Differential macrophage polarization promotes entheseal bone formation in AS

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Abstract

Systemic and focal bone loss is a common feature of many forms of arthritis. However, inappropriate new bone formation also occurs in inflammatory arthritis, including ankylosing spondylitis (AS), osteoarthritis, and rheumatoid arthritis. These features are important signs of systemic inflammation in AS.

In general, inflammation is thought to inhibit bone-forming osteoblasts whilst promoting the formation and activation of osteoclasts. More recently, macrophages have been recognized as another component of the bone formation unit. Macrophages (Mϕs) are actively involved in tissue remodelling and repair but also in tissue damage and fibrosis. Our research group demonstrated that the interaction between mesenchymal stem cells and monocyte/macrophages leads to an activation of STAT3 that drives osteoblast differentiation.

This project objective is to better explain the contribution of monocyte/macrophages to bone formation in a murine model of AS; the 1, 3 β-glucan (curdlan) challenged SKG mice which develop many features of AS including psoriasis, ileitis, enthesitis and arthritis. *In vitro*, the role of macrophages as regulators of osteogenic differentiation via direct cell contact with mesenchymal stromal cells (MSC) was investigated. OB differentiation was shown to depend upon contact with pro-inflammatory Mϕs rather than anti-inflammatory macrophages.

In conclusion, in the *in vitro* part, we evaluated the capability of GM-CSF Mϕs (pro inflammatory) to increase MSCs - OB differentiation using two different genetic strains of mice: SKG and IRF 5 (WT and KO). We assessed that both SKG - GM-CSF Mϕs and IRF 5 WT- GM-CSF Mϕs could synergize with, but not replace, osteogenic signals in the medium and enhancing MSCs capability towards OB differentiation. However, IRF KO - GM-CSF Mϕs showed no effect on MSCs.

Moreover, In the *in vivo* study, we evaluated the occurrence of enthesitis and the role of Mϕs in a murine model of AS (SKG). We assessed Mϕs activity at the entheseal site by depleting them using clodronate liposomes, which ameliorated the bone phenotype but not the clinical score (all the mice developed severe AS). Previous work in the laboratory showed that Mϕs promoted MSC osteogenic differentiation via oncostatin M (OSM). Therefore, OSM was blocked in SKG mice by injecting OSMR–Fc directly into the entheseal site. This led to the amelioration of ileitis however there was a dramatic exacerbation of inflammation and bone disease in the paws of the mice indicating that OSM is not a direct mediator of Mϕ-induced bone formation at the enthesis *in vivo*.

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Declaration of Originality

I hereby declare that all work described in this thesis is my own original work unless otherwise stated. Where the work of others has been used, it has been duly acknowledged.

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List of Abbreviations

ALP Alkaline Phosphatase

AS Ankylosing Spondylitis

CFU-F Colony forming unit-fibroblast

COX Cyclo-oxygenase

DMEM Dendritic cell Dulbecco's Modified Eagle Medium

FACS Fluorescence-activated cell sorting

FBS Foetal bovine serum

FLS fibroblast-like synoviocytes

IFN Interferon

IL Interleukin

iNOS Inducible nitric oxide synthase

LIFR Leukemia inhibitory factor

LPS Lipopolysaccharide

Mϕ Macrophages

MHC Major histocompatibility complex

GM-CSF Granulocyte macrophage colony stimulating factor

M-CSF Macrophage colony stimulating factor

MSCs Mesenchymal stem/stromal cell

OA Osteoarthritis

OB Osteoblast

OC Osteoclast

OPG Osteoprotegerin

OSM Oncostatin M

PBS Phosphate-buffered saline

PGE2 Prostaglandin E2

RA Rheumatoid Arthritis

RANK Receptor activator for nuclear Factor κ B

RANKL Receptor activator for nuclear Factor κ B ligand

Sca-1 Stem cell antigen-1

TNF α Tumour necrosis factor α

TRAP Tartrate-resistant acid phosphatase

T_H T helper lymphocyte

VCAM-1 Vascular cell adhesion molecule 1

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CHAPTER ONE

INTRODUCTION

1.1 Bone biology

Bone is a lively structure, which is continually being remodeled so that the skeleton can remain healthy. This is vital to ensure that skeletal functions occur efficiently throughout a person's lifetime. There are three crucial activities for bone: (i) It supports and acts as a site of connection for muscles; (ii) it protects critical organs including the brain and bone marrow; and (iii) it contains the main storage of calcium and phosphate, so can act as a metabolic organ. In every bone two important parts are identified: cortical, which is protective and has a mechanical function; and trabecular, which provides strength and, predominantly, most metabolic function. The trabecular part hosts the bone remodelling process, so it is the site of metabolic bone diseases, i.e. where diseases of bone remodel. The distribution of bone components is roughly as following: 10% cells, 35% organic matrix and 65% mineral crystals such as crystalline hydroxyapatite. The organic matrix is mainly constituted by type 1 collagen (89%); the other 10% includes proteins, while glycosaminoglycans and lipids represent the remaining 1–2%.

1.1.1 Bone remodelling and the cells of bone

During bone remodelling, as shown in (**Fig.1.1),** old or non-functional bone is physiologically eliminated by bone absorbing cells (osteoclasts - OC) and then replaced by new bone produced by bone forming cells (osteoblasts - OB)[2].

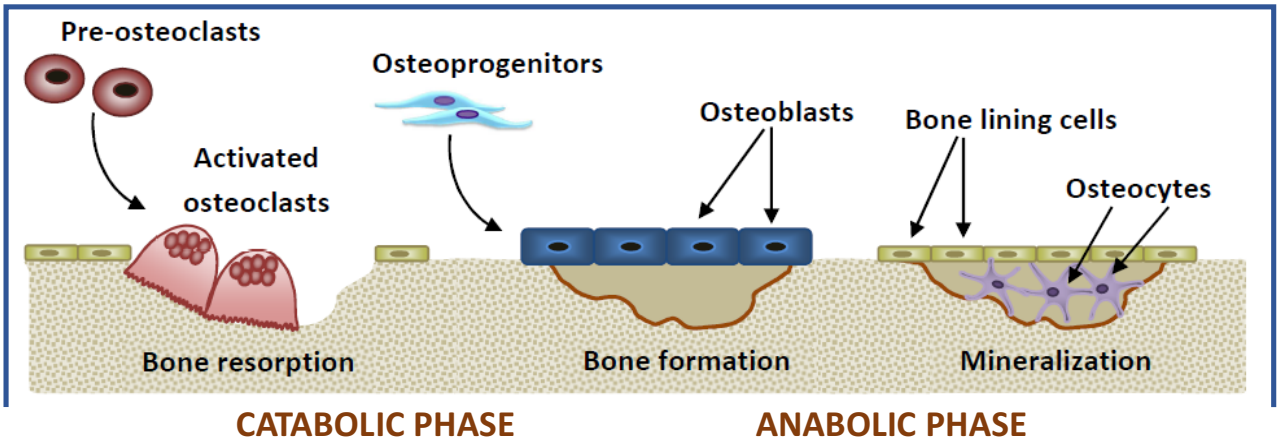


Figure 1.1: Schematic representation of bone dynamics and the cells of bone.
OB and OC cooperate together to maintain bone homeostasis. Bone remodelling consists of a series of sequential steps: bone resorption (catabolic phase) by OC is followed by OB migration to the bone resorption site and secretion of new osteoid. Mineralisation of osteoid leads to new bone formation in the anabolic phase. When OBs become part of the matrix they are known as osteocytes. Schematic adapted from Nicolaidou et al. (DPhil Thesis, May 2011).

From a molecular level, bone is removed and rebuilt constantly. The basis of many bone diseases is an unfavorable balance between bone formation and resorption, often caused by an exaggerated resorption. Resorption is achieved by OC; which are differentiated multinucleated cells derived from hematopoietic progenitors, whilst bone formation is due to bone matrix secretion by OB.

OC are the only bone resorbing cells in the body and they come from the pluripotent stem cells residing in the bone marrow, which are capable of producing all the blood cells. They are similar features with the monocyte-macrophage lineage as they share the same myeloid precursor[3]. The formation of OC is reliant upon the ligand of the nuclear factor kappa-B receptor activator (RANKL), which has also been defined as the ligand of tumor necrosis factor superfamily member 11 (TNFSF11), by OB, fibroblasts and other cell of the immune system like natural killer and T cells. RANKL is a type II membrane protein that can also be released as a soluble form, and it signals via receptor activator of nuclear factor kappa-B (RANK) on the osteoclast precursor to induce OC formation[2]. RANKL was discovered in 1997 following the earlier discovery of osteoprotegerin (OPG) which behaves as "a decoy receptor for RANKL"[2].

According to the literature RANKL is expressed in numerous tissues such as: bone tissues (osteocytes, OBs and bone parenchyma[4]), T lymphocytes and lung[5].

Moreover, during embryonic development, RANKL mRNA has been identified in many other tissues: kidneys parenchyma, brain tissue, heart, musculoskeletal tissues and particularly in E15 chondrocytes[6]. In physiological conditions the expression of RANKL can be induced by different factors such as: Wnt ligands[7], LPS[8], TNF-a[9], glucocorticoids and TGF-b[10]. During the bone remodeling phase MSCs, OB and osteocytes are the main sources of RANKL for OC precursors[11].

During inflammatory bone conditions, high levels of RANKL and TNF trigger the formation of OCPs (osteoclast precursors) in the bone marrow and their circulation into the bloodstream, and so supporting their proliferation where inflammation takes place. In response to RANKL and TNF, OCPs differentiate into OC creating a self-fueled autocrine cycle where OCs increase in size and numbers and interplay with immune cells involved in the bone turnover[12].

The “ruffled border” is a specialized area of the OC cell membrane where bone resorption occurs. In this area, a series of cellular events take place as following: a tight lysosomal compartment is formed, mineral is solubilized by acidic pH and matrix is digested by proteolytic enzymes. According with the literature, calcitonin and bisphosphonates directly inhibit OC’s activity. More recently, Denosumab (monoclonal antibody to RANKL) has been used to prevent osteoclastic resorption in the treatment of osteoporosis and cancer-induced bone loss[13]. However other molecules, such as hormones and other agents, would possibly interfere with bone turnover acting on the OB or cells that are OB derived.

In the bone marrow, mesenchymal precursors can develop and differentiate into OBs, also known as bone-forming cells. Mostly known for their bone-forming function, OB are cubicle cells based in the bone surface and they constitute 4–6% of the overall bone cells population [14]. They share similar morphological features with protein-synthesizing cells, such as extended Golgi apparatus and a considerable rough endoplasmic reticulum, but also numerous secretory vesicles[15]. The molecular stimulus that push MSCs to differentiate towards the osteoblasts pathway needs specific genes to be expressed and several proteins to be produced such as: bone morphogenetic proteins (BMPs) and molecular components of the Wingless (Wnt) pathways[16]. After the formation of OB progenitors (expressing Runx2 and ColIA1) there is a proliferation phase in which OB progenitors exhibit alkaline phosphatase (ALP) activity, in this phase they are named as pre-osteoblasts[14].

There are two main steps in the synthesis of bone matrix by OB: secretion of organic matrix and mineralization of the newly formed organic matrix. During the first phase, OB produce mainly collagen proteins, such as type I collagen, but also proteins which are non-collagen derived (osteocalcin, osteonectin and osteopontin), and proteoglycans which form the organic matrix. Subsequently there are two further steps in the mineralization of bone matrix: the vesicular and the fibrillar phases[17, 18]. The vesicular phase occurs when OBs release from their apical membrane domain matrix vesicles, which are portions with different size from 30 to 200 nm, into the newly established bone matrix. Once matrix vesicles reach the bone matrix, they bind to proteoglycans and other organic molecules. Sulphated proteoglycans have negative charge, therefore they are able to capture calcium ions that are kept inside the matrix vesicles[18, 19]. When calcium and phosphate ions inside the matrix vesicles become oversaturated, the fibrillar phase starts. When these ions become supersaturated, matrix vesicles break down and causes the release of

hydroxyapatite crystals in the surrounding matrix. Finally, mature OB can take several development pathways: go through apoptosis or differentiate into osteocytes or bone-lining cells[20].

It has been shown that, besides OC and OB, osteocytes are key players within the bone remodelling process[21]. Indeed, osteocytes orchestrate the bone-remodelling cycle by interfering with OB and OC activity through the production of exogenous factors[22]. For example, osteocyte are stimulated by mechanical forces to release molecules with anabolic effect on bone such as PGE_2, prostacyclin (PGI_2), NO, and IGF-1 [23]. Conversely, in absence of mechanical forces, osteocytes are more likely to secrete factors which inhibit OB activity such as sclerostin and DKK-1[24], as well as specific factors that stimulate local osteoclastogenesis[25]. The *SOST* gene encodes for Sclerostin which is known to act as inhibitor of bone formation, because it blocks the activity of Lrp5 in OBs, a key receptor of the Wnt/*β*-catenin signalling pathway[26]. Evenity (romosozumab) a monoclonal antibody to sclerostin has completed Phase III clinical trials and is now in the process of FDA approval for the treatment of osteoporosis as a bone anabolic agent[27].

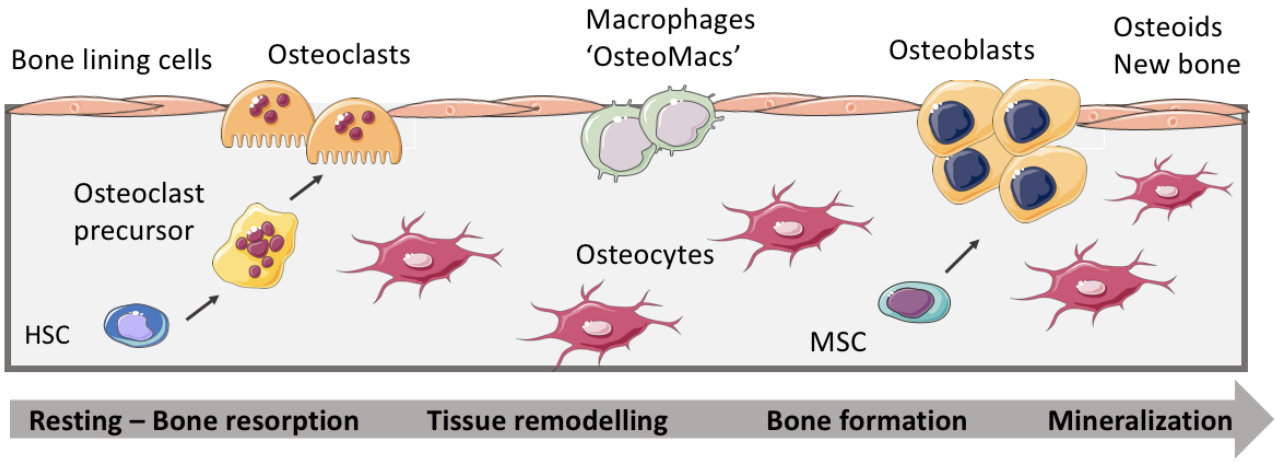


Figure 1.2: Anatomical location of cells of bone
Starting from the left side there are **Osteoclasts**, which derive from hematopoietic stromal cells (HSC) and drive bone resorption; **Osteomacs**, which are tissue resident Mϕs intercalated in osteal tissues between osteoclasts and osteoblasts, they maintain bone homeostasis; **Osteoblasts**, which derive from mesenchymal stromal cells (MSC), are key players in bone formation and mineralization; **Osteocytes**, which supports osteoblasts in the bone remodelling cycle.

1.1.2 Macrophages and Bone

Recent data from Chang et. al have highlighted the relevance of macrophages in bone homeostasis, remodelling as well as inflammatory situations due to their capacity of trigger healing processes after bone damage[28]. Moreover, other data suggest that macrophage interfere with both anabolic and catabolic stages of the healing process, leading to the concept they are actively involved in the fracture healing phases. Macrophages are heterogeneous cells which originate from the myeloid lineage, they develop from hematopoietic progenitors cells located in the bone marrow (BM)[29],[30]. They are "the chameleon cell of human organisms"[30] with vary and adaptive transcriptomes, they express several surface receptors such as growth factors, proteolytic enzymes and cytokines with both pro- and anti-inflammatory activity [30]. Due to their cellular plasticity and responsiveness macrophages have a dynamic phenotype. Indeed, they quickly respond to any molecular changes that occurs in the environment they are located. Given their multiple features and capabilities, classifying macrophages is quite challenging. Different macrophages subsets and phenotypes have been reported so far, therefore its classification is the subject of ongoing discussions[31].

The first branching of macrophages splits them into two categories, tissue repair or hematopoietic stem cell derived recruiting macrophages.

Recent data using lineage tracing techniques showed that tissue-resident Mϕs derive from embryonic precursors[32]. Resident tissue macrophages, during early development, penetrate several tissues such as immune and non-immune tissues and they continually circulate most of the tissues during adult life, with changing in turnover percentages, complexity and morphology[33]. When inflammation or damage does not occur, resident macrophages have beneficial effects for the related tissue such as tissue-specific development, tissue remodeling and homeostasis[29].

After birth, hematopoiesis takes place mainly in the bone marrow. Here, hematopoietic stem cells (HSCs) lineage undergo different differentiation stages which involve the loss of the self-renewal capabilities at every step. HSCs - derived recruiting Mϕs are pro-inflammatory Mϕs and they originate from the bone marrow[34]. These type of Mϕs behave as immune surveillance cells and during inflammation they infiltrate the damaged tissue to trigger both the innate and adaptive immune responses[34, 35].

For this study in bone, it is important to introduce a specific tissue-resident macrophage population termed 'osteomacs'. These cells are located in the endosteal and periosteal portion of the bone. They are physiological located mainly in osteal tissues and they increase in number during bone anabolism[28, 36].

Osteomacs have crucial roles maintaining homeostasis of bone and bone marrow (BM) supporting of OB functional activity[28] [37]. The myeloid lineage markers associated with osteomacs and expressed in mice are: F4/80, CD115, Mac-3 and CD68. Interestingly, osteomacs express in very low quantity tartrate-resistant acid phosphatase (TRAP - osteoclast marker) or Mac-2/galectin-3 (marker for inflammatory macrophage), therefore they need to be classified as tissue-resident macrophages[28]. The identification and characterization of osteomacs has led to significant interest in their uses for bone formation and in the repair of bone damage. Moreover, alongside OC, OB and osteocytes, osteomacs could act as principal participants in the balance of bone homeostasis.

1.2 Mesenchymal Stromal Cells (MSCs)

The first Friedenstein et al. were the first to definitively characterize "a bone marrow-derived, adherent, fibroblast-like heterogeneous population able to form distinct colonies (CFU-F)"[38]. These cells could also develop into OB. If they are subcutaneously transplanted they are capable of producing ectopic bone and reestablish a hematopoietic microenvironment[39]. Many other research groups have repeated and expanded Friedenstein et al.'s experiments. Thanks to these latter experiments it has been proved that these cells could be isolated from human's samples, and pushed towards an in vitro differentiation into a several mesenchymal lineages such as bone (OBs) cartilage (chondrocytes) adipose tissue (adipocytes), muscles (myoblasts) and other tissues[40].

The original source of mesenchymal stromal cells was bone marrow but different type of MSCs have since been identified in many other tissues such as: synovial membrane, skeletal muscle, tendons, dental pulp, amniotic fluid, placenta, umbilical cord blood and adipose tissue[41, 42].

Consequently, it has been shown that MSCs colonies that share analogous immunophenotypic, morphological, and functional features are located found in the brain, kidneys, liver, spleen, lungs,

thymus and pancreas of mice[43]. An innovative hypothesis states that MSCs are located in the body as pericytes, therefore they can act as the "perivascular zone as the MSC niche in vivo"[44]. The physiological proximity between MSCs and the vasculature system makes them capable of acting as a tool of new cells for the physiological cell turnover, therefore they support blood vessels homeostasis and immune system under physiological conditions. Furthermore, when tissue damage occurs MSCs are released from their perivascular site and actively acting in the healing of local tissue injury[45]. Although this hypothesis for pericytes acting as MSC niche in vivo is attractive, it is still unproved whether it can be established as a true niche or not. Recent studies have been investigated the periosteal part of the bones as a potential original source of MSCs also critical for fracture repair[36, 46]. The concept that MSCs are active contributors to immune pathology is supported by recent studies from other inflammatory disorders (RA and AS)[47]. Indeed, MSCs and other cell populations (such as fibroblast-like synoviocytes (FLSs)) derived from them have been found to affect AS and RA pathogenesis through the secretion of inflammatory molecules such as cytokines with pro-inflammatory activity, chemokines, and other growth factors[48].

In line with this hypothesis, Bottini et al. have extensively investigated the role of Fibroblast-like Synoviocytes (FLSs) as key players in the induction and continuation of joint inflammation and abnormal bone formation processes. FLS are fibroblasts derived from mesenchymal cells, they express proteins of the extracellular matrix including collagens proteins (both type IV and type V), vimentin, adhesion proteins such as integrin receptors and integrins belonging to the α5β1 class, and surface markers such as Thy-1 membrane glycoprotein (also named as CD90). Fibroblast-like Synoviocytes (FLSs) have been reported to be pathogenic in RA and AS due to their ability to secrete immunomodulating cytokines and a broad range of adhesion molecules and modelling enzymes of the extracellular matrix[47].

1.2.1 Cell surface markers and MSCs identification

Multiples methods and approaches for MSCs isolation and expansion have been recently carried out by researchers to characterize these cells. Therefore, progress in the field has been held back, as it has been quite challenging to measure and correlate study results. In order to tackle this problem, the Committee of Mesenchymal and Tissue Stem Cell of the International Society for Cellular Therapy established the general requirements needed to define human MSC. Firstly, MSCs must have plastic-adherent capabilities when are kept in standard culture conditions. Secondly,

MSCs must express CD90, CD73 and CD105, and must not express the following markers CD45, CD11b, CD34, CD79alpha or CD19 and HLA-DR extracellular molecules. Thirdly, under particular culture conditions and stimuli in vitro, MSCs must be capable of differentiate and develop into adipocytes, OB and chondrocytes[49].

The differences in surface marker expressions observed by various investigators might arise from several factors. Certainly, the tissue specific microenvironment from which MSCs come from is actively involved in the expression of their surface markers. In addition, gender and age of the donor of MSCs can be significant, although there are no research studies proving any specific differences in the expression of extracellular markers. Moreover, the capability of MSC differentiation it has been shown to be tightly linked with the age of the donors[50]. However, is still not clear how much the method used for isolation of MSCs can affect the expression of MSCs extracellular markers. Manipulating these cells might interfere with the increase or decrease expression of MSCs markers, and it may happen that the specific marker used for MSC identification is not produced during all the differentiation stages[51].

A growing case of studies have looked into the tricky issue of the reductive number of markers specific for MSCs. A solution to this would result in MSC isolation protocols with higher efficiency. For example, one of the most known MSCs marker is Stro-1. It was initially discovered by Simmons and colleagues, who demonstrated that the Stro-1^+ cells that exist inside the stromal fraction of the bone marrow has a high potential to form CFU-F colonies[52]. Stro-1^+ cells are able to develop into fibroblasts supporting-HSC, OBs, muscle cells, chondrocytes and adipocytes[53]. On the other hand, the expression of Stro-1 is gradually decreased during MSCs cultures and becomes expressed on other cell types. Data from recent studies showed that stage-linked embryonic antigen 1 and 4 (SSEA-1, SSEA-4), which were formerly posited to be limited to ES cells, are often used for the identification of murine and human bone marrow MSCs[54].

Even though these markers belong to multipotent cells, they are not used for isolation and identification protocols as they are expressed also on many other types of cells. Is good practice to use various markers at the same time in order to achieve a specific and precise isolation strategy. Gronthos and colleagues have shown a successful combination between Stro-1 and CD106, which is a molecule involved in vascular and adhesion pathways (also named VCAM-1). In cells that are positive for Stro-1 approximately 1.4% of these are also positive for VCAM-1 [55]. Another piece of research suggested the usage of FACS analysis (fluorescence-activated cell sorting) for the

identification of mononuclear cells of human bone marrow using the following positive markers CD90, CD105, CD49b, CD130, CD73, CD200, CD146 and αV/β5 integrin: there was an increase of each marker on CFU-Fs, by 23-, 50-, 60-, 100-, 256- 278-, 333- and 1750-fold respectively[56]. Finally, Horwitz et al. proved the existence of the GD2 nerval ganglioside to be used as a single surface marker for an accurate and unique identification of MSCs located in the bone marrow [57]. Further studies to validate this are yet to be done. Similarly, the correlation that these cell types show after being isolated with the same purification method, has yet to be established, and it also remains unproven if any of these population have beneficial effects or not when applied in clinical studies.

1.2.2 Mesenchymal stromal cells and Macrophages: cross-interaction in bone maintenance, fracture and healing

MSCs from bone marrow are viewed as good for regeneration and tissue engineering techniques because they are able to differentiate into OB, adipocytes and chondrocytes when they are kept in certain cell culture conditions. So far, human and murine MSCs have been extensively researched[58, 59]. MSC are the progenitor from which OB are derived [40]. Osteogenic differentiation of MSCs is regulated by OB-specific transcription factors, and other soluble paracrine, autocrine, and endocrine factors. Chang et al. recently described a new population of macrophages residing in the bone, also known as osteomacs, which are key player in bone physiology and homeostasis. The expression of macrophage markers, distinct from the markers found on the OC surface, is a characteristic of this peculiar myeloid population. In bone, osteomacs are in contact with OB, where they are involved in regulating bone homeostasis[37]. However, additional macrophage subtypes already existing in the bone marrow or taken from the blood circulation could have further functions, which could all be involved in maintaining bone architecture and its associated functions. Bone marrow macrophages have indeed been found to eliminate apoptotic cells, particularly apoptotic OB as a result of a process called efferocytosis. This phagocytic process is crucial for bone tissue homeostasis and new bone formation. Furthermore, hematopoietic stem cell (HSC) niches can be influenced by bone marrow macrophages. By secreting cytokines in a steady state or during stress conditions they help to regulate HSC progenitor cell maintenance, mobilization, and function. Molecules such as inflammatory mediators (G-CSF and IFNγ) and growth factors influence hematopoiesis either by a direct effect on HSCs or through the control of stromal cells that are vital for the HSC niches[60].

Moreover, it has been shown that macrophages are involved in bone fracture and repair[37]. The molecular circumstances that determine the fracture repair process form a complicated net which includes the following signalling pathway: cell death and recruitment, tissue damage, cell proliferation and differentiation, and tissue formation. The phases of the fracture repair process are known to occur in this chronological order: formation of hematoma in situ, inflammation, angiogenesis, cartilage production, and finally bone remodelling[61]. To coordinate the healing cascade, it is required the interaction between local and circulating cells. Knowledge around the contribution of different reservoirs of cells to fracture healing is much less.

As well as being abundant during inflammation, infiltrating macrophages (which are derived from blood monocytes) are present throughout bone fracture healing, development, and maintenance. It has been shown in vitro co-cultures of calvaria-derived OBs with macrophages promotes the mineralization of OB matrix[28]. Furthermore, it has been shown in vivo during intramembranous bone defect repair that macrophages support the secretion of collagen type I[36]. When molecular signals generated by pathogens, inflamed tissues or activated lymphocytes meet monocytes/macrophages, these cells are reprogrammed. What happens as a result is a range of different phenotypes: the pro-inflammatory phenotype (classical activated macrophages) and the tissue resident phenotype (alternative activated macrophages) depending on the local tissue environment and polarizing signals present at the timing of the disease[62]. Pro-inflammatory macrophages usually mediate inflammatory response and tissue resident macrophages mediate tissue repair and resolution of inflammation. In addition to that, macrophage polarization has been reported to be actively involved in many diseases and tissue regeneration [63].

1.3 The concept of Osteoimmunology

A new interdisciplinary research field named “osteoimmunology”, focused on understanding the bidirectional communication of the immune and skeletal systems, emerged two decades ago following the discovery of RANKL and the demonstration of a dynamic interdependence between the immune and bone cells[64, 65]. RANKL can be produced not only by OB but also activated T lymphocytes[66, 67]as well as fibroblasts, NK cells and B cells[67]. RANK/RANKL is also involved in T cell-DC interactions and is critical for lymph node development[68].

The immune system together with the bone system display distinct regulatory structures that were initially studied separately and now are analysed as integrated compounds[69]. Various

research studies have confirmed the tight correlation between skeletal and the immune system, not only in healthy subjects but also in those with disease. OC belong to the monocyte/macrophage lineage and therefore derive from the same myeloid progenitor as macrophages, monocytes and dendritic cells[70]. This is the first evidence of molecular interconnection between bone remodelling and the innate immune system. This is further supporting the initial hypothesis of RANKL, important player in the signalling pathway, linking the immune system (myeloid cells) with bone biology (OC and OB). ITAM-induced signalling, an immunoreceptor with tyrosine-based activation motifs occurs in OC but it is also able to activate macrophages, APCs, NK cells and T cells. This strengthens the idea that OCs are integral components of the immune and inflammatory responses as ITAM is also involved in the activation of the bone resorption process[71].

Secondly, cells of the immune system can interfere with osteoblasts development. Jung et al. have reported that all the immune cells progenitors, named as HSCs, were molecular supporters of MSCs differentiation into OBs through the secretion of HSC-derived BMP6 and BMP2. As a result, they are key players in the formation and maintenance of their niche[72]. Furthermore, when MSCs from bone marrow of Wistar rats were cultured together with cells from the monocyte/Mϕ lineage, Nakagawa et al. showed an increase in ALP activity in the co-culture system [73]. As previously mentioned, Chang et al., identified for the first time a type of Mϕs with anti-inflammatory and tissue resident features, named as osteomacs, which were found both in murine and human osteal tissues; they suggested that these cells could have an active role in bone homeostasis. Using murine bone explants and calvaria preparations, osteomacs were kept in co-culture with OBs, showing that their presence was an essential requisite for an effective mineralisation of mature OBs in vitro[28]. Therefore, osteomacs have been correlated with bone remodelling processes in situ.

Osteoimmunology is therefore an interdisciplinary biological and clinical subject which has facilitated the understanding of common disease, such as osteoporosis, trough new clinical interpretations [74]. Moreover, due to various connections and interactions between the skeletal and the immune system, the former is one of the main sign to spot the presence of chronic inflammation in rheumatoid arthritis (RA) and ankylosing spondylitis (AS). On one hand, inflammation leads to an increased bone resorption and decreased local bone formation in RA, on

the other hand it causes increased local bone formation in AS. Therefore, there is a broad range of bone engagement in both RA and AS[75].

1.4 Inflammatory diseases of bone

Inflammatory bone disorders such as RA, AS and systemic lupus erythematosus (SLE), are part of a diversified group of bone diseases which are all correlated with extra-articular secondary effects, such as bone destruction, fractures and excessive bone repair. Generally, there is a multifactorial pathogenesis of the diseases mentioned above. Common factors that significantly heighten the risk of bone fracture are mostly linked with age, genetic background, body mass index, gender and sedentary lifestyle[76].

Frequently, bone become inflamed in RA and AS. Indeed, inflamed and activated immune cells in situ release a broad range of cytokines that favor bone formation in AS and bone resorption in RA, leading to local and systemic bone loss[77]. In RA, peri-inflammatory bone formation is defective, therefore bone erosions are not effectively resolved, and this triggers inflammation trough a local negative circle which includes bone loss synovitis and osteitis. Conversely, in AS, peri-inflammatory bone formation is elevated, leading to erosions repair, ossification of inflamed enthesis, and consequent ankyloses of sacroiliac joints and intervertebral structures. These bone defections compromise the biomechanical activity of the spine, heightening the probability of vertebral cleavage (in both AS and RA) and non-vertebral cleavage (in RA)[76].

Consequently, most of the inflammatory bone disorders are further exacerbated by both elevated bone loss and destruction. When bone resorption is higher than bone formation, there final result is bone loss. This imbalance is behind most diseases of the skeleton: osteoporosis, displays an higher bone destruction than bone formation; hyperparathyroidism, is correlated with an accelerated turnover of bone cells; malignancy of hypercalcemia, cause an exaggerated bone resorption which leads to the hyper-secretion of calcium in the blood stream; Paget's disease is associated with bone loss in situ; metastatic bone disorders, show osteolytic lesions; periodontal disease, usually develop into local bone destruction of the jaws; rheumatoid arthritis, which is often associated with periarticular bone loss; permanent immobilization of the body, can lead to bone loss because of the absence of mechanical stimuli essentials for the maintenance of bone structures; and side effects associated with glucocorticoid treatments, are linked with bone loss as

they decrease bone formation. These pathological conditions could potentially be improved by blocking bone resorption and stimulating bone formation[78, 79].

1.5 What is Ankylosing spondylitis?

AS is an inflammatory rheumatic disorder linked with abnormal new bone formation and an elevated risk of osteoporosis and bone fractures. The prevalence of this disease is 5 per 1,000 in European populations[80]. The disease is gender dependent: "men are more likely to develop AS approximately two to three times more frequently than women"[81, 82].

AS is a chronic condition with unpredictable progression pathway: the history of the disease does not follow a defined-common course. Although findings from an early prospective study shown that a predictable pattern of AS become clear within the first 10 years of the disease[83]. The first clinical symptoms of AS often emerge in adolescence or early adulthood. The initial symptom is typically a tedious pain in the lower lumbar part of the body, followed by a generalized morning stiffness. The pain is attenuated during physical exercises and it increases again with physical inactivity. Unfortunately, the pain becomes persistent and bilateral with the passing of time and is normally more intense at night hours [84]. When inflammation of spine and sacroiliac joints further progress, causing pain and stiffness, there is the occurrence of ectopic new bone formation and joint ankyloses, which are the main clinical features of AS. Inflammation together with progressive structural damage leads to a negative evolution of the disease. Sometimes, AS is accompanied by arthritis in the shoulders and hips in the early phase of the disease. Arthritis occurs asymmetrically at any stage of the disease to other joints such as the lower limbs.

In addition to bone related symptoms AS exerts many extra-articular manifestations such as: acute anterior uveitis, unilateral pain, photophobia, elevated lachrymation and gut disorders as well as inflammatory bowel disease (IBD). Indeed, recent ileocolonoscopic studies, have been shown that asymptomatic IBD is seen in ~60% of patients diagnosed with AS[85]. In some cases, IBD can lead to further complications including aortic insufficiency and congestive heart failure. Moreover, the literature shows a strong relationship between gut and joint inflammation. Therefore, gut inflammation (and at a latest stage IBD) may play a crucial role in the pathogenesis of AS[86].

In addition to that, from a genetic point of view, AS is tightly linked with the human leukocyte antigen B27 whose presence indicates immune system dysfunction. However only 1–5% of HLA-B27-positive people further progress to AS, for this reason recent studies suggest that other genes

must be implicated in the initiation of AS[87]. Results from human and animal genetic studies report a pivotal role for the IL-23/IL-17 axis in the pathogenesis of AS[75]. Similarly, Sherlock et al. have been investigated the essential role of IL-23 in the development of enthesitis, which is one of the main features of AS and probably involved in the trigger of the disease [88]. The initial manifestation of AS is sacroileitis, followed by enthesitis of peripheral joints and extra-articular structures. In advanced phases of the AS, the spine become fused forming a long bony column known as “bamboo spine”[89].

1.6 Anatomy and tissue pathology of enthesis

Enthesis is identified as the anatomical site where tendons and ligaments meet the surface of the bone. Moreover, entheses are required for locomotion as they transduce biomechanical signals from muscles to bones. While joints allow the motility of the skeleton acting as physiological 'breaks between bones’, entheses trough ligaments ensure support to the rest of the body and trough tendons modulate the transmission of biomechanical forces to the skeletal system[90] Normally, entheses constitute the external part of the joints, they can be inserted either in the periarticular bone (this is the case of the insertion of the flexor tendon of the phalanges or the tendon inserting in the biceps), or far away from the synovial joint (as it happens with the Achilles tendon or with the fibrous insertions into the each vertebra).

Furthermore, 'functional entheses' is a term which was created to describe anatomical sites where the tendon is surrounding a bony protuberance (such as the peroneus tendons). According with what mentioned before, understanding the biological aspects of entheses is essential in order to target inflammation processes, which significantly differ from synovitis. On one hand enthesitis generally happens in the external part of the joint, on the other hand synovitis occurs in the inside of the joint leading to an intra-articular inflammation of the synovial membrane. However, in rheumatic diseases such as AS, these two inflammatory processes can take place either independently or simultaneously [91]. There are two forms of enthesis, namely: 1) fibrous; and 2) fibrocartilaginous. Fibrous attachments are found at the metaphysis and diaphysis of long bones, and in these collagen fibrils attach directly to the bone. In fibrocartilaginous enthesis a conspicuous transitional zone is present. Enthesis occurs in the following four regions:

1) Tendon with longitudinally orientated fibroblasts
2) A region of fibrocartilage where the cells are morphologically similar to chondrocytes
3) A transition region of fibrocartilage to calcified fibrocartilage

4) Bone

Enthesitis, defined as “the inflammatory changes of an enthesis”, leads to an abnormal new bone formation, and is considered a characteristic finding in AS[92]. Differently from other bony anatomical places, enthesis is a specific part of the body where various mechanical pressures come across. Thus, the external mechanical stimulation of enthesis can cause an inflammatory reaction which generate cytokines and leading to infiltration of monocytes. The net results is inflammation of either enthesis or synovium which clinically produce enthesitis or synovitis, respectively[93].

1.7 Pathogenesis of AS and the central role of enthesitis

The pathogenesis of AS is poorly understood. So far, it has not been descried any particular molecule or process able to trigger the initiation of the disease, however there is one hypothesis which identifies the enteric bacteria and the microbiota as the main cause of the disease, due to the tight correlation between AS and IBD [94]. The strong connection between AS and gut mucosa inflammation theorize that the physiological flora of the gut together with a subsequent immune response raised against it, can be actively involved in the pathogenesis of AS[80].

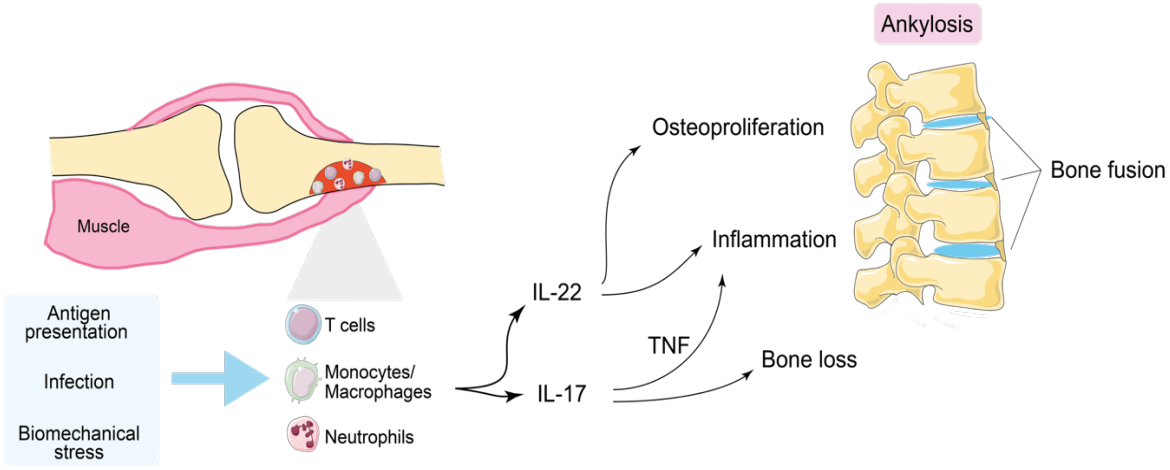


Figure 1.3: Pathogenesis of Ankyloses Spondylitis (AS)
Numerous pathways such as antigen presentation, infection and biomechanical stress, may cause IL-23 over expression. Sherlock and colleagues showed that IL-23 leads to the activation of entheseal T cells. Together with activated T cells, macrophages and neutrophil support local inflammation and bone remodelling through several mediators, such as IL-17 and IL-22. The final result of these activities is the ankylosis of the spine. The picture above it has been adapted from Sherlock et al. (Nature Med. 2012)

In addition to that, IL-23 has recently been identified as an initiation factor of inflammation and bone remodelling in AS. However, numerous pathways such as antigen presentation, infection and biomechanical stress, may cause IL-23 over expression (**Fig. 1.3**). This molecule is mainly secreted in the gut - especially in AS, the intestinal mucosa is the major producer of IL-23 - and is consequently activated by different signaling pathways such as biomechanical stress, antigen presentation, bacteria or viral infection. IL-23 production then leads to the activation of resident T cells, monocytes/macrophages and neutrophils within the enthesis.

This in turn boosts the initiation of inflammatory processes and bone remodeling, with the former regulated by IL-17 and the osteoproliferation phase modulated by IL-22. At this stage, the molecular result is ankylosing of spine and other inflamed parts of the skeleton. Sherlock et al. proved that hyper-expression of IL-23 leads to inflammation of the entheseal joints, and so this is the first clinical sign of disease. Consequently, inflammation is also spread to synovial tissues, with a successive progression to the joint tissues[95]. To sum up, the molecular consequence of IL-23 on bone is ubiquitous and pervasive, and other biological mediators are potentially involved in the syndesmophyte process, which is the heterotopic ossification that occurs between bone and ligaments, as seen in AS.

1.8 Hypothesis

There is evidence that describes a close bidirectional connection between macrophages and bone cells in both healthy and diseased states. Using a murine model of AS, the hypothesis is that immune cells such as macrophages promote bone formation at the enthesis by regulating MSC differentiation towards the osteoblast lineage.

1.9 Aims and Objectives

In vitro: Do polarized macrophages promote MSCs differentiation into osteoblasts?

- Confirm crosstalk between MSC and macrophages with murine cells;
- Define the differential effects of macrophage subsets on OB differentiation.

In vivo: Do macrophages promote bone formation in AS through direct interaction with MSC?

- Characterise when/where new bone formation at enthesis occurs in a mouse model of AS;
- Assess how the crosstalk, in vivo, between MSC and macrophages affects new bone formation at enthesis;

- Determine the role (presence or absence) of macrophages in new bone apposition at the enthesis.

CHAPTER TWO

MATHERIALS AND METHODS

2.1 Methodology in vitro

2.1.1 Murine Mesenchymal Stromal Cells: isolation protocol

In order to confirm the crosstalk between murine mesenchymal stromal cells (m-MSCS) and murine macrophages (m-MΦ), a protocol for the isolation and proliferation of m-MSCs was established. According to Zhu H. et al., digested bone chips flourish with progenitors of MSCs that are able to migrate in and out of the digested bone chips during culture and create mature stromal cells[96]. In this protocol, long bones are deprived of bone marrow, digested with collagenase type II, and bone fragments are seeded with a complete medium (DMEM Glutamax media supplemented with 10% FBS, 1% penicillin/streptomycin and 1% L-Glutamine). After 5 days in culture, FACS analysis (Fig. **2.1**) showed that the adherent cells migrating out of the fragments are mesenchymal stromal cells. However, MSCs were not sorted at the end and the purity used in the in vitro assays was 70% of $CD45^-$ MSCs with remaining $CD45^+$ cells, later identified as $CD11b^+$ and $F4/80^+$ myeloid cells.

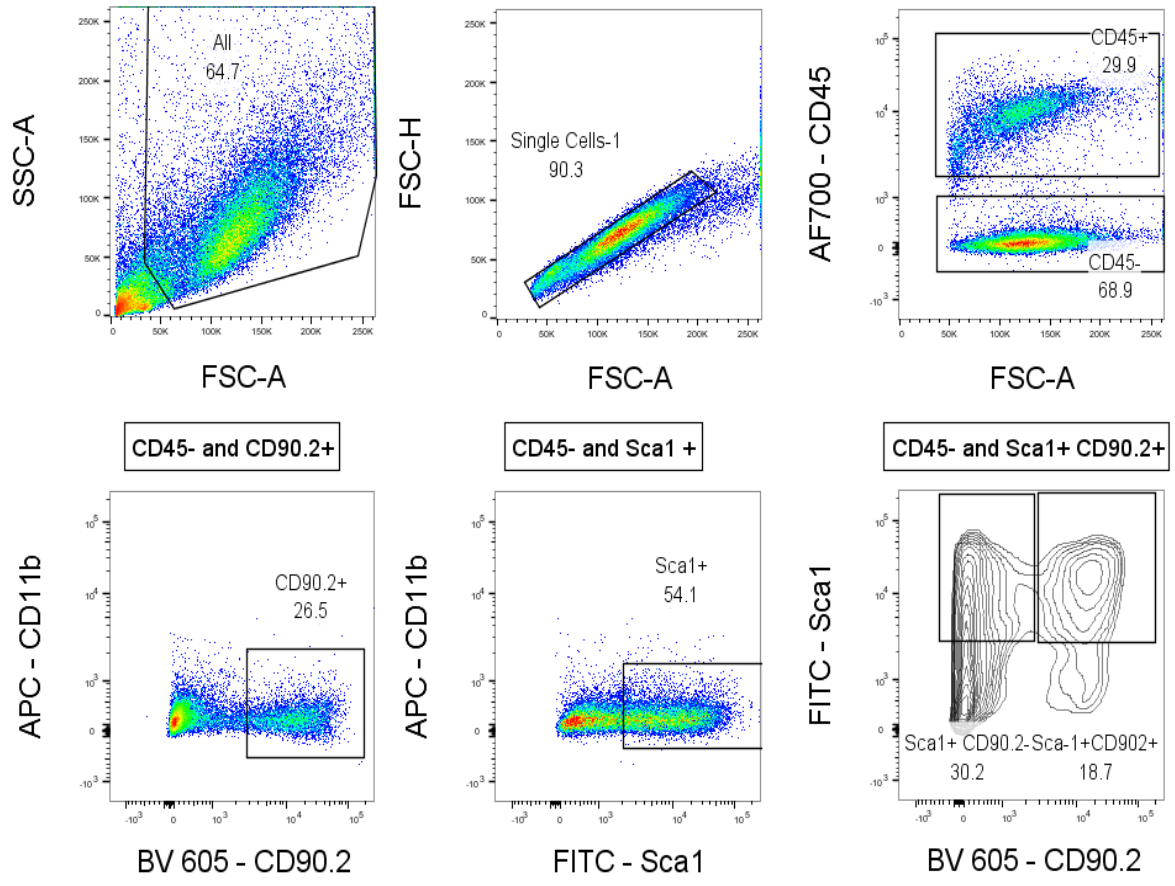


Figure 2.1: FACS analysis of Mesenchymal Stromal cells (MSCs) isolated from digested compact bone chips. FACS plots show (among 69% of $CD45^-$ cells): 30.2% of $CD45^-Sca1^+$ as early phase MSCs and $CD45^-CD90.2^+$ as mature MSCs.

All cell culture procedures were kept under sterile conditions in a laminar flow cabinet (Class II microbiological safety, Gelaire, Flow). To support propagation and proliferation, MSCs were maintained for 5 days in DMEM media (Glutamax) supplemented with 10% FBS, 1% penicillin/streptomycin and 1% L-Glutamine. Cell cultures were kept at 37°C in a humidified atmosphere of 5% CO_2. At day 5, when 70-80% confluent, these cultures were washed with PBS to eliminate the serum, and detached using 0.05% trypsin EDTA for 3 min at 37°C. MSCs were seeded in a 96-well flat bottom plate with both culture media (DMEM Glutamax media supplemented with 10% FBS, 1% penicillin/streptomycin and 1% L-Glutamine) and osteogenic media (dexamethasone 100nM, ascorbic acid 0.05nM and β-glycerophosphate 1mM) and tested for their ability to differentiate into osteoblasts.

2.1.2 Flow cytometer analysis of mesenchymal stromal cells MSCs

Cell surface marker expression of MSCs was evaluated by standard flow cytometry using a BD-LSR II flow cytometer and data obtained using FACS Diva software. Aliquots of $1x10^5$ cells were stained in FACS buffer (PBS containing 1% FBS) with the following antibodies: CD45-Alexa Fluor 700, Ly-6A/E(sca-1)-FITC, CD44-e Fluor450, CD11b-APC, F4/80-PE, and CD90.2-Brilliant Violet 605. Data was analysed using Flowjo software.

2.1.3 In vitro osteogenic differentiation of MSC

Murine MSCs can differentiate towards the osteogenic lineage with the addition of components (dexamethasone 100nM, ascorbic acid 0.05nM and β-glycerophosphate 1mM) in the culture medium (Gibco® DMEM 500mL, Gibco® FBS 50ml, Penicillin/Streptomycin 5mL)[40]. The timeline for the in vitro differentiation is 3-4 weeks and consists of 2 stages: a collagen matrix deposition phase (week 2) and a mineralization phase (week 3)[97].

2.1.4 Alkaline phosphatase quantification assay

For ALP evaluation, $1x10^4$ MSCs were placed in a 96-well plate in triplicate in 200µl of media and left overnight to adhere. Then the media was removed and replaced with DMEM media, osteogenic media and media containing macrophages. Every 3 days, this media was changed. ALP was quantified using the LabAssayTM ALP Kit (Wako pure chemical, Japan) at day 7. In the presence of ALP in the sample p-Nitrophenylphosphate (the substrate) is hydrolysed into p-nitrophenol and phosphoric acid in the carbonate buffer (pH 9.8). Released p- Nitrophenol, which exhibits a yellow colour, was measured at 405nm wavelength. Using p-Nitrophenol standard

solution, a standard curve was generated. Standard p-Nitrophenol solution (0.5mmol/L) was serially diluted with dH2O to concentrations of 0.25, 0.125 and 0.0625 mmol/L. Subsequently, 100µl of Substrate solution (p-nitrophenylphosphate disodium) was added to all of the wells and the plate was incubated at 37°C for 15 min. To terminate the enzymatic reaction, 80µl of stop solution (Sodium Hydroxide) was added to each well, and the plate was read in a microplate reader at 405nm. Using the standard curve, the ALP content was determined and then expressed as p-Nitrophenol (mmol/L).

2.1.5 Staining for bone nodule formation

For bone nodule formation, $1x10^4$ MSCs were seeded to wells of a 24-well plate in duplicate and left overnight to adhere. Cultures were either kept in normal media as control, or in media replaced with 500 µl/well of osteogenic media. Cultures were kept for 21 days, and every 3 days the media was changed. Subsequently, cultures were washed with PBS, fixed with 70% Ethanol for 1 h at -20C, and stained with a 40mM solution of Alizarin Red (adjusted to pH 4.1 using 10% v/v ammonium hydroxide) for 40min at room temperature. Bone nodules, appearing red, were visualised under a light microscope, photographed, eluted and quantified using microplate reader at 405nm.

2.1.6 Macrophage isolation, production and differentiation with M-CSF and GM-CSF

Macrophages were isolated from the bone marrow of murine long bones. Briefly, 3 male mice (6 to 8 weeks old) were sacrificed, back and front limbs were taken, and bone marrow was flushed out of the bone cavities, washed and plated. Cells were seeded at 2×10^6 cells/mL in square non-tissue culture-treated petri dishes (Thermo Scientific) in basal media composed of: RPMI (Gibco) supplemented with 10% FBS, 1% penicillin/streptomycin, 1% L-Glutamine, 1% of NaPyruvate, and 2.4% of Hepes. In order to further differentiate bone marrow macrophages, half of the cells were grown in the presence of 50 ng/mL of M-CSF (macrophage colony–stimulating factor) in the complete media, while the other half of the cells received 50 ng/mL of G-MCSF (Granulocytes macrophages colony-stimulating factor) in the complete media. On day 5, macrophages were fed, adding 5 mL of complete media, M-CSF and G-MCSF to each plate. On day 7, culture supernatants were removed and the cells were taken out of the dishes. Given that macrophages are very adherent and trypsin-resistant, 12 mL of frozen/cold PBS (Dulbecco's phosphate-buffered saline) was added to each plate for 5 minutes to remove them from the petri dishes in which they were

grown. Macrophages were then collected in the supernatant from each plate and used for co-culture experiments.

2.1.7 Co-cultures of MSCs and bone marrow-derived MCSF and GMCSF macrophages

Primary macrophages previously described were used in co-cultures with MSCs. Firstly, MSCs were plated in 96-well plates at a concentration of $1x10^4$ cells per 200µl and left overnight to adhere. The next day, differentiated Mϕs were added on top of MSCs along with fresh control or differentiation media. The MSC: Mϕs ratio was 1:1, 1:5, then 1:10. Wells that only contained MSCs were included as a control. Co-cultures were kept for 7 days for ALP staining and quantification or 21 days for bone nodule formation. Every 3 days, the media was changed.

2.2 Methodology *in vivo*

2.2.1 Mice, induction of disease and *in vivo* treatments

"SKG mice spontaneously develop T cell–mediated chronic autoimmune arthritis as a result of a gene mutation in the SH2 domain of ZAP-70, a key molecule in T cell receptor signaling"[98]. This mutation "impairs positive and negative selection of T cells in the thymus, leading to thymic production of arthritogenic autoimmune $CD4^+$ T cells"[98]. As a result, the mice experience symmetrical joint swelling, commencing in small joints of the digits progressing to larger joints. There is severe synovitis, accompanied by the formation of pannus that invades and erodes adjacent cartilage and subchondral bone. After injection of curdlan 3mg/mL (1,3 beta glucan), a fungal protein able to trigger the onset of AS in SKG mice, the clinical symptoms of AS occur in the following order:

1-2 weeks: Uveitis, psoriasis and redness of extremities
2-3 weeks: Redness and swelling of joints
3-5 weeks: abnormal bone formation at entheseal sites
4-6 weeks: Ileitis

SKG mice were kept under specific pathogen–free conditions. None of the SKG mice in the facility developed AS spontaneously unless previously challenged with curdlan. The University of Oxford and the animal ethics committee accepted and approved the experiments of this study. For the development of AS, female mice of 4-6 weeks old were used and then challenged with 3 mg of 1,3 β- glucan curdlan (derived from Alcaligenes faecalis, myxogenes variety; Wako), injected once

intraperitoneally. Mice were weighed and observed for clinical signs twice a week, for up to 6 weeks. Macrophage depletion was achieved using Clodronate Liposomes (from *Liposoma B.V,* The Netherlands) or saline as control 5mg/ml (0.125mg in the right enthesis of each mouse) injected subcutaneously once every two weeks [99]. OSM neutralization was performed using recombinant mOSMR-Fc (from Ray Owens, University of Oxford), or the appropriate vehicle as control. 10 ug was injected subcutaneously in the right enthesis of each mouse twice a week for 4 weeks.

2.2.2 Clinical scoring

Every week (2x/week) clinical scores were evaluated in all of the experimental mice, and monitored by the same person (the author); calipers were used to measure wrist and ankle widths. The peripheral enthesitis scoring system applied the following criteria (Fig. **2.2**): 1 = low inflammation at enthesis (insertion site of the tendon); 1.5 = low to medium inflammatory infiltration at the enthesis (insertion site of the tendon); 2 = high inflammation particularly at bone level; and 3 = severe ankylosing with loss and fracture of tendon–bone interface.

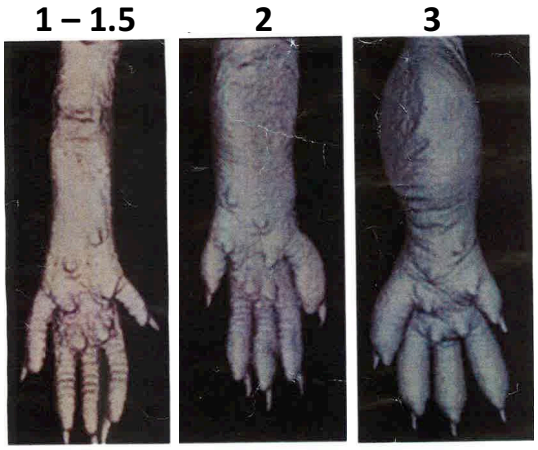


Figure 2.2: Pictures are representative of the peripheral enthesitis scoring system used during *in vivo* experiments.

2.2.3 Histology

At the experimental end point, joints from control and treated mice were fixed in formalin and embedded in paraffin. Joint sections were first decalcified in EDTA (OsteoSoft). Sections (4 µm) were cut and stained with hematoxylin and eosin for histological scoring and with Masson Trichrome. Sections were also stained with immunohistochemistry staining (IHC) for F4/80 –single colour DAB method. Photographs were taken with an Olympus BX51 Osteometric (fluorescence and bright field) microscope.

2.2.4 Flow cytometry

Using a stromal FACS panel, cells from enthesis, bone marrow and spleen were stained with the following antibodies: CD45-Alexa Fluor 700, Ly-6A/E(sca-1)-FITC, CD44-e Fluor450, CD11b-APC, F4-80-PE, and CD90.2-Brilliant Violet 605. Using a myeloid FACS panel, cells from enthesis, bone marrow and spleen were stained with the following antibodies: CD45-Alexa Fluor 700, CD11b-APC, Siglec F-PB, Ly6G-Brilliant violet 510, Ly6C-PE-Cy7, MHC-II-FITC, CD11C-PE, and F4-80- Brilliant Violet 605. Dead cells were excluded from analysis with a Live/Dead Zombie-Aqua (Biolegend). Data were obtained using BD-LSR II flow cytometer and analysed using FlowJo software.

2.2.5 Micro-Computed Tomography (micro-CT) analysis

Live mice where scanned using cone-beam X-ray Quantum FX (Perkin Elmer, Waltham, MA) mCT. The X-ray source was set at 90 V, 200 µA, 50 kV, 800 µA, 8.3 µm isometric voxel resolution and 0.7° rotation step. Samples were scanned up to a field of view of 5 mm diameter and were imaged with a 3D isotropic voxel size of 10 µm for a duration of 3 minutes per region of interest. The three-dimensional (3D) bone parameters were calculated using 3D image analysis software (Analyze 12.0/AnalyzeDirect) version 1.9.3.0. by selecting 10 mm × 6 mm × 1 mm volume of interest that included the parietal bones, lambda and coronal sutures. A threshold range of 30–255 density units was selected to distinguish mineralized from soft tissue. The following parameters were measured: the ratio of bone volume to total volume (BV/TV), cortical area fraction (Ct.Ar/Tt.Ar) and average cortical thickness (Ct.Th).

2.2.6 Statistical analysis

All statistical analyses were performed using GraphPad Prism software. Statistical tests performed for each data set and significance levels are indicated in the legends of the figures (One and Two-way ANOVA with Sydak's and Tukey's multiple comparisons test). For experiments with only two groups two-tailed *t* test was used. In all cases, data are presented as mean ± SEM., and a *P* value of 0.05 was considered significant.

CHAPTHER THREE

CO-CULTURES SYSTEM: MSCs and Macrophages

3.1 Introduction

The aim of this study was to investigate the cellular mechanisms that drive new bone formation at enthesis in a murine model of AS. It was hypothesised that abnormal bone deposition is triggered by interaction between MSCs and macrophages and that the effect can differ depending on the specific functional subset and on the activation state of macrophages. Macrophages are the major producers of cytokines in inflammation and the types and functions of macrophage biology have diversified over recent decades[100].

Generally speaking, "macrophages express specialized and polarized functional properties in response to cytokines and microbial products"[101, 102]. For a long while, it has been known that pro-inflammatory macrophages are induced by IFNγ alone or alongside pathogenic stimuli (e.g., LPS) or cytokines (e.g., TNF and GM-CSF)[101]. According to Murray PJ. et al, alternatively activated macrophages are exposed to IL-4 or IL-13, IL-10 and corticosteroid hormones[31]. Polarized macrophages show different receptor expression, metabolism, effector function and cytokine production compared to non-polarized macrophages. For example, while arginine metabolism is characterized by high levels of inducible nitric oxide synthase (iNOS) in pro-inflammatory macrophages, in tissue-resident cells the arginase pathway predominates, with the generation of ornithine and polyamines. Moreover, polarized macrophages differ in their cytokine production according to the environment. The pro-inflammatory phenotype includes production of IL-12 and tumor necrosis factor (TNF), whereas tissue-resident macrophages generally secrete "IL-10, IL-1 receptor antagonist and the type II IL-1 decoy receptor"[103].

The interaction between macrophages and OB and their mesenchymal precursors has also become apparent in recent years. Previous studies from the Horwood lab showed that MSCs can polarise Mϕs into an anti-inflammatory phenotype associated with Th2 response and tissue repair. It was found that this phenotype promotes bone regeneration by pushing differentiation of MSCs towards OBs lineage. The osteogenic effect of monocytes on MSCs was found to be mediated by COX_2 and PGE_2[104]. It is known that PGE_2 and COX_2 play significant roles in bone; regulating osteoclastogenesis[105] and regulating the expression of bone-specific transcription factors Runx2 and Osterix [106, 107]. Seven candidate genes (also encoded for soluble factors) namely IL-24, CXCL5, EREG, OSM, IL-10, MMP9 and LAMB3 were identified as potential mediators for the monocyte osteogenic effect. Of these, OSM could potently induce ALP activity in a dose-response manner when added to MSC cultures in increasing concentrations. This study aims to

research the effects of macrophage polarization on MSC osteoblast differentiation in vitro. Here, we show that pro-inflammatory macrophages promote MSC osteogenic differentiation, while tissue resident macrophages don't affect MSC osteoblast differentiation. These findings improve our understanding of cellular interactions between MSC and macrophage underlying bone formation to provide therapeutic targets for bone diseases.

3.2 Results *in vitro*

A previous PhD student in the Horwood lab showed that human monocytes/Mϕs could promote osteogenic differentiation of MSCs when cultured together[104]. Quantification of ALP activity was taken as a measurement of MSC capability to differentiate into OB. Alkaline phosphatase (ALP) enzyme plays an important role in the mineralization of bone and is often employed as an early marker for osteogenic differentiation[108]. Nicolaidou et al. showed that non-activated M-CSF and GM-CSF Mϕs could equally promote MSC differentiation[104]. When M-CSF and GM-CSF Mϕs where polarized respectively with IL-4 (alternative activation) and IFNγ (classical activation) IL-4-treated M-CSF Mϕs could increase ALP activity equally with the IFNγ treated GM-CSF Mϕs. It was also shown that with microbial stimulus such as LPS in the co-cultures, IL-4 treated M-CSF Mϕs caused a further increase in MSC ALP activity, while MSC ALP activity was completely inhibited by IFNγ-treated GM-CSF Mϕs.

The main aim of the study was to determine whether murine MSCs and Mϕs can promote OB differentiation in a similar way. Therefore, to ascertain the effect of Mϕs on osteoblasts differentiation, co-cultures of murine MSCs and murine BM-derived Mϕs were established with increasing ratios of Mϕs. Murine MSCs were generated from bone chips of SKG mice (as the in vivo work in the AS model is performed in this mouse strain) and after 5 days of culture in basal medium, MSCs were trypsinised and plated overnight in a 96-well plate at a concentration of 10^4 cells/well. Murine Mϕs were isolated from the BM of SKG mice and differentiated in vitro with either M-CSF or GM-CSF for 5 days. After 5 days in culture, Mϕs were then plated at increased concentration with MSCs, both in basal and osteogenic medium, for 7 days prior to cell lysis for ALP quantification.

On one hand, ALP quantification assay revealed that M-CSF Mϕs in osteogenic medium did not have any effect in ALP activity compared to mono-cultures of MSCs (Fig. **3.1**). On the other hand, the 1:1 ratio of GM-CSF Mϕs:MSCs (GM-CSF Mϕs and MSCs both at 10^4cells/well) generated a

clear increase in ALP activity. The ability of murine BM-derived GM-CSF Mϕs to induce osteogenesis was also confirmed in the bone nodule formation assays at the concentration of $5x10^4$cells/well, as visualized by alizarin red S staining and quantitative evaluated using a microplate reader (Fig. **3.2**).

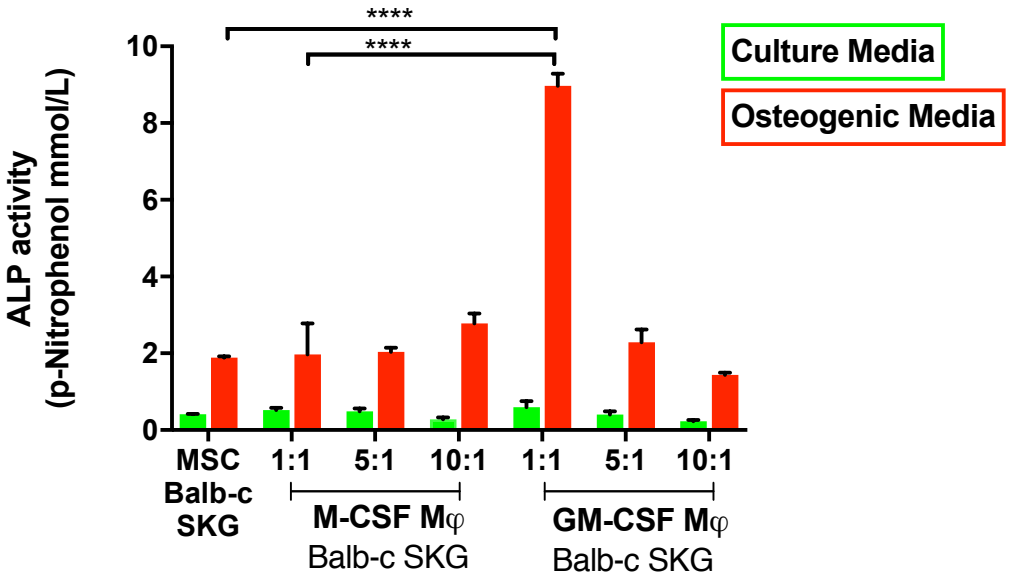


Figure 3.1: GM-CSF Mϕ promote MSC differentiation into OB. BM derived Mϕs, isolated from Balb-c SKG mice, were differentiated with either 50 ng/mL M-CSF or GM-CSF. $1x10^4$ MSCs were isolated from digested bone chips of 2 weeks old Balb-c SKG mice, then plated in 96-well plate and left 24h to adhere. Increasing amount (1:1, 5:1, 10:1) of BM derived macrophages were then added on the top of $1x10^4$ MSCs in control media or osteogenic media. Co-culture were kept for 7 days, with media changing twice per week. ALP activity was assessed at day 7. Graphs represent cumulative data of 3 experiments performed in triplicates, using BM derived Mϕ from different animals, and shown as mean +/- S.E.M. **** $p<0.0001$ Two-way ANOVA with Sydak's multiple comparisons test to MSCs alone.

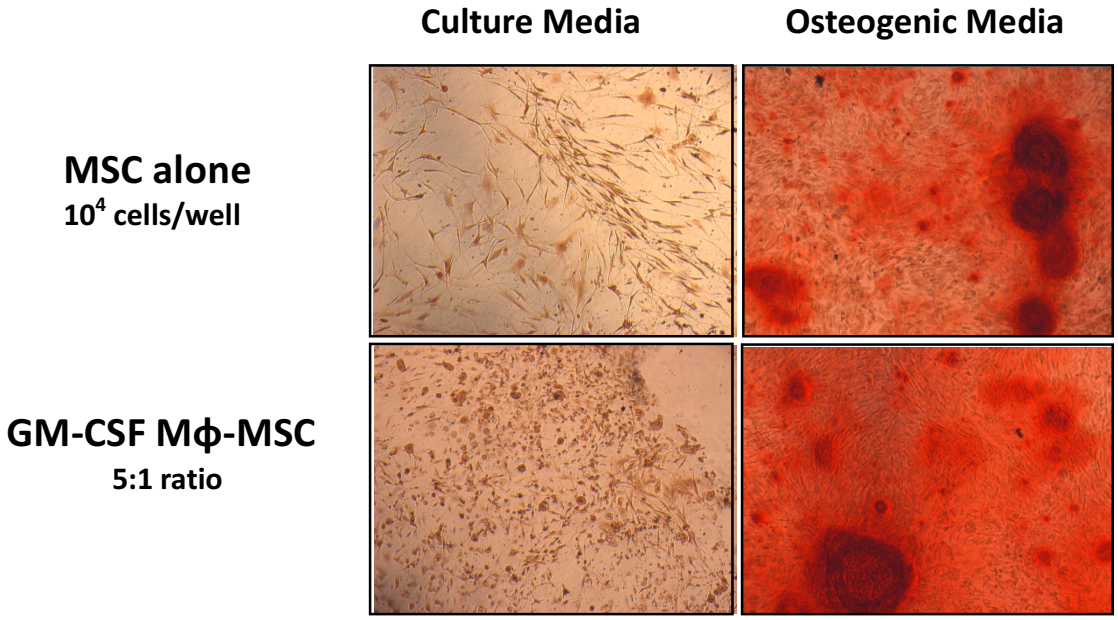


Figure 3.2: GM-CSF Mϕ promote bone nodule formation. 1x10^4 MSCs were plated in 48-well plate and left 24h to adhere. Culture basal media was then replaced either to osteogenic media for the wells containing MSCs alone, or osteogenic media and GM-CSF bone marrow derived Mϕs at a ratio of 5:1 (5x10^4). Co-cultures were kept for 21 days, changing the media every 3 days. Detection of bone nodules was evaluated using Alizarin Red staining. Images are representative of 3 experiments performed in triplicates using BM derived Mϕ from different animals.

This result indicated that GM-CSF BM-derived Mϕs synergize with, but do not replace, osteogenic signals generated by the differentiation medium. This type of Mϕs greatly enhances MSC capability to differentiate into osteoblasts, more than MSCs themselves. Moreover, co-cultures of MSCs and Mϕs where established using polarized Mϕs. Following that, Mϕs were polarized in vitro with IL-4 and IFNγ, prior to co-culture with MSCs. Macrophage polarization was performed in order to achieve either classical or alternative activation[30]. Classical activation mediated by the priming stimulus of IFNγ, followed by a microbial trigger such as LPS, leads to the production of pro- inflammatory cytokine and nitric oxide. Conversely, alternative activation mediated by IL-4, which is associated with Th2 responses, results in upregulation of arginase and has been associated with tissue repair[109, 110]. Although many different types of alternative activated Mϕs exist (depending on the polarization stimuli they receive – IL-4, IL-10, GC, immune complexes etc.-), in this study only one type of alternative polarized Mϕs was used (IL-4 polarized bone marrow derived MCSF Mϕs). This was done in order to keep this study consistent with the previous work in humans performed in the Horwood lab.

Polarized M-CSF and GM-CSF Mϕs were then added to MSC cultures in the presence of 10ng/ml of LPS, in every well, both in basal media and osteogenic media. Therefore, mono-cultures and co-cultures were exposed to 4 different conditions: basal culture medium +/- LPS and osteogenic medium +/- LPS. Previous studies showed that in response to LPS stimulation, GM-CSF and M-CSF Mϕs greatly differ in their cytokine production[111]. More specifically, GM-CSF Mϕs exhibit a pro-inflammatory phenotype with secretion of IL12/IL23, IL1β, IL-6 and TNFα without detectable IL-10, while M-CSF Mϕs produce anti-inflammatory IL-10 and lower levels of pro-inflammatory cytokines[112]. As shown in (Fig. **3.3 A-B**) non-activated GM-CSF Mϕs significantly increased ALP activity to the similar levels when compared to M-CSF Mϕs co-cultures and to mono-cultures of MSC. However, in the presence of LPS the effect on MSC differentiation differed; IFNγ-treated GM-CSF Mϕs caused a further enhancement of MSC-ALP activity compared to the levels of the MSC control alone.

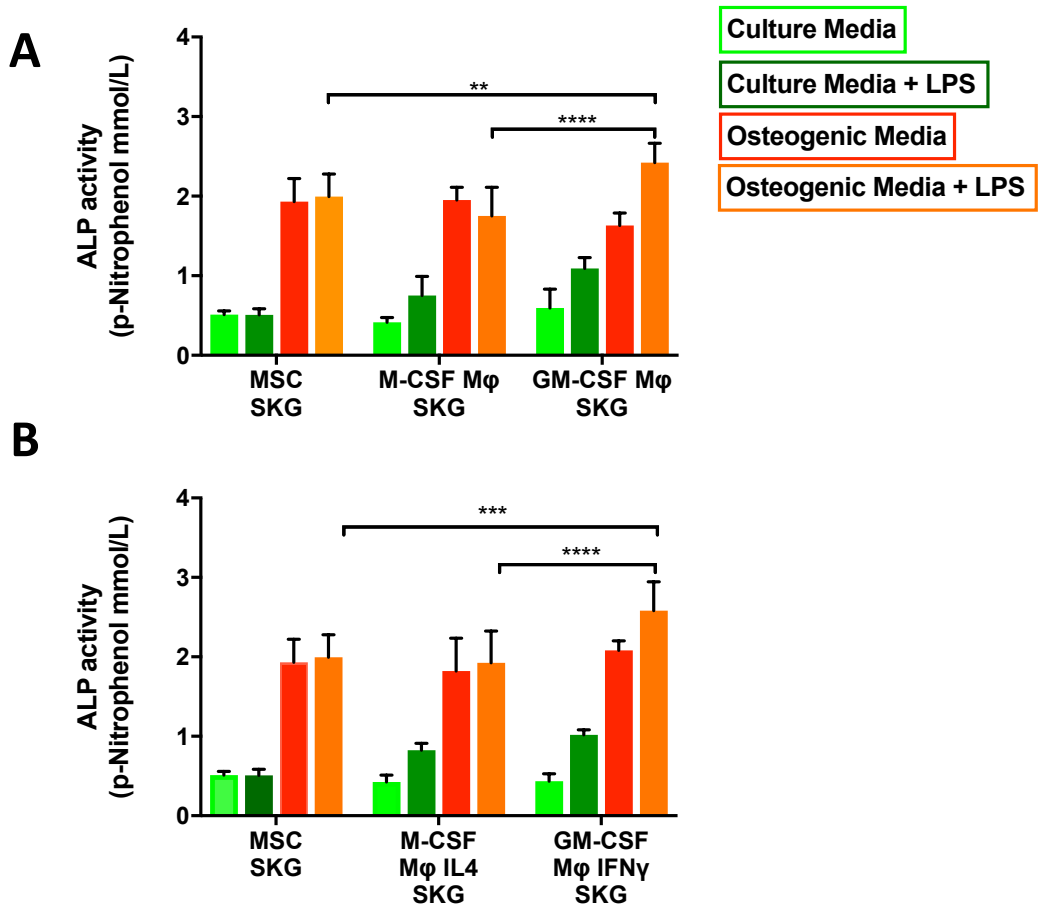


Figure 3.3: Polarized GM-CSF BM-derived Mϕs promote ALP expression by MSC. BM derived macrophages were differentiated with either 50 ng/mL M-CSF or GM-CSF for 5 days. During the last 24h of differentiation 20ng/mL of IL-4 was added to half of the M-CSF cultures, and 20 ng/ml of IFNγ to the GM-CSF cultures. **A**: $5x10^4$ of the differentiated Macrophages were added on the top of $1x10^4$ MSCs either in control or osteogenic media. **B**: A similar experiment was performed in the presence of 10ng/mL of LPS in all wells. Co-culture were kept for 7 days, with media changing twice per week. ALP activity was assessed at day 7. Graphs represent cumulative data of 3 experiments performed in triplicates, using BM derived Mϕ from different animals, and shown as mean +/- S.E.M. ** $p<0.0028$ *** $p<0.0002$ **** $p<0.0001$ Two-way ANOVA with Tukey's multiple comparisons test to MSCs alone.

Similar experiments were performed using BM-derived Mϕs from different background of mice: normal C57Bl/6 (IRF 5 WT and IRF5 KO - from Prof. Irina Udalova-) and SKG mice. For the following experiments, there were two main aims.

1) To test the capability of BM-derived GM-CSF macrophages (isolated from the bone marrow of mice with different genetic backgrounds) to enhance MSC differentiation towards the OB lineage; would the genetic background of GM-CSF macrophages affected their ability to promote MSC osteogenic differentiation.

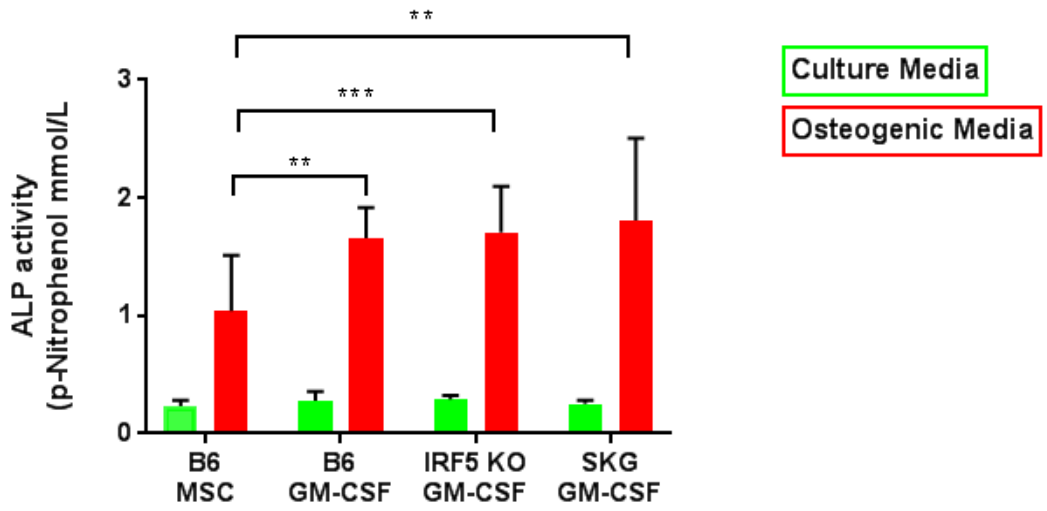


Figure 3.4: GM-CSF Mϕs from different strains are equally capable of promoting MSC differentiation. BM derived macrophages were differentiated from C57B6 IRF5 KO, WT and SKG mice with either 50 ng/mL M-CSF or GM-CSF. 1x104 MSCs were isolated from digested bone chips of C57Bl/6 mice, then plated in 96-well plate and left 24h to adhere. $5x10^4$ of the differentiated Macrophages were then added on the top of $1x10^4$ MSCs in control media or osteogenic media. Co-culture were kept for 7 days, with media changing twice per week. ALP activity was assessed at day 7. Graphs represent cumulative data of 3 experiments performed in triplicates, using BM derived Mϕ from different animals, and shown as mean +/- S.E.M. ** $p<0.0012$ *** $p<0.0004$ Two-way ANOVA with Tukey's multiple comparisons test to MSCs alone.

Interferon regulatory factor 5 (IRF5) plays a crucial role in Mϕs differentiation[113]. This transcription factor alters Mϕs polarization towards an inflammatory phenotype in both human and mouse models. IRF5-deficient mice produce less pro-inflammatory cytokines (IL-12, IL-23, TNF-a) and more IL-10 in response to LPS stimulus than WT mice. Quantification of ALP activity revealed that GM-CSF Mϕs derived from IRF 5 KO, C57B6 and SKG mice were all capable of inducing ALP expression in MSC co-cultures (Fig. **3.4**).

2) Are there any differences between BM-derived GM-CSF and M-CSF Mϕs (also isolated from the bone marrow of mice with different genetic backgrounds) in their capacity to promote MSC differentiation towards OB.

Murine MSCs were generated from bone chips of C57Bl/6 mice. Murine Mϕs, isolated from the BM of IRF 5 KO and WT mice and differentiated in vitro with either M-CSF or GM-CSF, were then plated with MSCs. At this stage, mono-cultures and co-cultures were exposed to 4 different conditions: basal culture medium +/- LPS, and osteogenic medium +/- LPS.

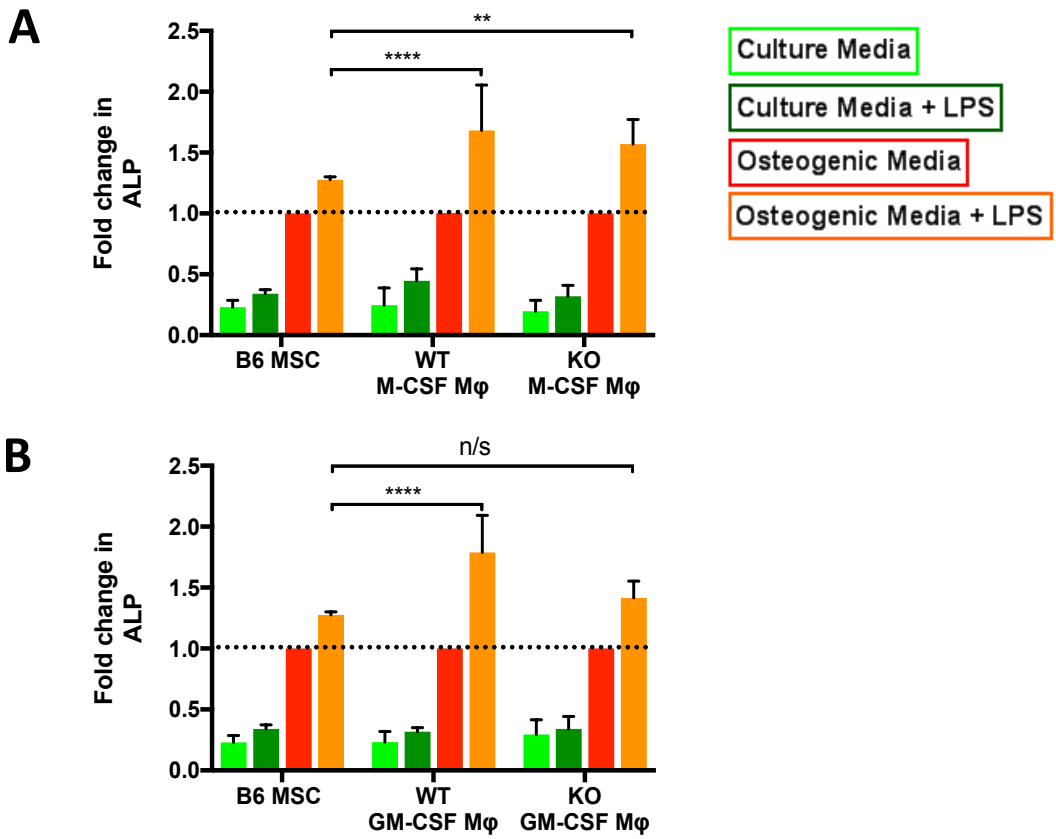


Figure 3.5: BM derived macrophages were differentiated from C57B6 IRF5 KO and WT mice with either 50 ng/mL M-CSF (Graph A) or GM-CSF (Graph B). 1x104 MSCs were isolated from digested bone chips of C57B6 mice, then plated in 96-well plate and left 24h to adhere. 5x104 of the differentiated Macrophages were then added on the top of 1x104 MSCs in control media and osteogenic media alone and control media and osteogenic media +/- 10ng/mL of LPS in all wells. Co-culture were kept for 7 days, with media changing twice per week. ALP activity was assessed at day 7. Graphs represent normalised data of 5 experiments performed in triplicates, using the red bar (OM) as the reference value, and BM derived Mϕ from different mice, and shown as mean +/- S.E.M. ** $p<0.0044$ *** $p<0.0001$ Two-way ANOVA with Tukey's multiple comparisons test to MSCs alone.

After 7 days of cell co- and mono-culture, quantification of ALP activity revealed that in the presence of LPS, M-CSF derived Mϕs from IRF 5 WT and KO mice were both able to induce ALP expression in MSC co-cultures. However, the graph **(Fig. 3.5 A)** shows that M-CSF Mϕs derived from IRF5 WT mice do not greatly enhances MSC capability to differentiate into osteoblasts, more than M-CSF Mϕs derived from IRF5 KO mice and MSCs themselves. This result is therefore aligned with the literature which states that M-CSF Mϕs derived from IRF 5 WT mice, does not express IRF 5 compared to GM-CSF Mϕs derived from IRF 5 WT mice[114]. Secondly, in the presence of LPS in the osteogenic medium, GM-CSF Mϕs derived from IRF5 WT mice largely increased MSCs' capacity to become osteoblasts in a co-culture system. Conversely, GM-CSF Mϕs derived from IRF5 KO

mice did not have any effect on MSC-ALP activity compared to WT Mϕs and to mono-cultures of MSC alone.

In conclusion, the assessment of ALP activity revealed that polarized and non-polarized M-CSF Mϕs could not enhance MSC differentiation compared to MSCs alone (even in the presence of LPS), when the genetic background of the bone marrow from which they derive is SKG. So LPS activation produced two main effects. Firstly, it greatly increased the effect of non-polarized GM-CSF Mϕs (from SKG bone marrow mice) without changing the osteo-inductive ability of the IFNγ-treated GM-CSF Mϕs (from SKG bone marrow mice). Secondly, GM-CSF Mϕs (pro- inflammatory macrophages) from the bone marrow of IRF 5 WT mice notably increased MSCs-ALP activity compared to monocultures of MSCs alone and to co-cultures with GM-CSF Mϕs from IRF 5 KO, which had no effect, as mentioned before.

3.3 Conclusions *in vitro*

To sum up this discovery it has been conducted in order to further investigate the role of MSCs and Mϕs in vitro, particularly focusing on the mechanism by which these populations influence an increased proliferation and differentiation capability of MSCs (**Fig. 3.6**). Bernardo and colleagues recently reported a study which explains the ability of MSCs to regulate Mϕs phenotype by switching from a pro-inflammatory to a tissue resident phenotype, in order to facilitate the wound healing process[115]. Moreover within this study, MSCs were shown to orchestrate the inflammatory response by increasing neutrophil migration towards the inflamed environment thus triggering an innate immune response and switching Mϕs phenotype in order to prevent further damage to the tissue[116].

MSCs have also been reported to directly affect the conversion of monocytes into a pro-inflammatory phenotype to a tissue resident phenotype, through direct cell contact and secretion of PGE_2 and IL-6[117]. The production of PGE_2 by MSCs directly interferes with the differentiation of epithelial cells; based on this MSC could potential act as immunomodulators and repair promoting cells [116]. Therefore, these soluble signalling proteins may be the major players in the transition of monocytes from a tissue repair to an inflammatory phenotype and vice versa.

Finally, it has also been shown the interdependency between polarization of Mϕs by MSCs and modulation of T-cell behavior. Activated T-cells secrete pro-inflammatory cytokines such as IFNγ and TNFα and modulate the expression of COX_2 in MSCs, which alters Mϕs polarization[118]. On

one hand, there is a lot of interest on the role of MSCs and Mϕs regulating the innate immune response however it is still not completely known how MSCs and Mϕs express in vivo their immunomodulatory effects. Therefore, the importance of these findings linking MSCs to Mϕs polarization will be further investigated in a murine model of AS in the next chapter.

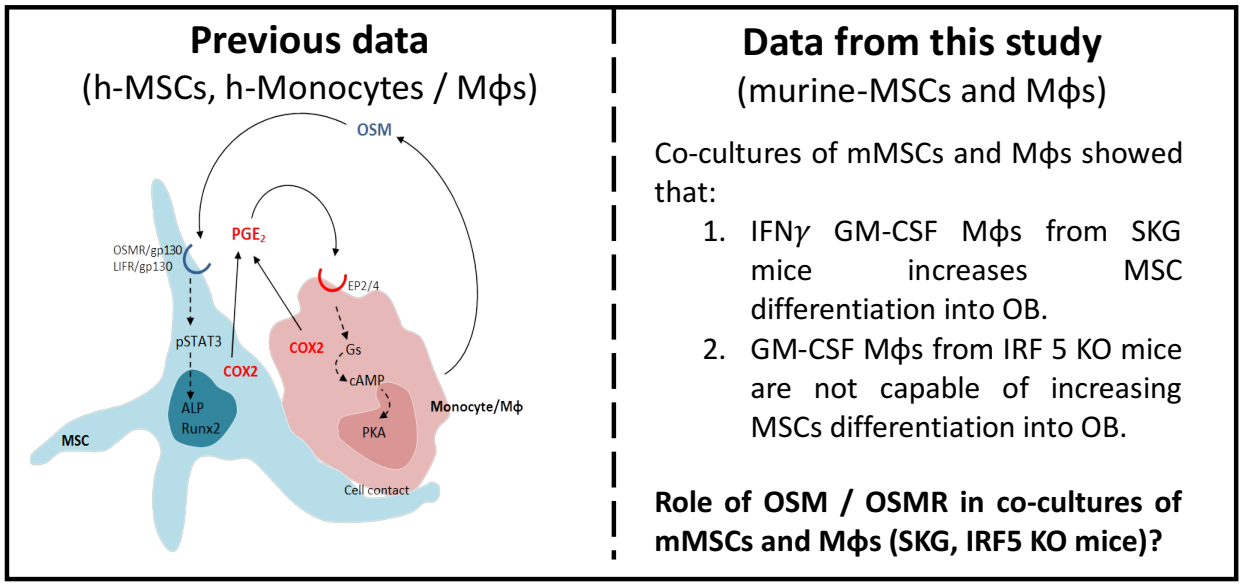


Figure 3.6: On the left side, the working model cartoon shows the MCS and Mϕs cross talk. Previous data from the Horwood lab proved that a direct cell–cell contact between h-Monocytes and h-Macrophages leads to MSCs differentiation into OB. More data showed that MSCs differentiation is driven by soluble factors such as: **OSM,** IL-24, CXCL5, EREG, IL-10, MMP9, LAMB3. However, only OSM it has been further investigated to have osteo-inductive abilities. On the right side of the picture are listed the main outcomes of the current study, including one future direction. Picture has been adapted from Nicolaidou et al. (Dphil Thesis, May 2011).

CHAPTER FOUR

NEW BONE FORMATION AT ENTHESIS

4.1 Introduction *in vivo*

The aim of this study is to evaluate the occurrence of enthesitis in a murine model of AS by undertaking a comprehensive time course study in the SKG mouse model. This model was chosen for its recapitulation of AS clinical features, including development of both inflammation and osteo-proliferation in the joints. To date, the understanding of enthesitis remains limited. Entheses are the insertion sites of tendons and ligaments to the bone surface and are essential structures for locomotion. Inflammation of the entheses (enthesitis) is a key feature of psoriatic arthritis and spondyloarthritis. In addition, the molecular steps that translate inflammation into resident tissue responses, result in new bone formation at the entheseal site. The in vivo study summarizes the clinical features of enthesitis, and the role of clinical and imaging instruments in detecting enthesitis are discussed together with their challenges and limitations.

4.2 Results in vivo

4.2.1 Enthesis development

For enthesis evaluation, several 4-week time course experiments were performed in SKG mice spanning from the start of the disease to its advanced stages with regular analysis, as described in the diagram below (**Fig 4.1)**.

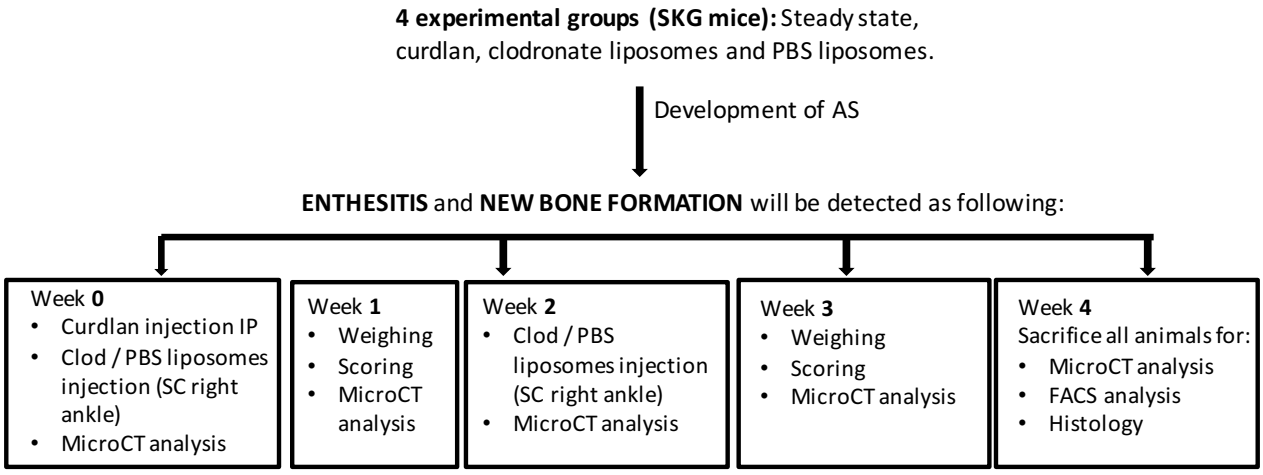


Fig. 4.1: In *vivo* timeline and the experimental design.

Firstly, microCt analysis was performed in order to detect variations in bone mineral density (BMD) and bone volume. Curdlan treated SKG mice showed a loss of thickness (**Fig. 4.2**) in the ankle bones (decreased cortical area fraction) and an increase of bone volume in the periosteal envelope of the joints (total cross-sectional area). This is due to an osteo-proliferation phase, one of the main features of the SKG – AS model.

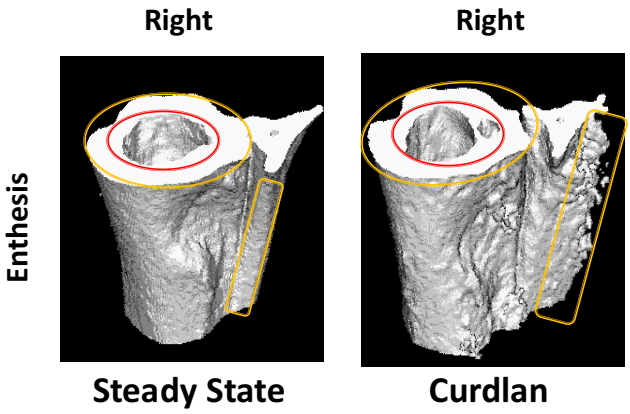


Figure 4.2: MicroCT analysis of cortical bone morphology of right enthesis of control mice (steady state) and curdlan treated mice. Entheses of curdlan mice show a loss of cortical thickness (cortical bone area -**marked in RED**-) and excessive bone formation at the periosteal envelope (total cross-sectional area -**marked in YELLOW**-) compared to the steady state.

Secondly, histological analysis (**Fig. 4.3 A-F**) helped to better comprehend the physiological location of Mϕs at enthesis. H&E staining on 4-week SKG-disease mice proved the presence of: chronic inflammation, great infiltration of inflammatory cells at enthesis, and erosion of synovial cartilage and membrane with migration of inflammatory cells towards enthesis. Masson Trichrome staining pictures indicated a clear inflammation expansion along longitudinal ligaments (e.g. for Peroneus tertius, brevis and longus ligaments). Moreover, Masson Trichrome staining pictures showed that excessive tissue formation in the entheseal joints was greatly increased in the more advanced stages of the disease (4 weeks). In order to detect the presence of macrophages at enthesis, immunohistochemistry staining (IHC) was performed for F4/80 –single colour DAB method on enthesis of 4-week SKG mice. However, the steady state image showed a great amount of aspecific binding (straight brown line) therefore it required further optimization steps.

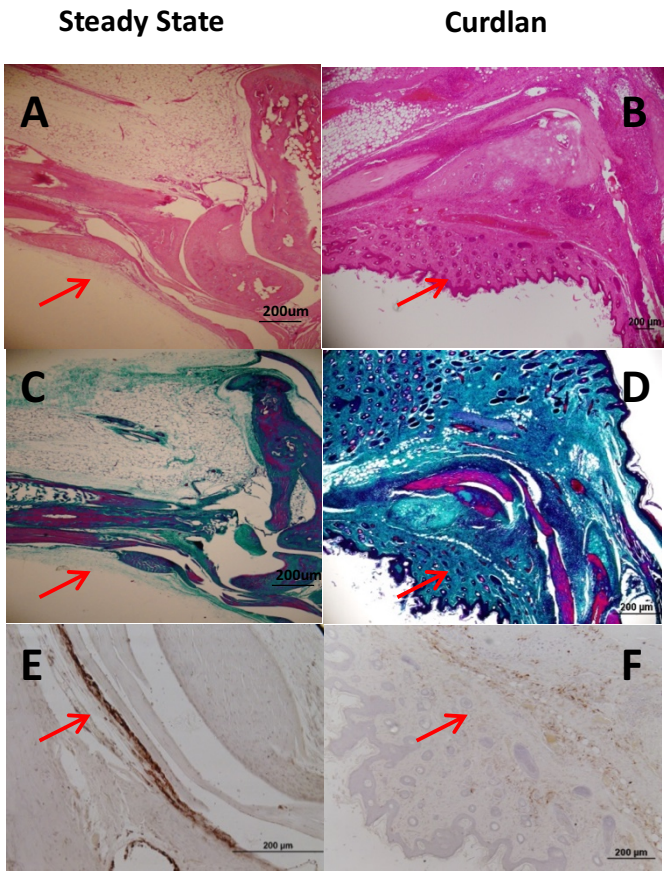


Figure 4.3: Histology analasys of right enthesis (4-week-old SKG mice) of Control mice and Curdlan treated mice. **(A-B)** H&E staining of right enthesis of Steady State and Curdlan treated mice. **(C-D)** Masson Trichrome staining on right enthesis of right enthesis of Steady State and Curdlan treated mice. **(E-F)** Immunohistochemical staining (brown) of macrophages (F4/80+) in 4-week-old SKG mice highlights their location in cortical bone (periosteal envelope).

Lastly, FACS analysis **(Fig. 4.4)** was performed to determine the absence/presence of macrophages at enthesis and to further characterize which subsets of macrophages are present at enthesis. However due to the heterogenic nature of the entheseal tissue and to the limited number of cells at enthesis, this technique did not reveal any significant changes between the disease and the steady state group.

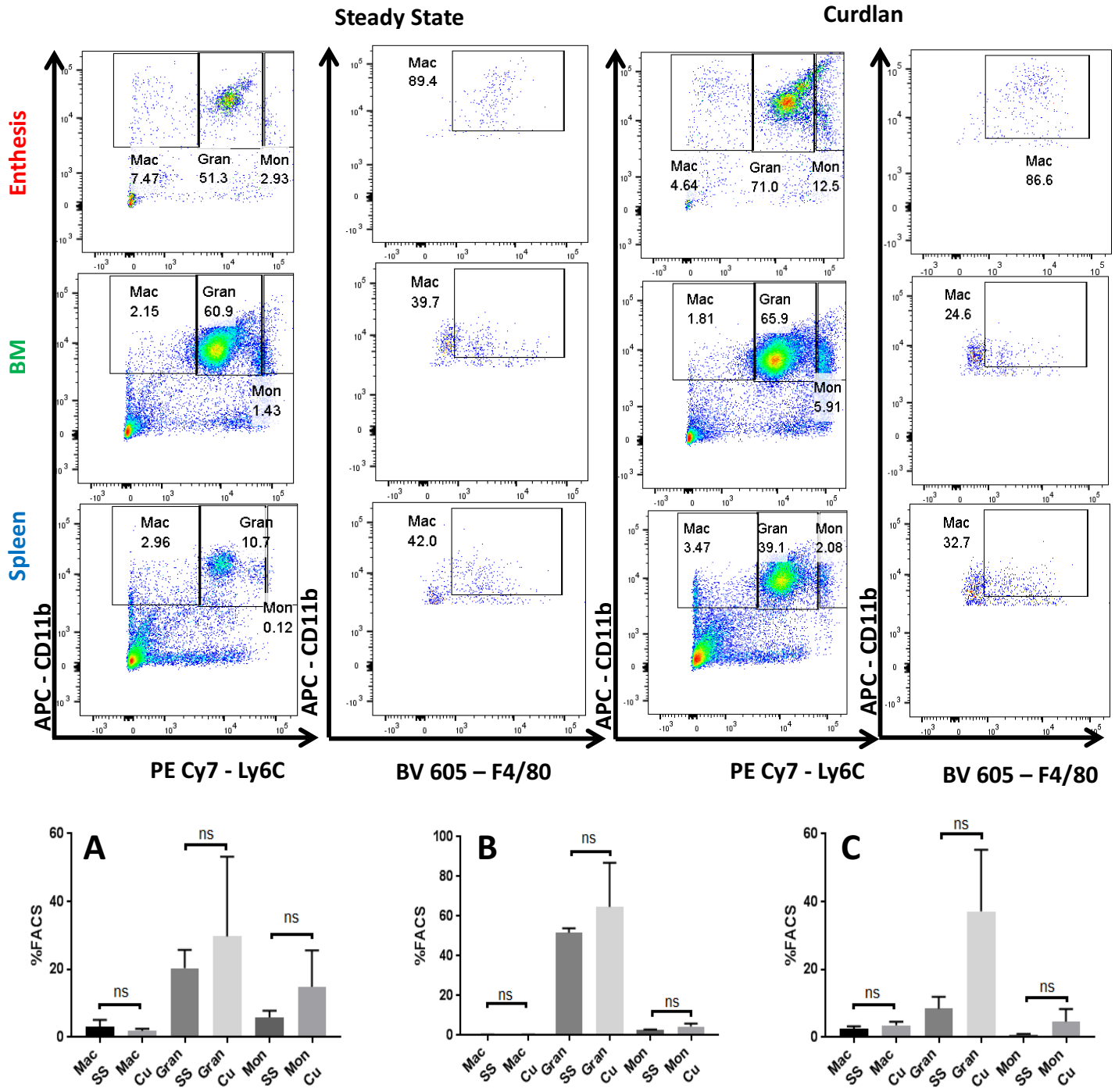


Figure 4.4: Evaluation of macrophages at enthesis through flow cytometry (FACS). FACS plots show (among $CD45^+$ entheseal cells): $CD11b^{low}Ly6c^+$ as macrophages (Mac), $CD11b^{int}Ly6c^+$ as granulocytes (Gran) and $CD11b^{high}Ly6c^+$ as monocytes (Mon)[1]. Graphs show during inflammation the behavior of inflammatory cells at enthesis bone marrow and spleen. (**A**, **B** and **C**) However percentages of macrophages, granulocytes and monocytes are not significantly different from the steady state (n/s).

4.2.2 In vivo Mϕ depletion at the enthesis

Recent studies have shown that macrophages can be detected in bone tissue and interact with bone cells. In terms of bone formation and repair, the interplay between macrophages and bone cells is vital [119]. Consequently, it was decided to deplete macrophages locally using clodronate liposomes and investigate their role in the pathogenesis of enthesitis in a murine model of AS. Once engulfed by phagocytic cells, clodronate liposomes do not escape from the cells. After disruption of the phospholipidic layer, the drug is dissolved in the aqueous compartments of Mϕs and released into the cell. Clodronate accumulates intracellularly, and once a threshold concentration is exceeded, the cell is irreversibly damaged and dies by apoptosis.

In accordance with the experimental design, animals were divided into 4 groups: steady state; curdlan; curdlan plus PBS liposomes; and curdlan plus clodronate liposomes. Apart from steady state, all animals received one intraperitoneal injection of curdlan at the beginning of the experiment. A subcutaneous injection of clodronate and/or PBS Liposomes was administered once every two weeks to the appropriate group. As shown in **Fig. 4.5 (A and B)** the clinical score was increased in all groups, which means all the mice developed AS. However, subcutaneous injections of clodronate liposomes did not affect neither the weight nor the clinical scores of the mice in the clodronate liposomes group. As shown in **Fig. 4.6 (A, B and C)** depletion of Mϕs was successfully achieved using local injections of clodronate liposomes at enthesis.

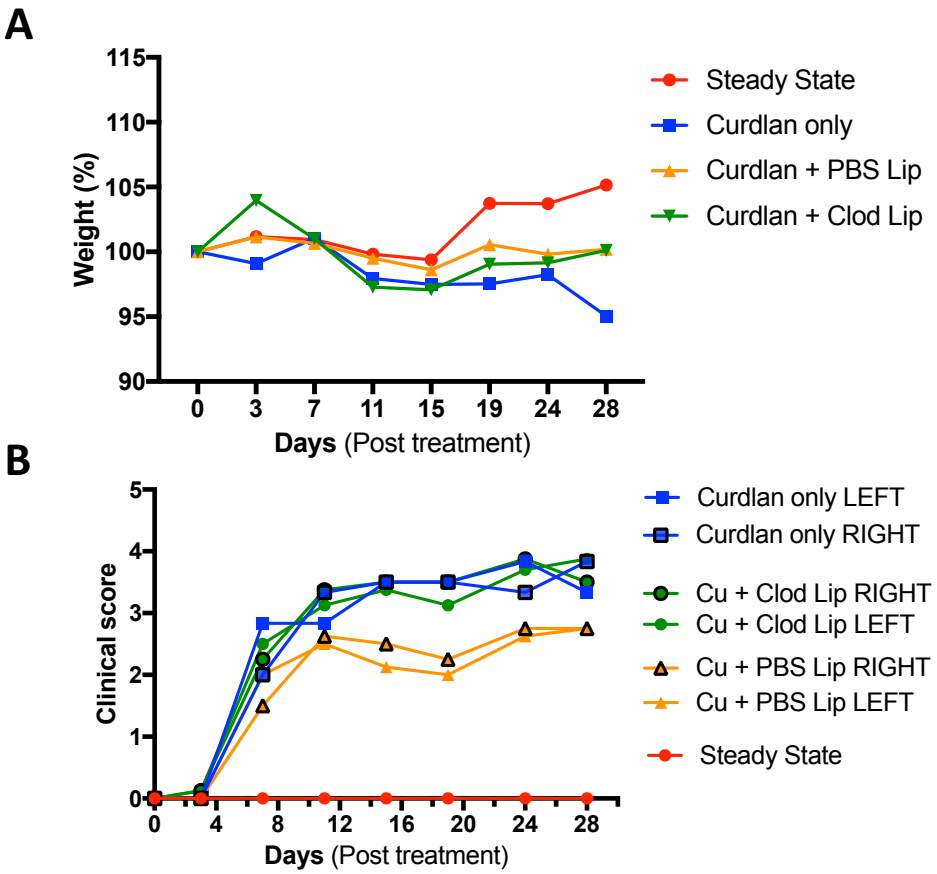


Figure 4.5: Mice were divided into 4 groups: steady state, curdlan, curdlan and PBS liposomes, and curdlan and clodronate liposomes. (**A**) shows each animal's weight over the time of the experiment. (**B**) shows the change of the clinical score over the treatment period for both legs: right-treated enthesis, which received 2 SC injections of clodronate liposomes, and left untreated enthesis, which did not receive any injection. Graph (**A)** represents normalised data of 1 experiments (n=4 mice/group) using all mouse starting weight as 100% all the data are plotted as a % change relative to the starting weight.

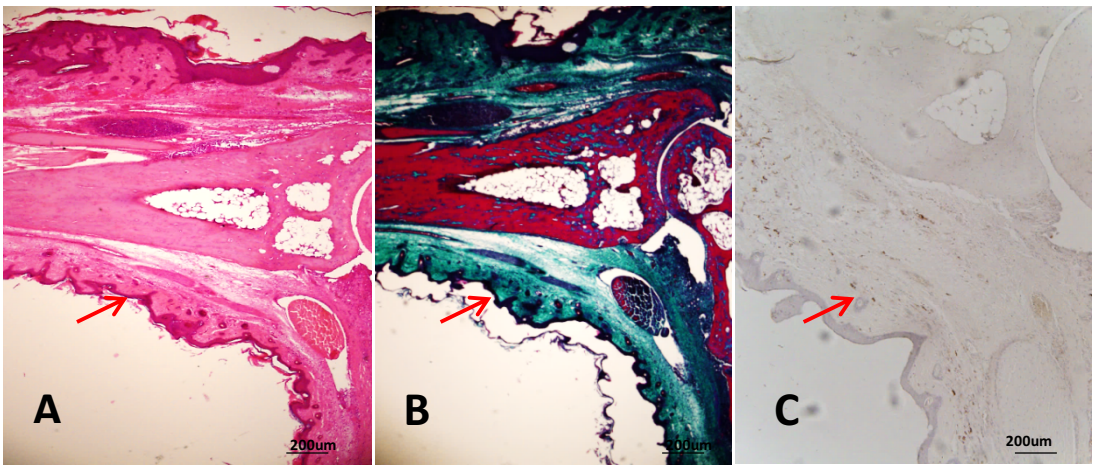


Figure 4.6: Histology analysis of right enthesis of curdlan and clodronate liposomes treated mice. **(A)** H&E staining on right enthesis of 4-week SKG curdlan-clodronate treated mice **(B)** Masson Trichrome staining on right enthesis of 4-week SKG curdlan-clodronate treated mice **(C)** Immunohistochemical staining (brown) of macrophages (F4/80+) in 4-week SKG mice highlights their successful depletion, using clodronate liposomes, in cortical bone.

As shown in (**Fig. 4.7; 4.8),** subcutaneous injection of clodronate liposomes in the right enthesis produced a clear local effect. The non-treated leg displayed a significant increase in abnormal bone deposition compared to time point zero. However, due to the subcutaneous injection of clodronate liposomes, the treated ankle did not show any significant bone changes compared to time point 0. One one hand, these findings suggest that clodronate liposomes have a local rather than a systemic effect. On the other hand, as the clinical scores of the clodronate liposomes group is increased (over the course of the experiment) there is no correlation between clinical score and effect of clodronate Liposomes at enthesis. Therefore, the absence of Mϕs at enthesis resulted to be critical in entheseal development and abnormal bone deposition at this site.

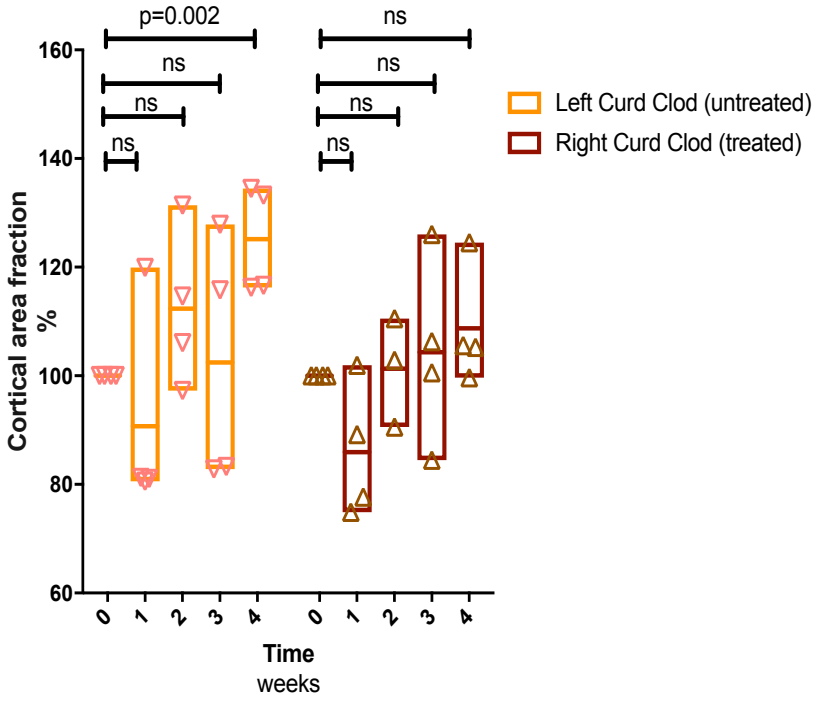


Figure 4.7: The graph shows the effect of clodronate liposomes on the cortical area fraction between the treated and non-treated entheses. At day 0 mice in the curdlan and clodronate liposomes group received one injection (IP) of curdlan. At days 1 and 14 the same group of mice received a single SC injection of clodronate liposomes in the right ankle. Graphs represent normalised data of 2 experiments performed with 3 different experimental groups, with 4 animals each, and shown as mean +/- S.E.M. * p<0.002 RM one-way ANOVA.

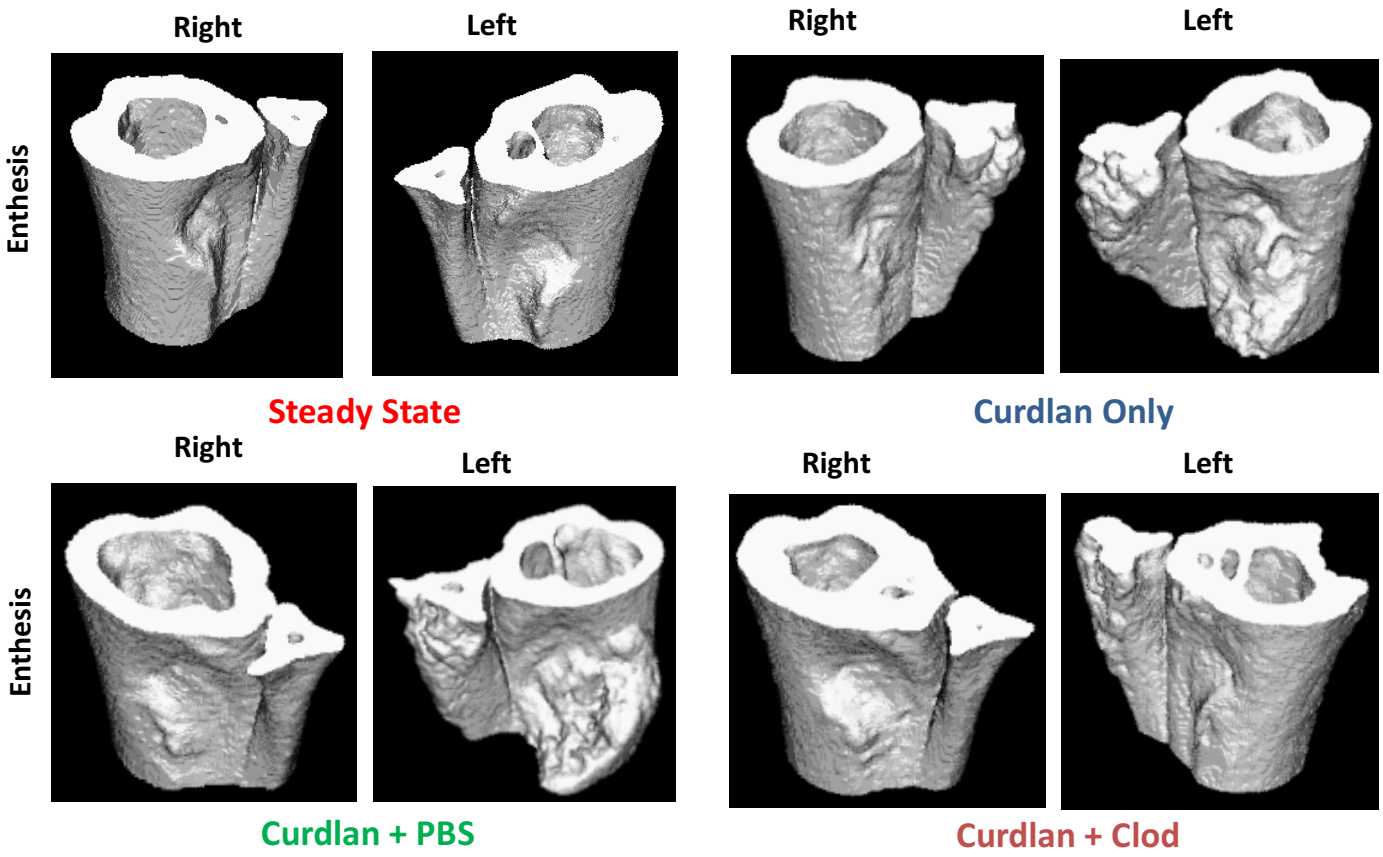


Figure 4.8: MicroCT analysis of cortical bone morphology of right and left enthesis of mice, belonging to the following experimental groups: curdlan only, curdlan + PBS liposomes, curdlan + clodronate liposomes. Enthesis of curdlan-only group clearly show a loss of cortical thickness (Cortical Bone area) and excessive bone formation at the periosteal envelope (total cross-sectional area). Enthesis of the curdlan and PBS group displays a loss of cortical thickness of the *right enthesis* (treated leg) and excessive bone formation at the periosteal envelope of the left enthesis (non-treated leg). Conversely, the subcutaneous injection of clodronate ameliorated the disease in terms of gain of cortical thickness at the right (treated) enthesis.

4.2.3 In vivo OSMR - Fc neutralization at the Enthesis

We have previously shown that macrophage-derived oncostatin M (OSM) is able to promote osteogenic differentiation of MSC and that injecting recombinant mOSM over the calvaria increases bone formation in vivo[104]. Recent studies from Guihard et al. showed that overexpression of mOSM in long bone induces woven bone formation that resembles intramembranous bone healing[120]. Moreover, it has been demonstrated that mOSM is expressed early during the inflammatory phase, primarily by macrophages, and that OSM or OSMR-deficiency delays bone healing. Besides that, new research published in Nature Medicine[121] highlights the important role the IL-6 family cytokine OSM plays in driving intestinal inflammation in IBD. Additionally, expression of OSM in intestinal biopsy samples from patients with IBD

predicts response to anti-TNF agents. Here, with the use of the SKG model of AS, it was decided to neutralize OSM signalling using an injected soluble 'decoy' receptor. This suppresses SKG-derived colitis in mice but increases abnormal bone formation at the entheseal site. According to the literature, OSM is a member of the IL-6 cytokine family, which also includes IL-6, IL-11, leukemia inhibitory factor (LIF) and cardiotrophin-1. Mainly produced by activated T cells, monocytes, and dendritic cells, OSM shows 27% amino acid identity with LIF[122]. Two different OSM receptors have been discovered so far: OSM type I, which consists of gp130 and the LIF receptor (LIFR); and OSM type II, which consists of gp130 and OSMR-β[123]. Intestinal pathology is mediated by hematopoietically derived OSM, as it promotes inflammatory behavior in gut-resident stromal cells. OSM is expressed as part of a core inflammatory cytokine module including IL-6 and IL-1α/β, with effects that are distinct from the closely related IL-6 and synergistic with those of TNF. While IL-23, through its actions on T cells, is a critical trigger of bacterially driven colitis [124, 125], OSM, by promoting chemokine, cytokine, and adhesion factor production by stromal cells, may act as an inflammatory amplifier and driver of disease chronicity. It is yet to be determined whether OSM can influence tissue fibrosis via the stromal compartment. Interestingly, OSM has been demonstrated to bind extracellular matrix components (including collagen, laminin, and fibronectin) in such a way that offers protection from proteolytic degradation and maintains biological activity for prolonged time periods[126]. In accordance with the experimental design (**Fig. 4.9**), animals were divided into 2 groups: Control group (curdlan plus SC injection at right enthesis of IgG2aFC, IgG2a or PBS), and OSMR–Fc group (curldan plus SC injection at right enthesis of OSMR–Fc).

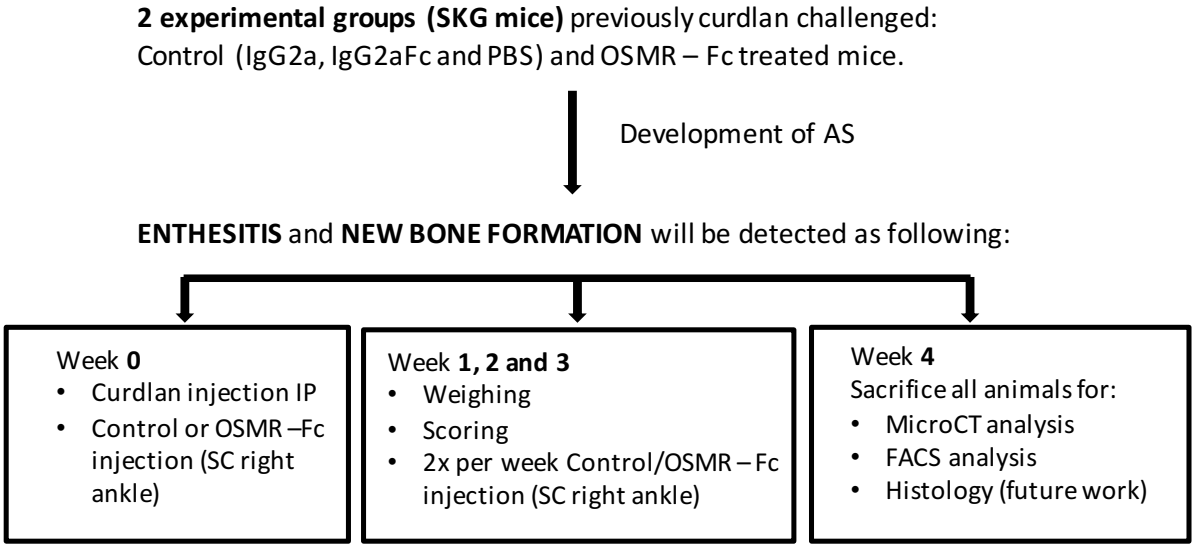


Fig. 4.9: In *vivo* timeline and the experimental design.

All the animals received one intraperitoneal injection of curdlan (in order to trigger the development of AS) at the beginning of the experiment. A subcutaneous injection of either OSMR–Fc or the corresponding vehicle was administered twice a week to the appropriate group for 4 weeks in total. As shown in **Fig. 4.10 (A and B)** the clinical score was increased in both groups, which means all the mice developed AS. However, subcutaneous injections of OSMR-Fc exacerbated the disease at a joint level, and so largely affected the clinical scores of the mice in the OSMR–Fc group compared to the control. As shown in **Fig. 4.11 (C and D)** the sum of front and rear paw measurements significantly increased in the OSMR–Fc treated group compared to the control, particularly when the disease became chronic: at days 24 and 28 (human end point of the experiment).

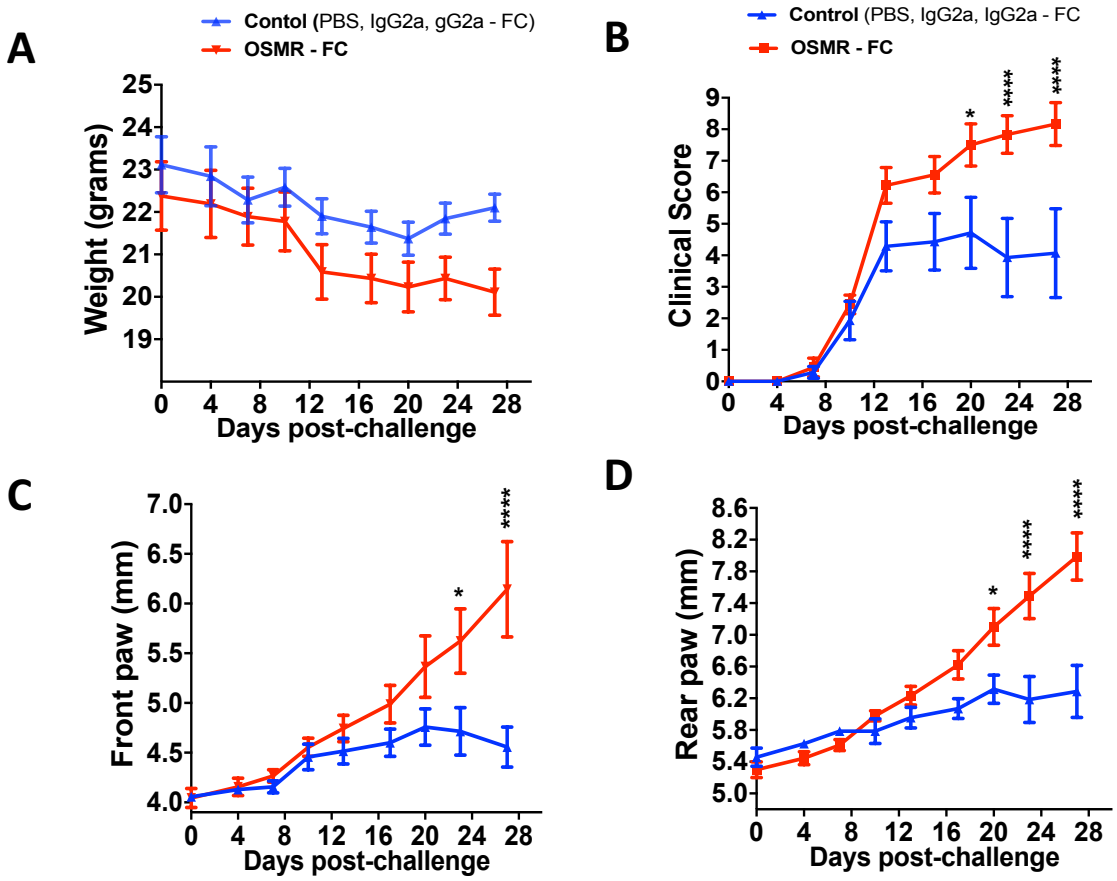


Figure 4.10: Animals were divided into 2 groups: Control (IP Curdlan and SC 2x /week IgG2a-FC, IgG2a and PBS in the right leg) and OSMR-Fc (IP Curdlan and SC 2x /week OSMR-FC in the right leg). (**A**) change in weight over time, (**B**) clinical score, over the treatment period of 4 weeks, of both legs together. Ankle diameter as well as clinical score and weight were measured weekly. Graphs **(C)** and **(D)** represent the sum of front paw and rear paw measurements. The graphs represent data of 1 experiment performed with 2 different experimental groups, with 9 animals each, and shown as mean +/- S.E.M.

As shown in **Fig. 4.11**, microCt analysis was performed to detect variations in bone mineral density (BMD) and bone volume. OSMR–Fc treated mice showed a loss of thickness in the ankle bones (decreased cortical area fraction) and an increase of bone volume in the periosteal envelope of the joints (total cross-sectional area). Enthesis of the curdlan-control group displays an increase of cortical thickness and no sign of excessive bone formation at the periosteal envelope (except for a minor bone increase in the IgG2aFc). Conversely, the subcutaneous injection of OSMR-Fc made the disease worse in terms of increased deposition of abnormal bone formation at both enthuses. Despite the local SC injection, the effects of OSMR-Fc were observed in all 4 joints and interestingly this treatment ameliorated the ileitis symptom associated with AS. Recent studies have shown that OSM may evoke distinct events during acute and chronic inflammation, which could relate to this cytokine's pro- and anti-inflammatory characteristics[127].

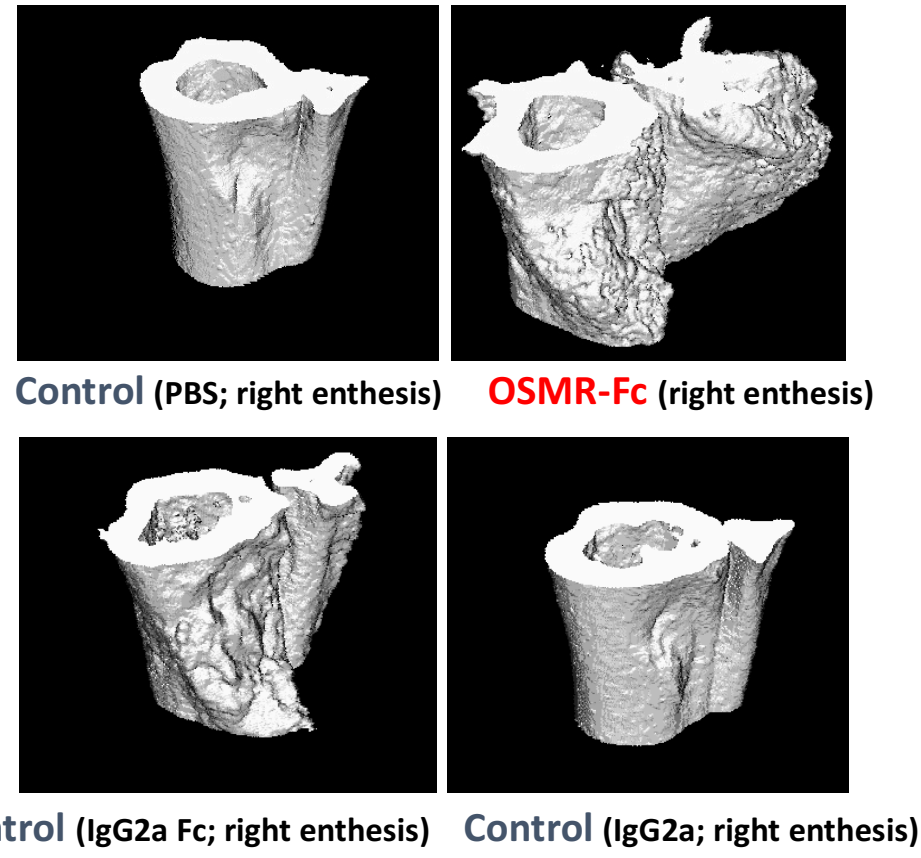


Figure 4.11: MicroCT 3D reconstruction of cortical bone morphology of right enthesis of SKG mice. Mice were divided into 4 groups: curdlan only; curdlan + SC injection IgG2a-Fc; curdlan + IgG2a; and curdlan + OSMR-Fc. Injection of both Control and OSMR-Fc occurred at the right enthesis. Images are representative of n=9 animals.

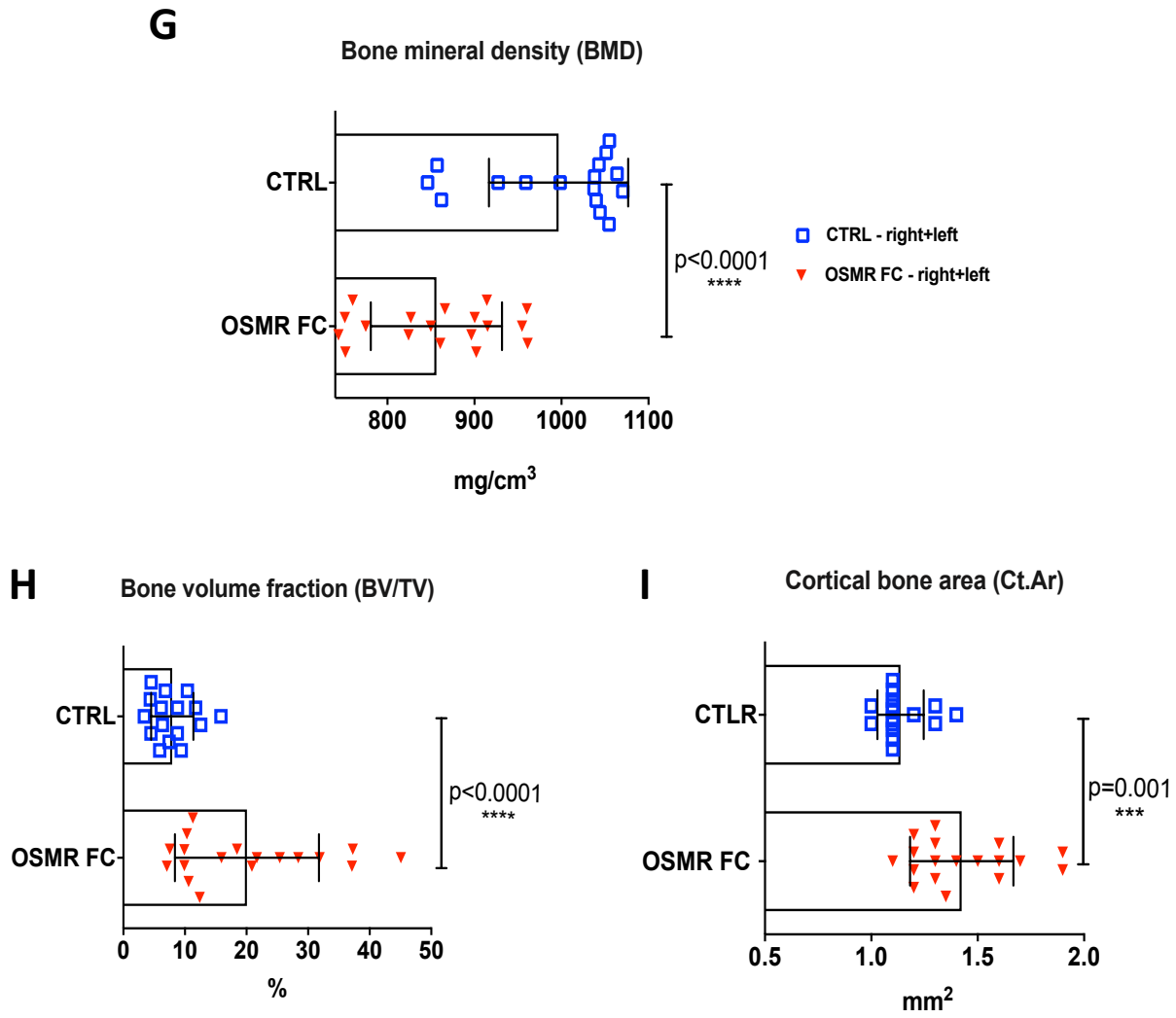


Figure 4.12: MicroCT analysis of BMD, bone volume fraction and cortical area fraction of right and left enthesis of mice, summed together, belonging to the following groups: Control (IP injection of curdlan and SC injection IgG2a-Fc right leg) and OSMR-FC (IP injection of Curdlan and SC injection of OSMR-Fc right leg). Enthesis of curdlan-OSMR-Fc group clearly show a decrease in the quality and the microarchitecture of the bone (**Graph G**) and excessive bone formation at the periosteal envelope as displayed by the significant increase in both bone volume fraction (**Graph H**) and cortical bone area (**Graph I**). Conversely, Enthesis of the curdlan-control group displays healthier bone quality (increase of BMD **Graph G**) and reduced abnormal bone formation at the periosteal envelope (**Graph H and I**). The graphs represent data of 1 experiment performed with 2 different experimental groups, with 9 animals each, and shown as mean +/- S.E.M. P value summary: *** p<0.001, **** p<0.0001 using Unpaired two-tailed T test.

As described in the above microCt analysis (**Fig. 4.12)**, subcutaneous injection of either OSMR–Fc or the appropriate vehicle at the entheseal site produced a clear effect on the ankle bone. In **Fig. 4.12 G** is displayed a significant difference in BMD (Bone mineral density) between the OSMR–Fc and the control group. This value measures the amount of mineral (mostly calcium and phosphorous) contained in a particular bone volume. Bone mineral density is used as an indicator of decreased bone mass in diseases like osteoporosis, to assess the efficacy of osteoporosis treatments, and to predict the likelihood of bone breakage. **Figure 4.12** tells us that the OSMR–Fc group underwent new bone apposition at the entheseal site after subcutaneous injection, however the quality of the bone apposed is very poor and lacks minerals, so the BMD measurement is decreased compared to the control group. Consistent with the pathophysiology of enthesitis in AS, which affects cortical structure and bone tissue mineralization, Bone volume fraction (BV/TV) and Cortical area fraction (Ct.Ar) behaved differently. As shown in **Fig. 4.12 H and I,** BV/TV and Ct.Ar were greatly increased in the OSMR–Fc group compared to the control. This increment is due to the excessive new bone formation that occurred at the entheseal site of the OSMR–Fc treated mice, and did not happen in the control mice.

4.2.4 Conclusions *in vivo*

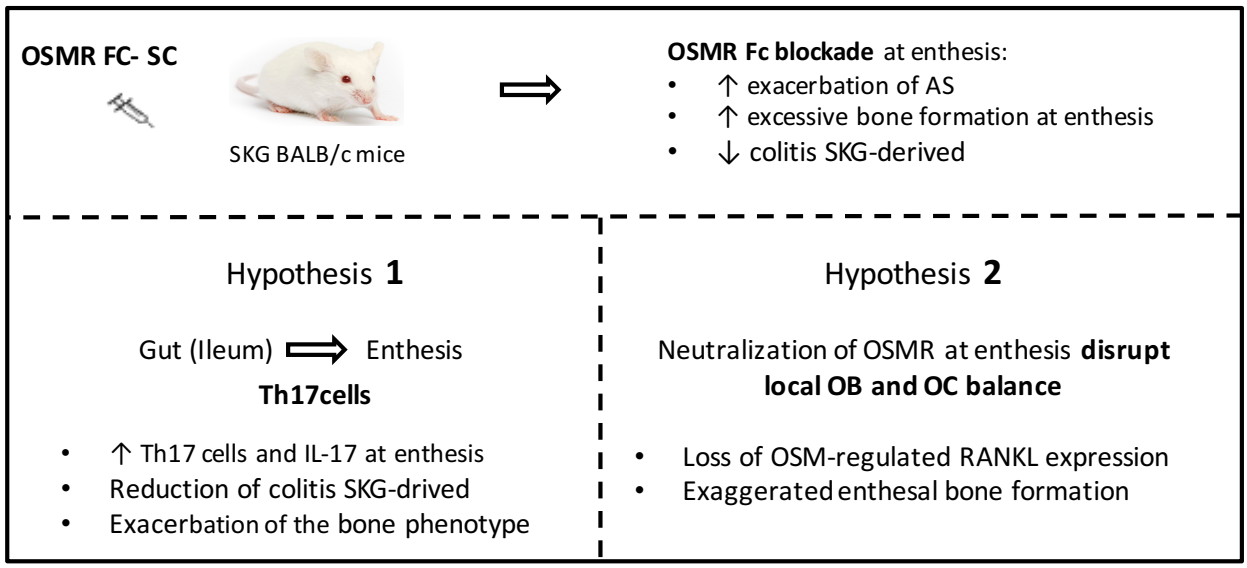


Figure 4.13: The working model represents a summary of the results obtained in the *in vivo* part. Injection of a soluble decoy receptor (OSMR-Fc) at the right enthesis of curdlan challenged mice led to: exacerbation of AS, abnormal bone deposition at the entheseal site and decrease of colitis SKG-derived in all the OSMR-Fc treated mice. The bottom part of the schematic shows two possible hypothesis that justify the absence of colitis (Hypothesis **1**) and the excessive bone formation at the entheseal site (Hypothesis **2**) in the OSMR-Fc treated mice.

CHAPTER FIVE

CONCLUDING DISCUSSIONS

5.1 Conclusions

Many of the immune processes associated with the pathogenesis of AS are mediated by an imbalance between pro-inflammatory and anti-inflammatory cytokines in the ankylosed joints. The continuous cytokine imbalance promotes disproportionate differentiation of osteoclasts and osteoblasts that leads to inflammatory bone disorders. Several studies have clearly demonstrated the contribution of monocyte Mϕs types of cells (lineages) in overall bone homeostasis[128]. Osteoblastic differentiation involved in bone modelling is supported by the presence of inherent Mϕs situated within the endosteal and periosteal tissue (in the bone lining). Physiological bone formation or bone repair is impeded by any defect in these cells [28]. Monocytes were shown to secrete various osteoinductive mediators including bone morphogenic protein 2, TGF-beta and OSM. Both in vitro and in vivo, these stimulate bone formation [129, 130].

The *in vitro* findings of the study showed that polarizing Mϕs from SKG mice towards a pro-inflammatory phenotype enhances MSC osteogenesis by increasing ALP activity, whereas anti-inflammatory Mϕs could not promote ALP activity compared to GM-CSF Mϕs and to mono cultures of MSCs. Moreover, it was demonstrated that GM-CSF polarized Mϕs increased MSC osteogenic differentiation in the presence of an inflammatory stimulus such as LPS. Therefore, this result suggests that they might be involved in the pathogenesis of AS through enhancing abnormal bone formation at enthesis. Moreover, it was interesting to observe that the differentiation signal used to generate Mϕs affected their ability to influence MSC differentiation[131, 132]. The capability of GM-CSF to augment MSCs' development into OB was tested using a different genetic background of mice: IRF5 $^{-}/^{-}$ and WT mice. IRF5 belongs to the IRF family, which includes transcriptor factors that regulate: Mϕs polarization, phenotypic changes and adaptation of Mϕs to different tissues. Though investigations on this topic are still ongoing, it has already been shown that *IRF-1, IRF-5*, and *IRF8* drive the pro-inflammatory phenotype, while *IRF-3* and - *4* support tissue repair Mϕs [133]. Both M-CSF and GM-CSF-derived WT bone marrow Mϕs were able to enhance ALP-MSC activity in the co-culture system and this was augmented by the addition of LPS. However, whilst GM-CSF-derived Mϕs from WT can significantly promote OB differentiation, GM-CSF-derived Mϕs from IRF5-/- mice cannot.

Therefore, we assert that the lack of IRF5 in GM-CSF Mϕs (pro-inflammatory) may lead to a significant decline in the secretion of chemokines and cytokines. This also illustrate that IRF5 deficiency may drive inflammation by modulating both Mϕs phenotypes and potentially the

recruitment of neutrophil. This means that for autoimmune diseases such as AS and RA IRF5 could be a future therapeutic target in clinical applications.

In an *in vivo* system Mϕs are actively involved in triggering the innate immune response and regulating the adaptive immune response. Mϕs dysfunction causes an inappropriate immune response, hence it has been implicated in many autoimmune diseases, such as AS[134].

To assess the role of macrophages locally in the SKG murine model, it was decided to deplete them from the complex environment. Clodronate liposomes, and PBS-liposomes as controls, were used to target and deplete entheseal macrophages during the osteoproliferative phase of AS in SKG mice (Fig. **5.1**).

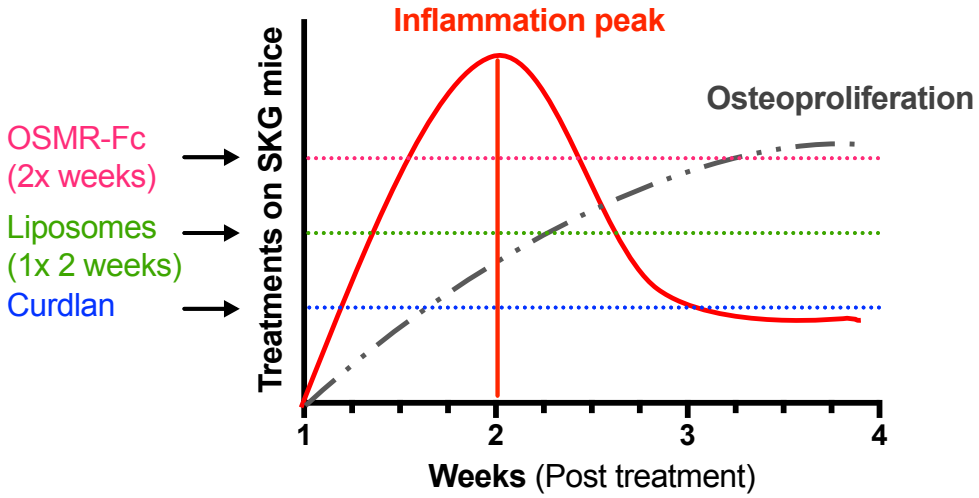


Figure 5.1: Early (1-2 weeks), intermediate (2 weeks) and late phases (3-4 weeks) of AS progression is characterized by inflammation, abnormal and excessive tissue and ectopic chondrocyte formation at enthesis. In advanced stages, inflammation is decreased, though bone deposition and osteoproliferation remained stable. The graph shows the natural pattern of AS in SKG mice as following described: on the **Y axis** are shown all the possible treatments (curdlan, clodronate or PBS liposomes and OSMR-Fc) that have been experimented so far during the in vivo experiments, on the **X axis** are displayed weeks post-treatments.

The *in vivo* research here showed that subcutaneous injection of clodronate liposomes ameliorated the disease in terms of gain of cortical thickness and lack of excessive bone deposition at the right treated-enthesis. This was proved by looking at the curdlan group where all the mice clearly showed a loss of cortical thickness and excessive bone formation at the periosteal envelope. However, the subcutaneous of clodronate liposomes brought several problems. The disadvantages of this technique were mainly: (i) local inflammation caused by the injection itself

(ii) the lack of specificity of the clodronate on Mϕs depletion, (iii) the technical complexity of checking the efficacy of the depletion, and (iiii) the incapability of this method to distinguish between the roles of resident versus recruited Mϕs in the new entheseal bone formation. Moreover, future interventions where the recruitment of monocytes at enthesis is blocked by using CCR2-blocking Ab, could improve the specificity of this technique.

As previously described, during inflammation and bone disorders, Mϕs seem to play a vital role in the regulation of bone formation. According to that, in culture models monocytes/Mϕs were able to produce OSM, important cytokine which belongs to the IL-6 family, that consequently supported MSCs differentiation into OB[135].

To define the entheseal role of OSM in the SKG murine model, it was decided to neutralize OSM signalling using an injected soluble 'decoy' receptor. This suppresses SKG derived colitis in mice but enhances abnormal bone formation at the entheseal site during the osteoproliferative phase of AS in SKG mice (Fig. **5.1**). The *in vivo* research here revealed that subcutaneous injection of OSMR–Fc ameliorated the disease in terms of disappearance of SKG-derived colitis and ileitis (ongoing investigation, data not shown). On the other hand, it caused exacerbation of the disease in terms of loss of cortical thickness and gain of excessive bone deposition at both entheses. This was proved by looking at the Control group (IP injection of curdlan and SC injection of IgG2a, IgG2aFc and PBS in the right leg), in which all the mice clearly showed a considerable exacerbation of the SKG-derived ileitis, a decrease of cortical thickness and decrease of bone formation at the periosteal envelope.

Moreover, subcutaneous injection of OSMR–Fc showed a tight interdependence between OSM signalling and Th17 cells activities. That AS leads to induction of Th17 cells, which express IL-17, is already known. Although Th17 cells has a strong pathogenic activity in mouse models of AS, in animal models of colitis and AS Th17 cells together with IL-17A show a combined pathogenic and tissue-protective role. Moreover, in mice models of colitis Th17 cell cytokines such as IL-17A and IL-22 have a protective role, while other cytokine such as IL-17F and IL-21 have a pathogenic role[136]. Indeed, FACS analysis of front limb of OSMR–Fc treated mice (data not shown) showed a significant increase of Th-17 cells both in the paws and in the spleen, compared to the control group. This result implies that OSM local blockade could be interfering with the homing and circulation of Th17 cells systemically. Potentially, the influx and proliferation of Th17 cells and the expression of IL-17 in situ is supported by neutralization of OSMR at the entheseal site. Expansion

of Th17 cells and consequently IL-17 at enthesis of OSMR–Fc treated mice worsens the disease at a bone level (reduced cortical thickness and increased abnormal bone apposition at the entheseal site).

To sum up, both the in vitro and in vivo results are promising for the following reason. It would be feasible to manipulate macrophages towards a phenotype that supports the resolution of inflammation and regulation of bone repair via enhanced differentiation of MSCs. However, the molecular interaction between MSCs and Mϕs is poorly understood in vivo. The discovery of this, would allow us to better understand the molecular mechanisms that regulate the excess of bone formation in AS.

5.2 Caveats of the approach and future directions

5.2.1 *In vitro*

The findings presented above provide a good starting point to study the direct regulation of murine MSC osteogenic differentiation by murine Mϕs in vitro. However, this mechanism should be further investigated with experiments such as the determination of cytokines levels through ELISA, Western Blotting, RNA isolation and RT-PCR. To identify the implication of soluble factors in the Mϕs/MSC co- cultures, a transwell system should be used. Moreover, it was previously demonstrated that monocytes/macrophages secrete a major cytokine of the IL-6 family, oncostatin M, to promote osteogenesis in MSCs[120]. OSM could increase osteogenic differentiation of MSC in vitro and in vivo when either injected or overexpressed in mice[137]. According to the literature it would be interesting to further validate the role of OSM as a key player in the Mϕs-induced osteogenesis, both in vitro and in vivo. In line with what mentioned above, it would be curious to test the effect of either mOSM (as a cytokine) or OSMR-FC into a co-culture system with MSCs using Mϕs from IRF 5 WT and KO mice. This will allow us to validate weather GM-CSF Mϕs from WT mice decrease their ability to promote MSC's differentiation and reach equal efficacy compared to GM-CSF Mϕs from IRF 5 KO mice.

Moreover, future caveats and technical improvements for the co-culture experiments are listed below. Firstly, it would be helpful to purify MSC's population, used for the in vitro assays, by sorting the $CD45^-$ portion from the $CD45^+$ ones, and then use the $CD45^+Sca1^+CD90.2^+$ population in the experimental assays.

Secondly, the ratio of MSCs and Mϕs, used in the co-culture systems, needs to be further optimized also in term of comparison between the 1:1 and 5:1 ratio of MSCs/ Mϕs.

Lastly, it would be interesting to test alternative polarization signals for Mϕs in addition to IL-4, when these cells are used in co-culture with MSCs. Indeed, Mϕs polarization should be then confirmed by monitoring the mRNA expression of iNOS (in pro-inflammatory Mϕs) and Arginase (in tissue repair Mϕs) in resulting Mϕs.

5.2.2 *In vivo*

The findings presented in this study provide preliminary data for potential treatments for the prevention of enthesitis and abnormal bone formation in AS. So far, very few new drugs for the treatment and the assessment of enthesitis have advanced in clinical trials. Current treatments aim to resolve entheseal inflammation and to prevent the inflammation-induced tissue responses. Drugs such as NSAIDs are generally used in rheumatic disorders (for example AS). They significantly reduce entheseal inflammation and osteitis by both blocking vasodilation of bone marrow vessels and inhibiting PGE_2-associated pain responses at the enthesis[138, 139].

As PGE_2 is a potent inducer of osteoblasts, NSAIDs suppress tissue responses associated with enthesitis[140]. In addition, TNF inhibitors improve the signs and symptoms of peripheral enthesitis, such as in the heels of patients with axial SpA and peripheral entheseal involvement[141]. The efficaciousness of TNF inhibitors in ameliorating peripheral enthesitis has been proven by a number of clinical trials in AS[142]. Newer data shows not only TNF inhibition, but also a significant response of enthesitis after the blockade of IL-23 and IL-17A.

Until now, the main goal of the pharmacological treatments for enthesitis is to block entheseal inflammation and to resolve clinical symptoms associated with it. However, is still not clear weather targeting and solving entheseal inflammation could lead to the systemic amelioration of the other symptoms of AS. Targeting Mϕs *in vivo* is one potential technique to block new bone formation at enthesis, so future studies should look to manipulate Mϕs in vivo at enthesis of SKG mice previously challenged with curdlan. This will involve the following techniques.

Firstly, it would be interesting to inject OSMR-Fc systemically (IP injection), and then test its therapeutic potential after the onset of the disease instead of before the developing of AS, as previously described. In parallel to that, it will be needed control experiments to measure how the

OSMR-Fc depletion would affect RANKL expression and consequently osteoclasts numbers and activity.

Secondly, new bone formation can be blocked by depleting Mϕs with experiments that better separate the roles of resident versus recruited Mϕs in AS by blocking their recruitment with anti-CCR2 or anti-Ly6G antibody respectively.

Thirdly, Mϕs can be targeted by using different approaches: blockage of Neutrophil migration (using macrophage-derived neutrophil chemotactic factor MNCF)[143], subcutaneous injection of M-CSF and GM-CSF-derived Mϕs (previously isolated from murine bone marrow and cultured), and blockage of M-CSF-receptor (anti-Fms antibody[144]).

CHAPTER 6

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