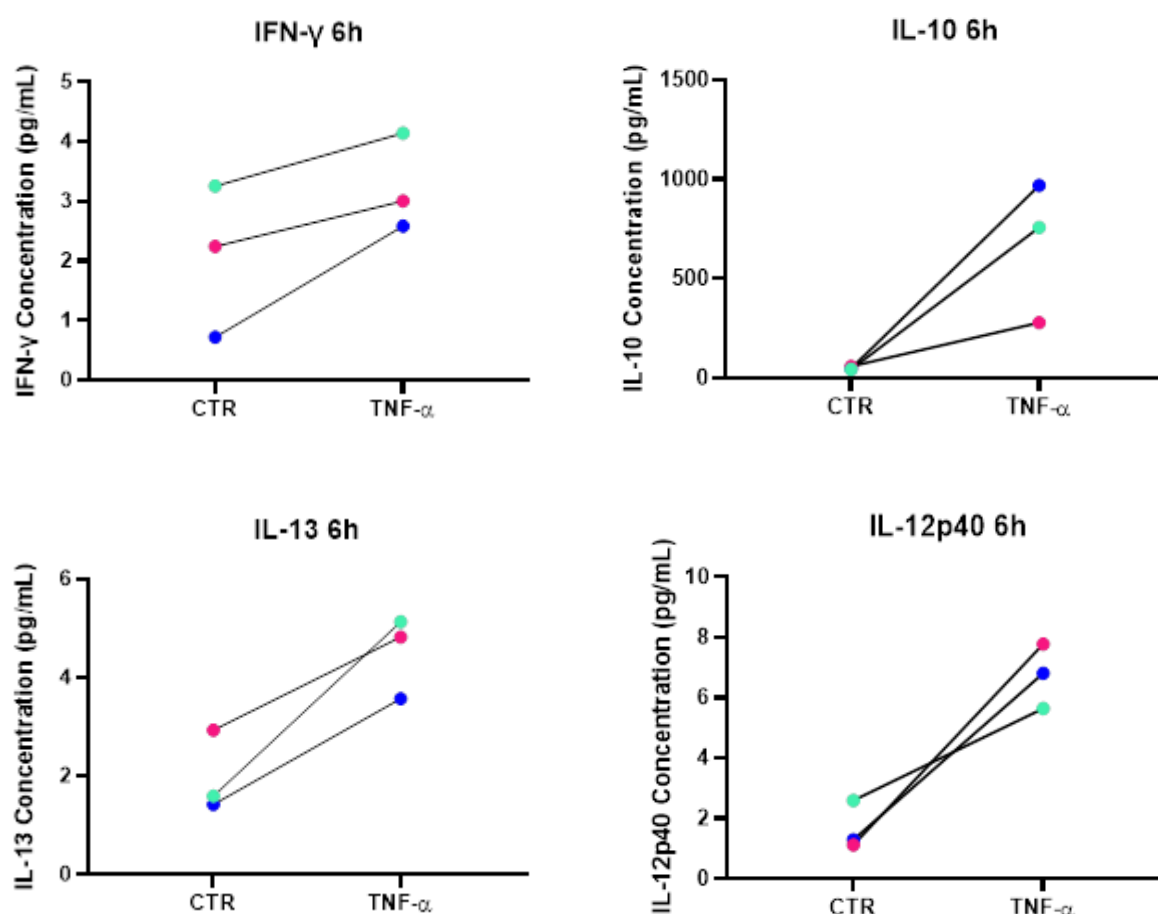


Figure 16. TNF- α -induced macrophages increase the secretion of IFN- γ , IL-10, IL-12p40 and IL-13. Human primary monocytes were differentiated into macrophages for 7 days and then stimulated for 6h with TNF- α . The levels of cytokines in the conditioned medias of control group and TNF- α -stimulated macrophages were quantified by Human Cytokine

Proinflammatory Focused 15-Plex Discovery Assay. Concentration of IFN- γ , IL-10, IL-13 and IL-12p40 released by TNF- α -stimulated macrophages after 6h (mean $\pm$ SD, n=3, Mann-Whitney test).

4.3 miR-195 is down-regulated in TNF- α -treated macrophages

Next, the effect of TNF- α treated macrophages on inflammiRs was assessed. To achieve this, miR-16, miR-195, miR-342 and let-7d were selected based on their inflammatory and osteogenic potential, according to previous studies and microarray data [180].

Results show that the expression levels of miR-16, miR-195, miR-342 and let-7d do not significantly change after 6h of macrophage stimulation with LPS, TNF- α , or IL-4, compared with the control group (Figure 17). On the other hand, after 72h of stimulation, miR-195 is significantly decreased in TNF- α -treated macrophages versus control group (FC=0.61 $\pm$ 0.23) (Figure 17). When macrophages were treated with TNF- α for 72h, results show a decrease in miR-16, miR-342 and let-7d expression, but with no statistical significance (Figure 17).

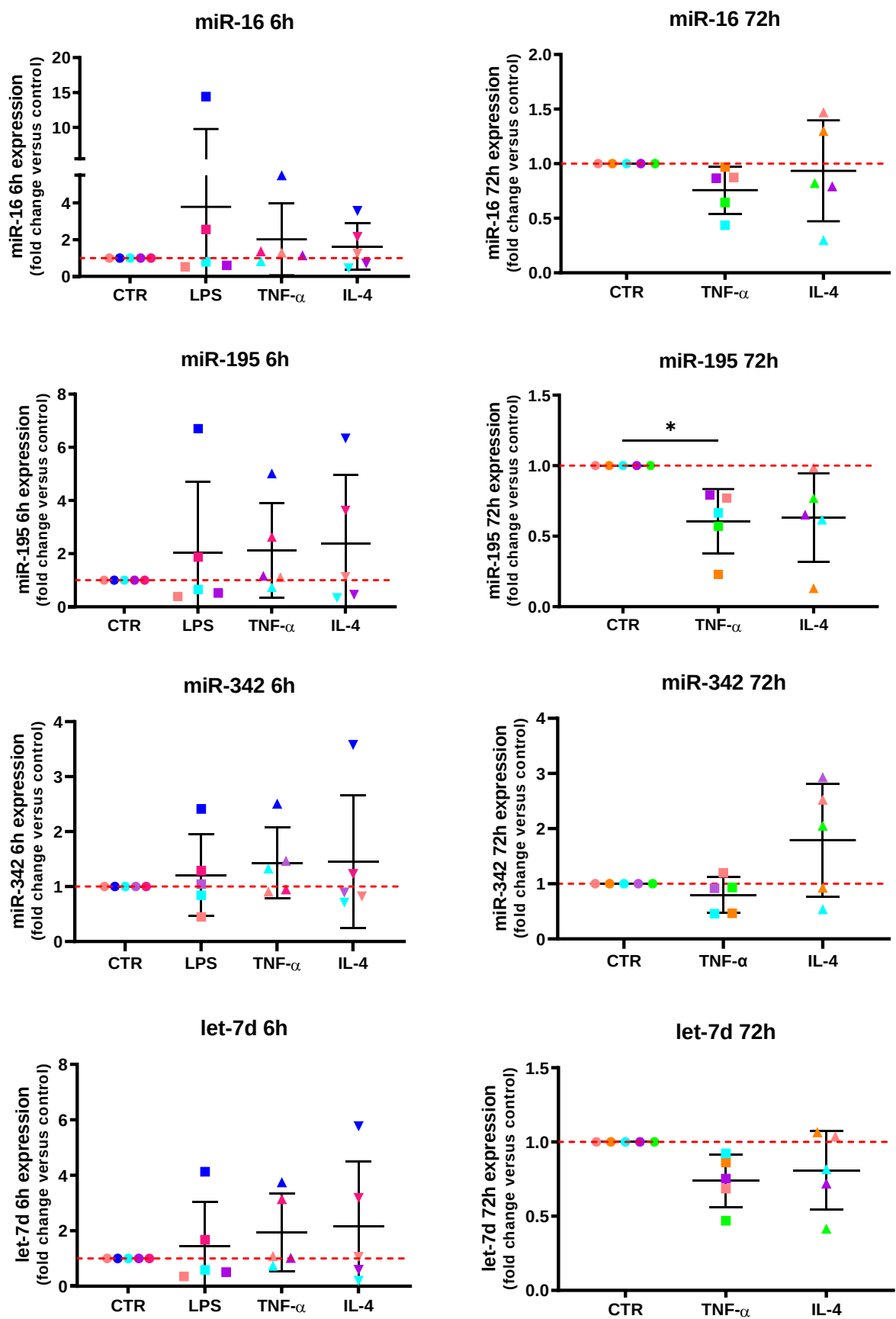


Figure 17. Expression of inflammiR. Human primary monocytes were differentiated into macrophages for 7 days and then stimulated for 6h or 72h with LPS, or TNF- α , or IL-4.

4.4 Conditioned media from TNF- α -treated macrophages does not impair MSCs migration

To test how TNF- α -treated macrophages influenced MSC recruitment, transwells assays were performed using the macrophages conditioned media.

Firstly, the migration protocol was optimized to determine the optimal time point to quantify MSC migration. Three different time points were initially tested using FBS as a chemoattractant. The percentages of MSC migration rate (stained area) are 18.62 %, 33.16 % and 54.45% after 6h, 12h and 18h, respectively (Figure 18). To avoid either excessive migration or reduced number of migrated cells, 12h was selected as the optimal time-point.

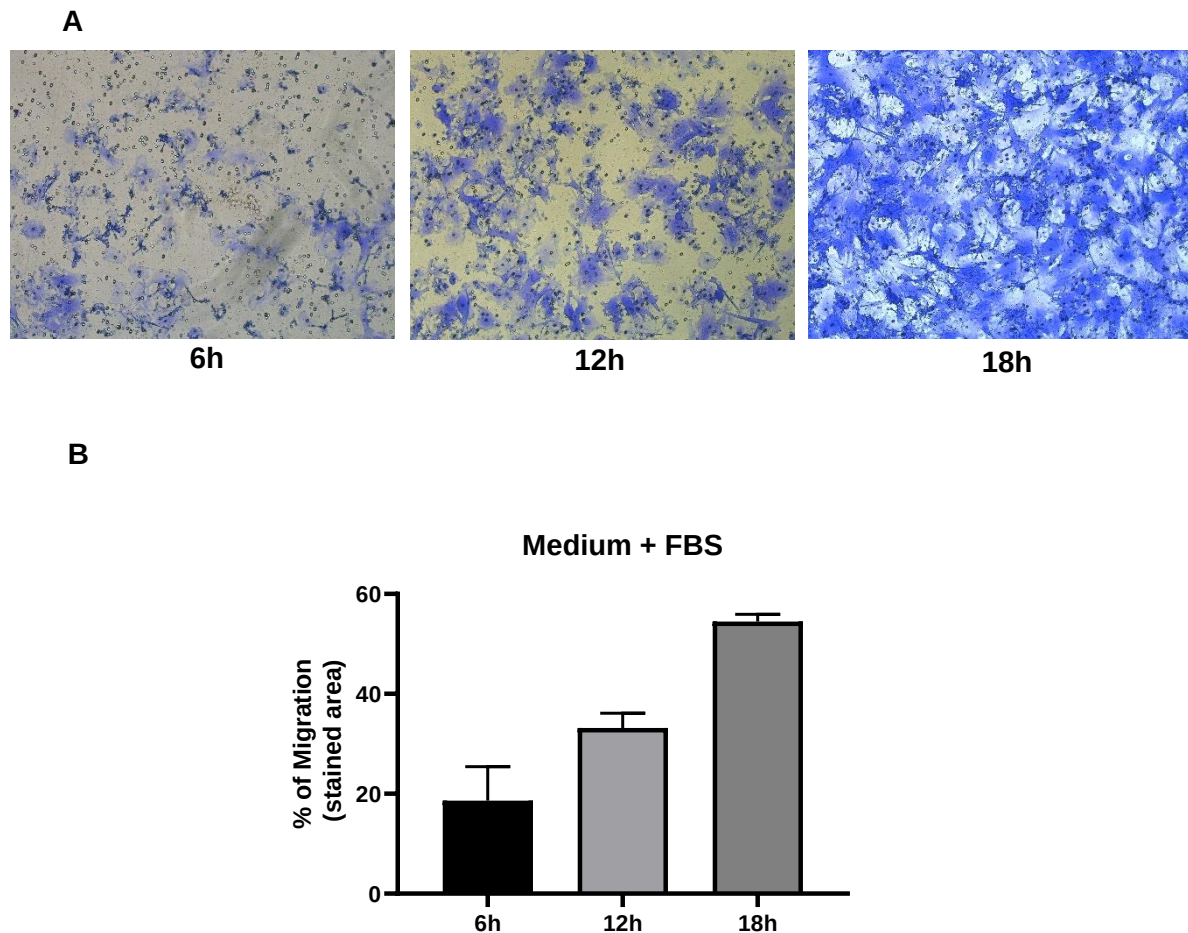
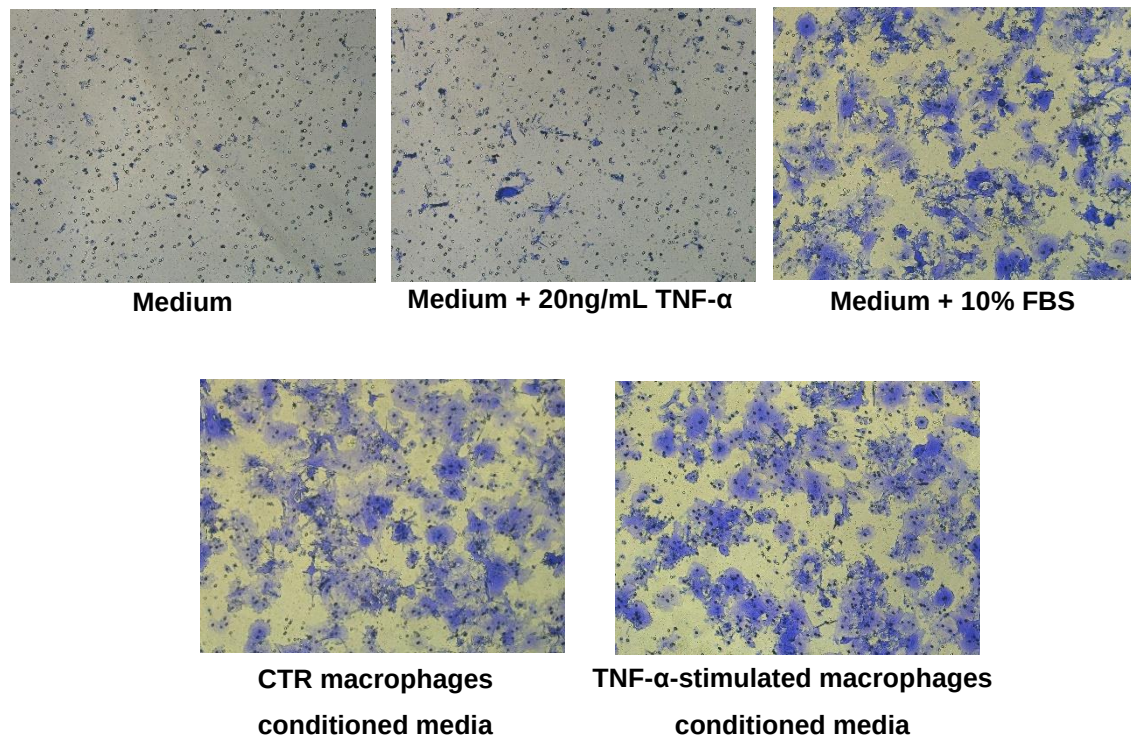


Figure 18. Optimal time point for human mesenchymal stem/stromal cells migration. **A** Microscope images representing migrated MSCs stained with Crystal Violet and allowed to migrate for 6 (left), 12 (middle) and 18 hours (right). **B** Percentage of MSCs migration. FBS was used as a chemoattractant.

Next, conditioned media of macrophages treated with TNF- α for 6h was collected and used as a chemoattractant for MSC. Results show no difference in MSC migration between conditioned media derived from TNF- α -treated macrophages versus control

macrophages (Figure 19). Also, similar MSC migration levels were found when using complete media that was not in contact with the macrophages. Therefore, results suggest that macrophages stimulation with TNF- α does not change MSC migration.

A



B

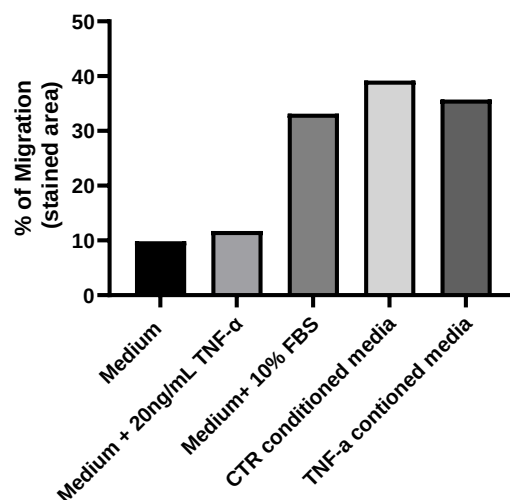


Figure 19. Migration of human mesenchymal stem/stromal cells. **A** Microscope images representing migrated cells stained with Crystal Violet. MSCs were allowed to migrate for 12h with medium, medium + 20ng/mL, medium + 10% FBS, CTR macrophages conditioned media and TNF- α -stimulated macrophages conditioned media (left to right). **B** Percentage of MSC migration after 12h.

4.5 Optimization of gene delivery by CaP particles

To assess the efficacy of a transfection protocol that could be used in alternative to the conventional lipofectamine method, monocytes were isolated and transfected with calcium phosphate (CaP) particles or CaP particles + siGLO Red. After 24h and 72h of transfection microscope images were taken. siGLO red is a fluorescent oligonucleotide duplex that localizes to the nucleus, allowing for visual evaluation of mammalian cell transfection. Fluorescence microscope images show that at both time points, neither non-transfected cells nor cells transfected with CaP particles display fluorescence. However, cells transfected with CaP particles + siGLO red show fluorescence (Figure 20). The detection of siGLO red in the macrophages at both 24h and 72h demonstrate that the protocol is effective at both time points.

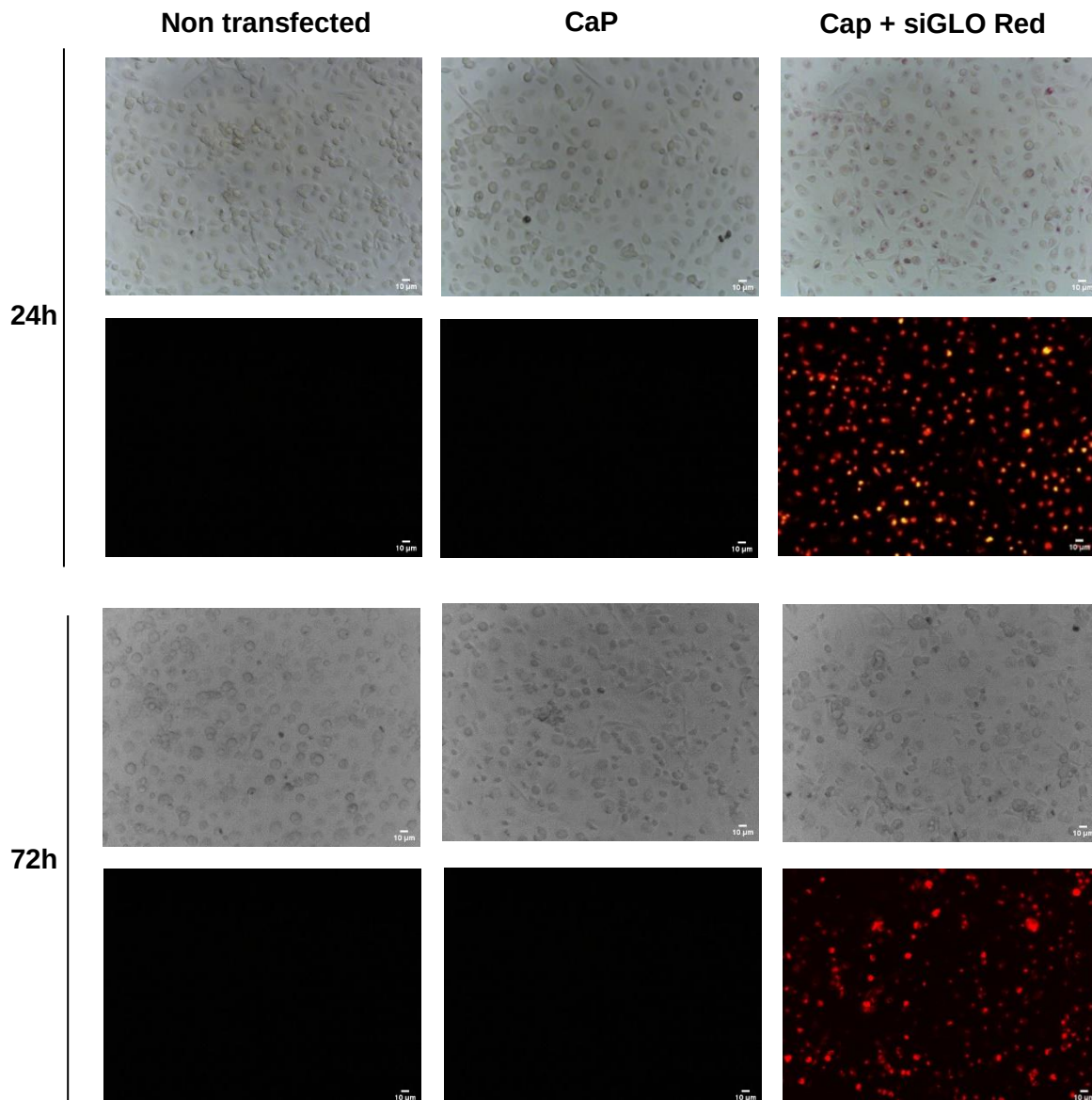


Figure 20. Detection of transfection efficiency. Monocytes were isolated and transfected with CaP particles (middle) or CaP particles + siGLO Red (right) at two time points, 24h (up) or 72h (down).

4.6 miR-195 overexpression in macrophages

Based on the results from section 4.3 and 4.5, miR-195 was used as a candidate to be delivered into macrophages, and a scramble sequence was used as a negative control.

Figure 21 shows that when miR-195 mimic was transfected into cells, miR-195 expression was increased relative to the control group (10142 ± 11330). Therefore, miR-195 delivery was efficient.

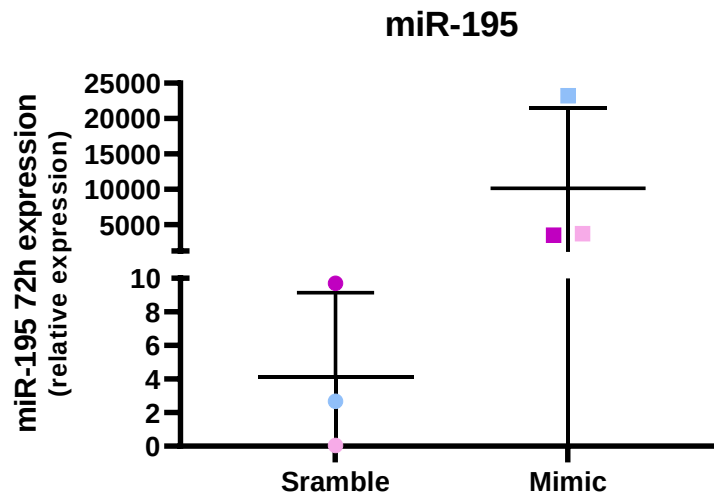


Figure 21. Expression profile of miR-195. Human primary monocytes were isolated and, after 3 days, were transfected with the miR-195 mimic or with the scramble control (SRC). After 72 hours of transfection, the expression of miR-195 was evaluated by RT-qPCR (mean $\pm$ SD, $n=3$, Mann-Whitney test). Results were normalized with U6 and are expressed in expression levels ($2^{-\Delta\Delta C_t}$) compared to CTR (Non-transfected macrophages) mean.

Macrophages were transfected with miR-195 and cells were stimulated with TNF- α for the following 72h. RT-qPCR analysis of miR-195 expression shows a significant increase in cells transfected with miR-195 mimic ($FC=8293 \pm 5574$, $p=0.0333$) compared with control group (TNF- α -treated macrophages, no transfection) and compared with SCR control. Cells transfected with SCR control show a slight increase in the levels of miR-195 compared with control group ($FC=1.99 \pm 1.48$, $p=0.0333$) (Figure 22).

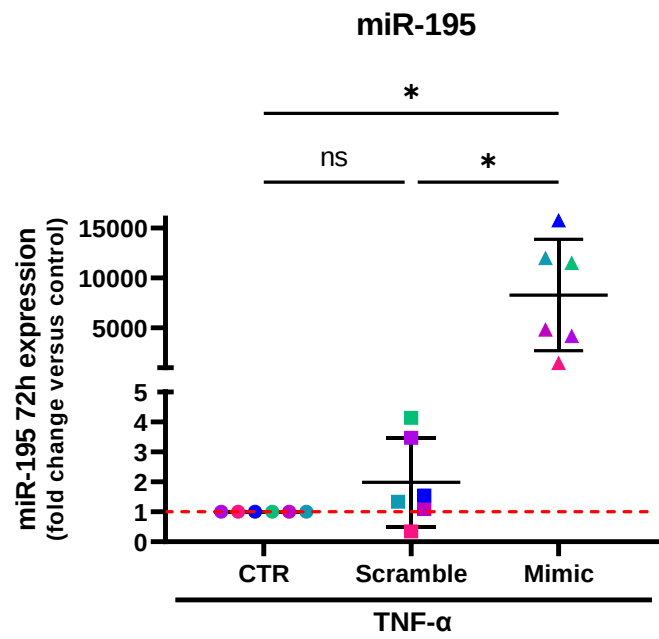


Figure 22. Impact of TNF-α on miR-195 transfection. Transfection efficiency for miR-195 mimic in macrophages compared with SCR and with control group. was evaluated by RT-qPCR. Small nuclear RNA U6 was used as reference gene (mean ± SD, n=6, one-way ANOVA, * p < 0.05). CTR (Non-transfected macrophages).

5. Discussion

Bone healing after injury is an important biological and biomechanical process [181]. Inflammatory cells, like macrophages, have a critical role in this process communicating with BM-MSCs via proinflammatory and anti-inflammatory cytokines, such as transforming growth factor, bone morphogenetic proteins, and growth factors to stimulate osteogenesis and angiogenesis [29]. This results in a reparative granuloma that serves as a template for bone growth [30]. As such, modulating macrophage function to promote bone regeneration is an exciting therapeutic prospect. miRNAs can modulate the magnitude of an inflammatory response, by changing expression levels of cytokines, surface receptors and other inflammation-related molecules, which will have consequences for macrophage phenotype, accelerating bone repair, particularly in cases of non-union [182].

Due to the importance of macrophages in bone regeneration, we started by isolating monocytes from blood donors, differentiating them into macrophages and stimulating with TNF- α to polarize the cells and understand how this mechanism could be engineered. The use of TNF- α can be explained by the fact that this cytokine acts during the first phase of the inflammation process. Macrophages are capable of polarizing into distinct functional phenotypes in response to environmental stimuli. Although the length of stimulation affects the expression of the majority of markers commonly used to evaluate M1 and M2 polarization, there is no consensus in the literature regarding the optimal time to examine specific markers during *in vitro* studies [183]. Importantly, this study was entirely performed using human primary cells, avoiding the use of cell lines. Primary monocytes/macrophages provide outcomes that are more similar to natural biological mechanisms than cell lines [184]. When cultured for short periods, primary cells preserve normal morphology, cellular function, growth characteristics, cellular markers, signaling, and genetic integrity, better replicating what occurs *in vivo* [185]. In this thesis, macrophages were induced to differentiate into M1 macrophages by stimulation with LPS or TNF- α for 6h or 72h, since this polarization stimulates the production of pro-inflammatory cytokines such as TNF- α , IL-1, IL-6, IL-12, and IL-23 [186]. As expected, our findings show an increase in TNF- α and IL-1 β expression at the transcription level in response to LPS and TNF- α stimulation, with this increase becoming more pronounced after 6h of stimulation. The ideal stimulation time for optimal TNF- α and IL-1 β gene detection, according to the literature, is 4-8 hours and 4-72 hours, respectively [183].

IL-4 was used to modulate macrophages into anti-inflammatory phenotype. TGF- β induces a M2-like phenotype characterized by up-regulation of the anti-inflammatory

cytokine IL-10, and down-regulation of the pro-inflammatory cytokines TNF- α and IL-12 [187]. At both time points, 6h and 72h, we observed a modest increase in TGF- β expression, which can be explained by the fact that this gene is more strongly stimulated by other molecules, such as IL-10, TLR agonists, or adenosine receptor activation [188].

Conditioned media produced from TNF- α activated macrophages was investigated to demonstrate macrophage polarization in an M1 phenotype after 6h of stimulation. Several cytokines were found to be secreted into the environment during this assessment. Among them, pro-inflammatory cytokines such as IL-1 β , IFN- γ , and IL-12p40 [189] were increased in conditioned media of TNF- α -stimulated macrophages. However, IL-10 and IL-13 were also increased, which contradicts prior findings since these anti-inflammatory cytokines are produced by M2 macrophages [188]. However, since macrophages are plastic cells, it is possible that a stimulus caused the phenotypic change from M1 to M2 in some macrophages and, as a result, anti-inflammatory macrophages present secreted these cytokines.

MSCs are multilineage cells that can self-renew and differentiate into a variety of cell types [190]. These cells are one of the most promising prospects in regenerative medicine, and their use might help in the repair and regeneration of skeletal disorders through homing, angiogenesis, differentiation, and inflammatory response [191]. The most prevalent stem/stromal cells used in cell therapy and tissue engineering are BM-MSCs. Nonetheless, throughout the healing process, BM-MSCs must migrate from bone marrow to injured tissues via peripheral circulation. Mechanical and chemical factors influence the migration of BM-MSCs in this trafficking process [192]. Therefore, next in this study, we investigated if TNF- α -treated macrophages influenced MSC recruitment. Results suggest that macrophage stimulation with TNF- α does not change MSC migration. As we have shown, conditioned media of macrophages stimulated with TNF- α increased secretion of IL-1 β , IFN- γ , IL-12p40, IL-10 and IL-13 and TNF- α . It is well known that IL-1 β , TNF- α , and IL-10 are major promoters of MSC migration [193-195], so the discrepancy between the results obtained and those previously reported may be due to differences in the exposure duration to these cytokines as well as the concentrations of cytokines secreted by the cells [196]. These differences are related to the functional repolarization of macrophages which is necessary for the inflammation process and their subsequent resolution.

miRNAs have essential functions in the development of immune system cells [197]. As previously mentioned, macrophages may exhibit distinct phenotypes based on the

cytokine milieu they encounter. A recent study tested the gene expression levels of each macrophage subpopulation to determine how these differentially expressed miRNAs affect the immunological capabilities of polarized macrophages [198]. For this reason, we evaluated the effect of TNF- α treated macrophages on inflammiRs.

Downregulation of miR-16 has been identified in a variety of cancers, including chronic lymphocytic leukaemia, prostate cancer, multiple myeloma, pancreatic cancer, ovarian cancer, malignant melanoma, colorectal cancer, and urinary bladder cancer [199]. Previous research has consistently identified many targets for miR-16, including BCL2, CCND1, and WNT3A, all of which are involved in tumor cell death or cell-cycle control, and hence directly affect tumor development [200-202]. However, little is known about the role of miR-16 in macrophage polarization and the possible targets engaged in this process. Our findings revealed that after 6h of polarization with TNF- α , miR-16 expression did not significantly change, and that after 72h, miR-16 expression decreased but with no statistical significance. Therefore, there is a need to increase the number of donors to confirm these results since there is great variability between donors. However, these findings contradict prior findings that miR-16 triggers M1 polarization [203].

The mature members of the let-7 family are the most abundant miRNAs in the cell and are controlled by many transcriptional and post-transcriptional processes. MiRNAs from the same family have the same seed sequence, hence members of the let-7 family may have overlapping targets [204, 205]. Due to the control of the cell cycle, proliferation, and apoptosis pathways, the let-7 family has multifaceted implications in cancer. In addition, the let-7 family affects differentiation and epithelial-to-mesenchymal transition (EMT) [206-208]. Cancers such as prostate, pancreatic, head and neck, primary pigmented nodular adrenal dysplasia, breast, ovarian, kidney, bladder, and retinoblastoma have dysregulated let-7d expression [209]. Our results showed that after 6h of polarization with TNF- α the expression of let-7d did not significantly change and that after 72h of polarization the expression decreased but with no statistical significance. Thus, it may be assumed that let-7d induces an M2 phenotype in macrophages, following the same pattern as miRs from the same family [210, 211].

Multiple cancers have been shown to have aberrant miR-342 expression, and downregulating or epigenetically silencing of miR-342 expression may cause the production of certain important oncogenic proteins [212]. In this thesis, miR-342 expression was evaluated in TNF- α -stimulated macrophages. Results show that miR-342 expression doesn't change in TNF- α treated macrophages after 6h of stimulation. Results in contrast with Bras et al that found that miR-342 was upregulated in primary

rat microglia activated with TNF- α [213]. However more experiments are needed to confirm these results.

Lastly, the expression of miR-195, which play a role in cancer, by inhibiting cell cycle progression and proliferation of different cancer cell types [214, 215], was also evaluated. miR-195 expression was decreased significantly in TNF- α -macrophages, indicating that miR-195 induces an anti-inflammatory phenotype in macrophages. These results are corroborated by Bras et al that described that miR-195 levels are increased in IL-10-treated human primary macrophages and also showed that miR-195 inhibits mechanisms that promote M1 macrophage polarization in THP-1 [216].

Finally, the effectiveness of the Cap-miRNA transfection protocol was assessed. Fluorescence microscope images demonstrated that the protocol was effective at both time points that were tested. The protocol used in this thesis has several advantages compared with other gene delivery methodologies. For instance, viral vector-based technology has several drawbacks, such as the possibility of insertional mutagenesis after viral integration and the unfavorable immunological reactions seen in patients [217, 218]. The lengthy and labor-intensive technique required to produce such viruses presents additional issues and limits their use in tissue engineering [219]. Alternatively, non-viral methods are presently used to preserve miRNA and improve delivery, such as nanoscale miRNA complexes with Lipofectamine® 2000, PEG (polyethyleneglycol), or PEI (polyethyleneimine) [220]. However, the detergent action of lipid vectors associated with these approaches might cause cell membrane damage, which restricts their clinical application [221]. Therefore, the strategy that we used has many advantages. Due to their chemical similarity to bone mineral, CaPs are the most extensively used bone substitutes in bone tissue engineering [222]. In addition, calcium phosphate nanoparticles have unique features that make them desirable as RNA delivery vectors. Calcium phosphate has a strong propensity for binding to a variety of molecules, including RNA, through electrostatic interactions between Ca²⁺ in the CaP carrier and phosphate groups in the RNA structure [223]. Calcium phosphate is efficiently endocytosed by cells via the lipid bilayer cellular membrane and dissolves in the acidic environment of endosomes and lysosomes, resulting in the release of nucleic acid in the targeted cell location [224]. In addition, calcium phosphate nanoparticles have shown superior cytocompatibility in comparison to Lipofectamine® 2000, indicating a greater *in vivo* applicability [225]. This approach is typically used for DNA delivery, but it is beginning to be utilized for siRNA delivery [226-228].

In the future we plan to use 3D methods, using collagen nano-hydroxyapatite scaffolds [220, 221]. As hydroxyapatite is a calcium phosphate mineral, it possesses many advantages for use as a gene delivery vector, principally in bone. As one of the primary components of bone, HA can raise the concentration of local Ca^{2+} , hence activating the proliferation of osteoblasts and promoting the growth and differentiation of MSCs [229]. Collagen is widely employed as a scaffold biomaterial owing to its biocompatibility and biodegradability [230-233].

6. Conclusions and Future Perspectives

In conclusion, the results of this thesis show that TNF- α -treated macrophages increase the expression of pro-inflammatory genes, but their secretome does not change MSC migration.

Also, we found that miR-195 is significantly decreased in TNF- α -treated macrophages when compared with untreated macrophages.

Finally, we show that the calcium/phosphate transfection method is efficient for the delivery of miRNA (specifically miR-195) into macrophages.

Future studies should address:

- TNF- α expression in macrophages transfected with miR-195 mimic to evaluate potential feedback loop mechanisms.
- The impact of miR-195 transfection on downstream molecular targets.
- Increase the number of donors and evaluate the impact of miR-342 transfection on primary macrophages.
- Test cytotoxicity in miRNA transfected macrophages.
- Implement the CaP-miR delivery in 3D scaffolds for future *in vivo* bone repair studies.

7. References

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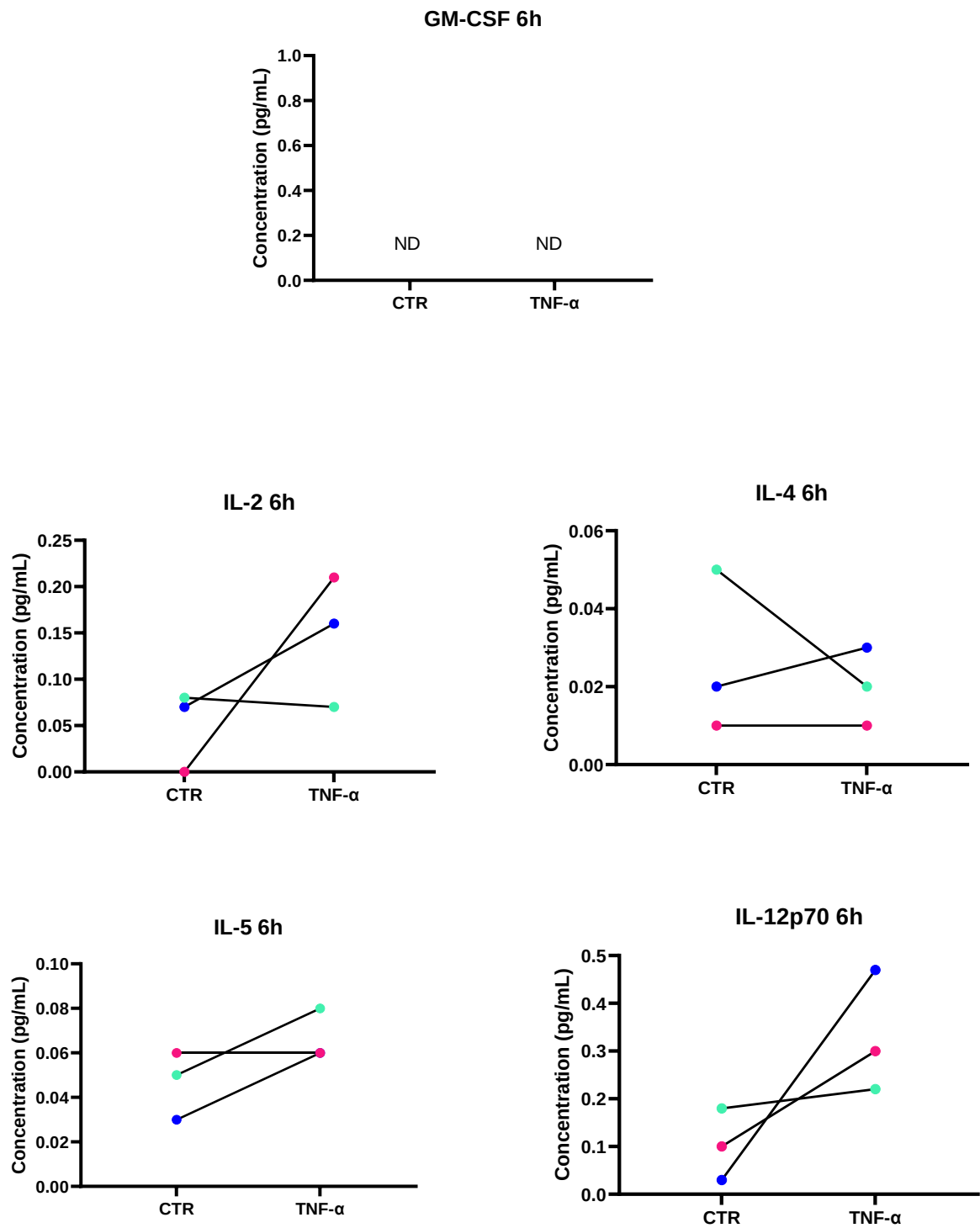
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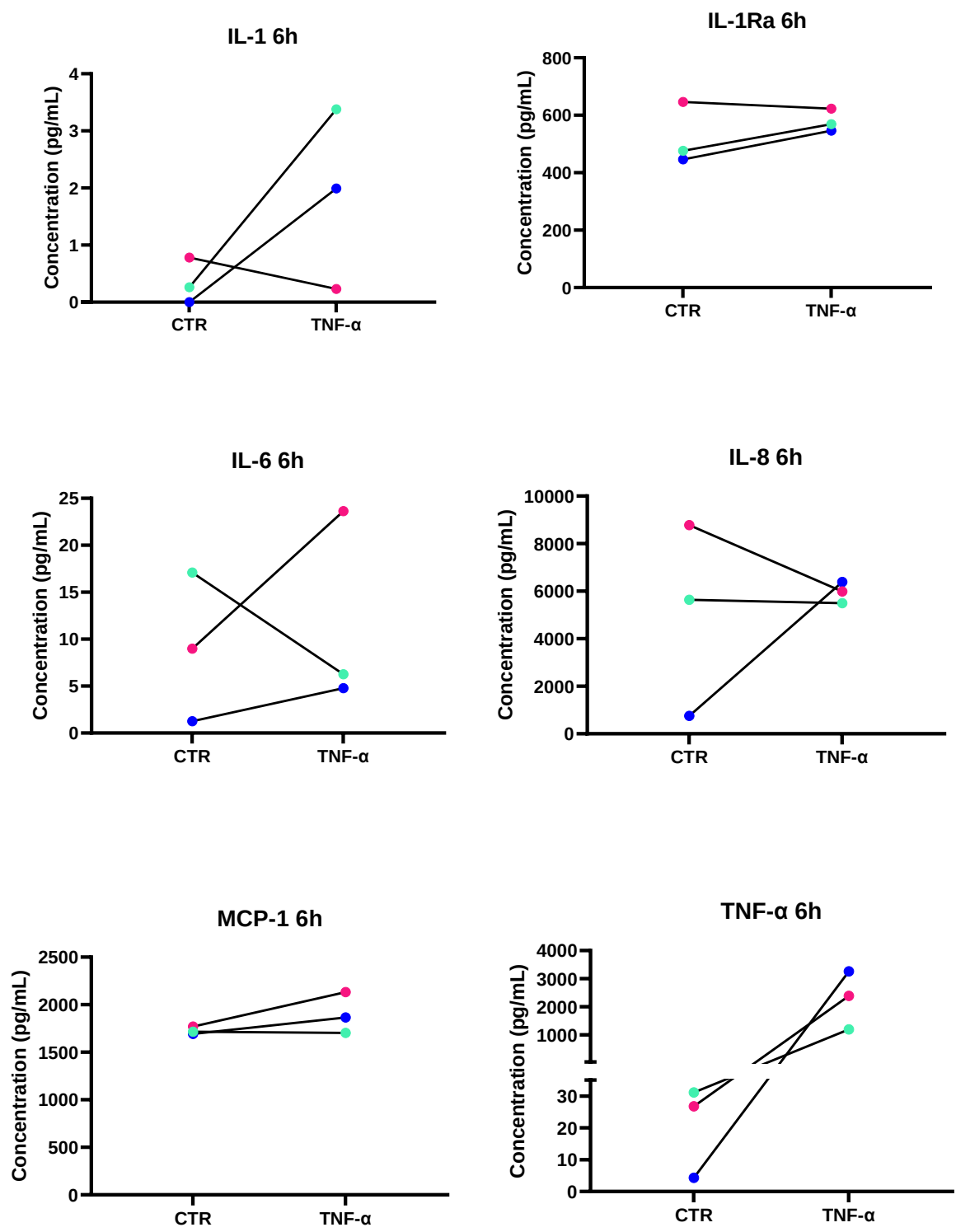
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8. Annexes

Annex 1





Supplemental Figure 1. Cytokines levels in the conditioned media of TNF-α-stimulated macrophages tested using multiplexing laser bead technology. Human primary monocytes were differentiated into macrophages for 7 days and stimulated for 6h with TNF-α. The levels of cytokines were quantified by Human Cytokine Proinflammatory Focused 15-Plex Discovery Assay (ND: not detected).