The Interaction of Regulatory T-cells and Osteoclasts in Health and Ageing

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Declaration

It is hereby declared that this thesis and the research work upon without it is based were conducted by the author, Raquel Lopera Burgueño

Raquel Lopera Burgueño

Por y para ti yayo 

Acknowledgments

Firstly, I would like to thank my supervisors Graham Wright, Katherine Staines, and Peter Barlow. Their support and advice have been vital for the completion of this project.

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Abstract

Regulatory T cells (Tregs) are part of the adaptive immune system, and their main role is to maintain peripheral tolerance. Emerging evidence suggests that Tregs play a key role in regulating healthy bone mass by driving bone repair and regeneration through inhibition of osteoclast formation. Age-related changes in the immune system can lead to dysregulations in the Treg population, which may ultimately cause bone remodelling imbalance. Bone remodelling is a complex process by which old and/or damaged bone is replaced by new bone. An imbalance in bone remodelling leads to several bone related diseases such as osteoarthritis.

The data in this thesis showed a decrease in $CD4^+Foxp3^+$ Tregs in peripheral blood in the aged population, as well as a decrease in the possible bone homing Tregs ($CXCR4^+CD4^+Foxp3^+$). CTX-1 levels, an indicator of bone resorption, in serum did not show any correlation with the levels of Tregs in peripheral blood.

In vitro studies supported the interaction of Tregs and osteoclasts, by revealing a lower functional activity of osteoclasts when cultured in the presence Tregs. Data suggested that aged Tregs may present a higher suppressive activity.

Spatial localisation of Tregs in murine knee joints showed co-localisation of Tregs and osteoclasts in the subchondral bone. Higher levels of Tregs were observed

in the subchondral bone in OA mice and aged WT mice suggesting migration of Tregs towards the site of bone remodelling. Higher presence of Tregs was also linked to higher numbers of osteoclasts during advanced osteoarthritis, which is an indicator of high bone remodelling.

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Abbreviations

ADAMTS Metalloproteinases with thrombospondin motifs

BD Becton, Dickinson and Company

BMPs Bone morphogenetic proteins

CALCR Calcitonin Receptor

cAMP Cyclic adenosine monophosphate

CAT K Cathepsin K

CFSE Carboxyfluorescein Succinimidyl Ester

CLA Cutaneous Lymphocyte Antigen

CTLA-4 Cytotoxic T-Lymphocyte Antigen

CTV Cell Trace Violet

DAMPs Damage Associated Molecular Patterns

DC Dendritic cells

ECM Extracellular Matrix

FACS Fluorescence-activated cell sorting

Foxp3 Forkhead Box Protein 3

GITR Glucocorticoid-Induced TNF-R Related

GPCR G- protein coupled receptor

H+-ATPase Vacuolar H+-adenosine triphosphatase

IDO Indoleamine 2,3-dioxygenase

IDDM Insulin-dependent diabetes mellitus

IL-1 Interleukin-1

INF Interferon

IPEX Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome

LFA-1 Leukocyte Function-Associated antigen-1

LPS Lipopolysaccharide

LRP Low-density lipoprotein 5/6

MCSF Macrophage Colony Stimulator Factor

MHC Major Histocompatibility Complex

MITF Microphthalmia Associated Transcription Factor

MMPs Tissue-destructive enzymes like Matrix Metalloproteinases

NFAT Nuclear factor of activated T cells

NK cells Natural Killer cells

OA Osteoarthritis

OARSI Osteoarthritis Research Society International

OC Osteoclast

OPG Osteoprotegerin

OSCAR Osteoclast-Associated Receptor

PBMCS Peripheral Blood Monocellular cells

PTH Parathyroid hormone

RANKL Receptor Activator for Nuclear Factor κ B Ligand

RT Room Temperature

RUNX2 Runt-related transcription factor 2

SDF Stromal Cell-Derived Factor 1

SNBTS Scottish National Transfusion Service

Shh Sonic hedgehog

Tcons Conventional T cells

TGF-β Transforming growth factor.

TGF Tumor Growth Factor

Th T helpers

TNFα Tumour necrosis factor alpha

TNF Tumour Necrosis Factor alpha

TRAP Tartrate-Resistant Acid Phosphatase

Tregs Regulatory T cells

WT Wild Type

Chapter 1

General Introduction

This thesis focuses on the interaction between bone-resorbing cells (osteoclasts) and immune cells called Regulatory T cells (Tregs), and the effects of Tregs on osteoclasts in healthy ageing and osteoarthritis. The introduction of this thesis will therefore provide i) a broad overview of bone biology, with a particular focus in bone resorption and osteoclast function, ii) an introduction to the immune system, with a deep focus in Tregs and their mechanisms of action, iii) the effects of ageing in both the skeletal system and the immune system, iv) an outline of osteoarthritis as a bone disease and the effects of the immune system in its development.

1.1 Bone Biology

1.1.1 Bone structure and functions.

Bone is often considered to be an almost inert or dead structure; however, it has been known to be highly dynamic for centuries. The skeleton is a tremendously complex organ that serves various functions. The bones of the skeleton allow structural support and provide movement and locomotion, protect the vital organs, act as a reservoir for calcium and phosphate, provide a crucial site for haematopoiesis inside the marrow spaces and can also act as an endocrine organ (Lee et al., 2007; Lee & Karsenty, 2008).

Bone structure has been adapted to its function. (Sommerfeldt & Rubin, 2001) Bone is formed by organic and inorganic components, which merge allowing the skeleton to be strong and stiff to withstand mechanical stress while allowing movement and elasticity to avert fracture. Two types of bone structure exist: trabecular (cancellous) and cortical (compact). Both types are made up of hydroxyapatite [$Ca_{10}(PO4)_6(OH)_2$] crystals, which is known as the inorganic material or mineralized matrix and account for of approximately 65% of the bone composition. The other 35% of bone is made of organic material, most of which is the fibrous protein collagen (**Figure 1.1**).

Cortical (compact) bone accounts for 80% of total skeletal mass and is predominantly found in the thin surface layer of long bones (the diaphysis). (Sommerfeldt & Rubin, 2001) Cortical bone is structured into concentric lamellae in Haversian systems with a central canal where blood arteries and nerves can be found. This efficient arrangement provides shape and support, and it is designed to absorb weight-bearing stress under acute mechanical demands which require a high resistance to tensile forces (**Figure 1.1**).

Trabecular (cancellous) bone, also known as spongy bone, makes up around 20% of total bone mass, is made up of thin lamellar bone struts lacking Haversian systems. It has a faster rate of turnover than cortical bone. (Sommerfeldt & Rubin, 2001) Trabecular bone contains a lot of haemopoietic tissue and fat, which helps the bone to be lighter. Because the primary role of trabecular bone is to endure the forces experienced during weight bearing, it is less thick and more elastic than cortical bone (**Figure 1.1)**

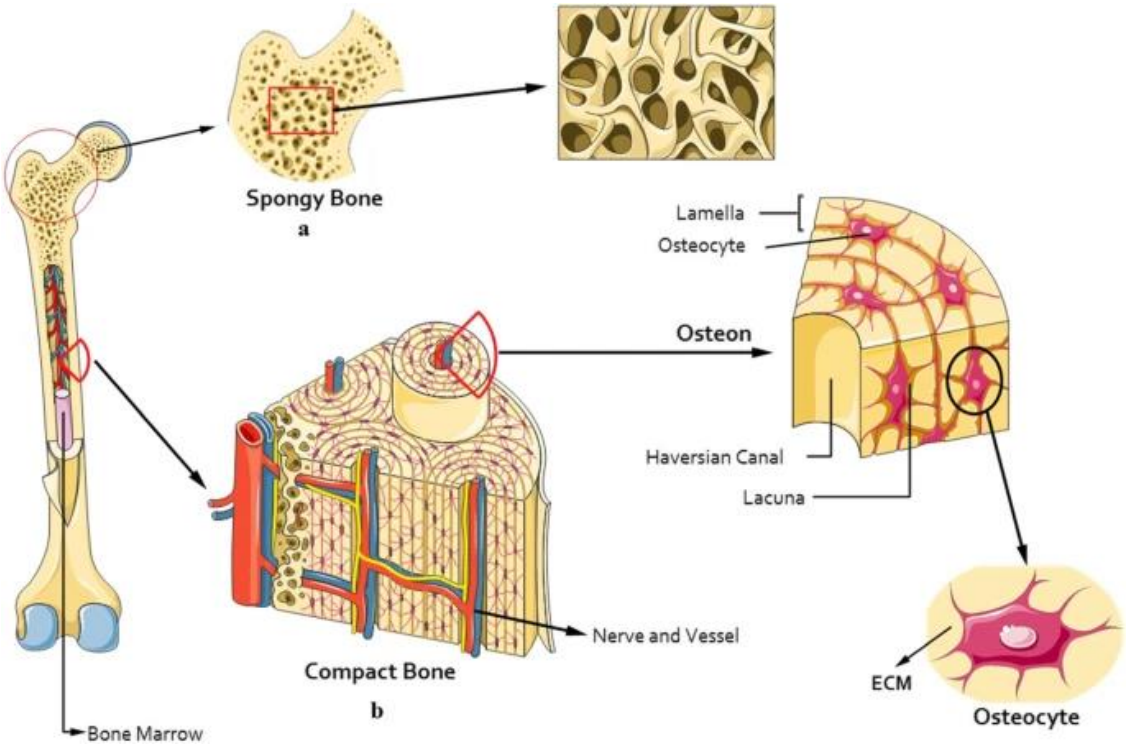

Figure 1.1 The structure of bone. Bone consists of both cancellous and cortical bone. Spongy bone, (trabecular bone) is characterised by struts of trabecular bone organised to withstand compressive forces (a). Compact bone (cortical bone) is found in the shaft of the bone. Centrally is the Haversian canal in which nerves and blood vessels reside and concentric to this there is layers of bone matrix and osteocytes (b) (Amirazad et al., 2022).

1.1.2 Bone Remodelling

Bone is a mineralized connective tissue, which despite its inert appearance is highly dynamic, being continuously remodelled (5-25% of bone surface is constantly under remodelling (Parfitt, 1994). Bone remodelling is a complex process by which old and/or damaged bone is replaced by new bone. Bone remodelling involves a co-ordination of bone resorption by osteoclasts and bone formation by osteoblasts. This requires a tight regulation maintaining an equilibrium of bone resorbed vs bone formed, such that bone disorders do not occur. Normal bone remodelling is necessary to maintain mechanical integrity and calcium homeostasis as well as restoration of fractures causing micro damages. Maintenance of normal mass bone and bone quality is achieved by a balance in between the amount of bone reabsorbed and the new bone formation (Boyce & Xing, 2006) and an imbalance in between these two causes several bone related diseases, including osteoporosis (Okamoto et al., 2017).

The bone remodelling cycle consists of three consecutives stages known as resorption, reversal, and termination (Owen & Reilly, 2018). The physical forces producing mechanical stress and micro damage to the skeleton are converted into biological signals that will start the bone remodelling cycle.

Resorption begins after pre-osteoclasts migrate to the bone surface where they form multinucleated osteoclasts induced by the expression of macrophage colony

stimulating factor (MCSF) and receptor for activation of nuclear factor kappa B ligand (RANKL) on osteoblasts, activated by parathyroid hormone (PTH).

Resorbing osteoclasts secrete hydrogen ions causing the pH to lower causing an acidic environment which enables dissolution of bone mineral break down for digestion by enzymes such as metalloproteinase and cathepsin K (Katsimbri, 2017; Owen & Reilly, 2018). Osteoclasts precursors (monocytes and macrophages) and pre-osteoblasts are then recruited to start bone formation. Bone formation phase is the longest phase and can take up to 6 months to complete. Osteoblasts synthesize osteoid, which will gradually be mineralize by osteoblasts and form the new bone.

The final phase in bone remodelling is mineralization of osteoid until the bone enters in a quiescent state and the amount of bone formed and bone resorbed is equal. The different stages of bone remodelling and each cell type involved are represented in (**Figure 1.2)**.

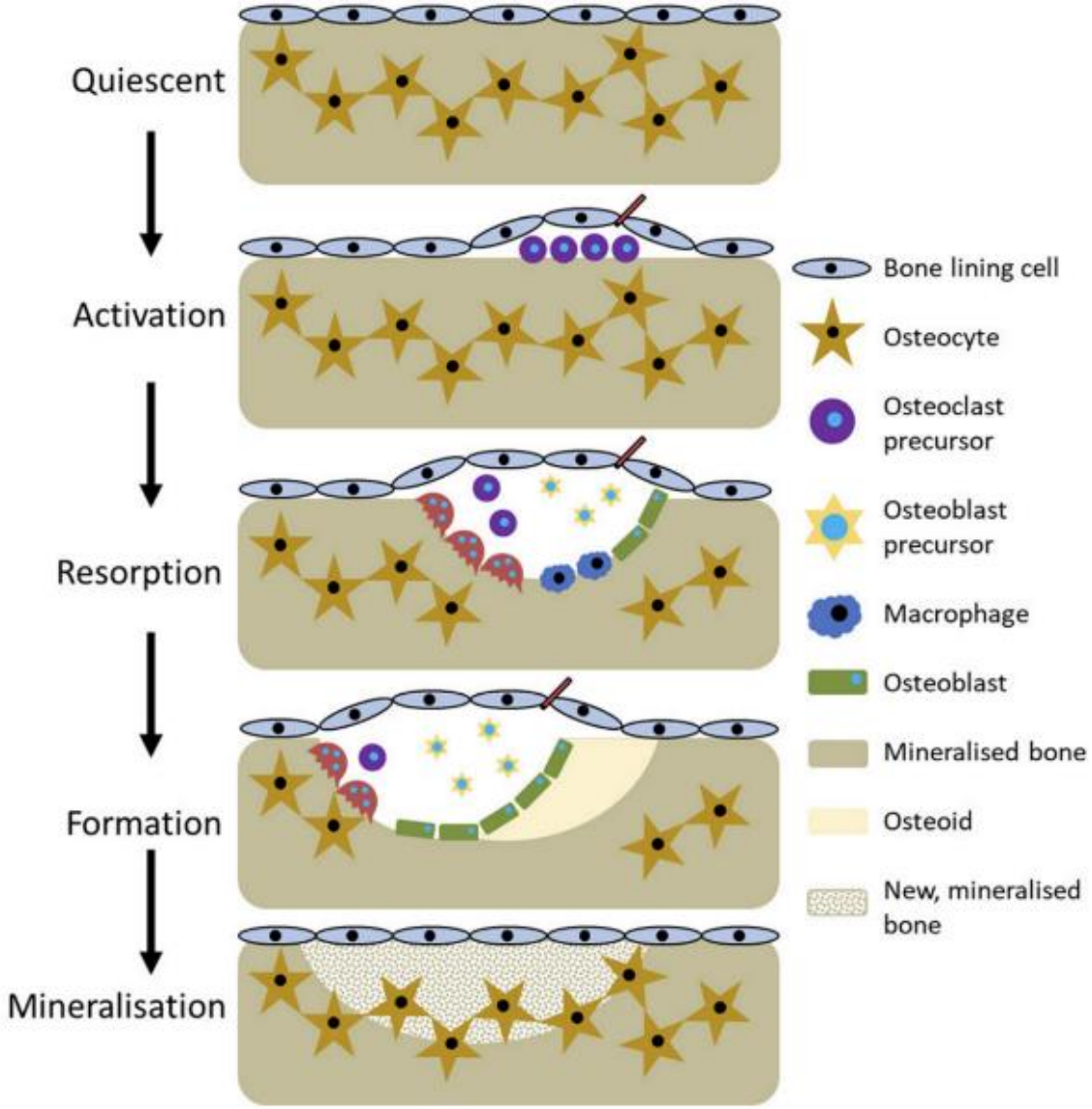

Figure 1.2 The stages of bone remodelling. Schematic representation of bone remodelling starting from quiescent to further activation, resorption, formation, and end stage mineralization (Owen & Reilly, 2018).

1.1.3 Bone Cells

Osteoclasts

Osteoclasts are bone-resorbing cells and originate from hematopoietic cells of monocyte/macrophage lineage. Osteoclasts are giant multinucleated cells (more than 20 nuclei) located in the bone surface and within a lacuna (Howship's lacunae) as an outcome of its own resorptive activity (Manolagas, 2000). They present a characteristic ruffled border, formed by a highly folded plasma membrane, which faces the bone matrix to secrete and absorb proteins and ions between the bone surface and the osteoclast (Sommerfeldt & Rubin, 2001).

Osteoclast differentiation and maturation (osteoclastogenesis) depends on MCSF and RANKL (Okamoto et al., 2017). Together MCSF and RANKL induce the expression of tartrate-resistant acid phosphatase (TRAP), cathepsin K (CATK), calcitonin receptor and the Beta 3-integrin that characterize the osteoclast lineage resulting in mature osteoclasts (**Figure 1.2**) (Boyle et al., 2003). Another key factor in osteoclast differentiation is osteoprotegerin (OPG), predominantly produced by osteoblasts, OPG acts by preventing osteoclasts formation and bone resorption by inhibiting the RANKL-RANKK receptor interaction.

Osteoclast formation is regulated by the bone microenvironment and by the presence of stimulatory molecules that mediate osteoclastogenesis. One of the first molecules required to start the process is transcription factors PU.1 encoded by SPI1 and the microphthalmia associated transcription factor (MITF). Both, PU.1 and MITF, regulate the expression of colony stimulator factor 1 (CSF-1) receptor

c-Fms at the early stage of pre-osteoclasts (macrophages) and mediates osteoclasts precursors' survival and proliferation (Tondravi et al., 1997). CSF-1 also induces differentiation of macrophages to osteoclasts precursors, which are from monocyte/macrophage lineage. CSF-1 main role in osteoclastogenesis is promoting the expression of RANK on osteoclasts progenitors allowing then the following interaction of those with RANKL which will fully induce osteoclastogenesis (Boyle et al., 2003)

Finally, differentiation of osteoclasts involves regulation of the expression of osteoclast-specific genes including TRAP, osteoclast-associated receptor (OSCAR), calcitonin receptor (CALCR), dendritic-cell-specific transmembrane protein (DC-STAMP), d2 isoform of vacuole ATPase (Atp6v0d2, V-ATPase D2) and integrin αVβ3 (vitronectin receptor) in cooperation with other transcription factors (Boyle et al., 2003).

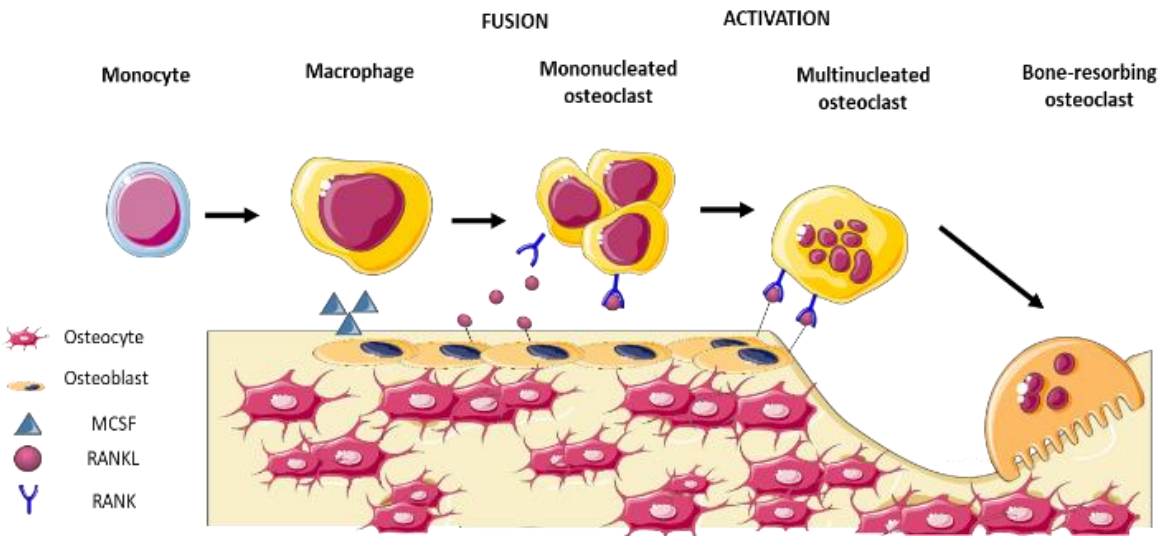

Figure 1.3 Osteoclast formation (Osteoclastogenesis) process. Schematic representation of osteoclasts formation from monocyte/macrophage lineage. From fusion of macrophages forming multinucleated osteoclast by RANK-RANKL interaction to activation of osteoclasts. Figure created using Smart Servier medical art.

Mature osteoclasts resorb the bone by demineralization of the inorganic components and further removal of organic bone matrix (Georgess et al., 2014). Osteoclasts form podosomes, cytoskeletal structures where the integrin αVβ3 binds to actin, crucial for the degradation of mineralized bone matrix which are responsible for the formation of the sealing zone between the osteoclasts and the bone/dentin matrix. The ruffled border transports protons and proteolytic enzymes into the sealing zone to acidify the resorption compartment via the Vacuolar H+-adenosine triphosphatase (H+-ATPase).

Hydrochloric acid is released by cathepsins or activated collagenases within Howship's lacunae generating a highly acidic environment that will cause dissolution of the inorganic material (bone mineral). The remaining organic material (mainly collagen) will further be degraded by proteases such as CTSK, and collagen fibres residues are then internalised or transported and secreted into the basolateral domain (Okamoto et al., 2017).

Osteoblasts

Osteoblasts are derived from multipotent mesenchymal stem cells originating in the bone marrow and comprise only 4-6% of the bone cells (Capulli et al., 2014). Osteoblast differentiation is coordinated by the expression and activation of transcription factors such as Runt-related transcription factor 2 (RUNX2), osterix, Ihh, sonic (Shh) hedgehog, Tumor growth factor β (TGFβ) and Bone morphogenetic proteins (BMPs) (Ducy et al., 1997).

Osteoblastogenesis is also dependent on the Wnt signalling pathway. Binding of Wnt ligands to the frizzled receptor (Frz) and low-density lipoprotein 5/6 (LRP) complex activates osteoblast differentiation therefore promoting an increase of bone formation (Capulli et al., 2014).

Osteoblasts are located in clusters along the bone surface, facing the layer of bone matrix that they are producing (Bellows et al., 1991). The main function of osteoblasts is to produce bone matrix constituent osteoid by synthesising and secreting collagen type I, which is the main bone matrix protein. Osteoblasts are also involved in the further mineralization of the osteoid. The process of osteoid

mineralization is regulated by the expression of phosphatases and inhibitors of mineralisation.

After osteoblasts have mineralized their matrix, they can remain either on the surface of the bone as inactive lining cells, undergo apoptosis or be embedded by the mineralized matrix and differentiate into osteocytes (Manolagas, 2000).

Osteocytes

Osteocytes comprise 90-95% of the total bone cells are the differentiated form of the osteoblast cell lineage (Bonewald & Johnson, 2008). Osteocytes are smaller in size than their osteoblasts precursors and present a higher nuclei number to cytoplasm ratio (Sommerfeldt & Rubin, 2001). Their function and development are coordinated by their shape and location. Osteocytes are located inside the bone within lacunae surrounded by mineralized bone and sense bone surface signals due to their characteristic dendritic shape (Florencio-Silva et al., 2015).

After bone micro-fractures caused by mechanical forces, osteocytes will detect the damage and respond stimulating the bone remodelling process to start (Bonewald, 2011). Mechanical stimulation also causes stimulation of osteocytes to produce secondary messengers which produce osteoblast. Moreover, osteocytes also regulate osteoclast formation by RANKL secretion as a consequence of bone damage, initiating bone resorption by osteoclasts (Nakashima et al., 2011).

Chondrocytes

Chondrocytes are mononuclear cells of mesenchymal origin and are the cells responsible for endochondral bone growth, the process in which a cartilage

scaffold is gradually replaced by bone (Ballock & O'Keefe, 2003). Chondrocytes are found in the growth plate which can be found at either end of long bones. Chondrocytes within the growth plate are organised in columns and embedded in a collagen rich membrane. Chondrocytes are defined by their state of differentiation. There are two main types of chondrocytes, non-hypertrophic and hypertrophic chondrocytes. Non-hypertrophic chondrocytes can be found in two different forms, resting and proliferating chondrocytes.

Hypertrophic chondrocytes are also found in the articular cartilage embedded in a matrix formed by collagen type-II and proteoglycans (Juneja & Hubbard, 2019). Articular cartilage is found at the end of long bones and synovial joints and acts as a shock absorber protecting the bone surface. Articular cartilage is avascular, and it is not involved in bone formation, however during bone diseases pathologies such as osteoarthritis vascular invasion can occur, leading to the subsequent damaged caused by the disease's development, this will be further explored in section 1.2.

1.1.4 Joints

Joints are complex organised structures that connect contiguous bones allowing the skeletal system to unite making it a whole functional system. Joints are classified by their structure (adjacent bones connected by fibrous tissue or cartilage) and function (degree of movement permitted in between bones). Based on the histological classification joints are known as fibrous, cartilaginous, and synovial.

Functionally the three types of joints are classified as immovable (synarthrosis), slightly moveable (amphiarthrosis) and freely moveable (diarthrosis) (Juneja & Hubbard, 2019).

The histological and functional classification offers a broad understanding of joints yet, every different joint type has a specific function in the body. Structural damage and/or dysregulation in function and in any of the components of the complex structure that comprises the joint leads to distinct bone pathologies associated to different joint types.

During osteoarthritis pathology the main joints affected are knee, hips and fingers, those joints are classified as diarthroses or synovial joints, which are freely mobile, and the bones are covered by a layer of cartilage encased in a synovial capsule (Kapoor et al., 2011). In diarthrodial joints the osteochondral unit, which main function is to transfer loads during joint motion, is formed by the articular cartilage, subchondral bone, and calcified cartilage, which is a thin layer in between articular cartilage and subchondral bone (Goldring et al., 2006). The different changes in the osteochondral unit during osteoarthritis and how this influence the pathology development are further explained in more detail in section 1.2.

1.2 Osteoarthritis

Osteoarthritis is the most common musculoskeletal condition, affecting 3.8% of the global population (Li et al., 2017). Osteoarthritis is a long-term chronic condition affecting the joints.

During osteoarthritis development alterations in the articular cartilage, subchondral bone, ligaments, capsule, and synovial membrane occur and for this reason, it is considered a disease of the whole joint (Kapoor et al., 2011). The condition most commonly causes joint debilitation and creating stiffness, pain, and reduced movement.

Despite the huge socioeconomic burden, there are currently no effective disease-modifying treatment options for osteoarthritis and patients largely rely on the use of symptom-modifying therapies, such as pain-modifying drugs or total joint replacement (Hunter et al., 2014).

1.2.1 Osteoarthritis Pathology

Osteoarthritis development occurs in two different phases defined as initiation and progression. However, the exact course of the disease remains uncertain and the distinction between the initiation and progression phases are still to be defined (Suri & Walsh, 2012). Most researchers use the early osteoarthritis and late osteoarthritis categories which also remain ambiguous. Even though osteoarthritis was once considered to be caused due to articular cartilage degradation, nowadays it is commonly known that structural, biochemical, and biomechanical changes in the entire osteochondral unit occur.

Articular cartilage

Articular cartilage is the top layer of the osteochondral unit, and its main function is to serve as a protective cushion by facilitating motion between joints and

reducing impact (Tamaddon et al., 2018). Articular cartilage is an avascular and aneural tissue composed mainly by water (90%), chondrocytes (1-2%) enclosed in an extracellular matrix (ECM), type II collagen, aggrecan and proteoglycan aggregates.

The high content in water, the proteoglycan aggregates and the fibrillar collagen allows cartilage to deform without failure under the presence of compressive load (Goldring et al., 2006). Friction on the other hand, is regulated by lubricants secreted by chondrocytes, such as lubricin and hyaluronic acid. When dealing with tension and stress occurring at the edges of the joint, articular cartilage is less capable of maintain the structure without splitting. In osteoarthritis where there are changes in the subchondral bone thickness, the regions where stiffness varies, are sites of high stress and the cartilage, more likely will fail in tension.

Osteoarthritis has historically been considered mostly as a cartilage disorder. Articular cartilage chondrocytes synthesise matrix molecules (such as: type II collagen and aggrecan), which ensure the integrity of this tissue throughout life. In osteoarthritis however, there is an increase in a variety of proinflammatory cytokines, including interleukin-1 (IL-1), tumour necrosis factor alpha (TNFα), tissue-destructive enzymes like matrix metalloproteinases (MMPs) and metalloproteinases with thrombospondin motifs (ADAMTS), all of which, contribute to matrix destruction and overall affect the structural integrity of the articular cartilage (articular cartilage degradation, **Figure 1.4**) Yuan et al., 2014). Changes in ECM also allows articular cartilage invasion by blood vessels coming from the subchondral bone, losing then its characteristic avascular property. These new formed vascular channels present hypertrophic chondrocytes, which express

genes encoding for collagen X, which regulates ECM formation, and MMP13, involved in collagen type II breakdown.

Subchondral bone

The subchondral bone is located immediately deep to the articular cartilage and remains connected to it through the calcified cartilage layer (Stewart & Kawcak, 2018). Subchondral bone is composed of hydroxyapatite crystals, which provide rigidity, and organic components such as type I collagen, proteoglycans, and water, which provide elasticity. Different regions of subchondral bone have been defined regarding its architecture and physiology. Adjacent to the calcified cartilage, a thin layer forms the subchondral bone plate and closer to the medullary cavity, a thicker area known as the trabecular bone zone is found (SB and TB in **Figure 1.4**).

Subchondral bone attenuates around 30% of the impact on the joints, enhancing then the articular cartilage capacity of reducing impact when joint loss as well as providing the articular cartilage with essential nutrients via synovial fluid (Mathiessen & Conaghan, 2017). However, even acting as a reduction-impact zone, subchondral bone is a zone of weakness, due to the lack of collagen fibres when connecting with the articular cartilage.

Subchondral bone association with osteoarthritis development is through osteochondral lesions and joint remodelling. Subchondral bone is modified in osteoarthritis by an increase in thickness and bone volume (G. Li et al., 2013b). It is still unclear whether subchondral bone remodelling precedes or follows articular cartilage degeneration. Although it is often considered secondary to articular cartilage changes, subchondral bone thickening in osteoarthritic joints is one of the

earliest detectable changes and is now considered a potential trigger for subsequent articular cartilage degeneration (das Gupta et al., 2022).

Osteophytes

Osteophytes are outgrowths of osteo-cartilaginous material that develop at the margin of osteoarthritic joints (**Figure 1.4**). Clinically, they can be the cause of pain and limitation of joint mobility. They are usually used as indicator to identify the disease status in humans (Brandt, 1999).

Osteophytes originate from periosteal and/or synovial tissue overlaying the margins of the joints. It is still uncertain if they are formed because of abnormal mechanical stimulation or are the product of modified joint environment (van der Kraan & van den Berg, 2007). During early stages of osteophyte formation, there is an increase level of cell proliferation and matrix components production, such as aggrecan, followed by endochondral ossification which forms bone in the central part.

Synovium

The synovium is the connective tissue present in diarthrodial joints sealing the synovial cavity and fluid from surrounding tissues (Scanzello & Goldring, 2012). Synovium produces lubricin and hyaluronic acid, which help to maintain the volume and composition of synovial fluid. The combination of those two molecules produce lubrication and reduce friction at the articular surface.

Two different layers compose the synovium; the subintima (outer layer) composed by a type I collagen network and contains mast cells, fibroblasts, adipocytes, macrophages, elastin and blood vessels and the intima (inner layer), which is

closer to the joint cavity (Tiwari et al., 2010). The intima layer cells (macrophages and fibroblasts) produce extracellular matrix components which regulates the production and composition of synovial fluid.

Even though the synovium is not the only tissue presenting an inflammatory change, it is the tissue where a higher inflammatory response is found during osteoarthritis development. Synovial inflammation results in synovitis. Extensive evidence suggest that synovitis is a highly related with worsening the progression of osteoarthritis (Liu-Bryan, 2013). Histology and imaging are the main techniques taken used in order to determine abnormalities in the osteoarthritic synovium (Mathiessen & Conaghan, 2017).

The histological changes in osteoarthritic synovium are increased vascularity, thickening of the lining layer by fibrotic tissue and inflammatory cell infiltration, consisting mainly of macrophages and lymphocytes (**Figure 1.4**) (Liu-Bryan, 2013; Wenham & Conaghan, 2010). At a macroscopic level, using imaging techniques, synovial hypertrophy and synovial fluid volume enhancement were determined as changes produced due to synovitis (Mathiessen & Conaghan, 2017).

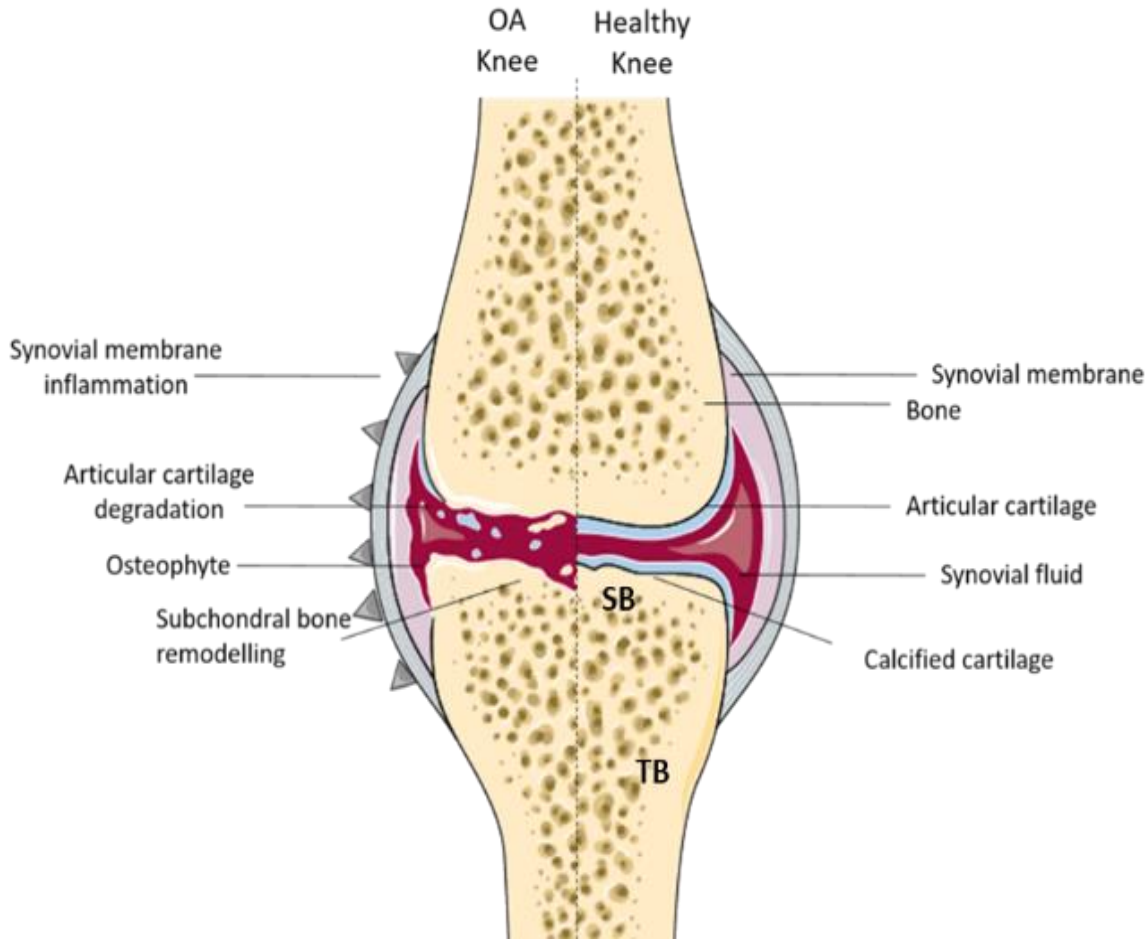

Figure 1.4 Structural change in knee during osteoarthritis development. Schematic representation of the structural changes undergoing osteoarthritis development in the knee compared to the structure of the healthy knee. Synovial membrane inflammation, articular cartilage degradation, osteophyte formation and subchondral bone remodelling are represented on the left side of the image. Subchondral bone (SB) and Trabecular bone (TB). Figure created using Smart Servier medical art.

1.2.2 Osteoarthritis treatment

To date, there is no non-invasive treatment for OA and the majority of the resources available are mainly to focus on symptoms management than a solution (Hermann W, et al., 2018). Guidelines for OA treatment is based on formal structured literature reviews and the two main sources are the National Institute of Health and Clinical Excellence (NICE) OA Guidelines and the OA Research Society International (OARSI). Based on their recommendations OA treatments can be classified as conservative or surgical treatments.

Conservative treatment

This is the first approach to developing OA therapy and it is based on the provision of lifestyle such as changes in lifestyle such as having an active life, include moderate exercise, weight loss and diet (Allaeys et al., 2020).

Regular use of paracetamol, intermittent use of Non-Steroidal Anti-inflammatory drugs (NSAIDs) and possibly weak opioids can achieve pain relief related to OA symptoms. Inflammatory symptoms can also be treated with corticosteroid injections; however, the effects are limited, lasting no longer than a few weeks.

Surgical treatment

The last resource to achieve pain relieve in osteoarthritic patients is performing surgery. Joint replacement has been the major surgical treatment for OA in the last century (de l'Escalopier N and Anract P, Biau D, 2016). Although relief of pain and recovery of function are not usually fully achieved, they are often improved and eighty to ninety percent of patients are very pleased with the outcome.

However, neither of these treatments are focusing on the disease mechanisms and are either based on pain relief or total removal of the injury. For this reason, other ways of therapies such as cell therapy are becoming popular in the area. Finding a non-invasive therapy that can solve the root causes of the disease progression and manage pain are much needed. Further implications of osteoimmunology in osteoarthritis development are expanded in section 1.5.

1.3 The Immune System

The immune system complies a complex interplay of cells, organs, proteins, and tissues. The main function of the immune system is to protect the host from invading pathogens. Even though barriers formed by the skin or lysozyme containing saliva can protect from infection, occasionally these can be breached by infectious organisms (viruses and bacteria). There are a vast variety of pathogens, all with different strategies for persevering within the host, as a result, the immune system has developed a variety of cell types with dedicated functions. The innate immune system - first line barrier of rapid response defence - is formed by the cells and mechanisms that protect the host from infections caused either by invading pathogens or by the immune response itself causing tissue damage (self-damage). The next line of defence is adaptive immunity, in which activation comes from actions previously taken by the innate immunity.

All immune cells are generated in the bone marrow from hematopoietic stem cells (Hato & Dagher, 2015). However, each cell type further develops through different routes of maturation which leads to different roles within the immune system.

Innate immune cells (neutrophils, macrophages, eosinophils, and natural killer cells (NK cells)) are able to recognise that a pathogen is likely to be dangerous and respond quick to infection by arriving at the site of pathogen invasion within the first hours of infection. On the other hand, the adaptive immune response is formed by T cells and B cells actions. They are highly varied and specific for each antigen. They are initially slow to respond to primary infection since they require that the cells specific for each antigen to proliferate prior to suppressing infection. However, on a second host invasion they are much faster because they have generated memory cells during the prior exposure.

1.3.1 T cells

T cells are the major cells of the adaptive immune response and are comprised of different subpopulations that control the immune response signal against a pathogen. T cells are divided in two major subgroups CD4+ (T helper cells) and CD8+ (cytotoxic T cells). CD8+ cytotoxic cells recognise MHC class I peptides and secrete perforin and granzyme to destroy infected cells. CD4+ T helper cells, on the other hand, recognise MHC class II peptides and play a more controlling role as opposed to cytotoxic T cells. CD4+ T helper cells, provide help to activate B cells and T cells in the following immune response steps by directing the immune response depending on the combination of cytokines they release on activation (Kumar et al., 2018).

1.3.1.1 Regulatory T cells

Tregs are a subpopulation of T helper cells important for their role in the immune system maintaining peripheral tolerance, protecting the individual from autoimmunity, and limiting chronic inflammatory diseases (Dias et al., 2017). The existence of a regulatory subset of T cells was first proposed in the early 1970s when Gershon and Kondo suggested the presence of more than one subset of T cells in the thymus derived lymphocyte populations (Gershon & Kondo, 1970).
Even though in the early 70s there was clear evidence of a T cell population presenting suppressive activity, the absence of a firm identification of these cells made impossible the evidence for the existence of a clear regulatory T cell population since no discriminatory markers could be identified.

The identification and isolation of Tregs dates from 1995 when Sakaguchi and colleagues identified a subset of cells with suppressive function constitutively expressing the α-chain of the IL-2 receptor, CD25 (Sakaguchi et al., 1995a). The expression of CD25 originally was thought to be expressed only by activated cells, however, resting cells with the marker extracted from the peripheral blood of mice were shown to have suppressive function and to be essential for the development of Tregs (Sakaguchi et al., 1995).

Since CD25 is constitutively expressed on most Tregs it has been identified, in combination with CD4 expression for T helper cells, to be used as a marker for isolating Tregs. However, using CD4+CD25+ combination for characterising and isolating Tregs has some limitations. Because of the IL-2 role in T cell inflammatory

responses, after activation other subsets of T cells also will express the CD25 marker, therefore using CD25 to isolate pure Treg populations can include a contamination of activated conventional CD4 T cells (Hatakeyama et al., 1989). In combination with CD4 and CD25, the surface marker CD127 is also used to obtain a purer population of Tregs. CD127 is the receptor for IL-7 and is downregulated on T-cells after activation and while, T cells re-express the receptor, Tregs demonstrate a reduced expression of CD127 (W. Liu et al., 2006).

Due to the limitations when using the combination of CD4, CD25 and CD127 surface markers, more unique markers are required for characterising and isolating Tregs. Many potential markers, which are expressed by Tregs and are essential for the functional capacity of the cells, have been assessed such as the surface proteins CTLA-4 and Glucocorticoid-Induced TNF-R Related (GITR). Even so, there are lack of evidence for those to be considered unique enough to qualify as effective Treg markers. $CD4^{+}CD25^{High}C127^{low}$ therefore remains the best markers for the isolation of the characterisation and isolation of Tregs (Seddiki et al., 2006).

The closest marker identified to isolate a pure population of Tregs is Forkhead Box Protein 3 (FoxP3), the human orthologue of the murine protein Scurfin (Khattri et al., 2003). Mutations of the gene FoxP3 in mice, causing a non-functional gene, cause scurfy, a systemic autoimmune disease (Bennett et al., 2001). In humans, the loss-of-function mutations of the FoxP3 gene lead to a similar syndrome known as immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), which is characterised by lymphoproliferation, neonatal insulin-dependent diabetes mellitus (IDDM) and eczema, among other symptoms (Bennett et al.,

2001). FoxP3 expression was subsequently found to be crucial to Treg development and function (Fontenot et al., 2003). FoxP3 functions by suppressing transcription of the IL-2 gene by interactions with NFAT (nuclear factor of activated T cells) and in the same way upregulates the inhibitory surface molecule CTLA-4 and the α-chain of the IL-2 receptor, CD25 (Wu et al., 2006). Even though Foxp3 has been proven to be a more specific Treg marker, its used is not always possible. Foxp3 requires fixation and permeabilization of cells to access it, since it is a nuclear factor. Hence, cells will require to be killed for Foxp3 staining and this is not always possible depending the nature of the study (i.e soting cells required to be alived).

1.3.1.1.1 Tregs Migration

Naïve T cells circulate in the blood and lymphatic system preferentially, homing to lymph nodes. Once activated, specific receptors get upregulated in T cells allowing them to migrate to inflamed tissue via the bloodstream following a gradient of chemokines to the appropriate site (**Figure 1.5**). During an immune response, Tregs migrate to both, inflammatory sites and draining lymph nodes. Treg migration to different tissues occurs in multiple steps, first step involves cell rolling on the vessel wall mediated by the interaction of selectins with their relative ligand, the main selectins being: L-selectin (CD62L), P-selectin (CD62P) and E-selectin (CD62E) (McEver et al., 1995).

L-selectin binds CD34 and mucosal vascular addressing MAdCAM1, P-selectin and E-selectin bind to ligands expressed by T cells such as, cutaneous lymphocyte

antigen (CLA), CD66 and P-selectin glycoprotein ligand-1 (PSGL-1) (Berg et al., 1993). Leukocytes express L-selectin while the endothelial cells express E-selecting when activated by cytokines (IL-1 or TNF) (Springer, 2003). P-selectin is located in the cytoplasm of platelets and endothelial cells, and it expresses on the cell membrane upon activation of the cells. P-selectin synthesis is increased by inflammatory mediators such as lipopolysaccharide (LPS) (Ley & Tedder, 1995; Ley & Zarbock, 2006).

The next step is mediated by integrins allowing a firm adhesion of the cell to the endothelial lining. Upon T cell activation, integrins receive signals to acquire an active form and allows the ligand to bind. Once the integrin is binding, the T cell forms a firm bound to the endothelium. The main integrins involved in T cell migration are leukocyte function-associated antigen-1; αLβ2 integrin (LFA-1), Mac-1 (αMβ2 integrin) or αdβ2 integrin and α4β7 and very late antigen-4; α4β1 integrin (VLA-4) (Kinashi, 2005).

The last step on the migration of T cells is diapedesis, where the T cell passes through the monolayer endothelial lining the blood vessel. T cells find an appropriate site, either a junction between cells or a region away from the nucleus, to pass through (Friedl & Weigelin, 2008). This can be done directly by the cell being able to pass the junction (paracellular transmigration) or mediated by podosomes (transcellular diapedesis) (Kvietys & Sandig, 2001).

Figure 1.5 Treg migration into tissues. Treg migration into tissues is characterised by a series of steps that can be categorised into rolling, tight binding, and trans endothelial migration. Factors released by immune cells and endothelium lead to upregulation of adhesion molecules, known as selectins. Glycosylated ligands on the immune cell bind to endothelial expressed selectins, resulting in a cell rolling across the endothelial surface. Chemokines in the surrounding environment, will bind to immune cells with the appropriate chemokine receptor. The cytoskeleton of the leukocyte rearranges, and the leukocyte passes through the gaps in the endothelial cells, aided by junctional adhesion molecules. Once within the tissue, chemokines direct cellular movement towards the desired location, assisted via chemokine gradients Figure taken from; (Ley & Zarbock, 2006).

Chemokines

Chemokines are molecules that support the migration of Tregs to specific tissues. They are small proteins (8-10kDa) containing four conserved cysteine residues and are known as chemotactic cytokines as they are responsible of creating a chemical gradient for cell migration. Chemokines and their receptors are named according to the pattern of cysteine residues in the chemokines: C, CC, CXC or CX3C (C standing for cysteine and the X for a different amino acid) (Ward & Marelli-Berg, 2009). In addition to being classified according to their structure, they can be divided into inflammatory, lymphoid, or homeostatic. Inflammatory chemokines direct cells to sites of inflammation, whereas lymphoid chemokines are constitutively present in lymphoid organs, maintaining normal lymphoid traffic (Ebert et al., 2005).

Chemokines play an important role in embryonic development, surveillance, angiogenesis, autoimmune responses, cancer progression and inflammation response (Gadhe & Kim, 2015). The interaction of leukocytes with chemokines initiates a series of coordinated cellular events that enables leukocyte migration, due to chemo attractant gradients, allowing them to serve as a first line of cell-mediated host defence against infection (Murdoch & Finn, 2000).

In order for Treg migration towards bone they must express specific receptors that will allow the binding to the endothelium and for the detection of the appropriate chemokines' gradient. Whilst I was unable to find previous studies that assessed Treg migration specifically to bone I was able to identify markers (CCR4 and

CXCR4) likely to be involved in the migration of Tregs to bone, based on bone migration in other cells type.

CXCR4

CXCR4 is a CXC chemokine type that belongs to the seven transmembrane G-protein coupled receptor (GPCR) superfamily of proteins and selectively binds to the extracellular chemokine ligand CXCL12 also known as Stromal Cell-Derived Factor 1 (SDF-1) (Wescott et al., 2016). CXCR4 is expressed in a variety of cell types (lymphocytes, endothelial, fibroblasts, epithelial and hematopoietic stem cells and cancer cells) and it is involved in different physiological processes. The role of CXCR4/SDF-1 in osteoarthritis pathology has been previously related to cartilage degeneration as SDF-1 regulates catabolic activity and stimulates production of metalloproteinases MMP-3 and MMP-13 which cause cartilage matrix degeneration (F. Wei et al. 2013). Recent evidence suggested that CXCR4/SDF-1 pathway may also play an important role in fracture healing (Yellowley, 2013). However, the role of CXCR4/SDF-1 signalling towards bone damaged areas and the role it may play in remains unclear.

CCR4

CCR4 is a receptor for CCL17 and CCL22 found on macrophages, dendritic cells, NK cells platelets and T cells, being highly expressed on human peripheral blood Tregs (Molinaro et al., 2015). High levels of CCL22 have been shown in the synovial fluid of osteoarthritis patients comparing with levels found in healthy control group (Iellem et al., 2001). CCR4 chemogradient property has more been studied in migration towards skin. However, expression of CCR4 by synovial

endothelial cells and the expression of CCL22 by sub-endothelial cells in the inflamed synovial membrane, links the ability of CCL22 to recruit Tregs to the inflamed area during osteoarthritis development (Flytlie et al., 2010).

1.3.1.1.2 Mechanisms of Treg cell function

Defining the mechanisms by which Tregs mediate suppression and their mechanism of action is crucial to provide an insight into the control Tregs have in maintaining peripheral tolerance. Suppressive activity on Tregs are antigen dependent, hence Treg cell activation requires antigen recognition by T cell receptor (TCR). TCR activation will lead to a series of signalling pathways that will determine cell fate through cellular proliferation, differentiation, cytokine production, and/or activation-induced cell death. (Corthay, 2009)

Tregs can supress via various suppression mechanisms which can be grouped into basics “mechanisms of action” grouped by contact dependent suppression and contact independent suppression. The different mechanisms of Treg suppression can work together or independently given the requirements of the immune response. Knowing the mechanism used according to the pathological condition can help identifying therapeutic targets.

Contact independent suppression

Suppression by inhibitory cytokines production

The inhibitory cytokines IL-10 and TGFβ have been the main focus of Treg cell mediated suppression. The role of these cytokines in Treg function is not entirely

clear as, some studies suggest that IL-10 and TGFβ are not required for Treg mediated suppression *in vitro* (Cao et al., 2007; Gondek et al., 2005). IL-10 functions by suppressing the expression of MHC class II and inhibits the production of inflammatory cytokines such as IL-12 (Bluestone & Abbas, 2003). One of the most known mechanisms of action of IL-10 is their action interfering with CD28 and inhibiting the proliferation of other T cells (Chaudhry et al., 2011; Loebbermann et al., 2012).

Similarly, TGFβ regulates the effect of other immune cells by inhibiting T cell, B cell differentiation and proliferation and inhibits the activity of macrophages, dendritic cells and natural killers. TGFβ also inhibits the production of IL- 2 which is essential for cell proliferation. Moreover, TGFβ also promotes differentiation of T naïve cells into Treg cells by inducing FoxP3 expression and differentiation of Th17 cells into Tregs by IL-6 secretion (Qin et al., 2009). On the other hand, IL-35 is an immunosuppressor cytokine produced by Tregs known to suppress proliferation of T helper cells and to promote the differentiation of Tregs from naïve T cells (**Figure 1.6 A**) (Perdigoto et al., 2016).

Granzyme and perforin

Treg cells produce granzyme, a serine protease, which induces apoptosis in effector T cells. Granules containing granzyme and perforin are secreted by direct exocytosis to the extracellular space upon Treg effector cell interaction.

Perforin molecules form transmembral cylinders in the lipid membrane of target cells allowing granzyme to enter the cell by endocytosis. Granzyme induces apoptosis on effector cells decreasing the number of these and regulation the immune response (**Figure 1.6 B**) (Gondek et al., 2008).

Metabolic interruption

Tregs metabolic disruption occurs by competing for IL-2, which is a key factor for cell survival and inhibit effector cells proliferation impairing the immune response. Recent studies have shown implication of cyclic adenosine monophosphate (cAMP) and adenosine on the suppressive mechanisms of Treg cells (Borsellino et al., 2007). cAMP accumulates to high concentration in the cytoplasm of Tregs and gets transferred to the target cell through gap junctions where they impact transcription factors, controlling their effector response (**Figure 1.6 C**).

Contact dependent suppression

Suppression by targeting dendritic cells

Dendritic cells (DCs) are a key component of the immune response, cell to cell contact of Tregs with DCs is one of the most important mechanisms of Treg suppression. DCs can activate or control the immune response and when interacting with Tregs, DCs promote Treg cell generation, creating a suppressive environment (Dhainaut & Moser, 2015) (**Figure 1.6 D**).

Tregs directly affect T cell function by modulating the maturation and/or function of DCs which are required for T cell activation. Tregs can interact with DCs through the interaction of CTLA-4 and CD80/86 (Klocke et al., 2016). This interaction stimulates the secretion of indoleamine 2,3-dioxygenase (IDO), which induces catabolism of tryptophan into pro-apoptotic metabolites suppressing effector T cells (Grohmann et al., 2002). Moreover, Tregs can also regulate the capability of DCs to activate effector T cells via downregulation of CD80 and CD86 on DCs

resulting in cell cycle arrest of T cells and induces death of activated T cells (Mahnke et al., 2002).

Recent studies have suggested that lymphocyte activation gene Lag 3, may also block DCs maturation. Lag 3 binds to MHC II and it is required for optimal Treg suppression. Lag3-MHC II affects the maturation of DCs, which produce an increase in costimulatory molecules and a decrease in antigen capture molecules (Triebel, 2003).

Currently, there is significant interest in the role of macrophages in maintaining immune tolerance and their role of interaction with Tregs has been shown to be similar to Tregs/DCs interaction.

Co-culture of macrophages and Tregs leads to a reduction in MHC II expression on macrophages and the production of proinflammatory cytokines. It is conceivable that macrophage/Treg crosstalk happens mainly via IL-10 stimulation, which plays a major role in macrophage function (Okeke & Uzonna, 2019).

Figure 1.6 Tregs mechanisms of action. Tregs can supress via various suppression mechanisms which are dependent on the target effector cell. This can be via inhibitory cytokines TGF- β and IL-10 (A), via cytolysis caused by granzyme/perforin (B), causing metabolic disruption (C) or targeting dendritic cells (D) Figure taken from; (Stone et al., 2009).

1.4 Ageing

The world's population is ageing, during the last 150 years the human lifespan has dramatically increased, according to the world health organisation (WHO) the number of people aged over 65 years will increase from 1 billion (as per 2019 data) to 2 billion by 2050. This expansion is increasing at an unprecedented pace and will accelerate in coming decades.

1.4.1 Ageing and the Immune System

Maintenance of the immune system to prevent the aged host from surrendering to infectious pathogens is required. Like many other organs, the immune system is prone to ageing, eventually leading to a deterioration of the immunological ability. This transformation, also known as immunosenescence, produces an imbalance between protective and pathogenic immune responses resulting in higher morbidity and mortality rates among the elderly population (Gavazzi & Krause, 2002). Immunosenescence changes are very diverse and a several components of the immune system are affected. The impact of ageing in both innate and adaptive immunity has been well recognized. Ageing changes in the immune system are likely to be due to a combination of intrinsic ageing and the impact of the senescent-ageing environment during proliferation and differentiation in response to an antigen. Moreover, ageing also causes changes in chemokine localisation and the micro architecture of lymph nodes and the spleen, which has an impact in cell trafficking and antigen recognition (Mahbub et al., 2011; Weigle, 1989).

Ageing effects on T cells are highly researched in comparison with other immune cells populations given that these are the regulators and effectors of the immune response. Predominantly age-related changes in T cells population are critical and bring significant challenges to the elderly population. However, even though not all cell compartments are affected equally, there is a considerable decrease in T cell numbers with age caused by thymic involution and the consequent T cell output (Goronzy & Weyand, 2005). During ageing the thymus undergoes a progressive degeneration (3% decrease per year until the age of 45 years) leading to a decrease of its capacity to generate T cells. T cell precursors are formed in the bone marrow and will normally undergo maturation in the thymus before entering periphery (Pido-Lopez et al., 2001).(Gregg et al., 2005) Therefore, it is likely that maintenance of the peripheral T cell population in aged individuals will mainly depend on proliferation of the already existing T cells by T cell expansion.

1.4.2 Ageing and Tregs

Changes caused by ageing in the Treg population in humans have rarely been explored and studies so far have suggested only minor changes in numbers to the circulating pool through age (Chougnet Huang et al., 2008; Gregg et al., 2005). Tregs production and regulation of function are age dependent (Rocamora-Reverte et al., 2021) Tregs are highly generated in the first week of age and then expand, colonizing secondary lymph organs and tissues, to protect the host from autoimmunity. During puberty, the Treg population is operational and thymic production of Tregs decline. Tregs generated during early life stages are maintained through life and are fundamental to maintain immune homeostasis.

Moreover, extra thymic Tregs, generated in the thymus but differentiated in peripheral organs, are produced during life and function protecting the organism against chronic inflammation.

Previous studies demonstrated an increase in peripheral blood presence of CD25+CD4+Tregs and CD4+Foxp3+ Tregs with age; however, their ability to maintain the suppressive activity remains uncertain. It is still not clear if ageing Tregs show a high suppressive function triggering a dampened immune response or if Treg suppressive ability gets reduced during ageing causing an imbalanced immune response (Gregg et al., 2005). It is for this reason, Treg changes during ageing, that it will be important in the future to determine to which extent these ageing processes contribute to an onset of autoimmune diseases, chronic inflammation, cancer, and potentially other pathologies affecting the elderly population such as bone diseases including osteoarthritis.

1.4.3 Ageing and Bone Health

As a result of the ageing process, the composition, structure, and function of bone deteriorates. The combination of these changes increases the predisposition to developing bone diseases such as osteoporosis or osteoarthritis. Age related bone loss can be caused by two opposing mechanisms which affect the bone turnover rate, subperiosteal apposition (taking place on the outside of the bone) or endosteal resorption (taking place on the inside of the bone). Increasing age causes bone remodelling imbalance which leads to a negative bone balance usually caused by a high osteoclast activity. This can be due to a variety of mechanisms which are accelerated during ageing (Demontiero et al., 2012).

In the elderly population an increase in the osteoclastic activity can be due to increased serum levels of parathyroid hormone (PTH), related to low levels of vitamin D, impaired renal function, oestrogen deficiency and calcium absorption. Low levels of calcium resorption can also be caused by reduction of intestinal resorption characteristic of ageing (Lips, 2001).

Moreover, vitamin D is also required for osteoblast activity and bone formation by stimulating collagen I formation and alkaline phosphatase Vitamin D can also regulate bone metabolism indirectly by controlling calcium and phosphate homeostasis(Kream & Lichtler, 2011). In women, during the early postmenopausal period, vitamin D levels are low. One of the main causes being suppression of PTH, a major Vitamin D regulator, related to a rapid phase of bone loss. In later stages, PTH secretion levels gradually increase leading to higher bone turnover (Duque & Troen, 2008).

Another key factor on skeletal health are sex steroids. The decrease of oestrogen levels, caused by the cessation of the ovarian function during menopause is the cause to a rapid bone loss in women. The consequences of oestrogen deficiency on bone are generally linked to an impaired function between oestrogen and bone resorption mediators (Hofbauer et al., 1999). Oestrogen has been shown to inhibit osteoclast formation and activity by boosting the production of OPG, a soluble receptor for receptor activation of RANKL (Lundberg et al., 2001). Oestrogen suppresses RANKL secretion by osteoblasts and T cells, directs apoptosis of osteoclasts precursors, and inhibits MCSF production leading to a decrease in

osteoclast differentiation from monocytes (Eghbali-Fatourechi et al., 2003). Indirectly, oestrogen also suppresses the production of bone resorbing cytokines, such as IL-1, IL-6 and TNF-α (Charatcharoenwitthaya et al., 2007).

1.4 Immunity in the Bone: Osteoimmunology

The immune system and bone relationship has been recognised since the 1970s when there were several pioneering studies on immune cell-derived osteoclast-activation factors. Osteoimmunology is the study of the immune system interactions with the skeletal system (Okamoto et al., 2017). The term “Osteoimmunology” was coined by Aaron and Choi in 2000 when they first identified the two-way communication between the immune system and bone in inflammatory bone diseases (Arron, J.R; Choi, 2000).

Bone marrow harbours hematopoietic stem cells, lymphoid and myeloid progenitors which share the microenvironment with bone cells, being part of both musculoskeletal and immune system (Terashima & Takayanagi, 2018) Bone is a central organ/tissue able to regulate a range of other organs and tissues as well as to be influenced by them. There is growing evidence corroborating that the interaction between immune cells and bone cells in the bone marrow is vital for homeostatic bone maintenance (Ponzetti & Rucci, 2019).

Osteoimmunology has become increasingly important for the development of new therapeutic strategies to diseases involving bone and immune system (Okamoto et al., 2017). The immune system is a clear example of the crosstalk between the bone system and other tissues as several immune cells are associated with bone

homeostasis maintenance as well as they are related with inflammatory bone diseases corroborating then a bidirectional crosstalk (Ponzetti & Rucci, 2019). Bone cells can influence the immune system, implying several immune factors for their physiologic function, and immune cells can influence bone health. Skeletal homeostasis is strongly influenced by the immune system, being lymphocyte and macrophage derived cytokines the main mediators of osteoimmunological regulation (Ponzetti & Rucci, 2019).

However, this intimate link between immune system and bone can turn out to be disturbed and lead to bone disease development, such as osteoarthritis. Therefore, establishing the interactions between immune and skeletal system and understanding how the dysregulation of those immune components needed to maintain bone homeostasis occur can and the consequent development of bone diseases can lead to novel approaches for prevention and treatment of diverse inflammatory bone diseases.

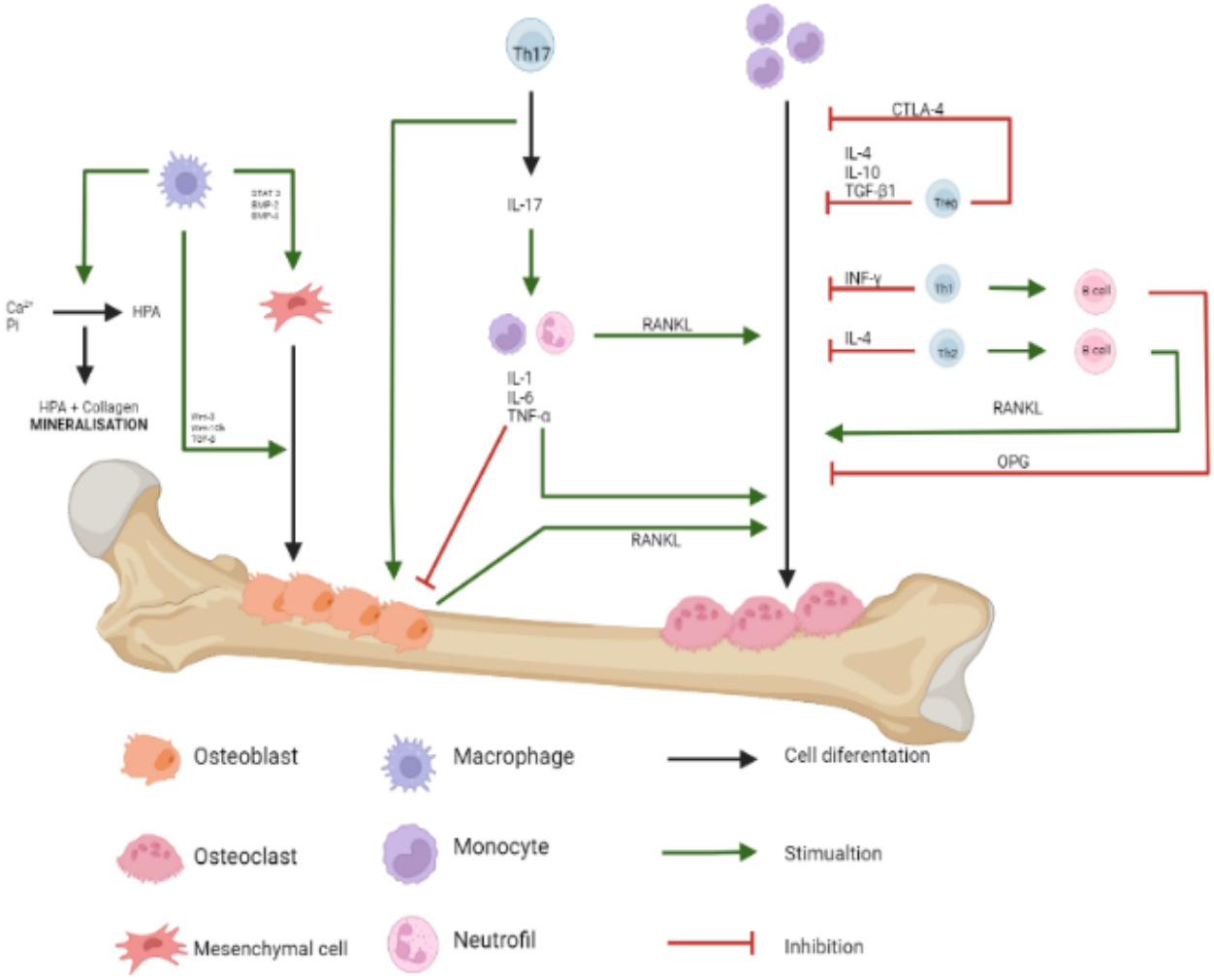

Figure 1.7 Bone homeostasis regulated by immune cells. The cross talk between the immune system and bone cells allows regulation of bone health. Th cell subsets Th1 and Th2 inhibit osteoclastogenesis by secreting IFN-g and IL-4 respectively. Treg cells secrete IL-4 and CTLA-4 which inhibits osteoclastogenesis and bone resorption. On the other hand, Th-17 cells secrete IL-1, IL-6, IL-17, TNF-a which act as pro-osteoclastogenic stimulating osteoclastogenesis and enhancing bone resorption. B-cells block RANKL expression by secreting OPG and therefore inhibiting osteoclastogenesis. Macrophages stimulate bone formation by regulating MSC to differentiate into osteoblasts and promoting bone mineralization. Figure adapted from (Dar et al., 2018).

1.5 Osteoimmunology in Osteoarthritis

Osteoarthritis historically has traditionally been considered a “wear and tear” and a “non-inflammatory” form of arthritis. The first evidence of osteoarthritis being an inflammatory diseases date back to 1959 when, Nettelbaldt and Sundblad, discovered abnormally high levels of inflammatory plasma proteins in both the synovial fluid and blood of patients with osteoarthritis (Nettelbladt & Sundblad, 1959). In the early 1980s, abundant inflammation in the synovium of osteoarthritis patients was further identified (Goldenberg et al., 1982.) with the presence of inflammation in osteoarthritis joints observed prior to the development of most bone and cartilage radiographic changes (Sokolove & Lepus, 2013). The presence of immune cells may not necessarily be attributable to acute or chronic inflammation and osteoarthritis development, as their function may be to maintain bone homeostasis as pointed out in previous sections. Hence, there is a need to identify when dysregulation of the immune system plays a key role in the development of the disease.

Growing evidence has revealed infiltration of cells from both the innate and adaptive immune system in osteoarthritis. Macrophages are the most abundant cell type found in osteoarthritis synovium, making up to 65% of immune cell infiltrate (Lopes et al., 2017). Macrophages are the major phagocytic cell of the body (Orlowsky & Kraus, 2015) being present in most of healthy organs and are essential to maintain immune homeostasis and organ function (Kurowska-Stolarska & Alivernini, 2017). However, they are also involved in the inflammatory response by initiating or resolving inflammation and restoring tissue damage

(Gordon & Martinez-Pomares, 2017). Synovial macrophages are activated by damage associated molecular patterns (DAMPs). These are produced when bone breakdown, cartilage and ECM components are released (e.g., hyaluronan acid, proteoglycans, alarmin and calcium phosphate crystals) (Majoska HM et al., 2018). Synovial macrophages can either be differentiated to macrophage type 1 (M1) (proinflammatory) when stimulated by IFN-γ, Toll like receptors, granulocyte macrophage colony stimulation factor and lipopolysaccharide, or macrophage type 2 (M2) (anti-inflammatory) when stimulated by IL-4 or IL-13 (Yunna et al., 2020). In osteoarthritis, the balance between M1 and M2 may be distorted, contributing to the initiation and progression of osteoarthritis pathology (B. Liu et al., 2018). Activation of M1 macrophages leads to the release of IL-1β, TNF-α and IFN-γ which are key players in cartilage breakdown in osteoarthritis due to their role in matrix MMP production and upregulation of IL-1b, IL-6 and ADAMTS5 (Zhang et al., 2020). Conversely, M2 macrophages secrete TGF-β which enhances cartilage repair through stimulating proteoglycan synthesis in vitro (B. Liu et al., 2018). Overexpression of TGFβ1 is related with subchondral bone sclerosis and osteophyte formation during osteoarthritis development. Overall, this results in hardening of the bone right below the cartilage and osteophyte formation (Shen et al., 2014).

The presence of NK cells has also been reported in the synovium of osteoarthritis patients (Jaime Y Z X et al., 2017) NK cells are also involved in osteoclast formation as they express RANKL and MCSF, which will stimulate the differentiation from monocytes to generate osteoclasts (Dimitrova & Ivanovska, 2012).

The role of NK cells in inflammatory diseases remains largely to be determined, however evidence suggest that interact directly with other immune cells, such as dendritic cells, macrophages, and lymphocytes (Boudreau & Hsu, 2018).

Moreover, presence of B cells in synovial membrane of patients with osteoarthritis is found in low numbers and studies are not showing a correlation between B cell infiltration and severity of osteoarthritis development (Sakkas & Platsoucas, 2007).

T cells represent 20-25% of the inflammatory cells in the synovial membranes of osteoarthritis. The presence of all T cells subsets are found in synovial fluid, synovial membrane, and peripheral blood of osteoarthritis patients. However, significant alterations occur on Th1, Th9, Th17, Cytotoxic T cells, Memory T cells and Tregs (Y. Li et al., 2017). T cells could contribute to the development of osteoarthritis via cartilage destruction produced by chemokine production or by inhibiting the osteoclastogenesis process due to secretion of anti-osteoclastogenic cytokines such as IFN-γ (Kikuta & Ishii, 2013).

Th17 and Tregs subsets show an important emerging role in osteoarthritis pathology. Th17 are mostly pro-osteoclastogenic, since they induce secretion of MCSF and RANKL by osteoblasts and stromal cells as well as increase RANK expression in osteoclast precursors by secretion of IL-17, IL-22 and INF-g. Conversely, the role of Tregs in healthy bone has been proven to be primarily anti-osteoclastogenic, by interacting with osteoclasts precursors via CTLA-4 and inhibiting osteoclast formation (Bozec & Zaiss, 2017).

Figure 1.8 Osteoimmunology in osteoarthritis. Osteoimmunology in osteoarthritis is represented by elevated levels of different helper T cells, Th17 secreting pro-inflammatory cytokines (IL-17, IL-22 and TNF-γ) which stimulate osteoblasts activity and Tregs, which secrete IL-4, IL-10, TGF-β1 and CTLA-4 that inhibit osteoclasts activity. These cytokines in combination with macrophages activity at the site of synovial membrane is characteristic of osteoarthritis condition. Figure adapted from (Dar et al., 2018).

1.6 Project Overview

In summary, there is emerging evidence to suggest that Tregs may play an important role in regulating healthy bone mass through regulation of osteoclast formation and function and have the potential to drive bone repair and regeneration (Zaiss, Sarter, et al., 2010).

Imbalance in the bone remodelling process can lead to bone diseases, such as osteoarthritis (Florencio-Silva et al., 2015). There is increasing evidence to suggest that the characteristic articular cartilage degradation during osteoarthritis development follows biochemical changes to the joint, and furthermore, there is clear evidence of immune cell invasion in the damaged joint (Weber et al., 2018). Whilst Tregs are thought to play a role in osteoclastic bone resorption, their role in osteoarthritis is unknown.

Moreover, ageing-related changes in both the immune system and the bone are related with bone loss and increase the risk of developing osteoarthritis (Demontiero et al., 2012). Changes in the Treg population during ageing may be related with the bone remodelling imbalance in the elderly population.

Given the limitations caused by COVID-19 restrictions for the duration of the project, making it was impossible to obtain osteoarthritis samples, the hypothesis and aims were forced to change based on the available resources. Therefore, the aim of this project was to explore the impact of Tregs on osteoclasts activity in healthy ageing and the spatial localisation of Tregs in osteoarthritis and aged bone.

Hence, we hypothesised that Tregs will suppress osteoclast function in vitro and colocalise in the site of high bone remodelling (related to healthy ageing or bone damage)

For this, I have completed the following aims to test the hypothesis:

1) Quantify Tregs, with a focus on those likely to be involved in bone turnover, in the peripheral blood of healthy young and healthy aged participants.
2) Examine the suppressive effects of Tregs from healthy young and healthy aged participants on *in vitro* osteoclastogenesis.
3) Determine the spatial localisation of Tregs in osteoarthritic bone in murine samples compared to healthy mice.

Together the data obtained from these aims may provide further evidence for Tregs to play a role in osteoclastic bone resorption, and this process to be disrupted by ageing. A better understanding of the effects of Tregs on osteoarthritis bone pathology will inform new mechanistic knowledge for improving health and wellbeing.

Chapter 2

Materials and Methods

2.1 Ethics and Sample Population

Human Blood and Serum

Ethics forms for working with human samples (whole blood and serum) were approved by the Ethics board at Edinburgh Napier University and the Scottish National Transfusion Service (SNBTS). Buffy coats obtained from SNBTS under the project's consent letter 20-06 and 21-05. Fresh blood samples were taken from volunteers by venepuncture at Edinburgh Napier University by a trained phlebotomist, and buffy coats samples were obtained from the SNBTS. Participants were fully informed of the study requirements and voluntarily signed a consent form and questionnaire (Appendix 2). Participants included males and females, of the age range 20-75 years. Participant's information can be found in Appendix 1.

Human Tissue

Human osteoarthritic samples were kindly provided by Mr Anish Amin and obtained from patients undergoing total knee replacement one female and one two males aged 59-78 year at the Edinburgh Royal Infirmary Hospital. Mr Amish Amin

(University of Edinburgh) diagnosed osteoarthritis based on clinical and radiographic osteoarthritis features. Samples are obtained with patient consent and all procedures with ethical approval by NHS Lothian in collaboration with Mr Anish Amin. The collection, storage, and subsequent use of human tissues are regulated in Scotland by The Human Tissue Act (Scotland) 2006.

Murine Tissue

Paraffin-embedded male murine knee joints from (i) destabilisation of the medial meniscus model (16 weeks old, surgery performed at 8 weeks and sampled 8 weeks post-surgery) and (ii) aged model (7 weeks of age), were kindly provided by Dr Katherine Staines (University of Brighton) (Samvelyan et al., 2021). The animal study was approved by the Roslin Institute Animal Users and Research Ethics Committees. The animals were maintained following the UK Home Office guidelines for the care and use of laboratory animals. Animal studies were conducted in line with the ARRIVE guidelines.

2.2 Cell counting and Viability

Viability of cells was assessed using Nigrosin dye 1:1 diluted with cells at room temperature (RT) and cells were visualised immediately after dying. Nigrosin dye will show dead cells in black allowing their differentiation from live cells. Cells were counted using FastRead counting slices (IMMUNE SYSTEMS Ltd, Devon, United Kingdom) and visualised using EVOS XL core microscope (Life Technologies, California, United States).

2.3 Purification of Mononuclear Cells Subsets from Whole Blood

Different populations of cells were isolated from whole blood samples by either gradient centrifugation, peripheral blood mononuclear cells (PBMCs), FACS sorting (Monocytes, Tregs and conventional T cells (Tcons)) or beads sorting (Monocytes, Tregs and Tcons).

2.3.1 Peripheral Blood Mononuclear Cells (PBMCs) Isolation

PBMCs were isolated from volunteer's whole blood by gradient centrifugation using Lymphoprep (STEMCELL technologies) and following the manufacturer's protocol. The whole blood was diluted 1:1 with sterile phosphate buffer saline (PBS) and was layered on top of 14mL of Lymphoprep using a Pasteur pipette. Samples were centrifuged for 30minutes, 800*xg*, RT and low break. PBMCs layer was carefully removed, trying to avoid any disruption in between layers, using a Pasteur pipette and transferred into a falcon tub. Cells were washed twice with sterile PBS by centrifuging 5 minutes, 400*xg* at RT.

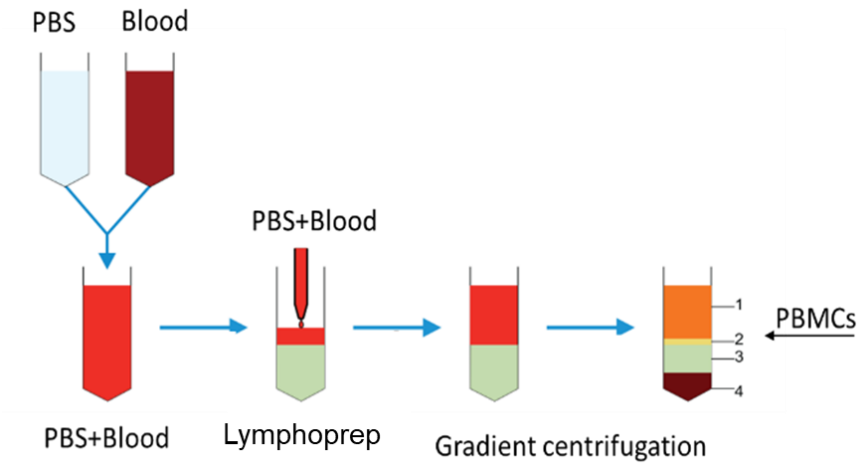

Figure 2.1 PBMCS Isolation from Whole blood Protocol. Diluted whole blood in PBS, Lymphoprep layered and gradient centrifugation steps are shown. After cell separation different populations can be differentiated, Plasma (1), PBMCs (2), Lymphoprep (3) and red blood cells (4).

2.3.2 FACS Sorting

2.3.2.1 Monocytes FACS Sorting

Monocytes were sorted using the BD FACS aria flow cytometer based on morphology gating and CD4intermediate population on PBMCs previously sorted from whole blood as in section 2.3.1.

2.3.2.2 Regulatory T cells (Tregs) FACS Sorting

PBMCs isolated from whole blood as in section 2.3.1 were further stained following the steps described in section 2.4.2 for anti-CD4, anti-CD127 and anti-CD25 antibodies to identify the Treg population. Samples were then processed through the FACS aria flow cytometer and selected based on the Treg gating to sort the $CD4^{+}CD25^{hi}CD127^{low}$ Treg cell population.

2.3.3 Beads Sorting

2.3.3.1 Monocytes CD14+ Positive Enrichment

PBMCs isolated from whole blood were further processed to obtain the CD14+ enriched cell fraction was obtained using the MagniSort™ Human CD14 Positive Selection Kit (Thermofisher, Oxford , United Kingdom) and following the manufacturer's protocol. Purity of selected cells was monitored by flow cytometry staining the cells for surface markers CD4 and CD14 following the protocol as in 2.4.2 and the antibodies conditions as in Section 2.6.

2.3.3.2 Regulatory T cells (Tregs) Beads Sorting

PBMCs isolated from whole blood were further processed using the CD127-/ CD25+ negative selection kit (STEMCELL technologies, Cambridge, United Kingdom) as per the manufacturer's instructions to obtain the Treg cells fraction. Purity of selected cells was defined by flow cytometry staining the cells for surface markers CD4, CD127 and CD25 following the protocol as in 2.4.2 and the antibodies conditions as in section 2.6.

2.3.3.3 Conventional T cells (Tcons) Beads Sorting

Following the CD127-/ CD25+ negative selection kit (STEMCELL technologies) as in 2.3.3.2, while sorting Tregs, Tcons (CD4-CD25-) where isolated by following the manufacturer's protocol. Purity of selected cells was proved by flow cytometry staining the cells for surface markers CD4, CD127 and CD25 following the protocol as in 2.4.2 and the antibodies conditions as in section 2.6.

2.4 Flow Cytometry Procedures and Analyses

Flow cytometry acquisition was performed by two different models of flow cytometer for these studies. Sorting was performed using FACS Aria (Becton Dickinson, BD Biosciences) which contains three lasers (488nm, 635nm and 350nm) and can detect up to ten different fluorescent chanels. As a cell analyser, FACS Celesta Aria (Becton Dickinson, BD Biosciences, Plymouth, United Kingdom) was used, which has 3 lasers (325nm, 488nm, and 640nm) and can detect twelve colours. Further analyses of acquired data were performed using FlowJo v.9 and FlowJo V.10 (FlowJo LLC BD Biosciences).

2.4.1 Spectra Compensation

Before running the sample, laser voltages were verified - to ensure consistency of lasers during data collection - by running the Cytometer Setup and Tracking (CST) beads (Becton Dickinson, BD Biosciences, Plymouth, United Kingdom) on the FACSDiva software. A further adjustment was carried out to counterbalance the spectra overlap by staining the compensation beads with one antibody at a time to identify the target population and set up the spectra to record on each channel. This ensured that no spectra overlap is recorded for the different antibodies on different channels and will avoid the presence of false positives.

2.4.2 Flow Cytometry Surface Staining

Isolated PBMCs cells were counted and a fraction of 10^5 cells were stained for multicolour flow cytometry by adding 100μL of sterile PBS and the pre-titrated optimal concentration of antibody. Incubation for 15 minutes at RT in the dark was done before washing twice with sterile PBS centrifuging 5 minutes, 400*xg* at RT. After centrifuging cells were re-suspended in 400μL of PBS for flow analysis.

Section 2.6 summarises the antibodies used and the optimal concentration of which was determined by preliminary titration.

2.4.3 Intracellular FoxP3 Staining

Following surface staining after centrifugation cells were fixed and permeabilised using the FoxP3 staining kit (eBiosciences, California, United States) as per the manufacturer's instructions. Cells were washed in PBS centrifuging 5 minutes, 400*xg* at RT and resuspended in the fixation/permeabilization buffer with the following modification: permeabilization incubation time was 30 minutes. After this, cells were washed in PBS centrifuging 5 minutes, 400*xg* at RT and 5µL FoxP3 antibody was added for 30 minutes (time modified from original protocol). Cells were washed in PBS centrifuging 5 minutes, 400*xg* at RT and re-suspended in 400µL of FACS buffer for flow analysis.

2.4.4 Division Dye Staining

Cell Trace Violet (CTV) and Carboxyfluorescein Succinimidyl Ester (CFSE); (Molecular Probes, Inc, Oregon, United States) was used to measure cell proliferation. Responder T cells used in the suppression assays (Tcons) were stained at a concentration of 10^6 cells/mL of cells following the manufacturer's instructions, with the following modification: after staining 5mL culture media containing FBS was added for 10 minutes at 37°C followed by two 5 minutes washes using culture media at 400*xg* RT.

2.5 Suppression Assays

Regulatory T cells ($CD4^+CD25^{hi}CD127^{lo}$) and conventional T cells (responder T cells) were isolated as described in section 2.3.2 or section 2.3.3. Tregs and

conventional T cells were activated by stimulation with CD3/CD28 Immunocult T cell activator (STEMCELL technologies, Cambridge, United Kingdom) as per the manufacturer's protocol. Tcons staining was done as per in 2.4.4. Cell cultures were set up in the following combinations: Tregs alone, Tregs and conventional T cells (1:1) and conventional T cells alone. To assess proliferation, cells were cultured for 7 days at 37°C, 5%CO2. On day 7, samples were analysed using flow cytometry. The proliferation of Tcons was analysed by calculating the division index (DI, average number of divisions undergone by a cell in the starting population) using FlowJo.

2.6 Antibody List

The following antibodies conditions were used as described in the previous sections for reference.

Table 1 List of antibodies with supplier, catalog, clone and concentration used per experiment.

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Antibody-Fluorochrome	**Supplier**	**Catalog No.**	**Clone**	**µg/10^6 cells**
CD4- Alexa488	Thermofisher	53004942	RPA-T4	0.25
CD127-PECy7	BD Biosciences	560822	HIL-7R-M21	0.25
CD25-PE	BD Biosciences	567214	BC96	0.25
CD25-PE	Stemcell	60153PE	2A3	0.25
CD14-v450	Thermofisher	48014942	61D3	0.2
CCR4-BV421	BD Biosciences	562579	1G1	0.2
CXCR4-BV605	BD Biosciences	740418	12G5	0.2
FoxP3-PECF594	BD Biosciences	562421	259D/C7	0.25

2.7 Human *In vitro* Osteoclast Culture

Monocytes (CD14+ enriched cells) were re-suspended in α-MEM containing 10% heat-inactivated FBS, 2 mM L-glutamine, 100 µg/mL penicillin, and 100 Units/mL streptomycin. $5x10^5$ CD14+ cells per well were plated on day 0 in a 96 well plate containing a dentine disc at the bottom. Cells were further stimulated with 25 ng/mL MCSF and 30 ng/mL RANKL (R&D systems, Oxford, United Kingdom) for osteoclast differentiation on day 0 and half media containing 25ng/ml human recombinant MCSF, and 30ng/ml human recombinant RANKL was changed every 3 days for 21 days.

2.8 TRAP Reactivity on Osteoclasts

Cultured osteoclasts after 21 days were washed with PBS and fixed with 2.5% glutaraldehyde for 5 minutes. TRAP reactivity was done using an Acid phosphatase leukocyte kit (Sigma-Aldrich Ltd, Dorset, United Kingdom). Cells were treated with the TRAP substrate kit prepared as per the manufacturer's instructions with some modifications. The final time of incubation was reduced to 30 min and no final counterstaining with haematoxylin for the samples was done. TRAP positive cells were imaged via light microscopy and visually counted. TRAP positive cells containing more than two nuclei were considered osteoclasts.

2.9 Scanning Electron Microscope (SEM) Imaging Osteoclasts

Osteoclasts cultured as in section 2.7 were imaged by Dr Callum Wilson, Edinburgh Napier University following the following steps. Samples were coated using the physical vapour deposition technique sputtering in preparation for

imaging. A Polaron E5100 sputter coater was used for 3 minutes at 18-20mA / 2.5kV to give a conductive Palladium coating. The samples were then imaged using a Tescan Vega 3 Scanning Electron Microscope (SEM).

2.10 Resorption Activity on Dentine Discs

Dentine discs obtained from elephant tusks, kindly provided by Dr Isabel Orriss (Royal Veterinary College, London, UK), were used as a source of mineralized substrate to measure resorption produced by osteoclasts cultured as per in section 2.7. Resorption pits were visualised by reflected light microscopy following TRAP reactivity as described in section 2.8.

After TRAP reactivity procedure (Section 2.8) on the dentine discs, these were left to air dry to improve visualisation of pits. Measurement of resorption pit area was performed by taking images of five representative areas on the dentine disc (centre and 4 corners). Area per osteoclast was measured using ImageJ and an average of the 5 areas per well was counted per sample.

2.11 Co-culture Tregs and Osteoclasts

CD14+ isolated monocytes were cultured as per in section 2.7. On day 0, $0.5x10^5$ Tregs obtained as in section 2.3.3.2 were added to the culture media containing MCSF and RANKL. Media was changed every 2-3 days as per in section 2.7, with the modification of spinning down the media (containing Tregs suspension) and resuspending in the culture media before adding to the wells (containing dentine disc and osteoclasts).

2.12 ELISA Serum Marker for Bone Turnover CTX

Serum samples were obtained ethically from the SNBTS, and ELISA was performed for serum markers CTX following the manufacturers protocol (Human CTX-1 ELISA Kit, Novus Biological, Oxford, United Kingdom). Absorbance at 450 nm was analysed using the Tecan Sunrise plate reader (Tecan).

2.13 Immunohistochemistry Procedures

2.13.1 Ethics and sample population

Ethics obtained as per in Section 2.1.

2.13.2 Tissue processing and paraffin embedding

Human bone samples were decalcified in 10% ethylenediaminetetraacetic acid (EDTA) at 4°C and changed every 2-3 days. Samples were dehydrated through a gradient of 70%, 95% and 100% ethanol for a total of 14 hours, before going into xylene for 4 hours and in paraffin for 4 hours, using the automatic tissue processor Leica TP 1020. Tissue was embedded in paraffin using Leica EG1160 tissue embedding centre. Tissue processing and embedding was performed by Dr Jasmine Samvelyan.

2.13.3 Toluidine Blue/Fast Green staining

Osteoarthritis phenotype was confirmed by histology using toluidine blue staining. Slides of 6 µM human sections and 5 µM murine sections were de-waxed and rehydrated as per in table 2. Sections were then incubated in 0.4% toluidine blue dissolved in sodium acetate and counterstained in 0.02% Fast Green in distilled

water. Sections were finally rinsed in H_2O and mounted. Images were taken using EVOS XL core microscope (Life Technologies, California, United States).
and analysed using ImageJ.

Processing stage	Reagent	Time (min)
Dewax	Xylene	5
	Xylene	5
	Absolute alcohol	2
	Absolute alcohol	2
Rehidrate	90% IMS	1
	70% IMS	1
	Water	5

Table 2 De-waxing and re-hydration steps for paraffin embedded tissue sections.

2.13.4 Immunohistochemistry and Foxp3 Staining

Samples were prepared as described in section 2.4.2. Antigen retrieval was performed using 1% Pepsin in 10 mM HCl buffer at 37°C for 30 minutes and RT for 10 minutes. For optimisation purposes, different unmasking methods were used, 0.1% Trypsin at 37°C for 30 minutes, 0.1 M citrate buffer pH 6.0 for 90 minutes at 70°C and 20 ug/ml Proteinase K in TE Buffer, pH 8.0 at 37°C for 30 minutes and RT for 10 minutes. Endogenous peroxidase was blocked using 0.3% hydrogen peroxidase in methanol for 30 minutes at RT. Further blocking was carried out using 2.5% normal horse serum from the Vectastain universal quick kit

(Vector laboratories, California, united States) for 30 minutes a RT. Primary antibody (Foxp3 Abcam ab20034) 5 µg/mL diluted at 1:100 (and 1:50 during optimisation steps) in blocking serum from the Vectastain universal quick kit (Vector laboratories, California, united States) and incubated at 4°C overnight. Sections were washed 3 times with PBS-T for 5 minutes and incubated in biotinylated Pan-specific antibody from the Vectastain universal quick kit (Vector laboratories, California, united States) for 10 minutes at RT. Sections were washed three times with PBS-T for 5 min and incubated with Streptavidin/peroxidase complex for 5 minutes followed by three times washes with PBS-T for 5 minutes. Labelling was developed using DAB substrate kit (Vector laboratories, California, united States) at RT for 2 minutes (or 4 during optimisation steps) producing a brown colour in positive sections. All samples were count-stained with haematoxylin prior to dehydrating, clearing, and mounting under PERTEX Mounting Media (CellPath, Newtown United Kingdom). Slides were imaged via light microscopy. Five randomised fields per section were imaged, positive and negative cells were counted and positive expression of Foxp3 was expressed as a percentage of total cells.

2.13.5 Osteoclasts Characterisation in Murine Bone

Murine bone samples embedded in paraffin were dewaxed and rehydrated as per in Table 2. TRAP reactivity was done using the Acid phosphatase leukocyte kit (Sigma-Aldrich Ltd, Dorset, United Kingdom) following the manufacturer's instructions with some modifications. The final time of incubation was reduced to 30 min. Slides were imaged via light microscopy, five randomised fields per section

were imaged TRAP positive cells, containing more than two nuclei were considered osteoclasts. Daa is expressed as total number of osteoclasts per field.

2.14 Statistical analysis

Data was checked to be normally distributed using a Shapiro-Wilk normality test using GraphPad. Data was analysed by one-way analysis of variance (ANOVA), or the student's t-test. All data is expressed as the mean ± standard error of the mean (SEM) and $P<0.05$ was considered to be significant.

Chapter 3

Characterisation of Regulatory T cells in the Peripheral Blood of Healthy Young, and Aged Individuals

3.1 Introduction

Emerging data indicates that Tregs may play a significant role in regulating bone turnover by accumulating at the site of tissue injury and inducing tissue regeneration (Zaiss et al., 2010). Bone, unlike most other tissues, is able to regenerate without scar tissue formation which indicates strong regenerative potential. Imbalance in the bone resorption process can lead to bone diseases, such as osteoarthritis. An increase in bone turnover is directly related to bone deterioriation and contributes to an increase of fractures (Dimitriou et al., 2011). Several bone turnover markers are being considered as a marker for detecting early bone diseases development. CTX-1, is a marker of bone resorption since it is a product of collagen type 1 degradation during the bone resoprtion phase of

bone remodelling and it is commonly used as an indicator of osteoclast activity. Hence, it is a potential marker for bone disease progression where there is an increase in osteoclast activity (Shaw & Högler, 2012).

The immune system is affected by age-related changes which alter its phenotype and functionality, leading to impaired immune responsiveness. Because of the decrease in functionality, there is an increase in the vulnerability to infection and malignancy and the process of immune ageing has detrimental consequences for the host, which considerably contribute to increased mortality and morbidity amongst the elderly population (Gavazzi & Krause, 2002). Both, the innate and adaptive immune responses are affected by ageing (Sadighi Akha, 2018). However, the changes in the T cell population cause considerable challenges to the elderly population since T cell population naturally declines with ageing as thymic involution causes a decrease of cell production (Goronzy & Weyand, 2005). The impact of immune ageing varies amongst the different T cells compartments and ageing progressively will affect thymic and peripheral differentiation of Tregs affecting numbers, subsets distribution and functional competency of Tregs (Darrigues et al., 2018).

Tregs contribution to immune homeostasis is based on maintaining unresponsiveness to self-antigens and suppressing excessive immune responses and resolving completed immune responses. Tregs are particularly versatile and can exert diverse effector functions depending on the health or disease context. The level of intricacy increases with ageing when the immune compartment is altered by thymic involution and dysregulation of immune factors and there is

emerging discrepancy in the field regarding the up- or down-regulation of Tregs activity with age. There is abundant data corroborating that the numbers of $CD25^{+}CD4^{+}$Tregs increase with age, implying then the possible role Tregs play in age-driven immune suppression (Gregg et al., 2005). Phenotypic evaluation of Tregs has shown that immunosuppressive capability is not affected, suggesting that Tregs function is not affected with ageing (Jagger et al., 2013.). Moreover, studies focused on FoxP3 expression demonstrated an enhanced FoxP3 expression in the aged population when compared with young individuals (Gregg et al., 2005). However, other studies suggested an increase Treg suppression function with ageing. Understanding the causes of increased or lower levels of Tregs in the older population is vital to understanding the role they may play in age-related diseases.

Tregs are usually found in the lymph, where immune response suppression by Tregs mostly occur, and circulate in the blood, but to exert their effects in the tissues, they must first migrate to them. Treg migration occurs in multiple steps. First, surface molecules on the lymphocyte contact selectins on the surface of the endothelium, a process known as rolling. The endothelium expresses E-selectin and this bind to CLA expressed on Tregs providing firm adhesion of the Tregs on the endothelium in the tissue. After adherence, diapedesis occurs. This is when the Tregs can move across the endothelium and travel through the tissue alongside chemokine gradients (Ward & Marelli-Berg, 2009).

The extensive diversity of Tregs in different tissues and the lack of clear markers to define Treg subtypes makes this area of study particularly complex. However, growing evidence suggests that Tregs from non-lymphoid tissues show a distinct functionality and phenotype that regulate the tissue resident immune cells (Almanan et al., 2018). Tregs have been identified as heterogeneous, expressing distinct chemokine receptors allowing their migration to different tissues as part of regulation of different inflammatory responses (Liston & Gray, 2014). For Tregs to reach the bone they must express an optimum combination of receptors to allow binding to the endothelium and for the appropriate chemokines gradient to ensure Treg migration towards bone.

In this chapter, the number of Tregs in the peripheral blood of young and aged healthy participants were analysed to observe possible changes in the Treg population related to ageing, and to use this as a healthy aged control population for bone diseases. Potential bone Tregs were characterised by expression of bone migration markers CXCR4 and CCR4, expression of bone markers is characteristic of possible bone. Moreover, this chapter will also correlate levels of peripheral blood circulating Tregs with levels of CTX-1 serum as a marker of bone remodelling in order to understand changes in the Treg population during imbalanced bone remodelling during ageing.

3.2 Hypothesis

There is a correlation between the number of Tregs in the peripheral blood and the amount of CTX in the serum as a consequence of Tregs influencing bone turnover. Additionally, the presence of serum CTX-1 will be higher in healthy aged participants when compared with the healthy young group, as a response of higher bone resorption.

Higher presence of Tregs in peripheral blood will be found in the healthy aged participants group when compared with young healthy participants. Moreover, Tregs from the healthy aged group will express higher numbers of bone homing markers (CCR4 and CXCR4) as an indication of higher migration towards bone injury caused by ageing.

3.3 Aims

I. Quantify the Treg population in the peripheral blood of healthy young and healthy aged individuals and identify possible bone Treg populations (expression of CXCR4 and CCR4) in the peripheral blood by multi-parameter flow cytometry.

II. Correlate Treg sub-populations with ELISA analysis of serum markers of osteoclastogenesis (CTX-1).

3.4 Materials and Methods

3.4.1 Samples and Ethics

Ethics were approved by the Edinburgh Napier University and the Scottish National Blood Transfusion Service (SNBTS). Samples were obtained as per in section 2.1 and grouped as healthy young (N=13, 5 female and 8 male, age 20-42 y/o), healthy aged (N=11, 7 female and 4 male, age 52-70 y/o). Healthy young and healthy aged grouping were determined in the results section as per the data obtained.

3.4.2 Purification of Lymphocyte subsets

3.4.2.1 PBMCs Isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by gradient centrifugation using Lymphoprep (STEMCELL technologies) as described in section 2.3.

3.4.3 Surface Staining and Intracellular Staining

PBMCs isolated from whole blood as in section 2.3 were stained with anti-CD4 Alexa 488, anti-CD127 PE-Cy7, anti-CD25 PE, anti-CCR4 BV421 and anti-CXCR4 BV605 surface markers as indicated in section 2.4.2 and for intracellular marker anti-FoxP3 PECF594 following section 2.4.3.

3.4.4 ELISA Serum Markers for bone turnover markers

Serum samples were obtained ethically from the SNBTS or by venepuncture at Edinburgh Napier University. Levels of CTX-1 in serum were analysed by ELISA following the manufacturer's protocol (Human CTX-1 ELISA Kit, Novus Biological) as per in section 2.11.

3.4.5 Statistical analyses

Data were checked to be normally distributed using a Shapiro-Wilk normality test using GraphPad. Data were analysed by unpaired Student's t-test. All data are expressed as the mean ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad and $P<0.05$ was considered to be significant.

3.5 Results

3.5.1 Gating Strategy and Definition of Cell Populations

The following gating strategy was used to identify the Treg population from the PBMCs isolated fraction from whole blood. Firstly, the lymphocyte population was identified depending on their morphology by plotting Forward Scatter Area (FSC-A) versus Side Scatter Area (SCC-A). This allowed the visual differentiation of the various cell population in the PBMCs fraction (lymphocytes, monocytes, and granulocytes), and the lymphocyte population of cells was gated (**Figure 3.1A**). Any doublet cells that could lead to false positive were discounted based on Forward Scatter pulse height to area ratio, gating then a population of singlets only (**Figure 3.1B**).

Further the T helper cell population was gated on the singlets by positive expression of CD4 (**Figure 3.1C**). Note that two clear populations of $CD4^-$ and $CD4^+$ should appear as this is previously gated on lymphocytes only. However, we can see the presence of an intermediate CD4 population which suggests that some monocytes have been included in the lymphocyte gate on **Figure 3.1A**. Sometimes this may happen as per sample variability and then, gating on T helper cells has to be done on $CD4^+$High cells, these will be referred to as the $CD4^+$ T cells.

Finally, two different strategies can be followed to determine the Treg population amongst the $CD4^+$ T cells. First by gating Tregs based on positive expression of intracellular marker Foxp3 and secondly, on high expression of CD25 (IL-2

receptor) and low (but not negative) expression of CD127 (IL-7 receptor) – denoted as $CD25^{hi}CD127^{low}$.

The gating strategy based on Foxp3 marker, is a more reliable way of selecting the specific Treg population in comparison to determine Treg population by $CD25^{hi}CD127^{intermediate}$ since activated T cells can express CD25 causing cross-contamination of populations (Sakaguchi et al., 1995b). However, these differences are minimal, and no significant changes could be seen between both gating strategies affecting the Treg population (data shown in Chapter 4, Figure 4.5). Gating based on $CD25^{hi}CD127^{intermediate}$ is the best option when needing a Treg sample population with live cells. Foxp3 staining (nuclear marker) requires for the cells to be fixed and permeabilised, causing cell death. This needs to be a consideration when follow-on functional experiments are to be carried out as outlined in Chapter 4.

In this chapter the gating strategy based on $CD4^{+}Foxp3^{+}$ expression (**Figure 3.1D**) was used to determine and quantify Tregs in peripheral blood from healthy young and healthy aged participants.

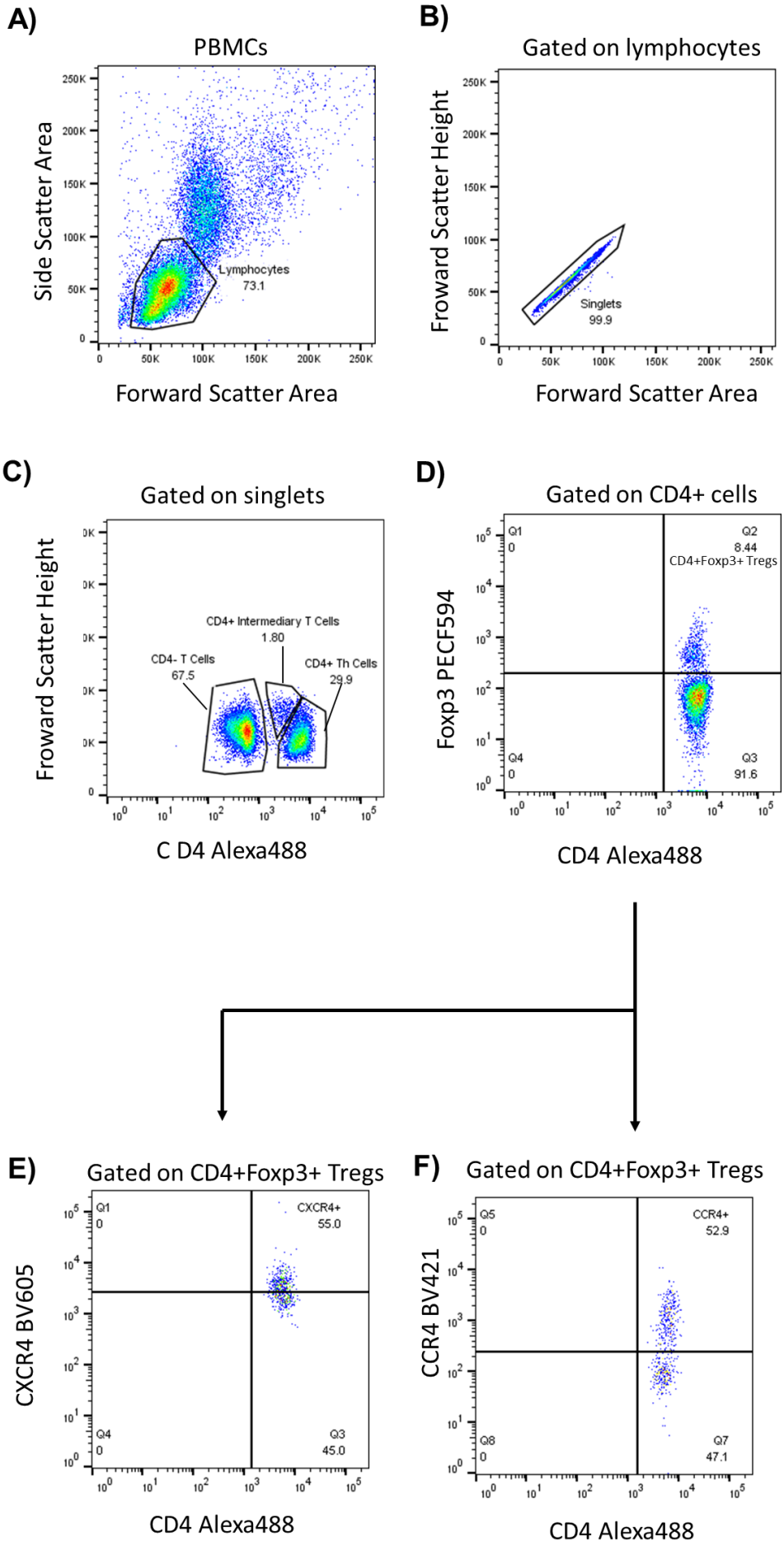
A)
PBMCs
Side Scatter Area
Forward Scatter Area
Lymphocytes
73.1
B)
Gated on lymphocytes
Froward Scatter Height
Forward Scatter Area
Singlets
99.9
C)
Gated on singlets
Froward Scatter Height
C D4 Alexa488
CD4+ Intermediary T Cells
1.80
CD4- T Cells
67.5
CD4+ Th Cells
29.9
D)
Gated on CD4+ cells
Foxp3 PECF594
CD4 Alexa488
Q1
0
Q2
8.44
CD4+Foxp3+ Tregs
Q4
0
Q3
91.6
E)
Gated on CD4+Foxp3+ Tregs
CXCR4 BV605
CD4 Alexa488
Q1
0
CXCR4+
55.0
Q4
0
Q3
45.0
F)
Gated on CD4+Foxp3+ Tregs
CCR4 BV421
CD4 Alexa488
Q5
0
CCR4+
52.9
Q8
0
Q7
47.1

Figure 3.1 Gating strategies and definition of Treg cell population and bone homing receptors (CCR4 and CXCR4). PBMCS isolated from human whole blood were stained with anti-CD4 Alexa 488, and anti-Foxp3 PECF594, anti-CCR4 BV421 and anti-CXCR4 BV605. Lymphocyte population was gated based on morphology plot using Forward Scatter versus Side Scatter (A), doublets of cells were discounted by gating on singlets based on Forwards Scatter Area versus Height (B). Singlet lymphocytes were gated on CD4High population (C). Tregs were gated based on $Foxp3^+$ expression (D). Further gating on CXCR4 (E) and CCR4 (F) bone homing markers was analysed from $CD4^+Foxp3^+$.

To further study the expression of potential bone homing markers on Tregs, these were identified as $CD4^+Foxp3^+$ cells and stained with anti-CXCR4 and anti-CCR4. The gating strategy followed to determine the chemokine expression was based on cells gated on $CD4^+$Foxp3 and expression of either CXCR4 (**Figure 3.1E**) or CCR4 (**Figure 3.1F**). The gating for positive population of both chemokines was set using $CD4^-$ population as there was a clear distinction in the CCR4 and CXCR4 positive and negative populations (**Figure 3.2A and Figure 3.2C**) the $CD4^+$ most times does not have a clear separation between the positive and negative populations for $CXCR4^+$ and $CCR4^+$ (**Figure 3.2.B and Figure 3.2D**).

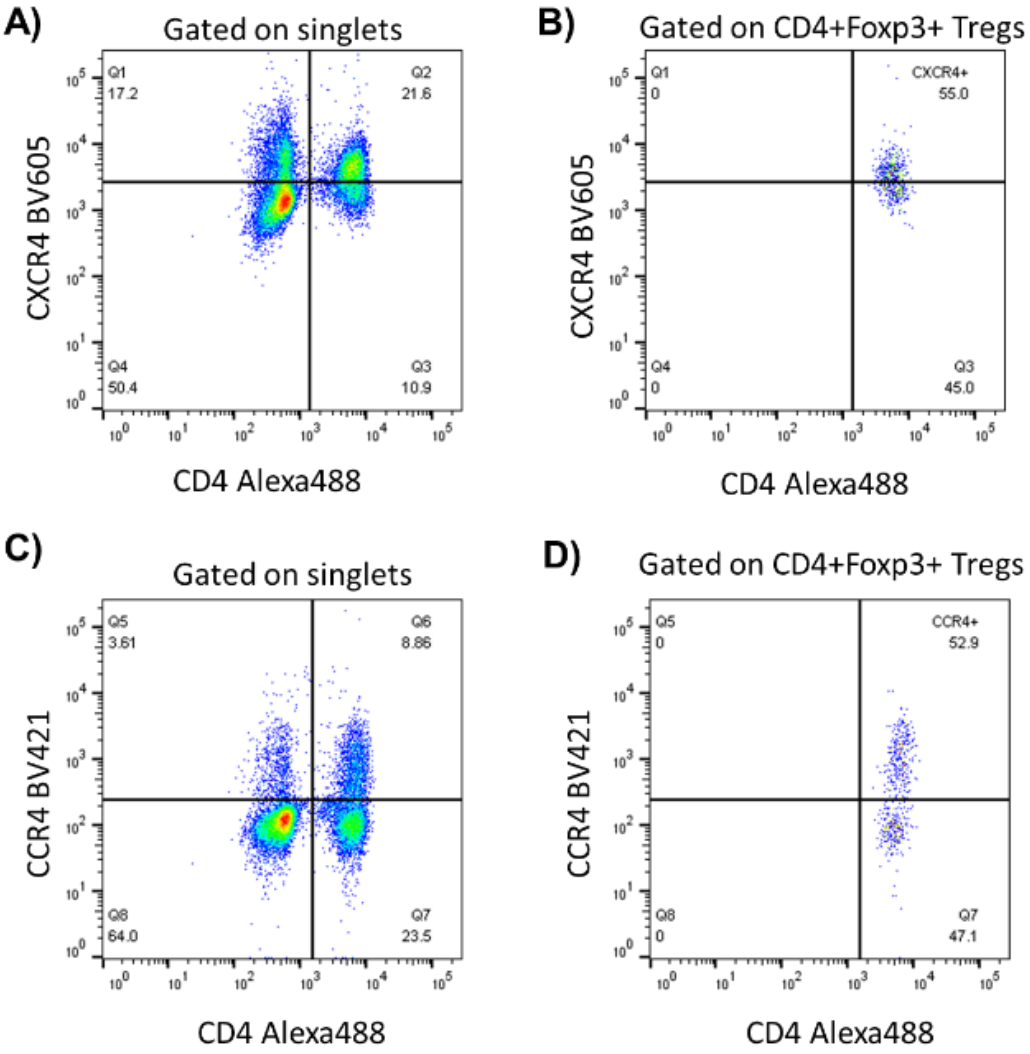

Figure 3.2 Gating strategy of bone homing receptors (CCR4 and CXCR4) on CD4+Foxp+ Tregs. PBMCS isolated from human whole blood were stained with anti-CD4 Alexa 488 (plots not shown), anti-CD127 PE-Cy7 (plots not shown), anti-CD25 PE (plots not shown), and anti-Foxp3 PECF594, anti-CCR4 BV421 and anti-CXCR4 BV605. Cells gated from singlets were used to identify $CXCR4^{+}$ (A) and CCR4 (C) positive cells. This same gating was applied to $CD4^{+}Foxp3^{+}$ Tregs population to identify the CXCR4 (B) and CCR4(D) expression on Tregs.

3.5.2 Treg quantification in peripheral blood of healthy young and healthy old participants

Following the gating strategy in the previous section, the numbers of Tregs were quantified in blood samples from individuals aged 20 – 70 years, to study any possible changes in the quantity of Tregs related to bone changes caused by ageing.

Quantification of Tregs based on the gating strategy previously described was based on $CD4^{+}Foxp3^{+}$ expression on the lymphocyte population from the PBMCs fraction (**Figure 3.3 A**). Number of Tregs were plotted in relation to the sample age to determine the ageing groups. Two clear clusters can be seen which suggest changes in Treg number may be age dependant (**Figure 3.3 B**). This therefore corroborated the age group separation into young (participants under 50y/o) and aged (participants over 50 y/o) as decided in the experimental plan in order to see age differences related to bone health. Quantification of Treg ($CD4^{+}Foxp3^{+}$) showed an average of 5.8% of cells from the $CD4^{+}$ population in the healthy young population while the average for the healthy aged population was 2.6% of cell from the CD4+ population, both values following the average percentage of Tregs in circulating blood (Niu et al., 2020; Sorrenti et al., 2016). Changes in Treg population ($CD4^{+}Foxp3^{+}$) suggest a significant decrease ($P<0.0001$) in Treg numbers associated with ageing (**Figure 3.3 C**).

Upon examining the generated data in Figure 3.3 a possible gender-effect was noted hence, data was also plotted by gender information (male in blue, female in purple) to visually determine any possible influences related to gender differences. Statistical analyses of Treg quantification based on the participant's gender did not show any significant difference between females and males without considering ageing as a factor. The average of Treg numbers for the female population was 3.8% of the CD4+ cell population when compared to the male population where the average on Treg number was 4.7% of $CD4^+$ cell population shown in **Figure 3.4 A**. However, a significant decrease in the number of Tregs could be observed in both sex groups as a consequence of ageing similar to findings in **Figure 3.3.** The average number of Tregs for the healthy young female population was 6.1% of the CD4+ cell population when compared to the healthy aged population where the average was 2.7% of $CD4^+$ cell population ($P<0.001$) **Figure 3.4 B**. A decrease in the Treg population was also found in the male population related to age even. (**Figure 3.4 C**).

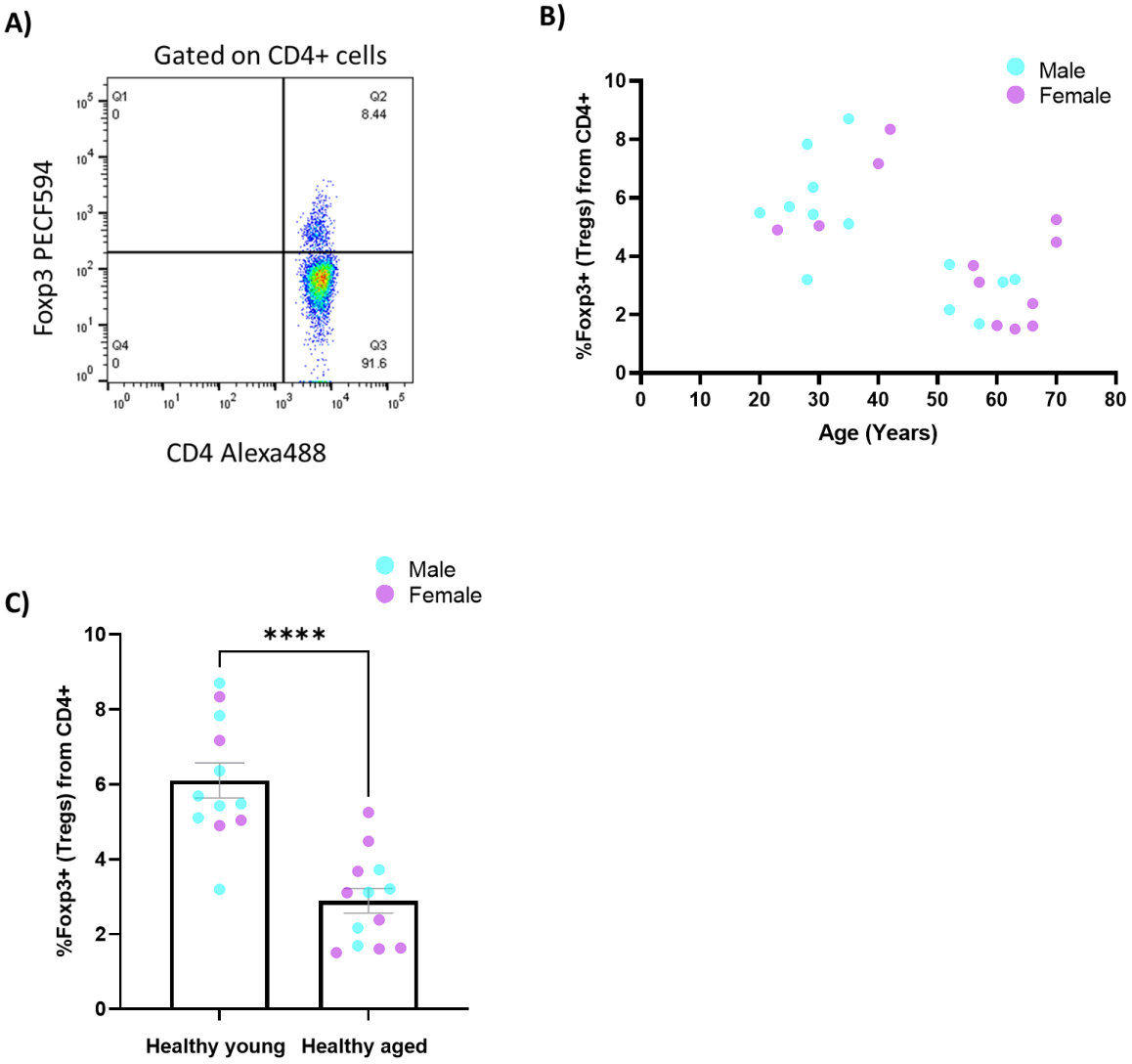

Figure 3.3 Quantification of CD4+Foxp3+ Tregs in peripheral blood of healthy young and healthy old participants. PBMCS isolated from human whole blood were stained with anti-CD4 Alexa 488, anti-CD127 PE-Cy7, anti-CD25 PE and anti-Foxp3 PECF594. Tregs were gated based on $CD4^{+}Foxp3^{+}$ expression (A). Treg ($CD4^{+}Foxp3^{+}$) quantification was plotted vs the age of the participant to determine a pattern of expression regarding ageing, two clear groups were determined healthy young (participants under 50y/o) and healthy aged (participants over 50 y/o) (B) and expression of Tregs ($CD4^{+}Foxp3^{+}$) was plotted based in the defined groups. Significant difference can be seen between healthy young and healthy aged groups. (C) Changes in Treg ($CD4^{+}Foxp3^{+}$) plotted by sex groups, female and male. Data shows mean with SEM and was analysed using unpaired Student's T-test ****<0.0001.

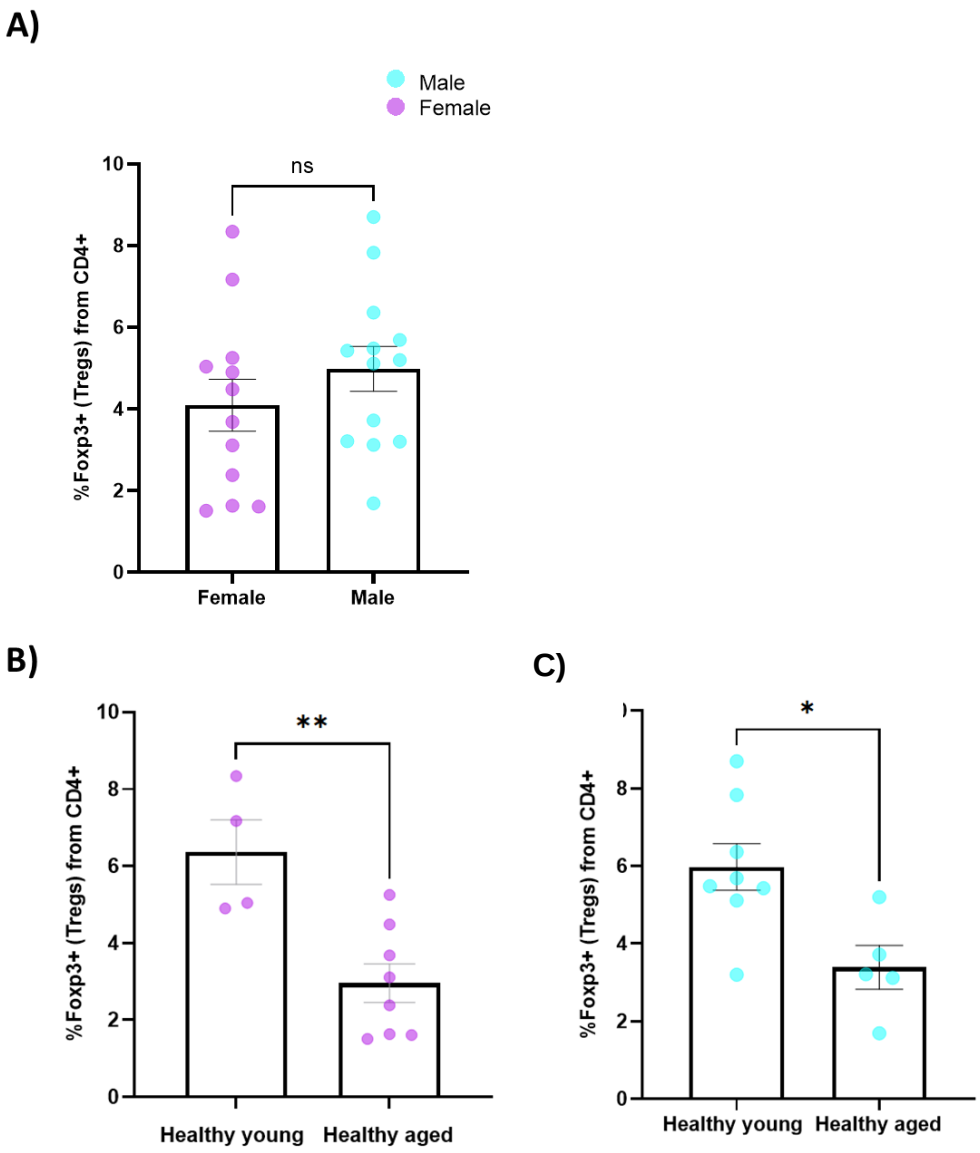

Figure 3.4 Quantification of CD4+Foxp3+ Tregs in peripheral blood of healthy young and healthy old participants based by gender (male and female). PBMCS isolated from human whole blood were stained with anti-CD4 Alexa 488, anti-CD127 PE-Cy7, anti-CD25 PE and anti-Foxp3 PECF594. Tregs were gated based on CD4+Foxp3+ expression. Treg (CD4+Foxp3+) quantification was based on the gender (male and female). No significance was found between Female and male groups p=0.23 (A). Changes in the same sex group based by age was shown in the female and male group (B and C). Data shows mean with SEM and was analysed using unpaired Student's T-test, **<0.001 and *<0.01.

3.5.3 Potential bone Tregs Quantification in Peripheral Blood of Healthy Young and Healthy Aged Participants

Quantification of possible bone homing Tregs in peripheral blood was done based on the expression of chemokines receptors CXCR4 and CCR4. Gating for CCR4 and CXCR4 was done on the CD4+Foxp3+ population (**Figure 3.5**).

Possible bone homing Tregs based on the expression of CCR4+, as a marker of Tregs migration towards the site of bone damage, were gated from CD4+Foxp3+ Treg based on the gating strategy in **Figure 3.2**. No significant difference was found between the numbers of CCR4+ Tregs (CD4+Foxp3+) amongst the different age populations. The average number of CCR4+ cells in the healthy young population was 57.29% of the cells while in the healthy aged group the population of CCR4+ cells was 51.07% of the CD4+Foxp3+ cell population.

Likewise, quantification of possible bone homing markers based on the expression of the marker CXCR4 was done based in the gating strategy in Figure 3.2. The average number of CXCR4+ cells in the healthy young population was 46.44% of the cells while in the healthy old group the population of CCR4+ cells was 48.14% of the CD4+Foxp3+ cell population. Altogether, no significant difference was found in the possible bone homing Treg population between healthy young and healthy aged based on age-related changes.

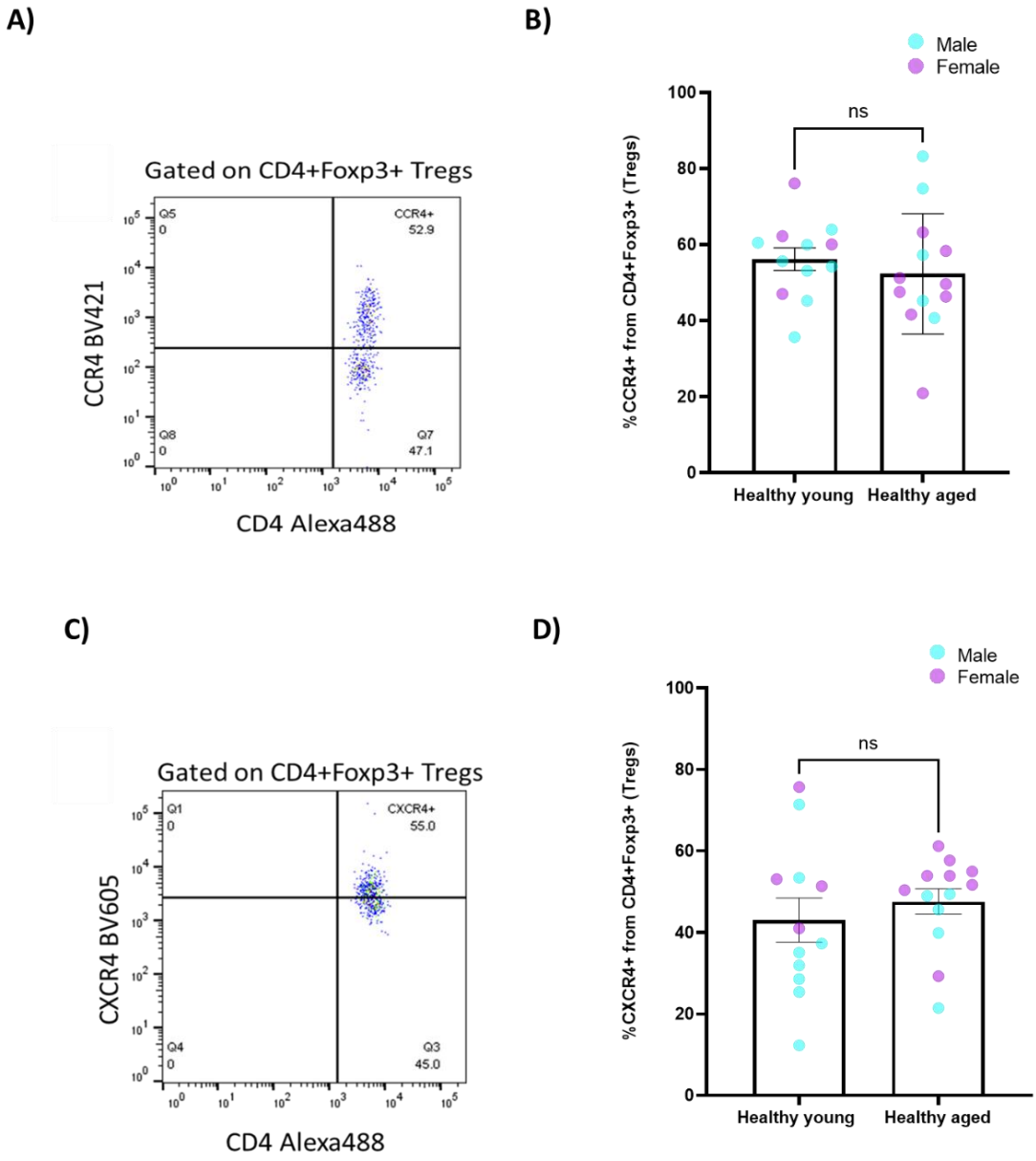

Figure 3.5 Characterisation of bone homing Tregs in peripheral blood of healthy young and healthy old participants based on the expression of bone homing markers CCR4 and CXCR4. PBMCS isolated from human whole blood were stained with anti-CD4 Alexa 488, anti-CD127 PE-Cy7, anti-CD25 PE and anti-Foxp3 PECF594. Tregs were gated based on $CD4^{+}Foxp3+$ expression and further expression of $CCR4^{+}$ and $CXCR4^{+}$ was gated from ($CD4^{+}Foxp3^{+}$) (A and C respectively). Data was plotted for $CCR4^{+}$ cells by group, healthy young and healthy old p=0.48 (B) and the same for $CXCR4^{+}$ p=0.46 (D). No significant difference can be seen between healthy young and healthy aged groups. Data shows mean with SEM and was analysed using unpaired Student's T-test.

Each sample shown in **Figure 3.5** is also assessed in **Figure 3.6** and **Figure 3.7** based on the gender information by colour identification (male in blue and female in purple).

The average expression of $CXCR4^+$ $CD4^+Foxp3^+$ Treg was 49.80% for the female group and 38.57% for the male group (**Figure 3.6**). Statistical analyses for $CXCR4^+$ from the $CD4^+Foxp3^+$ Treg population show a significant increase of $CXCR4^+$ $CD4^+Foxp3^+$ Treg in the female population compared to the male group (P=0.01) (**Figure 3.6 A**). However, further analyses of CXCR4+ Tregs based on gender and age showed no significant difference per group females (**Figure 3.6 B)** and male **(Figure 3.6 C).** This showed that expression of CXCR4 in CD4+Foxp3+ Tregs varies with sex, but it may not be related to ageing.

On the other hand, statistical analyses for $CCR4^+$ from the $CD4^+Foxp3^+$Treg population did not show any significant difference between male and female group. The average number of $CCR4^+CD4^+Foxp3^+$ Tregs for the female population was 50.09%, while the male population was 56.46% for potential bone homing Tregs based on the expression of $CCR4^+CD4^+Foxp3^+$ Treg (**Figure 3.7 A**). No significant difference was found in either sex group females (**Figure 3.7 B)** and male **(Figure 3.7 C)** when group by aged.

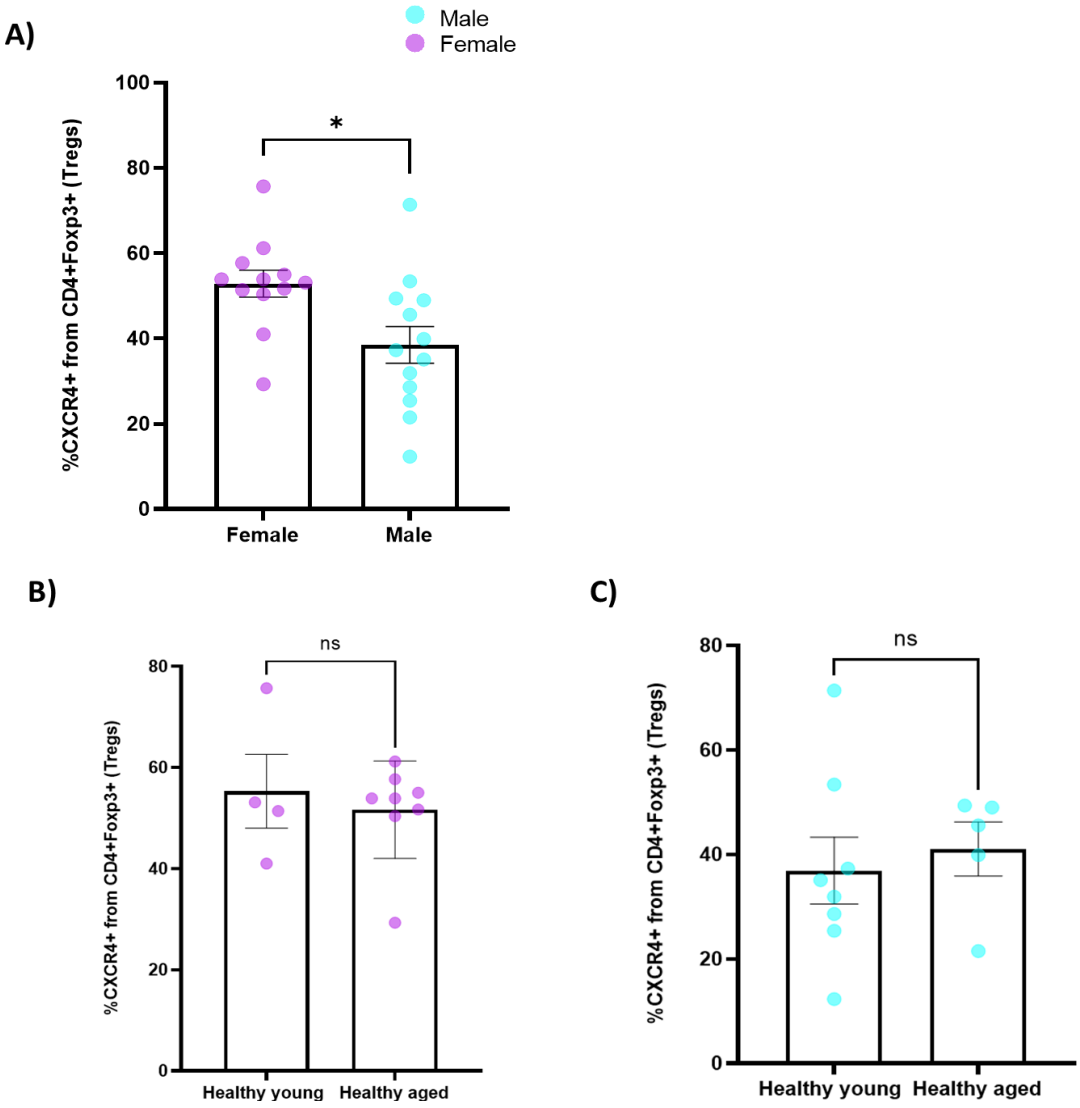

Figure 3.6 Characterisation of potential bone homing Tregs based in the positive expression of CXCR4 in peripheral blood of healthy young and healthy old participants based by sex (male and female). PBMCS isolated from human whole blood were stained with anti-CD4 Alexa 488, anti-CD127 PE-Cy7, anti-CD25 PE and anti-Foxp3 PECF594. Tregs were gated based on CD4+Foxp3+ expression and further expression of CXCR4+ was gated from (CD4+Foxp3+). Data was plotted for CXCR4+ cells by sex group, female, and male (A) and further grouping of sex groups females p=0.38 (B) and male p=0.69 (C) was done by age. Significant difference can be seen when comparing females versus males regardless of age (A). No significant difference can be seen between healthy young and healthy aged groups in either the female or the male groups (B and C). Data shows mean with SEM and was analysed using unpaired Student's T-test *<0.005.

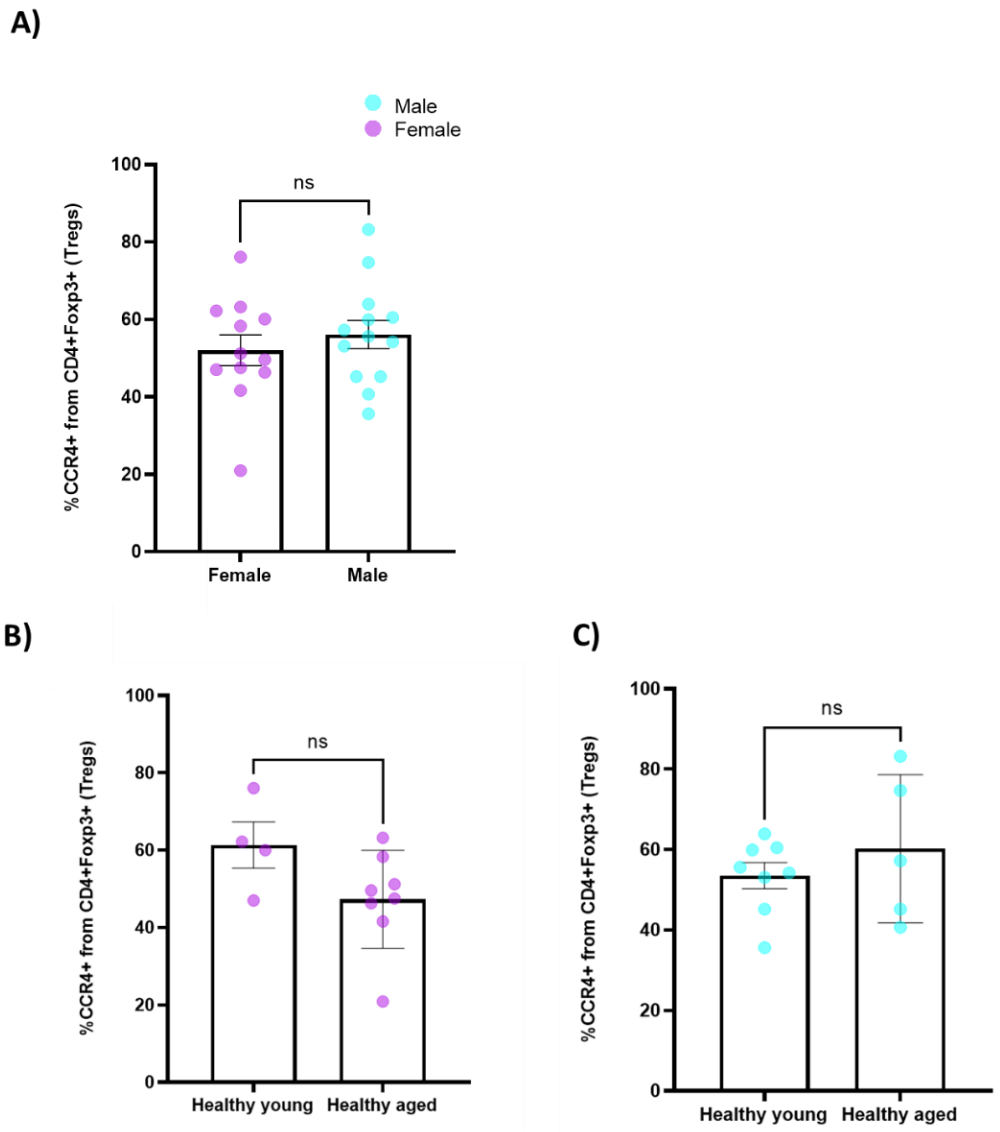

Figure 3.7 Characterisation of potential bone homing Tregs in peripheral blood of healthy young and healthy old participants based by sex (male and female). PBMCS isolated from human whole blood were stained with anti-CD4 Alexa 488, anti-CD127 PE-Cy7, anti-CD25 PE and anti-Foxp3 PECF594. Tregs were gated based on CD4+Foxp3+ expression and further expression of CCR4+ was gated from (CD4+Foxp3+). Data was plotted for CCR4+ cells by sex group, female, and male (A) and further grouping of sex groups females (B) and male (C) was done by age. No significant difference can be seen when comparing females versus males regardless of age p=0.45 (A). No significant difference can be seen between healthy young and healthy aged groups in either the female or the male groups B, p=0.13 and C, p=0.40. Data shows mean with SEM and was analysed using unpaired Student's T-test.

3.5.4 CTX-1 Quantification in Serum of Healthy Young and Healthy Aged Participants

CTX-1 levels in serum of healthy young and healthy aged participants were analysed in order to correlate differences in bone health and the presence of Treg in peripheral blood. CTX-1 was used as a maker of osteoclast activity since higher concentration of CTX-1 is an indicator of an increase of bone breakdown.

In serum samples obtained from the SNBTs, healthy young participants presented an average of 633 pg/mL of CTX-1 in serum while the healthy aged groups showed an average of CTX-1 level in serum of 698 pg/mL. No significant differences in the levels of CTX-1 in serum was observed between groups suggesting that the levels of osteoclast activity and hence the bone breakdown activity is not affected by healthy ageing in these individuals (**Figure 3.8**).

However, for these serum samples obtained from SNBTS, the donor information received was limited for more specific age and sex related observations. For this reason, further experiments were carried out where samples were collected freshly, and sex and date of birth information were available. However, due to time constraints the number of participants recruited was limited.

For these freshly collected samples, CTX-1 levels in serum were plotted by individual age to determine any pattern regarding ageing and CTX-1 levels in serum (**Figure 3.9 A**). No clear clusters were found based on healthy young or healthy aged participants and the levels of CTX-1 found in serum did not show a

significant difference between groups. However, this data cannot be conclusive given the low sample numbers per group (**Figure 3.9 B).**

Moreover, samples were plotted by sex (male in blue and female purple) to observe possible differences regarding sex affecting bone breakdown (**Figure 3.9 C**). However, no significant difference was found between groups (male and female) being the average of CTX-1 in serum 267pg/ml for the female group and 301pg/ml in males.

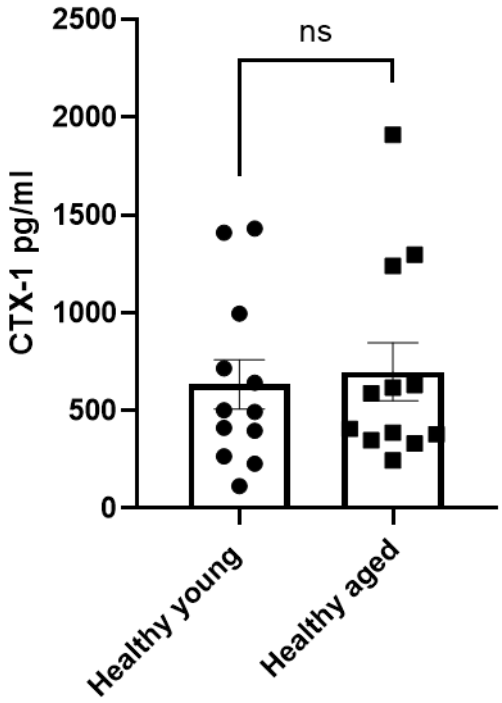

Figure 3.8 CTX-1 levels (pg/ml) in serum of healthy young and healthy aged participants. CTX-1 levels in human serum samples from healthy young and healthy aged were analysed by ELISA. No significant difference was found between groups (p=0.74) regarding the number of CTX-1 levels in serum. Data shows mean with SEM and was analysed using unpaired Student's T-test. N=24, biological replicates (12 healthy young and 12 healthy aged).

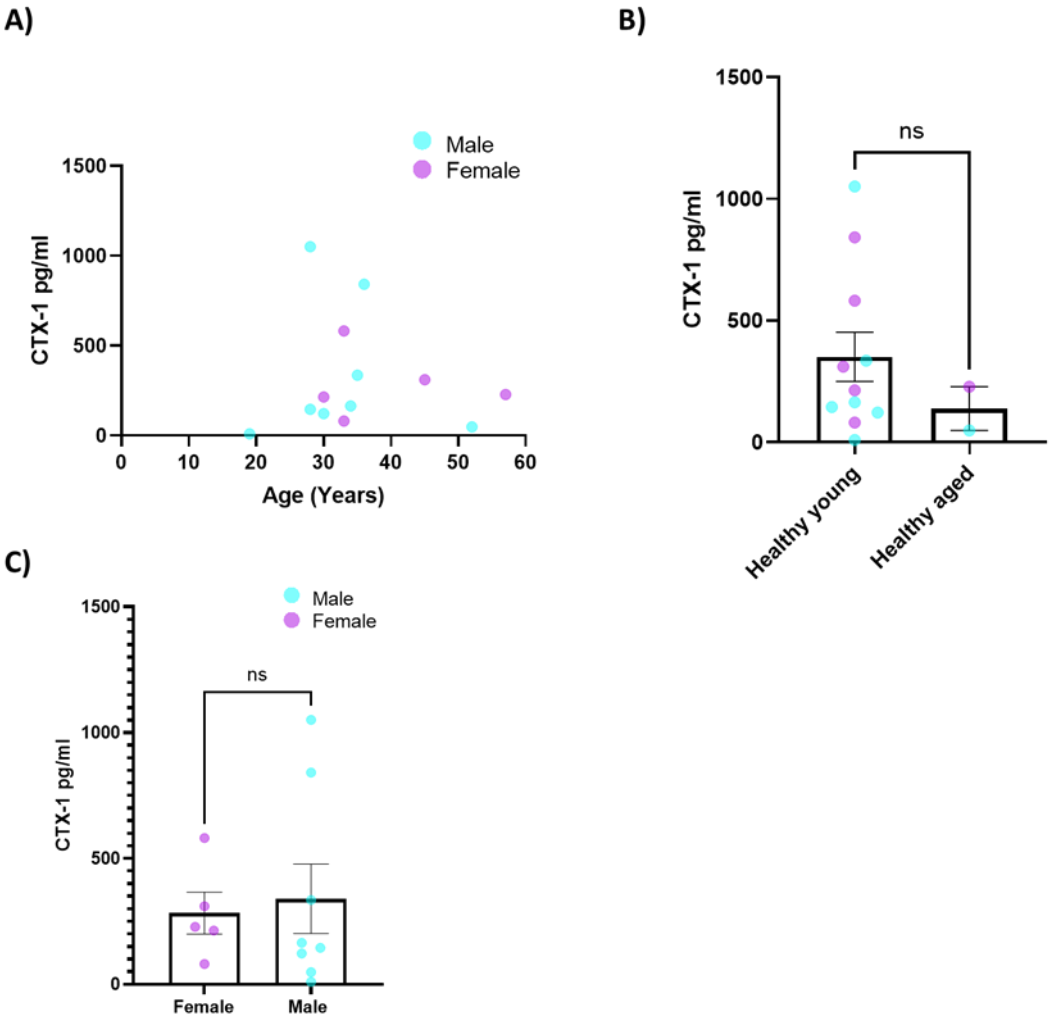

Figure 3.9 CTX-1 levels (pg/mL) in serum of healthy young and healthy aged participants. CTX-1 levels in human serum samples from healthy young and healthy aged were analysed by ELISA. Samples were plotted by age (A), grouped by healthy young and healthy aged (B) or grouped by sex (female or male) (C). No significant difference was found between groups regarding the number of CTX-1 levels in serum when comparing age groups, p=0.26 (B) or sex groups, p=0.76 (C). Data shows mean with SEM and was analysed using unpaired Student's T-test. N=13, biological replicates (11 healthy young and 2 healthy aged, 8 male and 5 female).

3.5.5 CTX-1 Levels in Serum Correlated with Tregs numbers in Blood from Healthy Young and Healthy Old Participants

Given the previous limitations explained regarding obtaining the required information for the participants (sex and age) or being able to collect fresh samples of serum and whole blood for Treg analyses from the same donor, studies to correlate CTX-1 levels in serum and Treg levels in peripheral blood were limited.

After COVID-19 restrictions were lifted, it was possible to obtain a few samples with matched serum and whole blood (n=7). Data were obtained for CTX-1 serum levels as following section 3.5.4 and Treg quantification was done based on the gating strategy from section 3.5.1.

Data were plotted for Treg numbers versus CTX-1 levels. Samples were also differentiated by sex (female purple and male blue), no further classification by age was done since all the samples fell into the healthy young group. No significant data was obtained from this, being the number of biological replicates is limited. No clear trend could be concluded from this data.

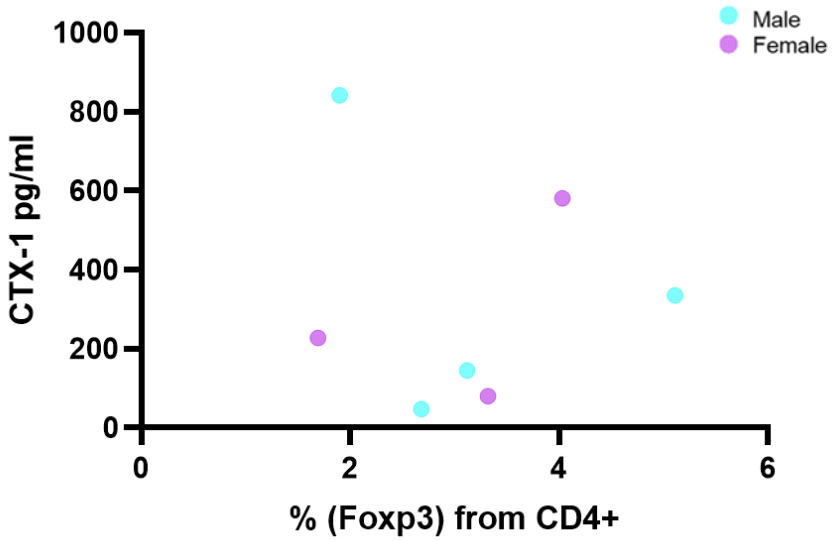

Figure 3.10 CTX-1 levels (pg/ml) in serum matched to Treg (%Foxp3+ from CD4+) in peripheral blood. CTX-1 levels in human serum samples were analysed by ELISA and Tregs in peripheral blood were characterised by flowcytometry. Samples were plotted for levels of Tregs against CTX-1 serum levels for the same participant, and samples were also differentiated by sex (purple female and blue male). N=7, biological replicates (7 healthy young, no healthy aged samples, 4 male and 3 female). Legend added

3.6 Discussion

In this Chapter I have quantified numbers of Tregs in peripheral blood and the numbers of potential bone Tregs, which have the ability to migrate towards the site of injury in bone, in healthy young and aged individuals. Moreover, this Chapter also explored the link between Treg numbers and bone turnover to study the correlation between both, by analysing serum levels of CTX-1, a marker of osteoclast activity. The analyses in this Chapter were done comparing healthy young and healthy aged participants to explore differences in Tregs population during healthy ageing. Overall, changes between the different age populations were expected to be found, since the ageing population will express age-related changes in both the immune system and the bone system.

Recent studies have highlighted the important role Tregs may play in regulating healthy bone mass, and their potential to drive bone repair and regeneration by accumulating at the site of sterile tissue injury (Burzyn et al., 2013). After tissue injury, uncontrolled inflammation can lead to impaired healing and increase tissue remodelling. In many tissues, Tregs are recruited to the site of damage where they regulate immunity post-injury. Tregs interact with many immune cells involved in the inflammatory process. In skeletal muscle injury, Tregs interact with neutrophils modulating neutrophil infiltration to the site of injury and promote secretion of anti-inflammatory molecules such as IL-10 and TGF-β (Lewkowicz et al., 2013). Tregs co-cultured with monocytes have shown a decrease in TNF-α and IL-6 in response to LPS, inhibited by Tregs secretion of IL-10, IL-4, and IL-13 (Taams et al., 2005). Tregs also interact with macrophages, which have shown to be involved in tissue repair and regeneration (Ding et al., 2019; Wynn & Vannella, 2016). In tissue

healing, Tregs can produce an M2 polarization of macrophages which shows higher expression of CD206, CD163, and decreased expression of HLA-DR (Tiemessen et al., 2007). Treg can modulate macrophage survival and activity as shown in a chronic kidney diseased liver where Treg-derived TGF-β inhibited macrophage activity (Venet et al., 2006). Previous studies have shown the potential for Tregs to maintain bone homeostasis. Hence, in bone diseases where there is an imbalance in bone remodelling e.g., osteoarthritis, we hypothesised that Treg number may increase to induce tissue regeneration by inhibiting osteoclast formation and activity as a result of the previous Treg-macrophage interaction (macrophages being the osteoclasts precursors during osteoclastogenesis).

Tregs may also exert direct effects on osteoclasts. Some studies have shown Tregs to inhibit RANKL (Pagliari et al., 2015) while others suggested that Tregs inhibit osteoclast differentiation by inhibiting TGF-β1, GM-CSF, IFN-γ, IL-5 and IL-10 (F. L. Yuan et al., 2010). In vitro studies have shown Tregs to inhibit osteoclast differentiation through paracrine signalling of TGF-β and IL-4 (Zaiss et al., 2007). Moreover, in vivo studies have shown Tregs to protect TNF-α-induced bone loss in TNF transgenic mice (hTNFtg) (Zaiss, Frey, et al., 2010). In a canine model (beagle dogs with induced periodontitis), Tregs have shown to migrate to the site of bone injury by CCL22 (CXCR4 ligand) which resulted in decreased bone resorption, through reducing inflammation, resulting in less bone loss (Glowacki et al., 2013). Even though Treg's protective role seems to be linked to regulating osteoclasts differentiation and bone resorption, Treg are also able to directly promote osteoblast differentiation by inhibiting secretion of IFN-γ and TNF-α by conventional T cells, increasing ICAM-1 expression, on MSCs (osteoblasts

precursors) which will lead to osteoblast precursors interacting with T cells and this will produce an upregulation of osteoblastogenesis (Liu et al., 2011). The combination of both actions on osteoclasts and osteoblasts will regulate bone homeostasis and will potentially restore the balance between bone resorption/formation which is characteristically imbalanced during bone diseases. Tregs are a highly complex population of cells and a lack of a consensus for a reliable form of identification by flow cytometry until the routine introduction of FoxP3 and CD127 marker makes the historical interpretation of the Tregs field complicated. Moreover, Tregs are a heterogenous population, containing populations with different functional characteristics and activation requirements, different populations of Tregs can be identified depending on the maturation and activation state (Sakaguchi, 2011). Emerging data has shown that naturally occurring Tregs (nTreg), are more prone to accumulate with advancing age, while inducible Tregs (iTreg) appear to be less available in the older host (Jagger et al., 2013). The data obtained in this Chapter showed a significant decrease in the number of $CD4^{+}Foxp3^{+}$ cells in the peripheral blood in the aged population (**Figure 3.3**). This may be related with bone diseases development since; bone damage may lead to migration of Tregs towards bone (and as a consequence less will be found in blood). Same way as, ageing-related changes in bone remodelling can lead to higher migration of Tregs towards bone site too as a consequence of the characteristic imbalance in bone remodelling caused by ageing.

Ageing associated decreases in immune system function have become a major health concern, and variations in the T cell population cause considerable challenges to the ageing population. The precise influence of ageing on Tregs

numbers is contradictory. Raynor et al., 2012 reported that only minor changes were found in blood circulating Tregs, while other studies suggest higher proportion of Tregs population through ageing (Hou et al., 2017). Differences in findings regarding Treg population may be due to the population heterogenicity and the markers used to identify the Treg population, some older studies are based on $CD4^{+}CD127^{low}CD25^{High}$ before Foxp3 was identified as a Treg marker.

Changes in the immune system related to age may also be affected by sex differences. Some immunological sex differences are present throughout life whereas others are only apparent after puberty and before reproductive senescence, which may suggest that hormones are involved (Klein & Flanagan, 2016). Previous human studies suggest there are higher numbers of Treg cells in healthy adult males compared with females which explains predisposition of female group to autoimmune diseases (Afshan et al., 2012). This may also correlate with predisposition of females to develop bone-related diseases with ageing, since lower levels of Tregs in a healthy female could mean less suppression of osteoclast activity and therefore increased bone turnover remodelling by osteoclasts suppression. Data in this chapter for Treg ($CD4^{+}Foxp3^{+}$) numbers was further grouped as female and male (Figure 3.4) in order to investigate the role sex ageing-related changes may play in the number of circulating Tregs. No significant difference was found regarding the number of Tregs between sex groups, Previous studies suggested that adult females have a lower level of Tregs in peripheral blood (Afshan et al., 2012).

Tregs circulate in the blood and lymph, however they need to migrate to different tissues to exert their function. Tregs that migrate specifically to bone will have a characteristic profile of chemokine receptors that will allow the migration (chemokine gradient) but also specific states and subpopulations of Tregs may show a preference to reside in specific tissues (Ward & Marelli-Berg, 2009). In this Chapter, potential bone homing Tregs were characterised by the expression of different chemokine receptors, which will allow migration of Tregs towards different organs based on its specific chemokine gradient. Tregs can present a variable chemokine receptor repertoire, including CXCR4 and CCR4, such variability may confer the specificity of tissue/organ homing of Tregs (Chakraborty et al., 2012; Chen et al., 2006).

Data showed no significant differences in the number of CCR4 expressing Tregs in either different age groups (Figure 3.5 B) or different sex groups (Figure 3.7B). CCR4 has mainly been implicated in Treg migration towards skin presumably also to bone, based on migration towards site of injury (X. Wang et al., 2010). The ability of bone to regenerate without scar tissue formation indicates strong regenerative potential, suggesting that Tregs involved in bone repair migrating towards bone will possibly present a similar chemokine profile to those migrating to skin, since skin is also tissue showing high regenerative function. The end result in both tissues, skin, and bone, is collagenous scar formation strengthening the bone and skin composition (Boothby et al., 2020).

Studies in mice have shown that blocking CCR4 ligand increased inflammatory bone loss and adoptive transfer of $CCR4^+$ Tregs to CCR4 knockout mice revert the increased inflammatory phenotype (Araujo-Pires et al., 2015). The participants who took place in the study were all healthy as per the ethics criteria (not diagnosed with osteoarthritis).

There is lack of studies in humans regarding CCR4 migration towards bone, however there is potential to believe $CCR4^+$ Tregs will exert an anti-inflammatory function in bone healing under damage repair. Further studies investigating $CCR4^+$Treg number in bone tissue will give a clearer understanding of the role of this specific population of Tregs in bone diseases.

Moreover, data presented in this Chapter showed no significant difference between healthy young and healthy aged participants in the numbers of $CXCR4^+CD4^+Foxp3^+$ Tregs (Figure 3.5). However, when looking at the different groups based by sex (female and male) this showed a significant increase of $CXCR4^+CD4^+Foxp3^+$ Tregs in the female group (Figure 3.6). This may be related with a higher incidence to develop bone disease in female.

Bone marrow is known as a reservoir for $CD4^+CD25^+$ Tregs. Bone marrow expresses functional stromal-derived factor (CXCL12), the ligand for CXCR4. Human Tregs traffic to bone marrow and are preserved in it through CXCR4/CXCL12 signals (Zou et al., 2004).

Data in this Chapter showed that females presented a high percentage of Tregs to be expressing CXCR4 which leads to the possibility of those Tregs migrating to bone where they could exert the anti-inflammatory function during bone diseases (**Figure 1.9**). One proposed mechanism of Tregs function is the suppression of osteoclast activity during bone diseases development where there's a higher bone breakdown caused by an impaired osteoclasts function (Zaiss et al., 2007). Thus, this highlights the need to fully understand the interaction between Tregs and osteoclasts *in vitro*.

Moreover, Treg effects on bone healing can also be measured by the levels of serum markers of osteoclastogenesis. Previous studies have shown numbers of Tregs in circulating blood to be correlated to CTX-1 levels in serum, in healthy individuals and in Rheumatoid Arthritis (Zaiss, Sarter, et al., 2010).

In this thesis, the levels of CTX-1 in serum were first analysed without any relation to the Tregs levels by age group (healthy young and healthy aged) and independently of sex (Figure 3.8) and correlated with sex groups (Figure 3.9). No significant difference was found regarding the levels of CTX-1 in serum in comparison with both groups either based on age or sex. This finding may be due to the small sample size and the lack of information per sample preventing better grouping. Previous studies suggested that CTX levels are low and remain unchanged between 20 and 49 years. After the age of 49 years, CTX levels rise and are elevated significantly until the age of 70 years (Rathnayake et al., 2021). However, this was not consistent with the findings presented here.

Whilst the project was not initially designed to take into account the influence of post-menopausal bone change mainly affected by an increase in bone resorption linked to a decrease in oestrogen (Karlamangla et al., 2018) the changes enforced by COVID-19, i.e. to explore age-related changes in bone health, also required consideration of the possibility that some of the participants in the older age group may be affected by menopause related bone changes. Because this was not the intention of the work and we did not have appropriate ethical permission, specific information whether they were or already undergone the menopause was not available. However, as the average age of onset of menopause is 45 y/o, 8 of the female participants in the study are over that age so this will need to be considered as a possible influence on the data. Women over 45 years old would be expected to present higher levels of CTX-1 in serum related to an increase in bone remodelling as a consequence to oestrogen decrease during peri- and post-menopausal stages (Desai et al., 2007) However, this effect may not be seen in menopause participants that are undertaking HRT medication. This study does not have any information regarding the menopausal or medication state for the participants due to samples being collected from the SNBTS bank.

Finally, a small study was conducted where enough information was obtained per participant allowing to correlate Treg numbers in peripheral blood with the levels of CTX-1 in serum, Given the small population size, samples where not grouped by either age group or sex group as per the data shown before. The data presented in this study was limited and no final conclusions can be taken due to the small sample size. Further collection of samples would help for a better understanding, since literature previously have shown that Treg levels in peripheral blood may

have an impact on bone breakdown since the levels of CTX-1 in serum decrease when the levels of Tregs in peripheral blood increase as previously shown (Zaiss, Sarter, et al., 2010). The literature suggests that Tregs will migrate towards the site of bone injury (Araujo-Pires et al., 2015) which may be linked to Treg function to reduce the levels of bone breakdown by suppressing osteoclasts activity. Findings in this chapter are not conclusive regarding a correlation between Tregs and bone breakdown (measured by CTX-1 levels in serum), given the small sample size.

A better understanding of the role of Tregs in bone remodelling, through interaction with osteoclasts, by suppressing formation and function is necessary to conclude if the *in vitro* studies done through this chapter may be representative of what is happening in the bone given that this are peripheral blood levels of Tregs. One possible explanation may be that a higher bone breakdown results in an increase of Tregs in blood circulation that will further migrate towards bone to regulate tissue injury.

During the study a major limitation came from the information for the samples provided by the SNBTS being limited (all the information we obtained was age and gender). These samples were considered healthy as per the ethics criteria set for the study, no osteoarthritis and no immunosuppressive diseases diagnosed (Appendix 2, section 2). In the same regard, participants were limited during the study, and this affected the size of the study population as well as the variability between groups, either age or sex. Levels of CTX-1 show circadian rhythm variabilities altered by food intake, which makes timing of sample collection an

important consideration and this is something that could not be controlled during this study (Williams & Sapra, 2022). Further studies with a larger number of participants and where more specific information need to be analysed to conclude this (such as post-menopausal status, medication status such as HRT, fasting samples or bone injury levels) and to obtain a better understanding of the correlation between Treg function in bone health.

In summary, this chapter has shown a decrease in $CD4^{+}Foxp3^{+}$ Tregs in peripheral blood with ageing. Moreover, change in potential bone Tregs expressing CXCR4 and CCR4 markers did not show any difference in peripheral blood populations regarding age groups.

$CXCR4^{+}$ $CD4^{+}Foxp3^{+}$ Treg population showed to increase in females compared to males, suggesting that migration towards bone injury sites may be sex dependent, given that there is a higher prevalence for bone diseases in the female population. Finally, CTX-1 studies showed similar levels in serum through age up until 45y/o where a slightly increase in CTX-1 levels in serum could be seen but no final conclusions could be taken regarding the correlation between Tregs levels in peripheral blood and CTX-1 serum levels. These findings therefore highlight the need to explore the correlation between age changes in the immune system related to sex differences during bone breakdown injuries and the future implications of Tregs in bone repair.

Chapter 4

Effects of Tregs on *in vitro* Osteoclastogenesis and Bone Resorption

4.1 Introduction

Bone remodelling is a stable process during life, and it is during the first three decades of life that bone turnover is tightly coupled to maintain a balanced state between bone resorbed and bone formed (Demontiero et al., 2012). Between 15-20 years of age, peak bone mass is achieved. After this, bone turnover continues at a slower pace and bone resorption predominates against bone formation. In females this is enhanced during menopause when bone resorption over bone formation occurs at higher levels, inducing accelerated bone loss. However, the consequences of ageing in the transition from a steady state to bone loss is poorly understood in both males and females (Møller et al., 2020a). Similarly, during bone diseases development such as osteoarthritis, there is an imbalance in bone remodelling, where increased bone remodelling is found in early stages of the

disease and a decrease on bone remodelling occurs during late osteoarthritis stage (Burr & Gallant, 2012).

Osteoclasts are the only cells in the body capable of resorbing bone. Their formation and function are regulated by MCSF an RANKL, which control the communication between the immune and skeletal systems (Amarasekara et al., 2015).

Tregs are immunosuppressive cells and may play a significant role in regulating bone turnover. Tregs population and suppressive function have been proven to be altered with ageing, higher levels of Tregs can be found in peripheral blood in the elderly as well as their suppressive function also increases (Gregg et al., 2005). However, this data is contradictory in the literature, and a better understanding of the Treg population changes through ageing will help understanding Tregs role in bone remodelling. Moreover, changes in Treg populations also occur during bone diseases such as osteoarthritis, where Tregs are found in higher amounts in peripheral blood and synovial fluid of people suffering from osteoarthritis when compared with healthy controls (Moradi et al., 2014).

One suggested mechanism by which Tregs may influence bone remodelling is by suppressing osteoclastogenesis through secretion of cytokines (TGF-β, IL-10 and IL-4) as well as by cell-to-cell contact via CTLA-4, which binds to monocytes (osteoclast precursors) through CD80/86 surface marker and can inhibit RANKL and TNF-a induced osteoclast formation (Bozec & Zaiss, 2017).

Hence, during ageing or bone diseases pathology where there is an imbalance in bone remodelling, we hypothesised that Treg number may increase to induce tissue regeneration by inhibiting osteoclast function. As previously shown in Chapter 3, Treg populations in peripheral blood differ amongst age populations. Therefore, a better understanding of the interaction amongst Tregs and osteoclasts is key to understand the role of Treg during bone remodelling imbalance.

Osteoclasts are terminally differentiated cells unable to proliferate, therefore, there is no available immortalised osteoclast cell line. Authentic osteoclasts can be obtained by differentiating them from their precursors, from isolation of primary osteoclasts whose successful isolation is limited to neonate animals (Orriss & Arnett, 2012) or from immortalised macrophage cell line such as RAW 264.7 cells (tumour mouse male origin, osteoclasts precursors, so differentiation by RANKL and MCSF stimulation is still required) (Collin-Osdoby & Osdoby, 2012). Due to this, research involving osteoclast has been difficult to undertake in the past and osteoclasts supply used to rely on giant cell tumours removed during surgery where the subsequent isolation of osteoclasts was done (Walsh et al., 1996).

The development of the techniques to routinely produce functional osteoclasts was based on the findings that co-activation of RANKL receptor and MCSF expressed by pre-osteoclasts results in their fusion generating multinucleated osteoclasts (Quinn et al., 1997). The technique has evolved from using co-culture of osteoblasts (secrete MCSF and express RANKL) with PBMCs (contain osteoclast precursors), to the culture of PBMCs with commercial human recombinant RANKL

and MCSF, which removes the need to use co-culture of osteoblasts with PBMCs to successfully generate osteoclasts (Agrawal et al., 2012).

The possibility to isolate and culture primary human osteoclasts from PBMCs has been revolutionary and has developed a powerful tool to study formation and function of osteoclasts in health and disease. In this Chapter, I exploit this culture system and through the co-culture of osteoclasts and Tregs, investigate the effects Tregs may have on osteoclastogenesis when comparing healthy young patients and healthy aged patients.

4.2 Hypothesis

Hypothesis

Tregs will suppress osteoclast formation and function *in vitro*. Tregs from healthy aged participants will have a higher suppression effect on osteoclasts formation and function when compared with the healthy young control group.

4.3 Aims

I. Validate an in-vitro human osteoclastogenesis model.

II. Optimise an in-vitro co-culture model for osteoclasts and Tregs.

III. Study the effects of Tregs on osteoclast form and function during healthy ageing.

4.4 Materials and methods

4.4.1 Ethics and Sample Population

Samples were obtained as per in section 2.1 and grouped as healthy young (N=8, 3 female and 5 male, age 23-41 y/o), healthy aged (N=7, 5 female and 2 male, age 56-72 y/o) based on the results from Chapter 3.

Ethics forms for working with human samples were submitted and approved by the Ethics boards at Edinburgh Napier University and to the Scottish National Blood Transfusion Service (SNBTS).

4.4.2 Purification of Lymphocyte Subsets from Whole Blood

PBMCs were isolated from whole blood samples as per section 2.3.1. Further, monocytes, Tregs and Tcons cell populations were isolated from PBMCs by FACS sorting following section 2.3.2 and magnetic beads sorting as described in section 2.3.3.

4.4.3 Osteoclast Culture and Characterisation

Osteoclasts precursors (monocytes) were obtained from human PBMCs by either FACS sorting or magnetic beads sorting. Cells were differentiated into osteoclasts according to the protocol described in section 2.7.

4.4.4 Osteoclasts Characterisation and Functional Assays (Resorption pits)

To characterise osteoclasts, TRAP reactivity was performed as described in section 2.8 and further SEM images were taken as per section 2.9. Resorption pits (indicators of osteoclasts activity) were visualised by light microscopy and the area resorbed was calculated using ImageJ.

4.4.5 Tregs Functional Assays (Suppression assays)

Cell proliferation of Tcons was analysed by staining with CTV or CFSE according to the protocol section 2.4.4.

0.50 x10^5 Tcons were cultured in the absence of Tregs or with Tregs at a ratio 1:1 in a 96 well plate. Tregs were activated by adding Immunocult STEMCELL CD3/CD28 T cell activation as per the manufacturer's protocol. The cells were incubated for 7 days in standard incubation conditions before wells were harvested and analysed by flow cytometry and the suppressive ability of Tregs was analysed using the division index (DI) as per in section 2.5.

4.4.6 Tregs Co-culture with Osteoclasts

To test the effect of Tregs on osteoclasts formation and activity, osteoclasts were seeded as in section 2.10 (10^5 Monocytes, osteoclasts precursors) and 0.5x10^5 Tregs were added to the culture at the same seeding point as the monocytes (Day 0). Tregs and Monocytes were added at a ratio 1:2 (Treg:monocyte). 25 ng/mL MCSF and 30 ng/mL RANKL were added to the culture for osteoclast formation

and Tregs were activated by immunocult for Tcell proliferation CD3/CD28 added to the culture as per the manufacturer's protocol.

4.4.7 Statistics

Data were checked to be normally distributed using a Shapiro-Wilk normality test using GraphPad. All data are expressed as the mean ± standard error of the mean (SEM). Statistical analyses, T-test or ANOVA, were performed using GraphPad and $P<0.05$ was considered to be significant.

4.5 Results

4.5.1 Development of an *In Vitro* Model of Human Osteoclastogenesis

Human osteoclasts can be generated *in vitro* from their precursors (monocytes) which are differentiated into osteoclasts by stimulation with MCSF and RANKL.

The original experimental plan was to isolate monocytes from peripheral blood (PBMCs) by FACS sorting from fresh blood. However, as a result of COVID-19 access to the FACS sorting facilities was restricted part way through the project, and so instead magnetic beads sorting of monocytes from PBMCs was explored. Both methods, are compared in the following sections.

4.5.1.1 Isolation and Characterisation of Osteoclasts Precursors

4.5.1.1.1 FACS Sorting of Osteoclasts Precursors (Monocytes) from PBMCs

Monocytes isolated from PBMCs by FACS sorting were sorted based on cell morphology, on Forward Scatter (FSC) and Side Scatter (SSC), and expression of CD4. PBMCs stained with anti-CD4 and sorted using FACS Aria could be differentiated for sorting by morphological changes used as the gating for sorting (**Figure 4.1 A, Pre-sorting**). First, the PBMC population was gated (**Figure 4.1 A PBMCs graph**) followed by gating the monocytes fraction of cells. Monocytes are recognised to express CD4 at intermediate levels compared to T cells (Filion et al., 1990).

The $CD4^+$ intermediate population of monocytes can clearly be seen when sorting the cells as compared to the lymphocytes which are $CD4^-$ or $CD4^+$ (**Figure 4.1 A gated on PBMCs graph**).

Purity of the sorted population of monocytes was determined by flow cytometry and morphology of the cells contained within the samples could be seen to be distinct when comparing the post sorting with the pre-sorting sample. In the pre-sorting population of PBMCs lymphocytes and monocytes could be seen (**Figure 4.1 A**) while post sorting the lymphocyte population is not present anymore and only the monocyte fraction can be seen in the PBMCs gating (**Figure 4.1 B**). Similar comparison can be seen when looking at the $CD4^+$ intermediate population since before sorting, $CD4^-$, $CD4^+$ and $CD4^+$ intermediate still appear in the sample (**Figure 4.1 A**) and post sorting, only the $CD4^+$ intermediate population can be detected in the sample, demonstrating that only the monocytes have been sorted as intended by the gating selected (**Figure 4.1 B**).

After sorting the monocyte population, the purity of the sample was analysed. There was a significant difference ($P=0.008$) in the number of monocytes per sample when comparing the pre-sorting sample, containing the full PBMCs population, and the sorted monocytes sample. The population of $CD4^{intermediate}$ cells pre-sorting was in average 15% of the total PBMCs cells while the post sorted sample of monocytes contained an average of 86.9% monocytes of the total sample (**Figure 4.1 C**).

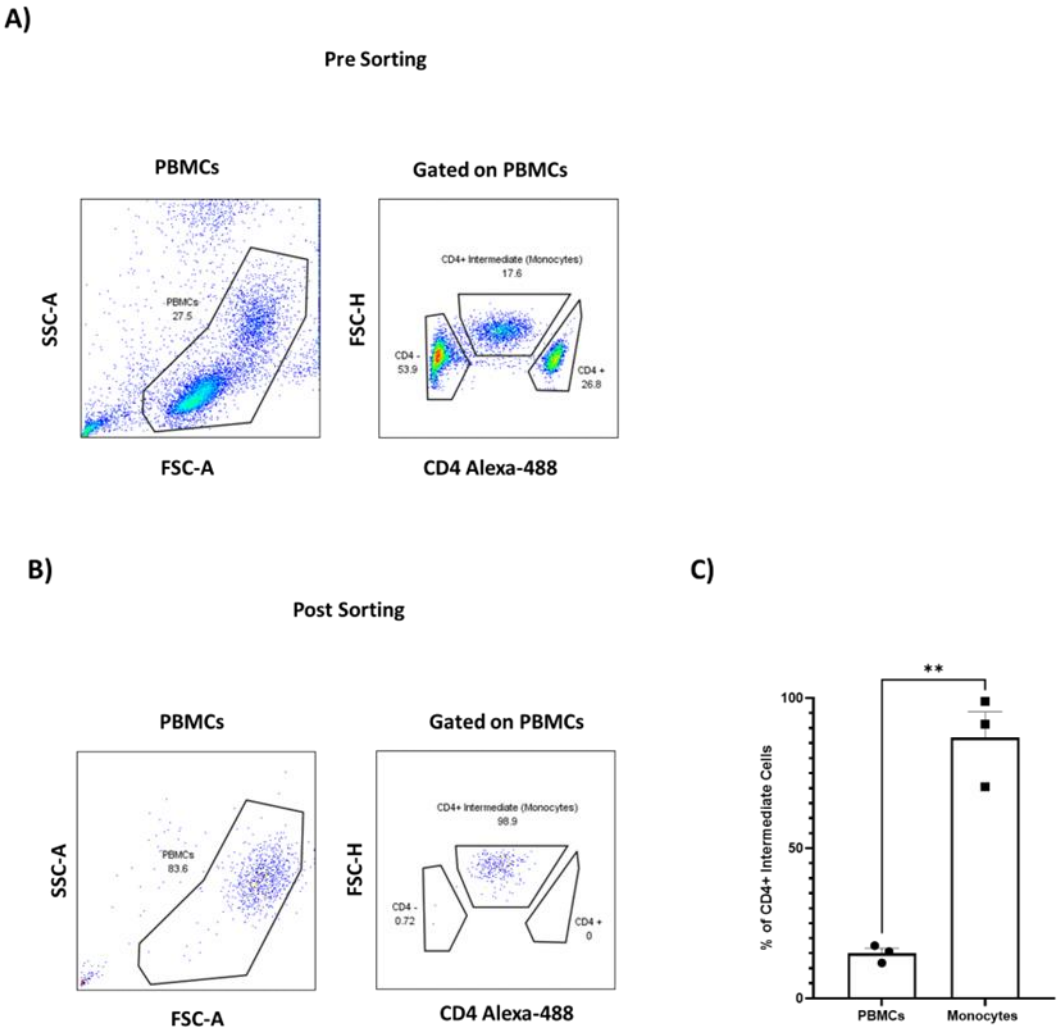

Figure 4.1 Isolation of Osteoclasts precursors (monocytes) from PBMCs by FACS sorting. PBMCs were stained with anti-CD4-Alexa.488 antibody. Monocytes were gated from PBCs as $CD4^{intermediate}$ population. Changes in the monocyte population can be seen pre-sorting (A) and post sorting (B**).** Purity of sorting showed an average of 86.9% of monocytes sorted. (C) Graphical representation of CD4+ quantification in PBMCs and Monocytes samples. Data shows mean with SEM and was analysed using paired Student's T-test ** = $p<0.008$. Analyses were repeated in N=3 biological samples.

4.5.1.1.2 Magnetic Beads Sorting of Osteoclasts Precursors (Monocytes) from PBMCs

Monocytes isolated from PBMCs using the Magnisort human $CD14^+$ positive selection kit (Thermofisher) were then characterised by flow cytometry to determine the purity of the enrichment for further osteoclast culture.

Both the PBMCs before sorting and the fraction of monocytes after sorting were stained with anti-CD14 and analysed using flow cytometry. This indicated the sorting purity of monocytes.

Morphological differences can be observed between the pre-sorted sample (containing the full PBMCs population) and the post sorting, monocytes sample. Before sorting, the PBMCs sample contained lymphocytes and monocytes. In the post sorting samples the monocyte population in the $CD14^+$ cells (enriched fraction) can be seen. Moreover, in the depleted fraction the monocyte population is not present and only the lymphocytes remain in the sample ($CD14^-$ cells) (**Figure 4.2 A**). $CD14^+$ expression was further tested on the PBMCs sample before and after the enrichment in both $CD14^+$ (enriched population) and $CD14^-$ (depleted population) populations showing enrichment of osteoclast precursors cells as CD14 was higher expressed on the $CD14^+$ enriched sample (**Figure 4.2 B**).
The purity of enriched $CD14^+$ cells was always above 90% corroborating the enrichment and the quality of the sample to further generate osteoclasts (**Figure 4.2 C**).

Both methods proved to be efficient in producing a pure sample of sorted monocytes from PBMCs. However, given the limitations to access the FACS sorting facilities during COVID-19 lockdown, all experiments following from here were carried out using the Thermofisher magnetic beads separation kit.

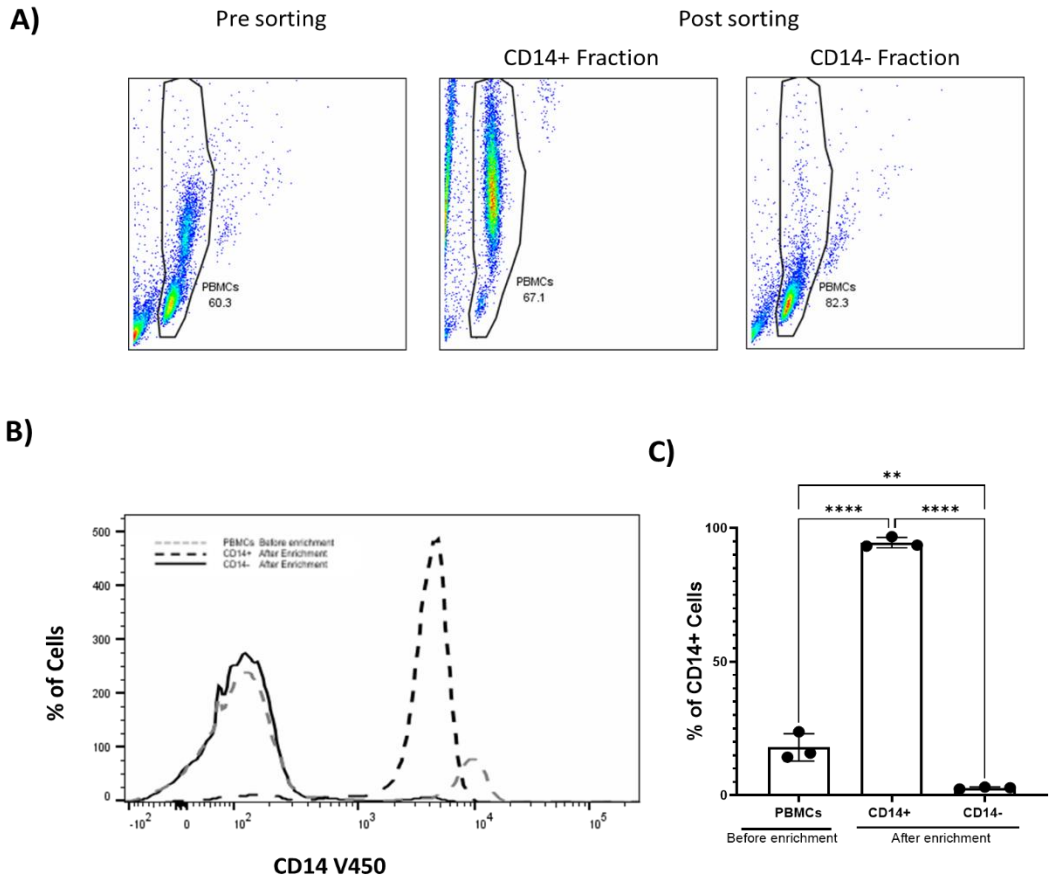

Figure 4.2 Isolation of Osteoclasts precursors (monocytes) from PBMCs by magnetic beads isolation (CD14⁺ enrichment kit). Morphology changes between PBMCs sample pre-sorted showed lymphocytes and monocytes populations while after the sorting the monocyte fraction could be differentiated as the CD14⁺ cells (osteoclast precursors) and the depleted fraction showed the CD14- population of cells (A). CD14 expression tested by flow cytometry showed a higher expression of CD14 on the CD14⁺ enriched population (B). Purity of enriched population showed above 90% of the cells expressing CD14 on the CD14⁺ enriched population. (C) Graphical representation of CD14+ before and after enrichment, ** = p<0.02 and ****= p<0.001.

4.5.1.1.3 Human Osteoclast Culture and Characterisation

To study the functional impact of Tregs on osteoclastogenesis an human *in vitro* osteoclast model was established based on the methodology previously used in the literature (Agrawal et al., 2012). This was done by stimulating monocytes ($CD14^+$ monocytes sorted from the PBMCs whole fraction) with MCSF, which stimulate macrophage formation (osteoclasts intermediate), and RANKL, which promote macrophages to fuse and generate osteoclasts (Quinn et al., 1997). This section will explore the optimisation steps (different cytokines concentrations and days of culture) to successfully obtain osteoclasts.

$CD14^+$ monocyte population of cells isolated from PBMCS using the Thermofisher magnetic beads kit were seeded at the density $5x10^5$ cells/well in a 96 well plate. Further, stimulation with different concentrations of MCSF (20 ng/mL, 25 ng/mL and 30 ng/mL, based on previously described methods (Agrawal et al., 2012)) were studied to determine a formation of macrophages, RANKL was also added from day 0 of culture and concentration was kept consistent as 30 ng/mL, and cells were cultured for 21 days as this is the previous proven optimal time for human osteoclasts to appear and be functional (Agrawal et al., 2012).

Morphology changes during culture indicate the differentiation of cells from monocytes to macrophages from day 1 to day 7. On day 7 cells have become larger and do not have a clear round shape like monocytes. At this stage cells present a more macrophage-like shape, such as characterised by irregular cell shape, oval-shaped nucleus, higher number of cytoplasmic vesicles, and high cytoplasm-to-nucleus ratio (Italiani & Boraschi, 2014) (**Figure 4.3 A and B**).

More notable changes can be seen after day 7, where macrophages start to fuse producing osteoclast precursors. These are larger in size, and are multinucleated, indicated by arrows (**Figure 4.3 C**). Lastly, from day 14 to 21, mature osteoclasts will become active, however in this case since cells are cultured on plastic and not on a bone like surface such as a dentine disc, these will not become activated given the lack of substrate to resorb. Instead, the osteoclasts will keep fusing and generating bigger osteoclasts with high numbers of nuclei (**Figure 4.3 D**).

When monocytes were stimulated with 20 ng/mL of MCSF there was an observed reduction in differentiation to macrophages on days 7 and 14, as there was still a considerable number of cells with morphology consistent with monocytes, and equivalent to that observed on day 1 of culture. This indicated that the MCSF concentration used might be too low to stimulate macrophage formation from monocytes and consequently the formation of osteoclasts (**Figure 4.3 A** and **B**, 20ng/ml).

Monocytes stimulated with 30 ng/mL MCSF after day 7 started to show a similar elongated shape as in comparison to the round shape presented when using 20 ng/mL MCSF. Polarisation of macrophages towards different phenotypes in vitro exhibit dramatic changes in cell shape. M2 cells exhibit an elongated shape compared with M1 cells and since M2 macrophages are known to be pro-healing in comparison with the proinflammatory function of M1 this was considered a beneficial shape change in our experiments to achieve osteoclasts (McWhorter et al., 2013). However, after 14 days stimulation cells presented apoptotic

morphology, suggesting that the 30 ng/mL MCSF concentration may not be optimum for osteoclast differentiation (**Figure 4.3 A** and **B**, 30ng/mL MCSF).

Finally, the culture condition using 25 ng/mL MCSF showed a morphology change from monocytes (day 1) to macrophage like morphology (day 7) and on day 14 mature osteoclasts could be seen already, with cells significantly larger and with high numbers of nuclei.

After 21 days of culture, cultured cells were stained with TRAP to positively determine formation of osteoclasts (**Figure 4.3 D**). Even though all concentrations of MCSF tested ended with formation of osteoclasts, we determined that 25 ng/mL MCSF and 30 ng/mL RANKL were the optimum conditions to induce osteoclast formation, given the morphology changes observed. Hence, further experiments shown in this Chapter were done using those culture conditions.

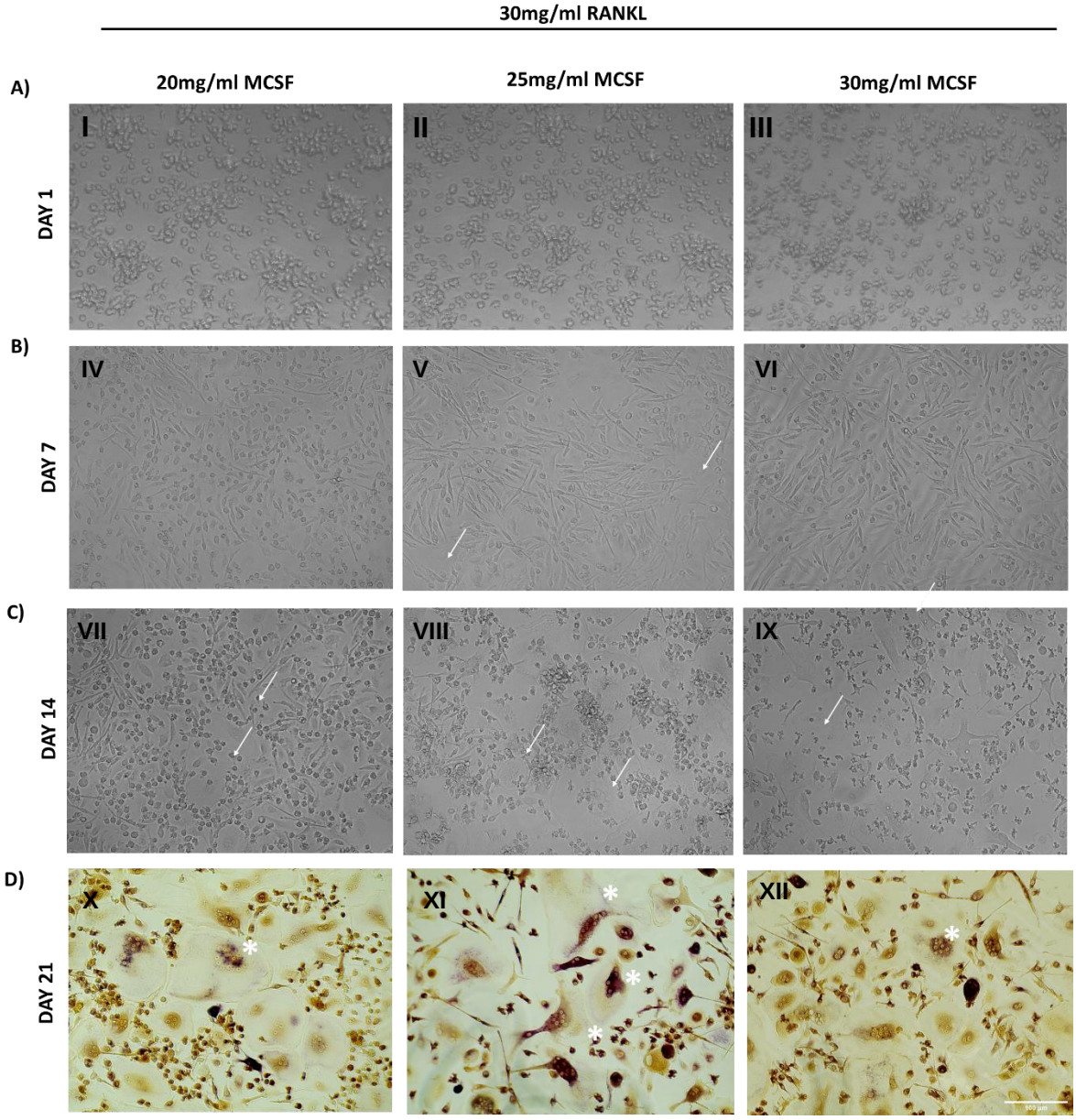

Figure 4.3 Osteoclasts formation from monocytes ($CD14^+$) sorted cells from PBMCs fraction stimulated with 20ng/ml MCSF, 25ng/ml MCSF or 30ng/ml MCSF and 30 ng/ml RANKL. Images of $CD14^+$ monocytes cells were taken on day 1 (A),7 (B),14 (C) and 21 (D) of culture and morphological changes can be seen as they start to differentiate into osteoclasts precursors. On day 21 cells were stained with TRAP to confirm osteoclast formation. Arrows show fused macrophages on day 14 and asterisks show multinucleated osteoclasts on day 21. Scale bar 100 µm.

Previous culture optimisation steps were carried out on a plastic 96 well plate. However, for functional assessment of osteoclast resorption activity, monocytes on day 0 were cultured on mineralized substrate (dentine discs) which allows osteoclast activation and the subsequent resorption (Boyle et al., 2003). In addition, resorbed areas can be visualised by light microscopy following TRAP reactivity as a measure of osteoclast activity.

Osteoclasts precursors, $CD14^+$ monocytes sorted from PBMCs, were seeded on dentine discs as per in section 4.4.3. Monocytes cultured with 25 ng/mL MCSF, and 30 ng/mL RANKL were incubated for a period of 21 days and osteoclast precursor formation assessed on days 7 and 14. Dentine discs were stained with TRAP (**Figure 4.4 A,** marked by asterisks). Morphological changes can be seen when culturing cells on dentine discs as compared to culturing on plastic. Cells appear smaller and rounder. This is likely caused by osteoclasts acquiring a 3D structure on top of the dentine disc and the formation of the ruffle border which will allows for resorption.

When comparing with the results obtained from culturing on plastic in the previous section, it can also be determined that osteoclast precursors will become mature osteoclasts and start resorbing as shown in **Figure 4.4 A** where resorption pits can already be seen around active osteoclasts after 14 days in culture. Quantification of resorption showed a significant increase from day 7 where cells are still not osteoclasts or capable of resorption, to day 14 where the resorption per osteoclast

average is 0.004 mm^2 and day 21 where the resorbed area per osteoclast average is 0.034 mm^2 (**Figure 4.4 B**).

For further corroboration that the resorption pits could be visualised, samples were analysed under SEM. Images taken under SEM allowed the visualisation of resorption pits and the differentiation between a non-resorbing mature osteoclast and an active resorbing osteoclast (**Figure 4.4 D**).

Overall, after this series of optimisation steps, we can conclude that the optimum concentration of cytokines for osteoclast differentiation from $CD14^+$ sorted monocytes and effective resorption is 25 ng/mL MCSF and 30 ng/mL RANKL for 21 days of culture. Hence, in further sections of this Chapter those will be the culturing conditions used and referred to.

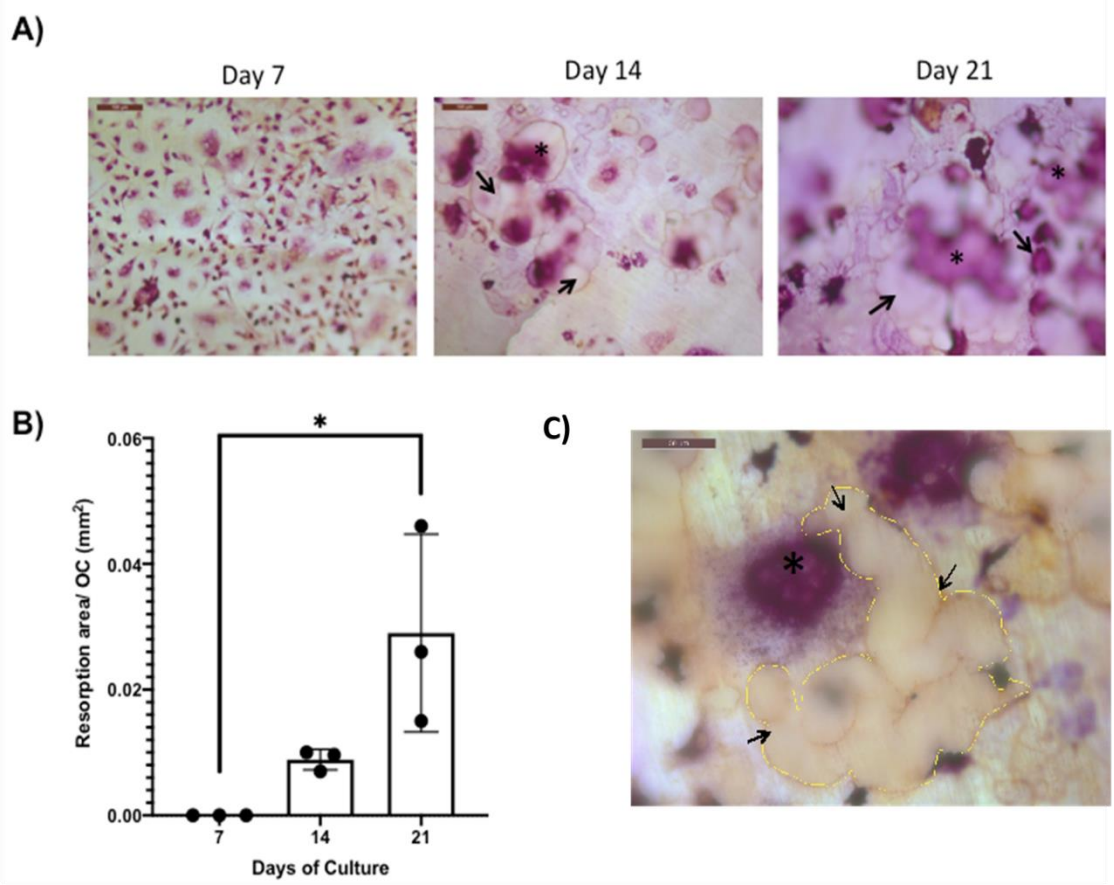
A)
Day 7
Day 14
Day 21
B)
Resorption area/ OC (mm²)
0.06
0.04
0.02
0.00
7
14
21
Days of Culture
*
C)
D)
Non-resorptive Osteoclast
Resorptive Osteoclast

Figure 4.4. Optimisation of in vitro human osteoclast differentiation at different days of culture. TRAP positive osteoclasts generated from $CD14^{+}$ cells cultured on dentine discs and stimulated with MCSF and RANKL during 7,14 or 21 days. Multinucleated osteoclast (asterisk) and resorption pits (arrows) can be seen. Image taken on a light microscope at 400X magnification, scale bar 50mm (A). Resorption area measurements for osteoclasts at 7, 14 and 21 days of culture (B). Methodology used to delimitate the area of the resorption pit per osteoclast (C). SEM images of osteoclast after 21 days of culture, representation of area analyses by ImageJ by highlighting in yellow area of resorption (D). Data shows mean with SEM and was analysed using ONE-WAY ANOVA *$p<0.005$. N=3 Biological replicates.

4.5.2 Isolation of Treg from PBMCs and Assessment of Functionality

To study the effects that Treg exert on osteoclasts, a co-culture of both cell types is needed. Tregs can be isolated from PBMCs using Fluorescence activated cell sorting (FACS) or magnetic sorting beads, and both these methods were investigated due to the COVID-19 restrictions regarding access to the FACS sorting facilities. Therefore, to be able to use Tregs in further experiments in this thesis, the isolation of Tregs from PBMCs was optimised and their functional suppression was assessed. Moreover, Tcons were also sorted to determine the functional suppression of Treg cells.

The original intention of the project was to isolate Tregs from fresh samples of blood. However, the COVID-19 restrictions, brought in part way through the project, regarding access to participants, this limited the possibility to obtain blood samples and thus blood components after centrifugation (buffy coats) from the Scottish Blood Transfusion Service were used instead. Because of this enforced change it was necessary to confirm the Tregs obtained initially from fresh peripheral blood and latterly from buffy coats were equivalent. This is particularly important given the documented tendency not to survive storage as well as other peripheral blood cells as Tregs are extremely sensitive to temperature changes (Golab et al., 2013).

Given the limitations in accessing fresh blood, it was necessary to sort and compare Tregs from fresh blood and buffy coats to prove that buffy coats could be used as an alternative to accessing fresh blood samples when needed.

4.5.2.1 Gating Strategy for Treg Sorting

Tregs defined in the previous Chapter (section 3.5.1) were further stained for anti-CD25 and anti-CD127 to determine the Treg population amongst the $CD4^+$ (or $CD4^{High}$) cells based on high expression of CD25 (gamma chain of IL-2 receptor) and low expression of CD127 (IL-7 receptor). Gating Tregs only based in $CD4^+CD127^{low}CD25^{high}$ is a limitation to identify Treg populations, since activated T cells also express CD25. Moreover, some Tregs express low CD25 and can be excluded from the $CD127^{low}CD25^{high}$ phenotype (Yu et al., 2012). FoxP3 is the most reliable approach to identify Tregs, however since to access FoxP3 cells need to be fixed and permeabilised, and this causes cell death when needing a Treg sample population with live cells $CD127^{low}CD25^{high}$ can be used to identify functional Tregs. Tregs used for co-culture with osteoclasts were sorted using the gating strategy from $CD4^+$ $CD127^{low}CD25^{high}$ (**Figure 4.5 B**).

In order to confirm CD4+Foxp3+ Tregs and $CD4^+CD127^{low}CD25^{high}$ Tregs are an equivalent population Treg previously gated as described in section 3.5.1 as $CD4^+Foxp3^+$ (**Figure 4.5 A**) were also analysed by $CD4^+CD127^{low}CD25^{high}$ (**Figure 4.5 B**). When comparing the Treg population based on both methods of identification numbers of Tregs slightly differ between gating strategies being 8.44% $CD4^+Foxp3^+$ and 8.05% $CD4^+CD127^{low}CD25^{high}$ however, this variation may be due to the gating strategy followed being slightly different and the cells included in each gate can vary.

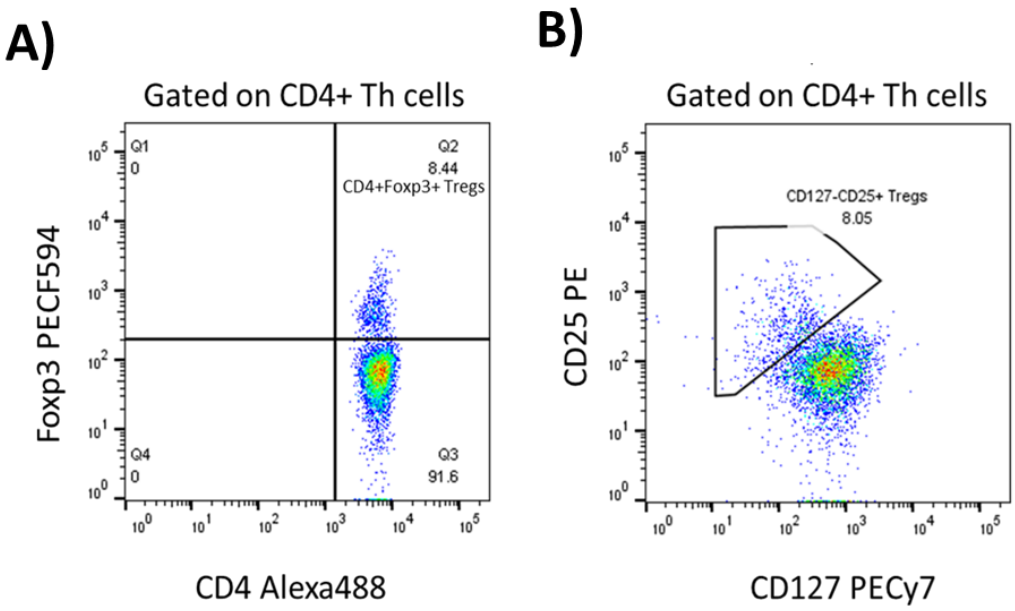

Figure 4.5 Gating strategy for sorting Tregs sorting based on CD127low/CD25high expression. Figure 3.1 Gating strategies and definition of Treg cell population. PBMCS isolated from human whole blood were stained with anti-CD4 Alexa 488, and anti-Foxp3 PECF594, anti-CCR4 BV421 and anti-CXCR4 BV605. Lymphocyte population was gated based on morphology plot using Forward Scatter versus Side Scatter, doublets of cells were discounted by gating on singlets based on Forwards Scatter Area versus Height. Singlet lymphocytes were gated on CD4High population. Tregs were gated based on Foxp3+ expression (A) or CD127Low/CD25High (B).

4.5.2.2 FACS Sorting of Tregs and Functionality Assay (Suppression Assay)

PBMCs isolated from fresh blood were further stained with anti-CD4, anti-CD25 and anti-CD127 and sorted using BD FACS Aria. Pre-sorting and post sorting samples were also stained with anti-CD4, anti-CD25 and anti-CD127 to confirm the sorting purity. The purity of the isolated cells can be seen in **Figure 4.6 A** by morphological characteristics. Before sorting, lymphocytes and monocytes are still in the PBMCs sample, while after cell population enrichment by sorting only Tregs and Tcon populations are shown. Moreover, the different population of cells were also analysed regarding the expression of surface markers used to define Treg population ($CD4^{+}CD127^{Low}CD25^{Hi}$). Morphological changes show successful sorting, since in the pre-sorting sample Treg and Tcon population can still be seen. After sorting, each population presented either only Tregs or Tcons regarding the cell type selected for sorting (**Figure 4.6 B).**

After sorting, the purity of the cells of interest was almost 100% for Tregs, being an increase in the purity of Tregs post sorting compared with the amount of Tregs in the whole sample pre-sorting (average, 5% of Tregs in the total cell population) (**Figure 4.6 C**)**.**

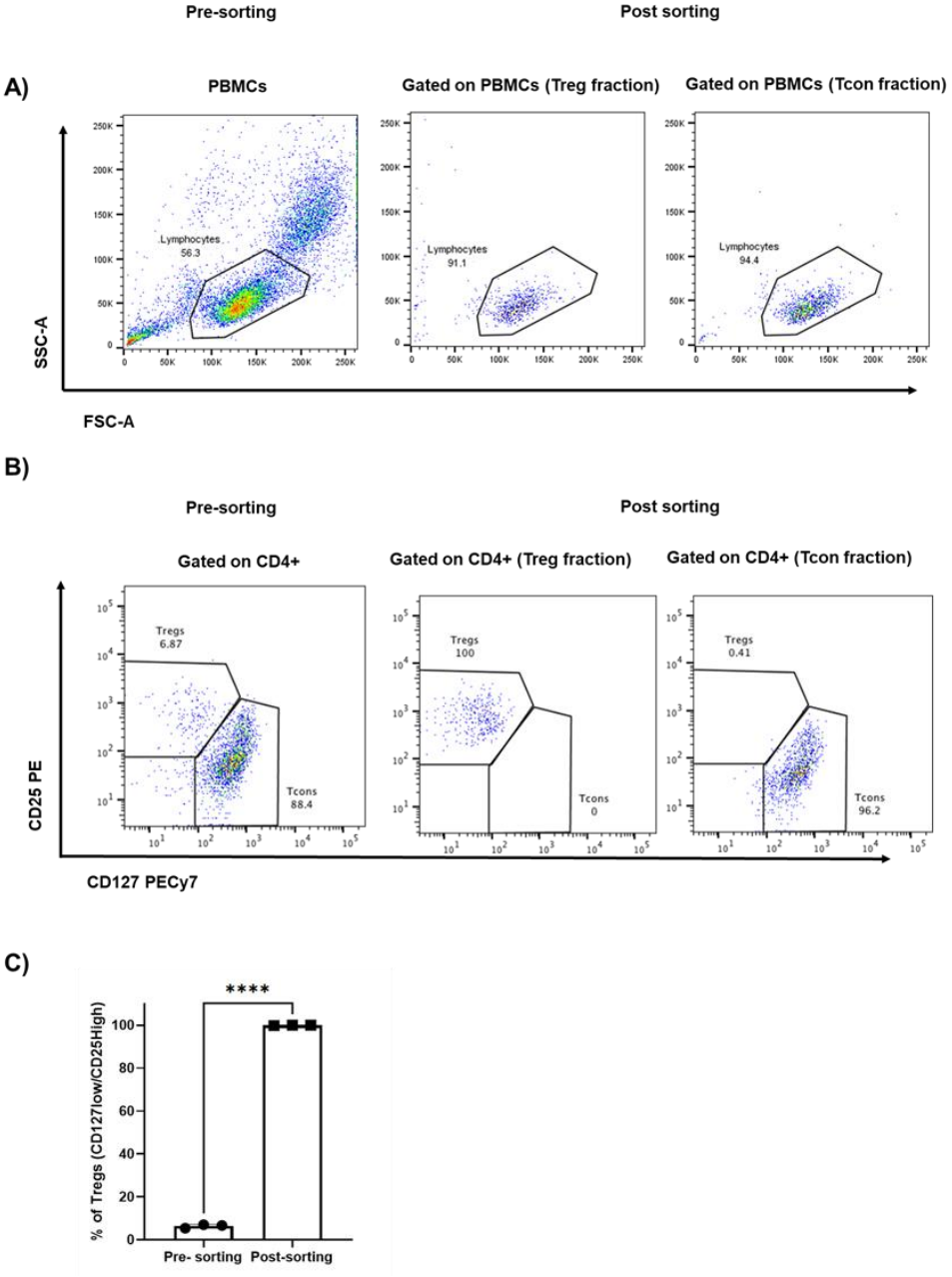

Figure 4.6 FACS Isolation of Tregs and Tcons from PBMCs isolated from fresh whole blood samples. Isolated PBMCs from whole blood were further stained with anti-CD4, anti-CD25 and anti-CD127 and sorted using BD FACS Aria. Purity of isolated cells can be seen by cell morphology (A) and by expression of surface markers (B). Moreover, the percentage of isolated cells was analysed and shown in (C). Data shows mean with SEM and was analysed using paired T-test. Analyses were repeated in N=3 biological samples, ****=<0.0001.

Further functionality assays (suppression assays) of sorted Tregs were done to determine the suppressive function of Tregs in the proliferation of responder cells (Tcons) from FACS sorted Tregs.

The proliferation of responders alone, measured as the division index can be seen when compared with inactivated cells samples with 65% of Tcons proliferating (**Figure 4.7 A**). The suppression of this proliferation is shown when adding Tregs to the culture, as the proliferation of Tcons reduced to 26.6% when co-cultured with Tregs obtained from fresh blood samples (**Figure 4.7 A**). This demonstrated that FACS sorted Tregs were functional, suppressing Tcons proliferation by more than 50% (**Figure 4.7 B**).

Whilst this sorting method obtained pure and functional Tregs and would have been taken forward in the following experiments, I could not continue with this approach for the entirety of this PhD as access to the FACS sorter facilities was not possible during COVID-19 restrictions.

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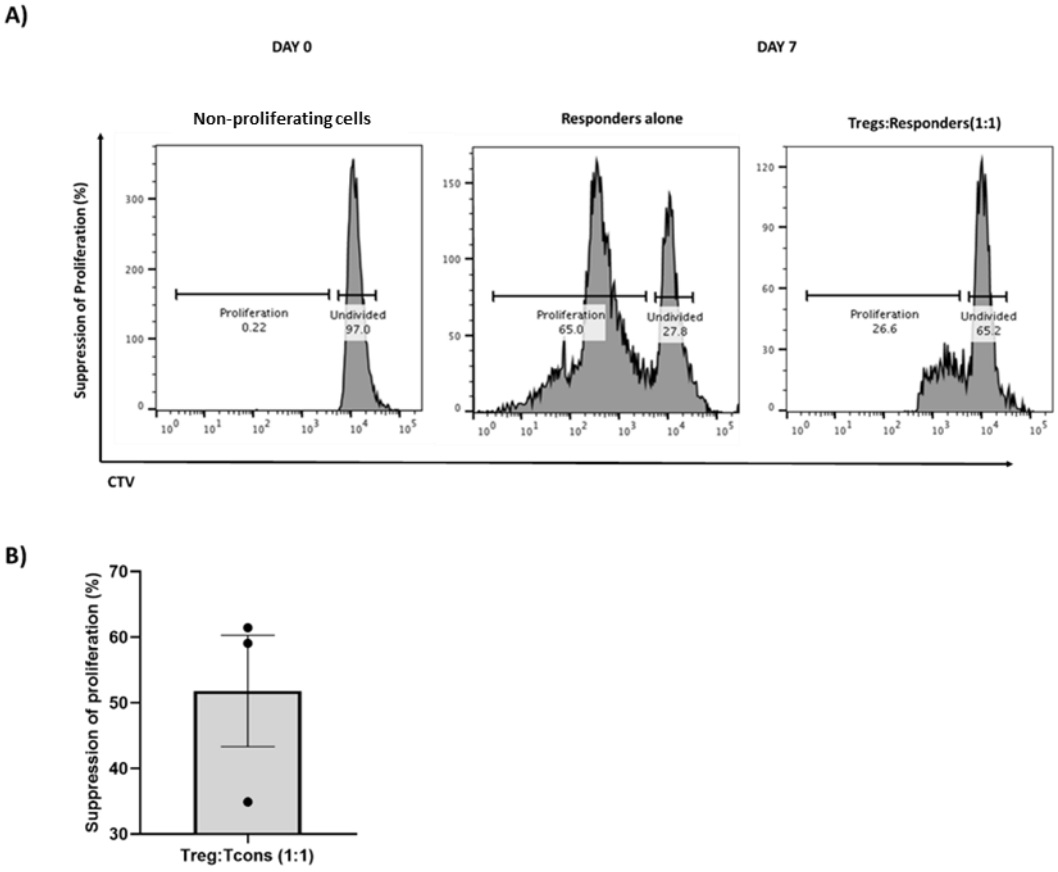

Figure 4.7 Suppression assay of FACS sorted Tregs. Proliferation of Tcons (responders) showed undivided cells on day 0 of culture and proliferation after 7 days of culture without Tregs. Suppression of the proliferation of Tcons caused by Tregs can be seen after 7 days of culturing in the presence of Tregs (A). Suppression of proliferation calculated from the division index on each sample (responders alone and Tcons:Tregs) showed a suppression of proliferation of 50% (B). Data shows mean with SEM. Analyses were repeated in N=3 biological samples.

4.5.2.3 Magnetic Beads Sorting of Tregs and Functionality Assay (Suppression Assay)

Since FACS sorting was not possible, the next step was to explore magnetic beads sorting of Tregs. Moreover, at this stage of the project, COVID-19 restrictions also made it impossible to obtain fresh blood samples. Therefore, Buffy coats were used to optimise magnetic beads sorting of Tregs and Tcons and the subsequent suppression assays to test functionality of sorted Tregs.

Tregs and Tons were sorted from buffy coats samples using EasySep Human $CD4^{+}CD127^{Low}CD25^{+}$ Regulatory T Cell Isolation Kit and purity was assessed by flow cytometry. The purity of the isolated cells can be seen in by changes in the forward scatter **Figure 4.8 A**. Before sorting, the sample contains lymphocytes and monocytes, while after cell population enrichment only Tregs and Tcon populations are shown in each sample respectively.

The different population of cells were also analysed regarding the expression of surface markers used to define Treg population ($CD4^{+}CD127^{Low}CD25^{Hi}$) (**Figure 4.8 B).** After enrichment populations the post-sorting sample showed an increase in the amount of Tregs compared to other cell populations, showing Tregs to be over 90% of the total amount of cells in the sample **(Figure 4.8 C).**

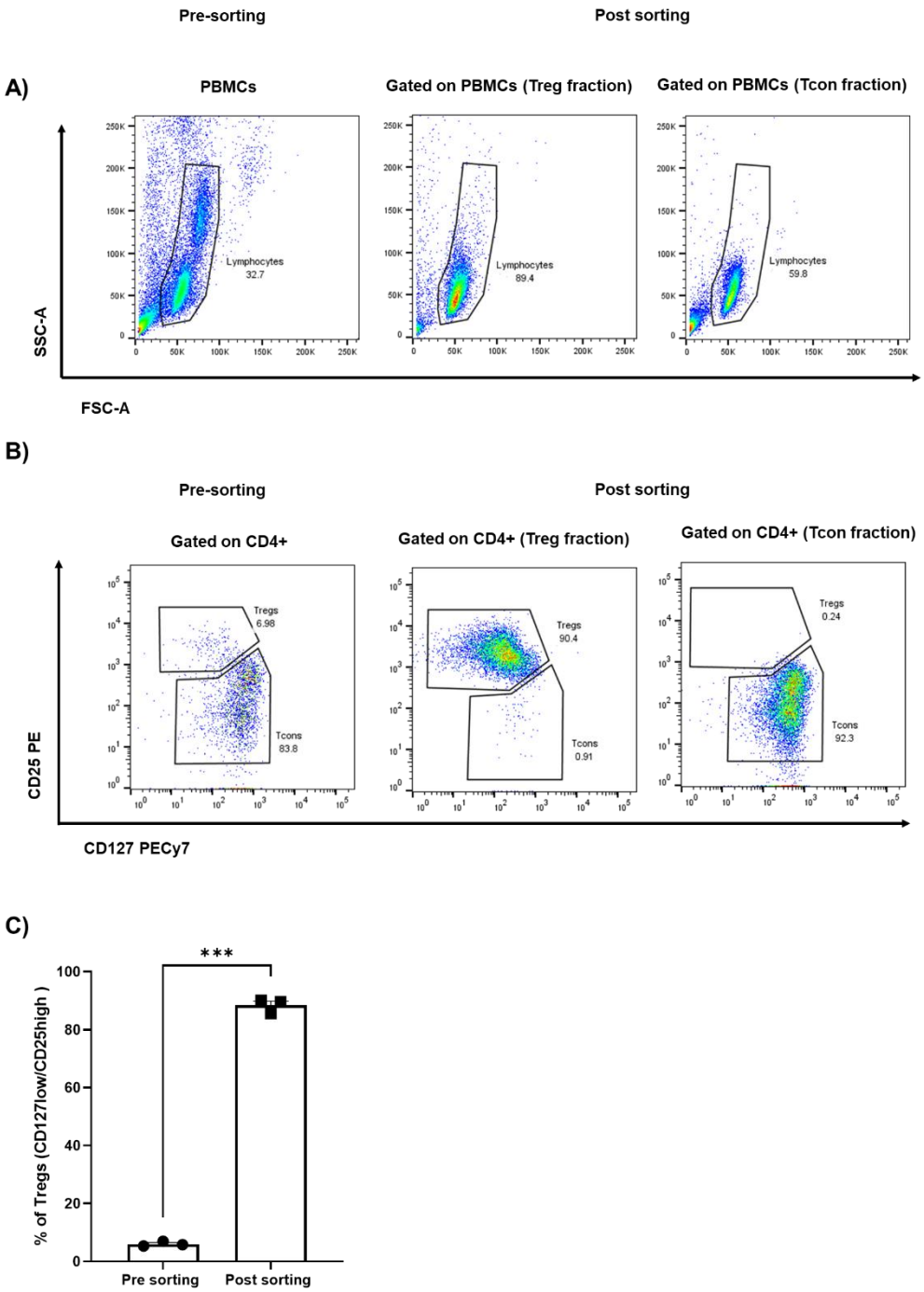

Figure 4.8 Magnetic beads Isolation of Tregs and Tcons from PBMCs isolated from fresh whole blood samples. Isolated PBMCs from whole blood were further isolated using the EasySep Human $CD4^{+}CD127^{Low}CD25^{+}$ Regulatory T Cell Isolation Kit. Further cells were stained with anti-CD4, anti-CD127 and anti-PE to check purity of isolated cells. Purity of cells can be seen morphology (A) and by expression of surface markers (B). Moreover, the percentage of isolated cells was analysed for Tregs and Tcons (C). Data shows mean with SEM and was analysed using paired T-test *$p<0.05$. Analyses were repeated in N=3 biological samples.

Finally, the suppressive function of beads sorted Tregs was assessed. For these experiments the staining of Tcons to visualise proliferation was done using CFSE instead of CTV as in the previous sections when looking at proliferation from FACS sorted cells due to other technical issues not described in this thesis. After using CTV to stain Tcons sorting using the magnetic beads, no proliferation could be detected. In repeated experiments we found that CTV stained Tcons from buffy coats would not reliably proliferate (data not shown), this suggested that CTV was not the optimum staining method to quantify cell proliferation. As a consequence of buffy coats being more fragile than the fresh blood used previously, due the prolonged storage and additional processing, CFSE as a method of cell staining to measure proliferation of magnetic sorted Tregs was explored.

After 7 days of co-culturing Tcons and Tregs, the suppressive effect of Tregs could be seen in the proliferation of Tcons. The proliferation of responders alone can be seen when compared with inactivated cells samples with 43.3% of Tcons proliferating (**Figure 4.9 A**). The suppression of this proliferation is shown when adding Tregs to the culture, as the proliferation of Tcons reduced to 34.4% in Tregs obtained from buffy coats and using magnetic beads to sort them (**Figure 4.9 A**). This proved that magnetic beads sorted Tregs were functional, since they are performing their suppressive function of Tcons proliferation, showing above 20% of suppression (**Figure 4.9 B**). Even though Tregs are performing their suppressive function this is not as high as expected based on the existing literature. This may be due to the conditions used for the co-cultures not being optimal for culturing Tcells as the culturing conditions have been previously optimised to derive osteoclasts (section 4.3.2). Since this limitation cannot be changed for the purpose

of this Chapter, and the effect of Tregs was still visible when used in further experiments on Chapter 4, the fact that we are not seeing a high suppression can be related to the culturing conditions and must be also considered in future experiments.

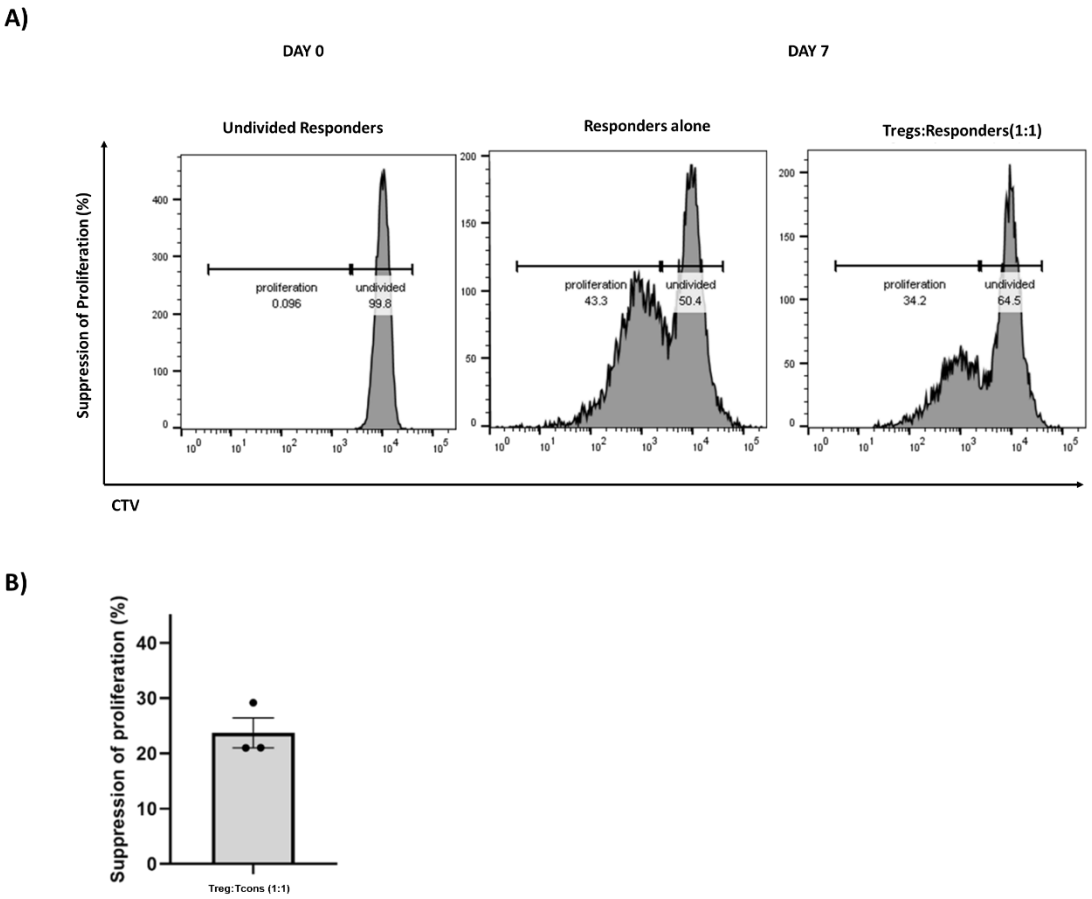

Figure 4.9 Suppression assay of magnetic bead sorted Tregs. Proliferation of Tcons (responders) showed undivided cells on day 0 of culture and proliferation after 7 days of culture without Tregs. Suppression of the proliferation of Tcons caused by Tregs can be seen after 7 days of culturing in the presence of Tregs (A). Suppression of proliferation calculated from the division index on each sample (responders alone and Tcons:Tregs) showed a suppression of proliferation of 20% (B). Data shows mean with SEM. Analyses were repeated in N=3 biological samples.

4.5.3 Co-culture of Tregs and Osteoclasts

Isolated Tregs and monocytes (osteoclasts precursors), both by magnetic bead sorting as previously shown in this Chapter, were co-cultured to study the effects of Tregs on osteoclast formation and their resorptive potential.

To determine if conventional Treg activation via their T cell receptor is required for Tregs to influence osteoclast function, co-culture with activated or non-activated Tregs was explored. Moreover, osteoclasts cultured in the absence of Tregs but with the activation antibodies CD3/CD28 was also tested to determine the influence of the activation method for Tregs on osteoclasts.

Co-culture of osteoclasts with activated Tregs significantly decreased the area resorbed per osteoclast in comparison to osteoclasts cultured in the absence of Tregs ($P<0.05$; **Figure 4.10**). Similarly, area resorbed per osteoclast was also significantly decreased in osteoclasts co-cultured with activated Tregs in comparison to those co-cultured with inactivated Tregs ($P<0.05$) and osteoclasts cultured with just the activation cocktail ($P<0.01$; **Figure 4.10**). There was no effect of inactivated Tregs on osteoclast resorption in comparison to osteoclasts cultured without Tregs (**Figure 4.10**). Overall, this concluded that when seeing reduction in osteoclast function this can be determined to be due to activated Tregs and that the activation method (CD23/CD28 stimulation of Tregs) was not directly influencing the osteoclast function (**Figure 4.10**). Therefore, experiments in the following sections were carried out comparing osteoclasts function in the absence of Tregs and in the presence of activated Tregs.

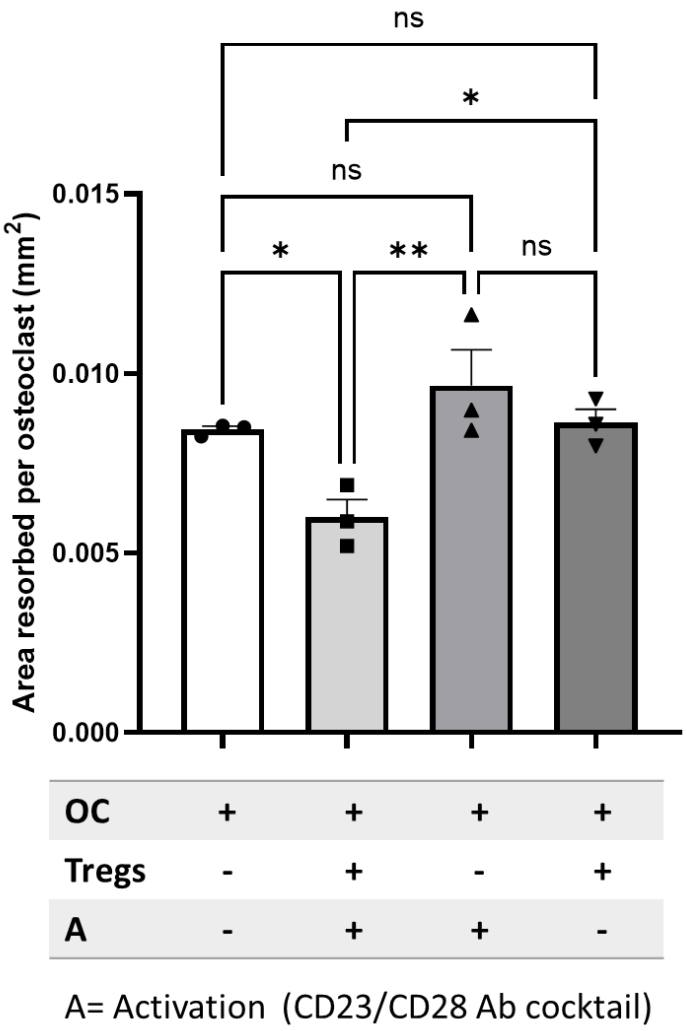

Figure 4.10 Experimental set up for co-culture of Tregs and osteoclasts. Isolated Tregs and monocytes from PBMCS by magnetic beads were cultured together in a 96 well plate containing a dentine disc and stimulated with MCSF and RANKL for osteoclast formation. Osteoclasts were cultured in the absence or presence of activated and inactivated Tregs, and the combinations reveal a reduction of osteoclast function was caused by activated Tregs and that the activation of Tregs alone (CD3/CD28 stimulation) did not affect osteoclast function directly. Data are represented as mean ± SEM and were analysed using ONE-WAY ANOVA. Analyses were repeated in N=3 biological samples, *p<0.05

4.5.4 Effects of Tregs on osteoclastogenesis

This section explored the effects of Tregs from different age groups (young under 50y/o and aged over 50 y/o) on osteoclast formation and function by co-culturing osteoclasts in the absence and presence of activated Tregs for 21 days on dentine discs. Analyses of osteoclasts numbers formed from monocytes and the resorption pits produced per osteoclast was indicative of osteoclast activity.

4.5.4.1 Osteoclastogenesis in the absence of Tregs

It was necessary to first understand the effects of ageing on osteoclast form and function before establishing the effects that activated Tregs may have on this.

Number of osteoclasts formed from monocytes after 21 days of culture with MCSF and RANKL was plotted against the age of the participants to visualize the distribution of the number of osteoclasts formed depending on the age of the donor. No clear clusters for any age groups were seen and it appeared to be that, in our sample population, osteoclasts formation was therefore not affected by ageing (**Figure 4.11 A**). Further analyses of samples as healthy young and healthy aged was done and statistical analyses showed no significant difference between groups (**Figure 4.11 B**). Moreover, when looking at differences caused by sex (female or male) this showed also no significant differences (**Figure 4.11 C**). Hence, the data presented in **Figure 4.11,** based on our sample population, showed that *in vitro* formation of osteoclasts was neither affected by ageing or sex.

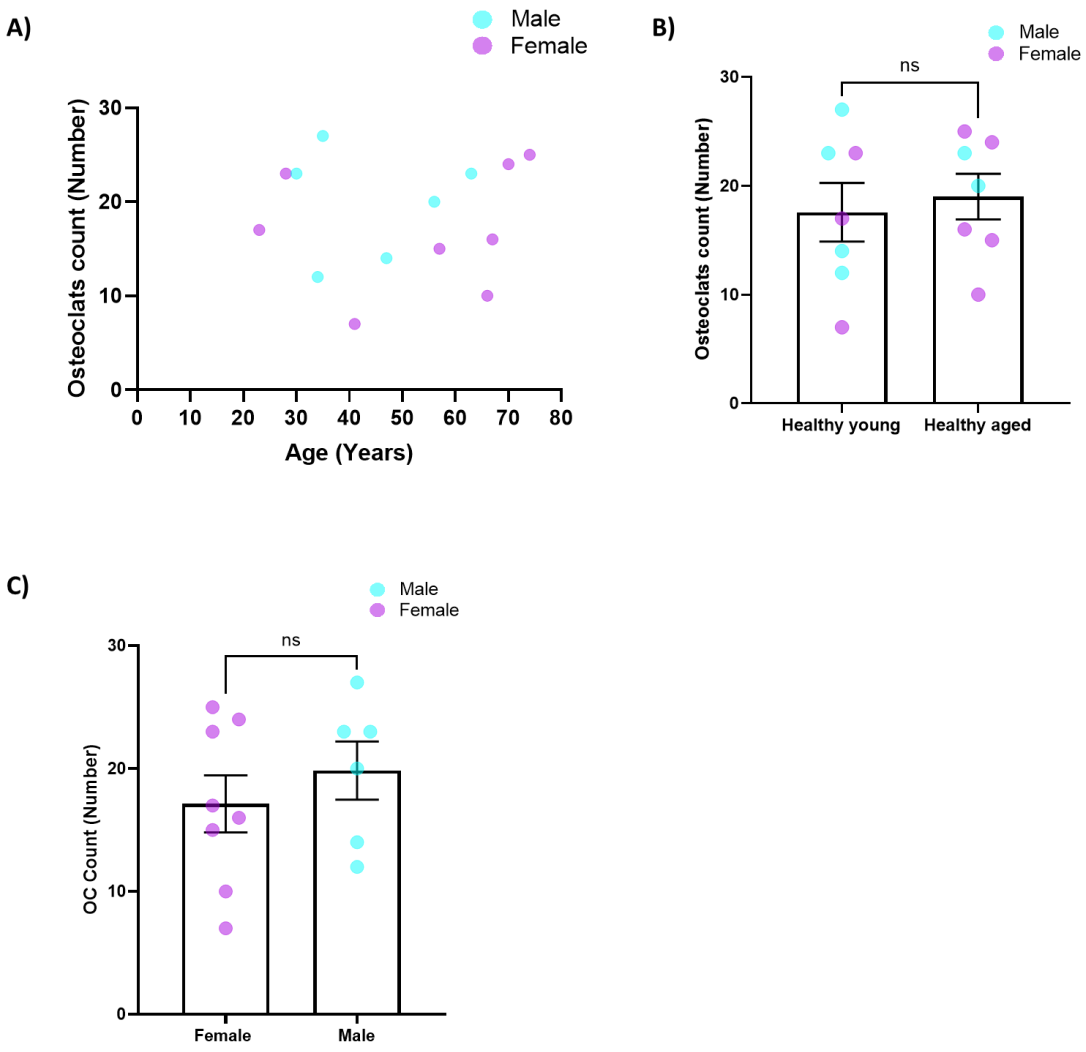

Figure 4.11 Osteoclast differentiation from precursors in the absence of Tregs effects of ageing and sex. Monocytes were cultures on dentine discs for 21 days and stimulated with 25 ng/mL MCSF and 30 ng/mL RANKL for osteoclasts differentiation. After 21 days osteoclasts were assessed for TRAP reactivity and counted. Numbers of osteoclasts were plotted by aged of participant and no clear cluster of aged were determined (A). Numbers of osteoclasts formed was not significantly different between healthy young and healthy aged participants (B). Sex differences (female or male) did not show any significant difference in the number of osteoclasts differentiated from monocytes (C). Data shows mean with SEM and was analysed using Student T-test. Analyses were repeated in N=14 biological samples.

Next, the ability of those osteoclasts formed to resorb the mineralized matrix from dentine discs was assessed.

Osteoclast function represented by area resorbed per osteoclast was plotted by age to visualise the distribution on osteoclast function during ageing. When samples were represented by colour to differentiate female (purple) and male (blue) a trend showing a decrease on osteoclast activity in the female group could be observed when comparing with the male group in the same age group (**Figure 4.12 A**).

Statistical analyses for osteoclast function when comparing healthy young and healthy aged groups showed a significant decrease (P=0.001) in the area resorbed per osteoclasts in the healthy aged where the average of osteoclasts function was 0.005mm^2 resorbed per osteoclasts while the healthy young group resorption was 0.0078 mm^2 per osteoclasts (**Figure 4.12 B**). The difference in osteoclasts function may be related to sex differences since when looking at differences caused by sex, this showed a significant difference (P=0.04) with the female groups showing a decreased area resorbed per osteoclast (0.0059 mm^2) when compared to the male group (0.0079 $mm^{2)}$ (**Figure 4.12 C).**

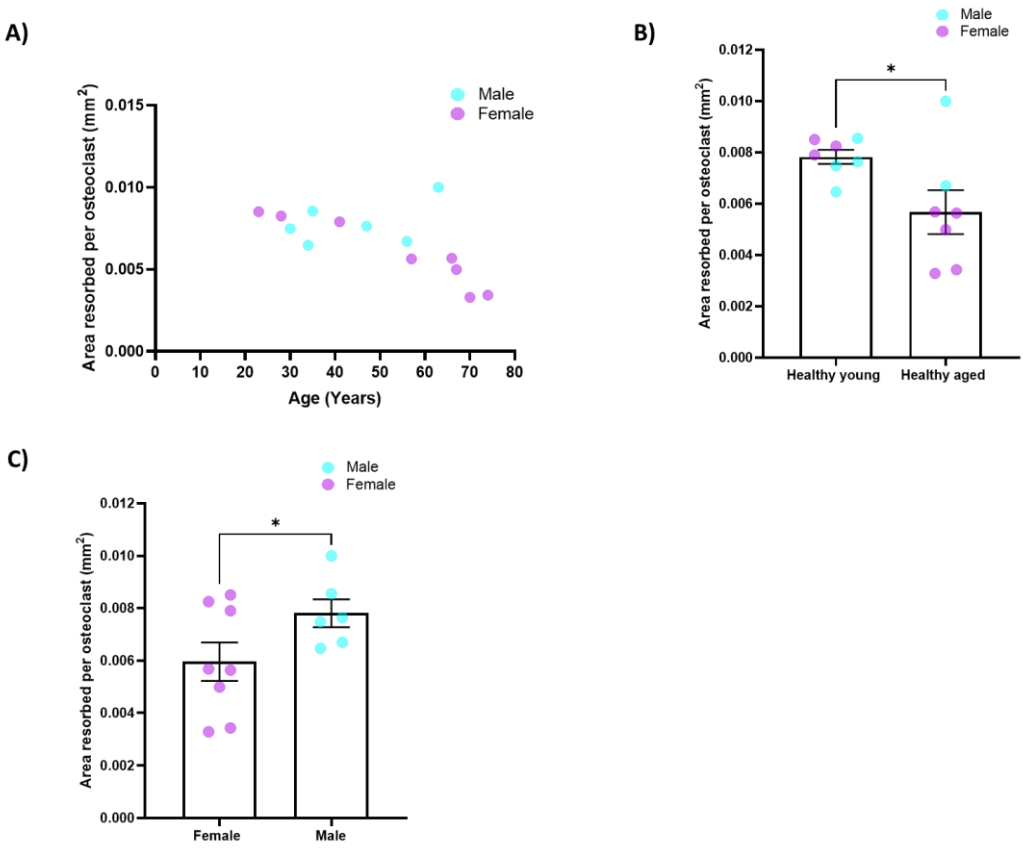

Figure 4.12 Osteoclast function and the effects of ageing and sex. Monocytes were cultures on dentine discs for 21 days and stimulated with 25 ng/mL MCSF and 30 ng/mL RANKL for osteoclasts differentiation. After 21 days osteoclasts were stained with TRAP the resorption pits area was measured using imageJ. Osteoclast function was plotted by aged of participant and no clear cluster of aged were determined (A). Osteoclast's function showed significant decrease in healthy aged participants compared to healthy young (B). Sex differences (female or male) showed a significant difference in osteoclast function. Osteoclasts from the female group presented a lower function than the male group (C). Data shows mean with SEM and was analysed using Student T-test. Healthy young participants (N=7) and healthy aged participants (N=7) biological samples, biological samples, Male N=6 and Female N=8 *p<0.05

4.5.4.2 Effects of Tregs on osteoclastogenesis in healthy young and healthy aged participants

Having previously demonstrated in section 4.5.2.1, age, and sex differences in osteoclast function (**Figure 4.12 A and B**) the next step was to study the effects of Tregs on osteoclasts. Monocytes (osteoclasts precursors) were co-cultured for 21 days in the presence of MCSF and RANKL and Tregs added and activated (by CD3/CD28 stimulation) on day 0 of culture.

Osteoclast formation from monocytes was not affected by co-culture with activated Tregs. The number of osteoclasts formed were not significantly different when cultured in the presence of Tregs from when osteoclasts were culture in the absence of Tregs in either group, healthy young ($p=0.36$) (**Figure 4.13 A)** or healthy aged ($p=0.17$) (**Figure 4.13 B**). Even though both groups did not show significance difference in the number of osteoclasts in the presence of Tregs, a trend can be seen where a reduction in osteoclast number is observed when co-cultured with Tregs. No statistically significant difference is found between this groups, this may be due to the small sample population and the donor variability amongst conditions.

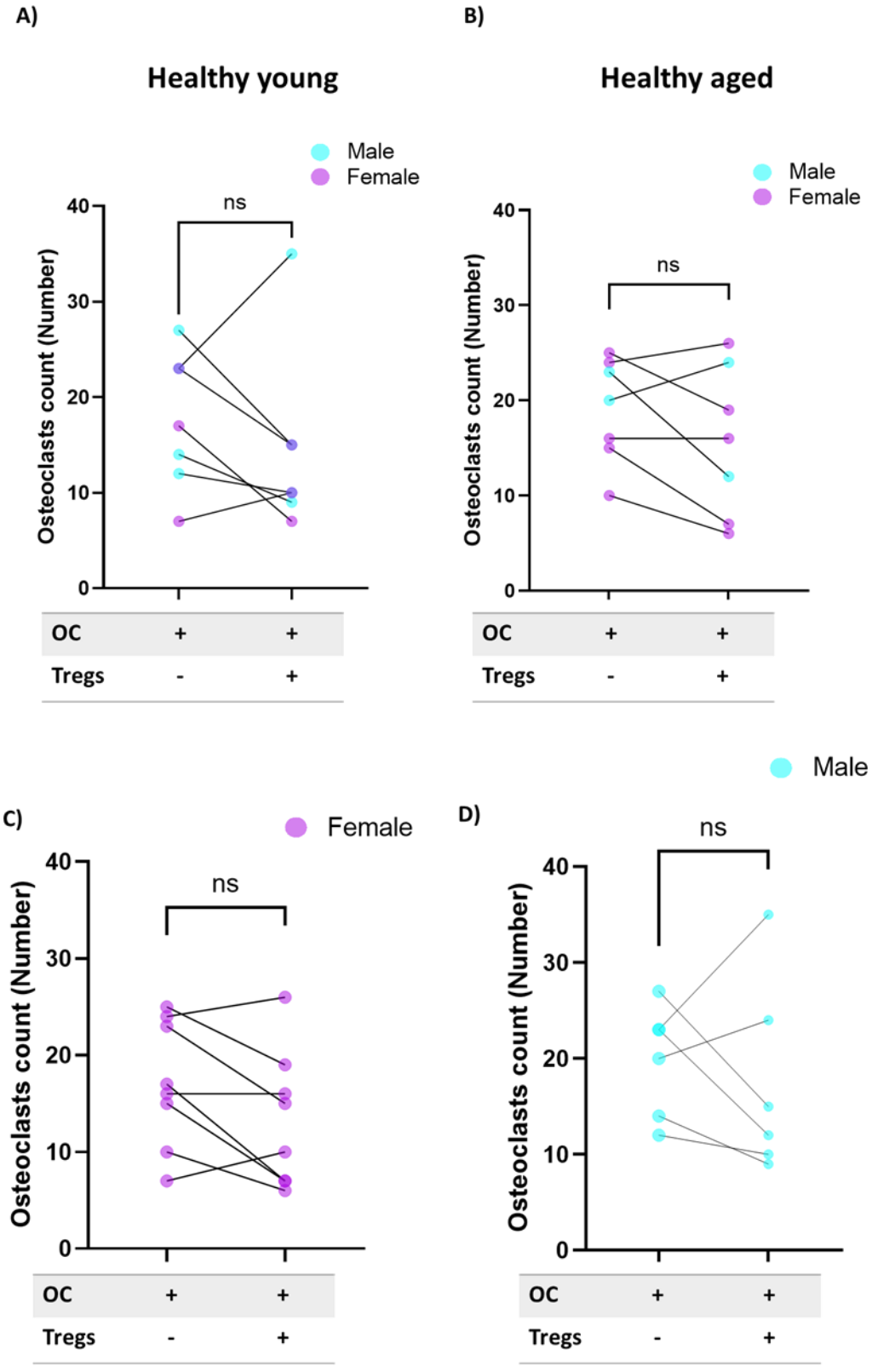
A)
Healthy young
Male
Female
ns
Osteoclasts count (Number)
40
30
20
10
0
OC + +
Tregs - +
B)
Healthy aged
Male
Female
ns
Osteoclasts count (Number)
40
30
20
10
0
OC + +
Tregs - +
C)
Female
ns
Osteoclasts count (Number)
40
30
20
10
0
OC + +
Tregs - +
D)
Male
ns
Osteoclasts count (Number)
40
30
20
10
0
OC + +
Tregs - +

Figure 4.13 Effects of Tregs in osteoclasts formation caused by ageing and sex. Monocytes were cultured on dentine discs for 21 days and stimulated with 25 ng/mL MCSF and 30 ng/mL RANKL for osteoclasts differentiation and CD3/CD28 Ab for Treg activation. After 21 days osteoclasts were stained with TRAP and counted. No significant difference was seen in the number of osteoclasts differentiated from monocytes in either healthy young (A) or healthy aged participants (B). Sex differences did not show any significant difference in the number of osteoclasts differentiated from monocytes in either female (C) or male (D) group. Data shows mean with SEM and was analysed using paired Student T-test. Healthy young participants (N=7) and healthy aged participants (N=7) biological samples, biological samples, Male N=6 and Female N=8 *$p<0.05$

Moreover, differences in osteoclast formation caused by sex were also analysed independently of ageing, to look at the effects of Tregs on osteoclasts in female and male participants.

The number of osteoclasts formed was not significantly different when cultured in the presence or absence of Tregs when comparing both groups, female (p=0.06) (**Figure 4.13 C**) and male (p=0.56) (**Figure 4.13 D**). Females showed an average of 17 osteoclasts per mm^2 formed in the absence of Tregs and 13 osteoclasts per mm^2 in the presence of Tregs (**Figure 4.13 C**). Also, no significant difference on the number of osteoclasts formed was seen for the male group where the average of osteoclasts formed when cultured in the absence of Tregs was 19 osteoclasts per mm^2 and 17 osteoclasts per mm^2 when culture with Tregs (**Figure 4.13 D**).

In order to assess the ability of Tregs to suppress osteoclast function the resorption pit area formed after 21 days of culture on dentine discs for osteoclasts culture in the presence or absence of Tregs for healthy young and healthy aged participants. Further the same samples were also analysed regarding the sex to determine changes caused by sex during ageing.

A higher effect on osteoclasts function was found in the healthy aged group when co-culturing osteoclasts and Tregs, shown by a significant decrease in the resorbed area per osteoclast (P=0.0001) when comparing osteoclasts in the absence and presence of Tregs. The average of resorption per osteoclasts was

0.005 mm^2 per osteoclast in the absence of Tregs and 0.003 mm^2 per osteoclast in the presence of Tregs (**Figure 4.14 A**).

A significant decrease in osteoclast activity (P=0.0002) was found when osteoclasts were cultured with Tregs in comparison of osteoclasts cultured in the absence of Tregs in the healthy young group. The average of resorption per osteoclasts was 0.007 mm^2 per osteoclast in the absence of Tregs and 0.005 mm^2 per osteoclast in the presence of Tregs (**Figure 4.14 A**).

Moreover, data was analysed by gender (female in purple and male in blue) and for both groups, male and female, Tregs produced a significant decrease in osteoclasts activityIn the female group the average of resorption per osteoclasts was 0.005 mm^2 per osteoclast in the absence of Tregs and 0.003 mm^2 per osteoclast in the presence of Tregs (**Figure 4.14 C**). For the male group, the average of resorption per osteoclasts was 0.007 mm^2 per osteoclast in the absence of Tregs and 0.005 mm^2 per osteoclast in the presence of Tregs (**Figure 4.14 C**).

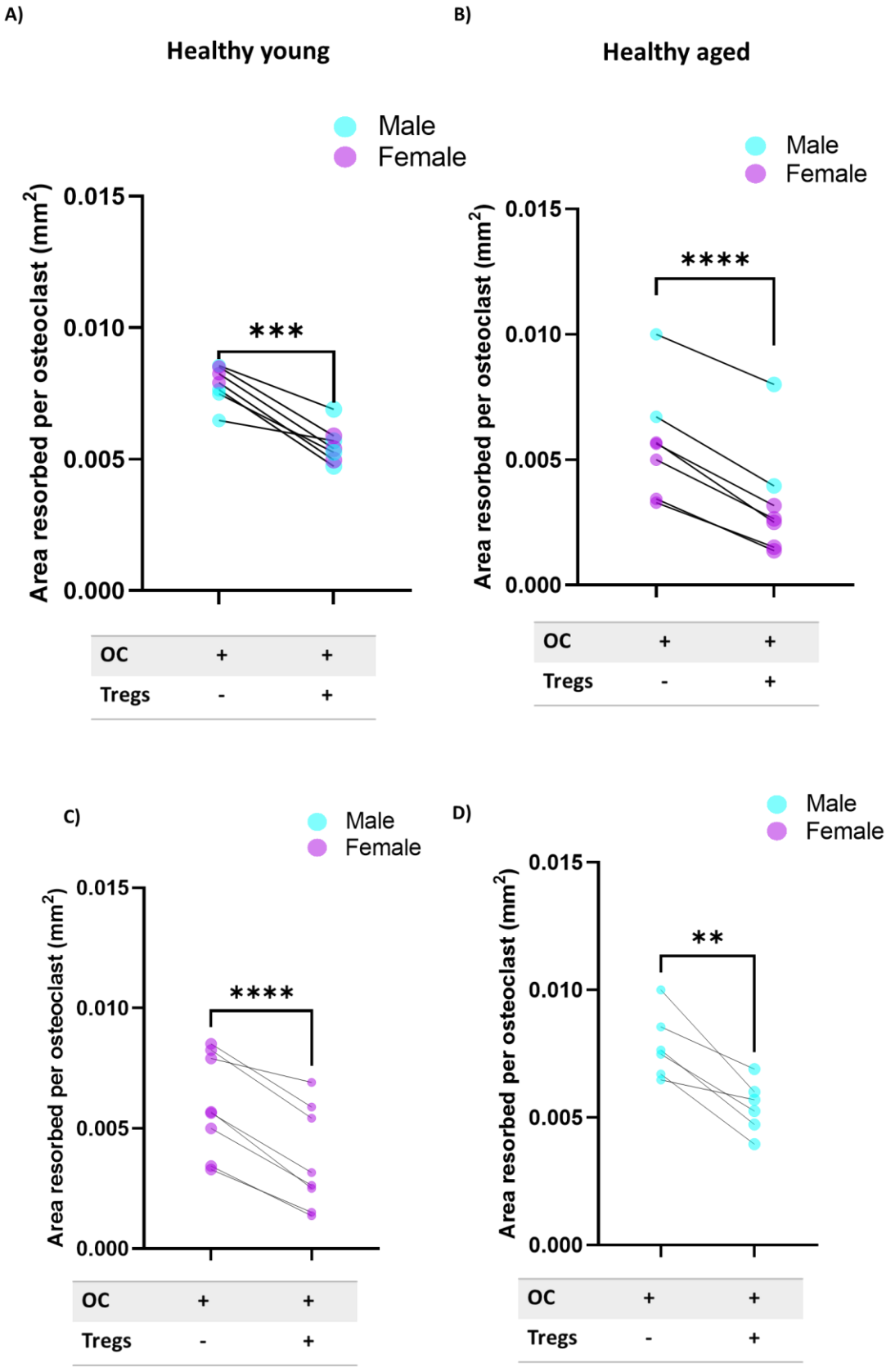
A)
Healthy young
Male
Female
Area resorbed per osteoclast (mm²)
0.015
0.010
0.005
0.000
***
OC + +
Tregs - +
B)
Healthy aged
Male
Female
Area resorbed per osteoclast (mm²)
0.015
0.010
0.005
0.000
****
OC + +
Tregs - +
C)
Male
Female
Area resorbed per osteoclast (mm²)
0.015
0.010
0.005
0.000
****
OC + +
Tregs - +
D)
Male
Female
Area resorbed per osteoclast (mm²)
0.015
0.010
0.005
0.000
**
OC + +
Tregs - +

Figure 4.14 Effects of Tregs in osteoclasts function ageing and sex (female and male). Monocytes were cultures on dentine discs for 21 days and stimulated with 25 ng/mL MCSF and 30 ng/mL RANKL for osteoclasts differentiation and CD3/CD28 Ab for Treg activation. After 21 days osteoclasts were stained with TRAP and counted the resorption pits area was measured using imageJ. Osteoclast's function showed significant decrease when cultured in the presence of Tregs in both, healthy young participants (A) and healthy aged (B). Sex differences also showed significant difference in osteoclast function when cultured with Tregs in female (C) and males (D). Data shows mean with SEM and was analysed using Student T-test. Healthy young participants (N=7) and healthy aged participants (N=7) biological samples, biological samples, Male N=6 and Female N=8 *$p<0.05$

From these results it can be concluded that in this *in vitro* system, Tregs do indeed impact on the osteoclast function (**Figure 4.14**) but not on the number of osteoclasts formed.

When looking further into the changes considered by age, healthy aged osteoclasts function may be more affected by Tregs than healthy young osteoclasts. This can relate to the previous data shown in Figure 4.11 where osteoclasts function was analysed without the impact of co-culturing them with Tregs. Those results showed already a decrease in osteoclast function with ageing *in vitro*, which may relate to *in vivo* changes in bone health related to the female group caused by menopause.

These findings do not clarify if the higher impact of Treg-mediated osteoclast activity suppression is due to osteoclast activity already being reduced with ageing and thus perhaps they are easier to suppress, or if it is due to an increased suppressive function of Tregs during ageing.

Therefore, to determine if the results seen are due to the decrease in osteoclasts function during ageing or a higher suppression activity of Tregs during ageing, a further set of experiments was carried out where a combination of co-cultures from both groups young and aged was interchanged.

Osteoclasts were cultured as described before in the absence or presence of Tregs. Only in this case, Tregs were either age matched (from the same donor) or interchanged with the respective aged group (healthy young or healthy aged).

Healthy young osteoclasts were cultured with autologous Tregs and with healthy aged Tregs (from a different donor) (**Figure 4.15 A**). Healthy young Tregs had an impact on function of osteoclasts from young participants, as shown by a significant decrease in area resorbed per osteoclast ($P<0.003$). The average area of resorption produced by osteoclasts was 0.008 mm^2 per osteoclasts in the absence of Tregs and 0.005 mm^2 per osteoclasts when cultured with healthy young (age-matched, same donor) Tregs (**Figure 4.15 A**).

Moreover, when the same healthy young osteoclasts were cultured in the presence of healthy aged Tregs (from a different donor) these showed a higher decrease in osteoclast function caused by the presence of healthy aged Tregs ($P<0.0001$). The average of resorption produced by osteoclasts was 0.008 mm^2 per osteoclasts in the absence of Tregs and 0.003 mm^2 per osteoclasts when cultured with healthy aged (from a different donor) Tregs (**Figure 4.15 A**). These results suggest that Tregs from healthy aged participants present a higher suppressive capacity than the Tregs from healthy young donors since the osteoclasts function is being suppressed at a higher level.

In order to corroborate that, the same combination of cultures with Tregs from healthy young and healthy aged interchanged with healthy aged osteoclast was also explored.

Healthy aged osteoclasts were cultured with healthy young Tregs (from a different donor) and with autologous Tregs (**Figure 4.15 B**). Healthy young Tregs did not exert any significant effect on healthy aged osteoclast's function. A trend can be identified showing a reduction in the osteoclast activity; however, no significant difference was found in the area resorbed per osteoclast when healthy aged osteoclasts were cultured in either absence or presence of healthy young Tregs. The average of resorption produced by osteoclasts was 0.007 mm^2 per osteoclasts in the absence of Tregs and 0.006 mm^2 per osteoclasts when cultured with healthy young Tregs (from a different donor) (**Figure 4.15 B**). However, when the same healthy aged osteoclasts were cultured in the presence of healthy aged Tregs (aged matched, same donor). These produce a significant decrease in osteoclasts activity (P=0.001). The average of resorption produced by osteoclasts was 0.007 mm^2 per osteoclasts in the absence of Tregs and 0.002 mm^2 per osteoclasts when cultured with healthy aged Tregs (aged matched, same donor) (**Figure 4.15 B**).

Altogether, these data suggest that the effects of Tregs in osteoclast function is not caused by the natural decrease of osteoclast function occurring with healthy ageing, and rather, it is in fact caused by an increased ability of Tregs to suppress osteoclast function, which has been shown in the previous results to be greater with ageing (**Figure 4.14**).

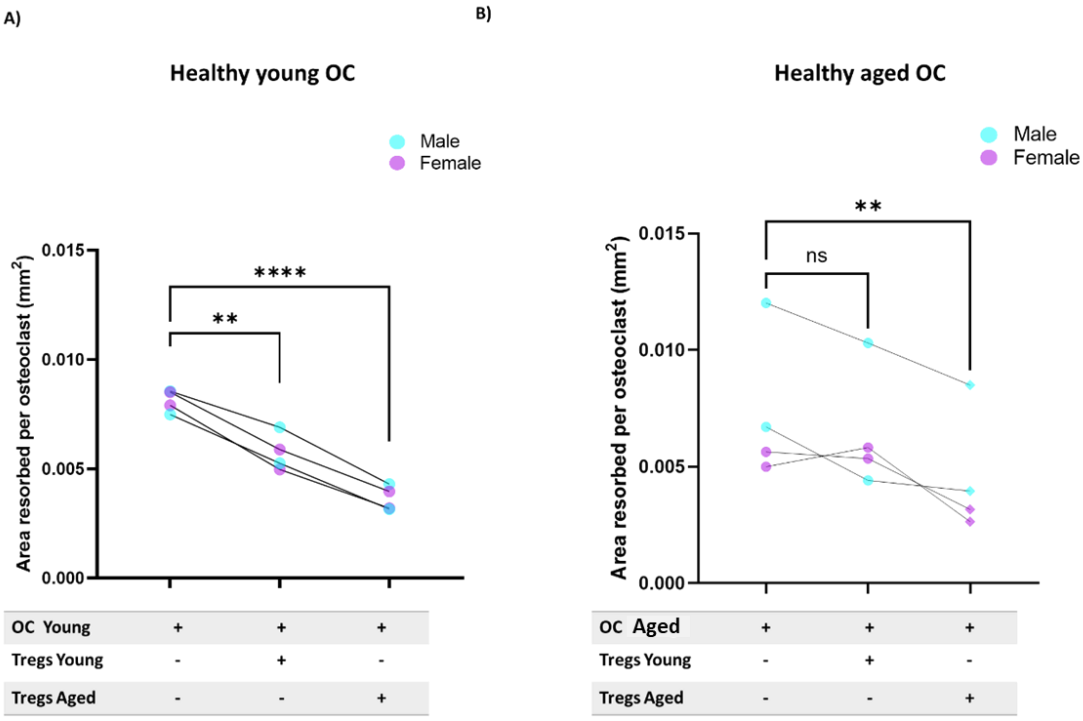

Figure 4.15 Effects of aged-matched or non-aged matched Tregs in osteoclasts function. Monocytes were cultured on dentine discs for 21 days and stimulated with 25 ng/mL MCSF and 30 ng/mL RANKL for osteoclasts differentiation and CD3/CD28 Ab for Treg activation. After 21 days osteoclasts were stained with TRAP and the resorption pits area was measured using imageJ. Osteoclasts were cultured with either aged-matched Tregs (from the same donor) or non-aged matched Tregs (from another donor). Osteoclast's function showed significant decrease in healthy young osteoclasts when cultured in the presence of both, healthy young (A) and healthy aged Tregs (B). Data shows mean with SEM and was analysed using Student T-test. Healthy young participants (N=4) and healthy aged participants (N=4) biological samples, *p<0.05

4.6 Discussion

Osteoclasts are the bone resorbing cells. Together with osteoblasts and osteocytes they maintain bone turnover by keeping a balance in the bone remodelling process. During the development of bone disease, there is an imbalance in the bone remodelling process which can be affected by the rate of osteoclast formation, leading to a high resorption of bone. Osteoclast's function can be regulated by several factors as discussed in Chapter 1, however in this chapter, I focused on examining the interaction of osteoclasts and Tregs. Tregs, are part of the immune system and their function is to suppress and regulate other immune cells' function. Tregs have been shown in the past to interact with osteoclasts consequently suppressing osteoclasts activity (Bozec & Zaiss, 2017).

In this Chapter I have validated a method to study osteoclast function *in vitro* using monocytes (osteoclasts precursors) from whole blood and differentiating them into osteoclasts by stimulation with RANKL and MCSF by following the previous worked done by (Agrawal et al., 2012). This is key to study osteoclast's function and to develop novel treatments for bone diseases.

Two different methods of monocytes sorting were explored during this Chapter FACS sorting (**Figure 4.1**) and magnetic beads sorting (**Figure 4.2**). Both showed a high level of purity for monocytes obtained, with both methods the purity of the monocytes obtained was over 90% of monocytes in the sample. Here I have validated that both methods are reliable to isolate a population of pure monocytes from PBMCs and either could be used to further culture them for osteoclast

formation. However, given the restriction to access the FACS sorting facilities during the pandemic all the following experiments were done using magnetic beads sorting. Similarly, this approach was also adopted for Treg sorting.

Suppression assays of Tcons proliferation showed that Tregs sorted by both methods were able to suppress Tcons proliferation. Tregs sorted by FACS sorting showed a higher level of suppression (**Figure 4.7**) than Tregs sorted using the magnetic beads kit (**Figure 4.9**). Levels of suppression were low in both methods. This could be because the culture conditions used were those previously optimised for setting up the osteoclasts assay, and the media used differs from what has previously been used for Tcells suppression assay. In this case, α-MEM media was used for osteoclast culturing since it was used in previous literature as it contains ascorbic acid which stimulates differentiation of murine osteoclasts (Ragab et al., 1998). Treg assay based on literature are usually performed using RPMI medium, which contain the reducing agent glutathione and PABA protein not found in α-MEM. Several studies have demonstrated the importance on selection of the appropriate culture media to support efficient expansion and function of the cell population of interest (Arora, 2013). Even though the data presented in this Chapter showed that Tregs cultured in α-MEM presented proliferation, ascorbic acid in the media has been shown to reduce their suppressive function (Oyarce et al., 2018). During this study, I prioritised culturing conditions for osteoclasts, and I can conclude the Tregs were able to suppress Tcons proliferation, even if it was less efficient as expected based on literature as a consequence of the culturing conditions.

Osteoclastogenesis was first studied in the absence of Tregs and differences between groups (healthy young and healthy aged) were observed. Further analyses grouping the samples by sex independently of age were done given some of the findings in the age group. Two different analyses were done through the osteoclastogenesis process, first the ability of osteoclasts to form from monocytes (osteoclasts count) and finally the osteoclast activity (resorption produced per osteoclast).

In the absence of Tregs, the number of osteoclasts formed was not affected by either age or sex (female or male) since no significant difference was found in between any group (**Figure 4.11**). However, the function of those osteoclasts formed was lower in the healthy aged group compared to the healthy young group. Becerikli, M et al., 2017 showed that, expression of osteoclastogenesis markers RANKL, NFATc1 and TRAP were enhanced in young bone when compared to aged individuals. Moreover, they also showed that young bone shows elevated osteoblast activity by an increased expression of RUNX2 and osterix (Becerikli et al., 2017). Overall findings in this Chapter, suggesting a dysregulation in osteoclast function linked with aged matched the literature.

In the age group the samples from female participants seem to follow a trend of being lower, representing less functional osteoclasts in the female group, while in the younger group male and female samples seem to be grouping around the same values, showing similar function on osteoclasts regardless of the sex of the participant.

Changes in osteoclast function during ageing may be related to a decrease in oestrogen levels during menopause or age. During menopause there is an increased osteoclastic resorption activity(Møller et al., 2020b) . Oestrogen inhibits osteoclast activity, hence lower levels of oestrogen due to menopause will result in higher resorption activity (Kameda et al., 1997).. Oestrogen also causes downregulation of FasL on osteoclasts, which will result in a prolonged life span of osteoclasts. Moreover, a decrease in oestrogen has also been shown to trigger an increased expression of MCSF and RANKL in osteoblasts. This is linked with a boost in osteoclast formation since both key cytokines driving osteoclastogenesis (Novack, 2007). Findings in this Chapter suggested a downregulated osteoclast activity *in vitro,* however the experimental design did not control for oestrogen levels hence we believe that changes found in osteoclast activity may have been inherited from osteoclasts precursors, monocytes and macrophages, this may be explained by sexual dimorphism correlated to structural gender differences in bone mass (Lorenzo, 2020).

The literature suggests an increase in resorption activity in post-menopause females. However, the data in this Chapter has shown a decrease in osteoclast activity with ageing in the female population. This study does not have information regarding the menopausal status of the participants or HRT status and no final conclusions as to whether the lower osteoclasts activity in the female group is due to oestrogen imbalance could not be taken. The sample population in this study was not big enough in participants number to be able to do statistical analyses based in aged differences caused by sex. In this case, all samples were grouped together and further analyses by sex showed a lower osteoclast function in the

female population when compared to the male population (**Figure 4.12**). Even though this cannot be conclusive data given that it was not possible to look at aged sex matched differences, it is a possibility that lower osteoclasts activity is related to sex differences more than age causes and that the differences seen in the healthy aged population compared to the healthy young population is caused by the sex of the participants.

When studying the effects of Tregs on osteoclast numbers this Chapter showed that neither age nor sex had an impact on the number of osteoclasts formed from monocytes in the presence of activated Tregs. Tregs have been shown to inhibit osteoclasts formation from their precursors (macrophages) by the release of inhibitor cytokines (IL-4, IL-10) or by direct cell contact. The cell contact inhibition is mediated by CTLA4, which binds directly to CD80/86 on macrophages (osteoclasts precursors) (Axmann et al., 2008) inhibiting osteoclast formation and inducing apoptosis of osteoclasts precursors by TNF-*α* inducing Fas expression and IFN-*γ*, IL-12, and IL-18 induce FasL (Kitaura et al., 2013)

Tregs showed to produce a high impact on osteoclasts function in both groups healthy young and healthy aged. Moreover, osteoclast function was also affected by Tregs in regard to sex. Osteoclasts on the female group had a higher level of suppression produced by Tregs (**Figure 4.14**).

The higher suppression levels from female aged participants may be related to menopause related changes. Previous studies showed that Treg suppressive function on osteoclasts was enhanced in the presence of oestrogen, suggesting

then the role of oestrogen in mediating regulation of bone metabolism and its protective function (Bhadricha et al., 2021). This suggest that Tregs isolated from peripheral blood in higher oestrogen levels (i.e. young population), may have developed a higher suppressive function as we are seeing in *in vitro* cultures. Data in this Chapter showed a higher suppressive function in the aged population, which theoretically will have lower levels of oestrogen (female menopause). However, since the information regarding menopausal or HRT state levels is not available, we cannot assume the oestrogen levels being lower.

Moreover, mice studies have demonstrated that Tregs from aged mice produce higher impact on dendritic cells when compared to Tregs from young mice (Garg et al., 2014). Since dendritic cells present a similar mechanism of action as macrophages (osteoclasts precursors), we speculated that Tregs effect seen in osteoclasts may be due to the higher suppressive function in aged Tregs, following the similar mechanism of action as dendritic cells.

Oestrogen is also involved in activating vitamin D, declining levels of oestrogen (characteristics of menopause) could lead to vitamin D deficiency. Vitamin D can induce Foxp3 transcription factor involved in the development and function of Tregs. Hence, lower levels of vitamin D during menopause may be linked to with a lower suppressive activity of Tregs (Fisher Id et al., 2019).

Overall, this Chapter has shown that Tregs suppress osteoclast activity, with this suppressive activity being higher in the aged and female groups. However, it could not be concluded as if this effect is caused by Tregs effects on osteoclasts or mainly by the decrease of osteoclasts function caused by natural ageing.

Finally, this Chapter concluded that the suppressive effects of Tregs in osteoclast function is not caused by a natural decrease of osteoclast function with ageing, but rather it is dependent on the age of the Tregs as shown in the final experiment in this Chapter (**Figure 4.15**).

Chapter 5

Spatial Localisation of Tregs in healthy and pathological ageing and osteoarthritis

5.1. Introduction

Bone is a living organ that undergoes changes with development and ageing. With ageing the normal skeletal functions become impaired, bone becomes fragile affecting its ability to perform the mechanical functions (Florencio-silva et al., 2015). However, distinguishing between normal ageing related changes and those caused by diseases is a complex process. This contradiction can cause controversy over the diagnosis and treatment of some of the most common bone diseases.

Bone related diseases share a dysregulation of the immune processes that target the bone tissue causing adverse effects on bone structure and function (M. Wang et al., 2013). Regulatory T cells (Tregs) are part of the adaptive immune response and play an important role in maintaining peripheral tolerance, protecting the individual from autoimmunity, and limiting chronic inflammatory diseases (Dias et

al., 2017). Tregs may also play an important role in the development of bone related diseases by regulating bone remodelling. Tregs can inhibit formation of osteoclasts by secretion of suppressive effector IL-4 and IL-10 (Bozec & Zaiss, 2017), or by direct contact via CTLA-4 which binds to monocytes through the CD80/CD86 surface marker and can inhibit RANK and TNF, resulting in a decrease in osteoclast formation (Axmann et al., 2008). Moreover, Tregs can also affect the function of osteoclasts by inhibiting the sealing zone formation (Dohnke et al., 2022; Roscher et al., 2016). This is the mechanism by which osteoclast form a complex with actin and “seal” the osteoclast and the bone matrix allowing for the release of hydrogen ions and proteolytic enzymes within the Howship’s lacunae at the resorption site, creating a highly acidic environment that results in the bone breakdown (Song et al., 2014).

Tregs are required for immunological homeostasis and have been proven to reside in specific tissues (Chen et al., 2006). Specifically, Tregs exhibit a distinctive ability to return to and persevere in their tissue of origin (bone marrow). In the homeostatic condition, bone marrow harbours high levels of $CD4^{+}Foxp3^{+}$ Tregs (Camacho et al., 2020). Treg cells may create an immune suppressive niche in bone marrow. This is important during potential inflammation and for Tregs interaction with other hematopoietic cells.

However, the presence of any alteration in the Treg cell compartment due to ageing or osteoarthritis development remains unknown. As shown in the previously Chapters, Tregs inhibit osteoclast function when co-cultured however, this may differ when looking at the tissue level in diseases such as osteoarthritis. Tregs are

found in the bone marrow, hence they are naturally in proximity with osteoclasts in bone areas of the joint.

To support temporal studies on osteoarthritis pathology and development, various animal models have been established to help overcome the limitation of studies on human patients, which only present at late stages of osteoarthritis. Animal models are particularly considered to be beneficial in studying the early processes of the disease, where access to human samples is almost impossible. The most well characterised animal models of osteoarthritis are inducible - surgical destabilisation of the joint, or natural - where osteoarthritis development happens spontaneously with ageing. These are all available in mice, which is a well-characterised model to study osteoarthritis development and diseases progression (Bendele, 2001).

The surgical destabilization of the medial meniscus (DMM) in mice is one of the most frequently used for osteoarthritis models. DMM is an instability model and involves transecting the anterior attachment of the medial meniscus to the tibial plateau. This releases a small part of the medial anterior horn, increasing osteoarthritis development by increasing loading on the tibia (Culley et al., 2015). The advantages of using DMM model for osteoarthritis is that this model simulates a gradual development over a long time replicating the human condition and allows the study of osteoarthritis pathophysiology from early to late stage. Moreover, DMM is an invasive procedure, which may be associated with additional inflammation and cartilage damage because of the procedure (Alves et al., 2020).

In addition, the short life span in mice also allows for ageing studies. This allows the study of naturally occurring osteoarthritis, thought to reproduce many characteristics of primary human osteoarthritis. One well-recognized model which develops a natural form of osteoarthritis is the STR/ort (Staines et al., 2017). However, these models present some limitations since mice will naturally develop osteoarthritis naturally with ageing, and this factor cannot be experimentally controlled (Fang et al., 2018a).

Being able to determine the area of bone where the Treg niche localise, and the possible changes during ageing and osteoarthritis development, is crucial to understanding their interaction with osteoclasts. Hence, in this Chapter I sought to examine the spatial localisation of Tregs and osteoclasts in bone using osteoarthritic human samples from patients undergoing total knee replacement, and the DMM mouse

5.2. Hypothesis

Spatial changes in Tregs will be observed during ageing and osteoarthritis development when compared to healthy young samples. Higher presence of Treg populations may lead to a decrease in osteoclasts numbers.

5.3. Aims

I. Determine the quantity and spatial localisation of Tregs in the subchondral bone and trabecular bone in healthy young, healthy aged and osteoarthritic murine samples.

II. Determine the number of osteoclasts in the subchondral bone and trabecular bone in healthy young, healthy aged and osteoarthritic murine samples.

5.4. Materials and Methods

5.4.1. Human and mice samples

Human osteoarthritis samples (one female and two males aged 59-78 year) were kindly provided by Dr Anish Amin (Edinburgh Royal Infirmary Hospital) and obtained from patients undergoing total knee replacement. Fresh bone samples were decalcified and processed as per section 1.12.1.

Embedded murine samples, sex male, from DMM-model (16 weeks old, surgery performed at 8 weeks and sampled 8 weeks post-surgery) and, C57/Bl6 wild type aged model (1 year old) where kindly provided by Dr Katherine Staines (University of Brighton) (Samvelyan et al., 2021).

5.4.2. Toluidine Blue/Fast Green staining

Osteoarthritis phenotype was confirmed by histology using toluidine blue staining. Slides of 6 µm human sections and 5 µm murine sections were de-waxed and rehydrated as in section 2.12.2. Sections were then incubated in 0.4% Toluidine blue dissolved in sodium acetate and counterstained in 0.02% Fast Green in distilled water following the methods in section 2.12.3. Sections were finally rinsed in H_2O and mounted. Images were taken using EVOS microscope and analysed using ImageJ.

5.4.3. OARSI Grading Murine Samples

The OARSI system consists of a grading component, where a more aggressive biologic progression will score a higher value (Figure 5.1). Assessment of osteoarthritis severity was performed using multiple sections (6-11 sections per slide) from different intervals across the whole joint using the OARSI grading scale (Glasson et al., 2010). Scoring was conducted blindly by two observers, Dr Katherine Staines and Dr Jasmine Samvelyan.

Figure 5.1 OARSI Grading Scale. The recommended semi-quantitative scoring system for osteoarthritis diseases stages Glasson et al, 2010

5.4.4. Immunohistochemical staining of murine samples

Murine tibias from DMM and control (non-operated groups) were immunostained for Foxp3 as described in section 2.12.4. Immunohistochemical staining of 5 µm thick sections was performed using the Vectastain ABC kit. Foxp3 primary antibody was used at 1:100 dilution, whilst an anti-rabbit secondary antibody was used at 1:400 dilution. Immunohistochemical labelling was visualised using DAB chromogen. For control sections, IgG at the same concentration as primary antibody was used instead. Quantification was performed by manually counting positive stained cells and shown as a percentage of the total number of bone marrow cells.

5.4.5. Characterisation of Murine Osteoclasts

Tartare resistant acid phosphatase (TRAP) reactivity was performed using the phosphatase leukocyte kit (Sigma-Aldrich Ltd) as in section 2.12.5 according to the manufacturer's protocol. TRAP positive cells were imaged using EVOS microscope and manually counted using ImageJ.

5.4.6. Statistical Analyses

Data was checked to be normally distributed using a Shapiro-Wilk normality test using GraphPad. Data was analysed by one-way analysis of variance (ANOVA) for which suitable post-tests for multiple comparisons were conducted, or the Student's t-test. All data are expressed as the mean ± standard error of the mean

(SEM). Statistical analysis was performed using Graphpad and $P<0.05$ was considered to be significant.

5.5. Results

5.5.1. Human osteoarthritic bone

Tibia osteoarthritic samples from individuals undergoing total knee replacement surgery were used to determine the spatial localisation of Tregs in subchondral bone during osteoarthritis pathology. Samples were first stained with Toluidine Blue/Fast Green to study the cartilage and bone structure in three different human samples (**Figure 5.2)**. Histological differences were found when comparing the samples, the internal control side (lateral knee side) showed presence of cartilage (dark blue stained) when compared with the osteoarthritis site (DMM knee side, presenting osteoarthritis) where the cartilage has been slightly degraded. This therefore corroborates cartilage degradation as a consequence of osteoarthritis pathology where either total loss of cartilage can be seen (**Figure 5.2 Sample #1 and #2)** or a reduction of cartilage thickness when compared to the control site (**Figure 5.2 Sample #3).** Another characteristic change which can be seen on the osteoarthritis site samples (**Figure 5.2**), is an increase in subchondral bone thickness, caused by an imbalance in bone remodelling (Uygur et al., 2015).

Even though the samples obtained from patients undergoing total knee replacement gave a clear understanding on morphological changes during osteoarthritis development, these did not present any bone marrow in the sections. Given that Tregs are known to localise at the bone marrow (Zou et al., 2004), this samples were therefore not of use for the aforementioned aims of this Chapter.

Subsequently, further experiments were carried out using murine samples which were known to contain bone marrow.

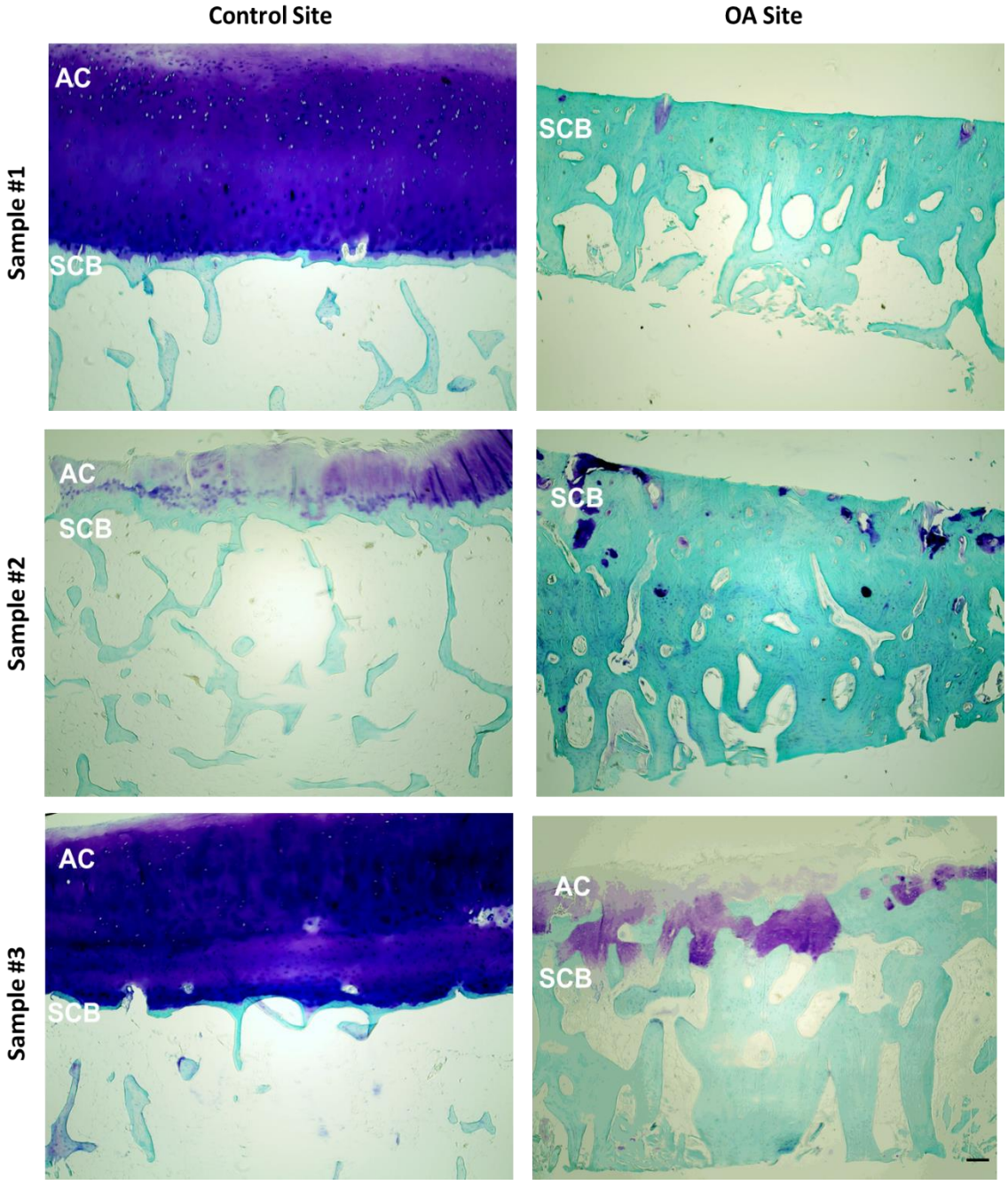

Figure 5.2 Human Osteoarthritic Tibia. Human tibia samples obtained after total knee replacement surgery, decalcified and paraffin embedded, were stained with Toluidine Blue/Fast Green to analyse cartilage and bone histology. Characteristic changes in articular cartilage (AC) and subchondral bone (SCB) are shown, such as AC degradation and increase in SCB thickness. Scale bar 100 µm.

5.5.2. Murine Samples Osteoarthritis Gradient

Histological changes in tibia sections from young DMM-operated model and aged-wild type (WT) mice were visualised by staining using toluidine blue. Changes related to the development of osteoarthritis such as articular cartilage degradation and the erosion of cartilage creating an uneven surface are seen in the DMM-model medial side in young mice (showed by arrows in **Figure 5.3 B**) when compared to the control non-operated mice (**Figure 5.3 A**), where the surface and cartilage morphology was intact presenting a normal architecture of the matrix and appropriate orientation of chondrocytes.

Moreover, the stage of the disease was calculated following the OARSI histologic scoring of murine osteoarthritis following the grading system detailed in Figure 5.1 (Glasson et al., 2010) to determine the mean osteoarthritis score and the max osteoarthritis score. Comparing the medial side of DMM-operated versus the medial side of the non-operated control there was a significant increase on the disease scoring by a 2-fold increase ($P<0.05$; **Figure 5.3 C**). On the other hand, no significance difference was found when comparing the lateral side between groups (**Figure 5.3 C**). When looking at the max osteoarthritis score, the highest value was for the DMM-operated medial side, since that side presented surface discontinuity at the superficial zone and fissures in the matrix towards the mid zone, however no changes in the subchondral bone appeared (**Figure 5.3 D**).

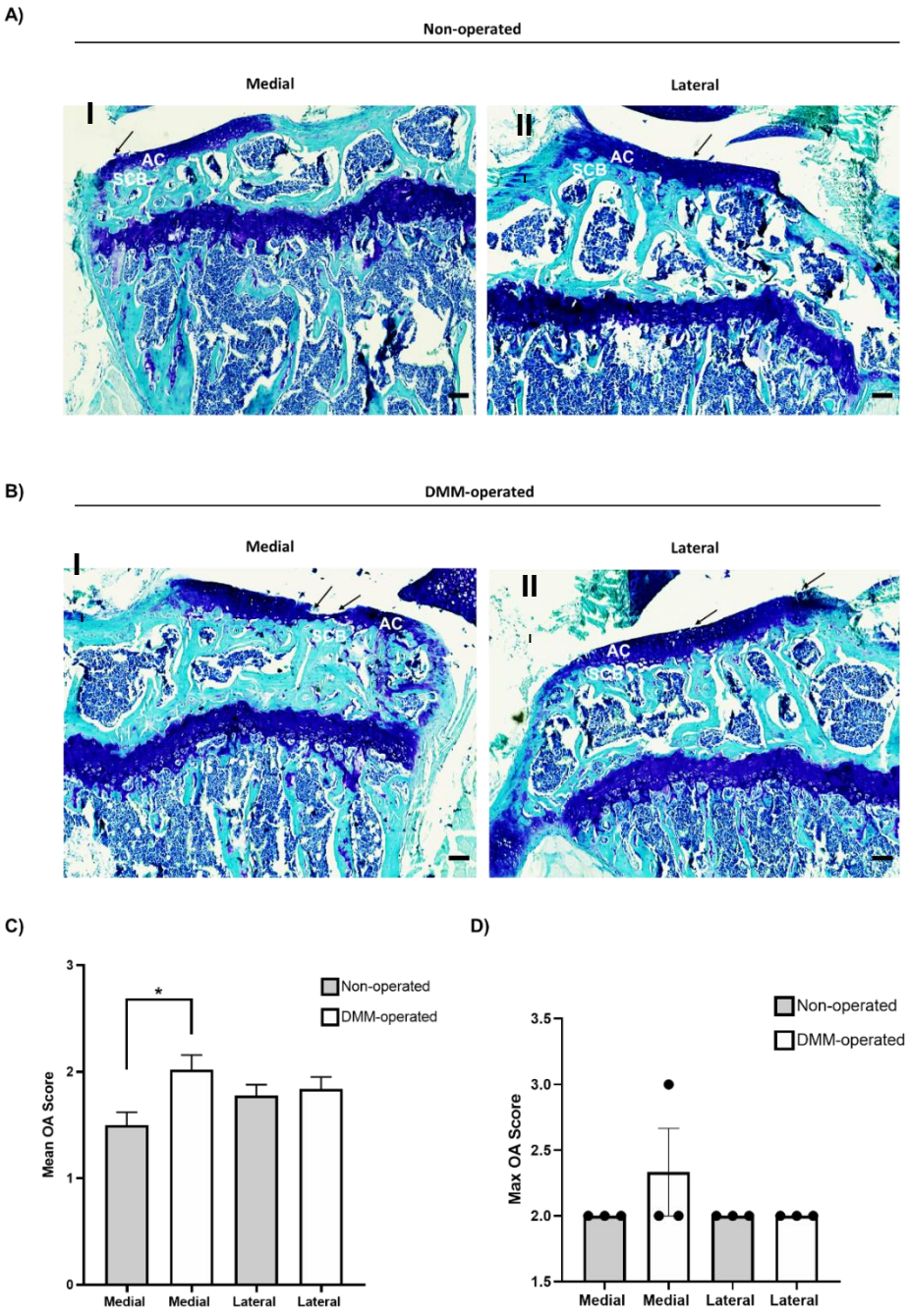

Figure 5.3 Histological analyses of the tibial plateau in young mice and OARSI microscopic scoring. Toluidine blue staining of the medial and lateral tibia in the control, non-operated group (A) and DMM-operated group (B). Histological score of the medial and lateral tibia in different groups showing the mean of osteoarthritis score (C) and the max of osteoarthritis score (D). Data is represented as mean ± SEM and were analysed using Kruskal-Wallis test, arrows point at structural changes, N=3 biological replicates scale bar 100 µm.

WT aged samples (~ 1 year old) were also stained with toluidine blue to determine the ageing-related structural changes associated with osteoarthritis grading using the OARSI method. Changes such as articular cartilage degradation, matrix vertical fissures and delamination of the superficial layer of the cartilage matrix, cartilage erosion, bone microfractures and deformation with cyst formation are present in the medial side when comparing to the lateral side (**Figure 5.4 A**).

No significant difference between medial and lateral side in the aged-WT mice were found on the osteoarthritis scoring when using the OARSI method was found. Both sides, medial and lateral presented high osteoarthritis scoring, being the average of the score 5 for the medial side and 4 for the lateral side (**Figure 5.4 B**). Moreover, the presence of late-stage osteoarthritis was confirmed by the max scores, being this 6 for both sides, medial and lateral (**Figure 5.4 C**).

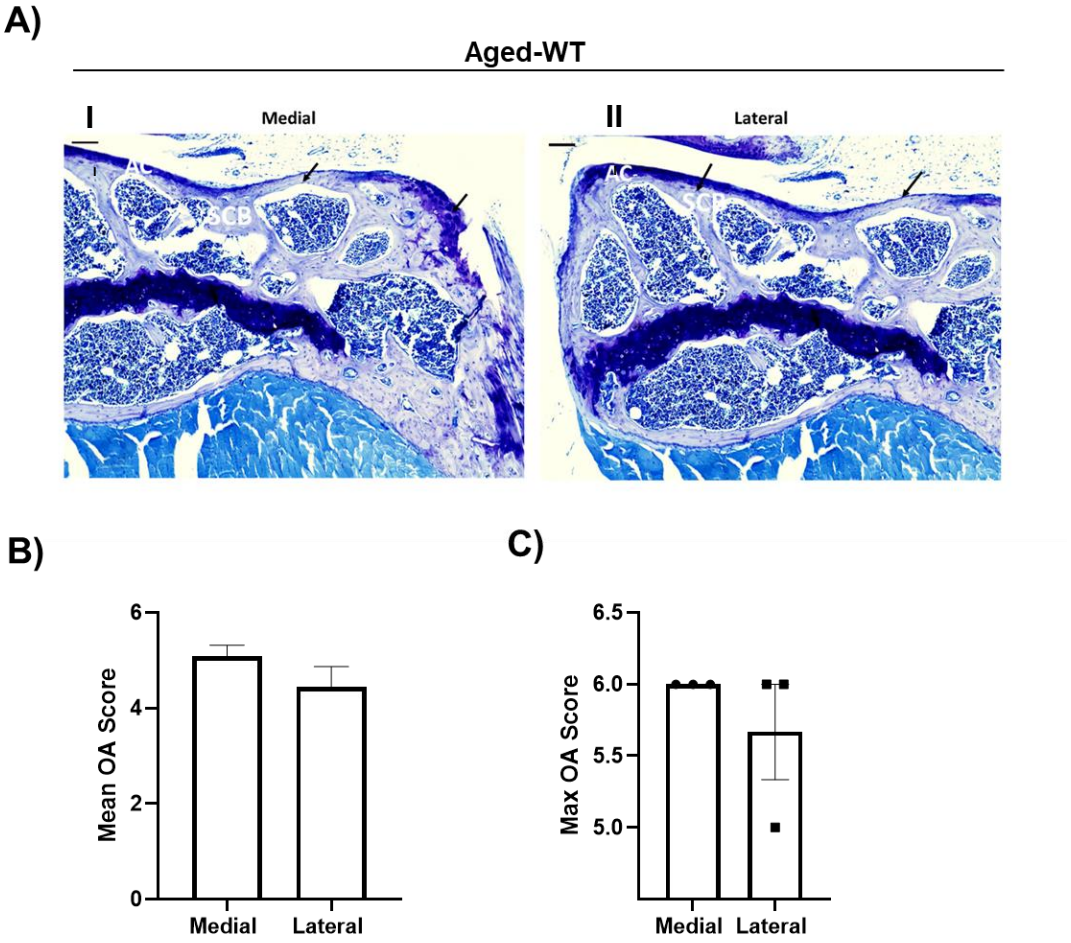

Figure 5.4 Histological analyses of the tibial plateau in aged-WT mice and OARSI microscopic scoring. Toluidine blue staining of the medial and lateral tibia in the aged WT mice (A). Histological score of the medial and lateral tibia in different groups showing the mean of osteoarthritis score (B) and the max of osteoarthritis score (C). Data are represented as mean ± SEM and were analysed using Kruskal-Wallis test, arrows point at structural changes, scale bar 100 µm.

5.5.3. Optimisation of Anti-mouse Foxp3 Antibody for Immunohistochemistry Localisation of Tregs in Healthy and Osteoarthritic Bone

Mice tibia sections were immunostained with 5 µg/ml anti-mouse Foxp3 stock at 0.05 µg/ml and 0.1 µg/ml concentrations to optimise the method for labelling (**Figure 5.5**). The best labelling was obtained when using 0.1 µg/ml concentration of anti-mouse Foxp3 antibody since this concentration allowed a clear identification of Foxp3 in the bone marrow **(Figure 5.5 D**). However, this was accompanied with strong non-specific background, when using only PBS as a negative control (**Fig. 5.5 A and C**).

Following this, several conditions for antigen retrieval were tested in order to establish the best method to reduce unspecific and background staining. The use of IgG as a negative control was also incorporated into subsequent optimisation. Different epitope retrieval buffers were trialled with the anti-mouse Foxp3 antibody on mice samples (**Figure 5.6**). This determined that the use of 0.5% pepsin buffer enhanced the identification of Foxp3 **(Figure 5.6 D**). A reduction of 30% of the concentration recommended on the manufacturer's instructions for the kit for the secondary antibody gave the optimum staining with a clear background and no non-specific staining present (**Figure 5.6**).

This concluded that the further experiments carried out during this Chapter were done using 0.1 µg/ml of anti-mouse Foxp3, using pepsin buffer as the antigen retrieval method with a 30% reduction of secondary antibody and IgG as a negative control.

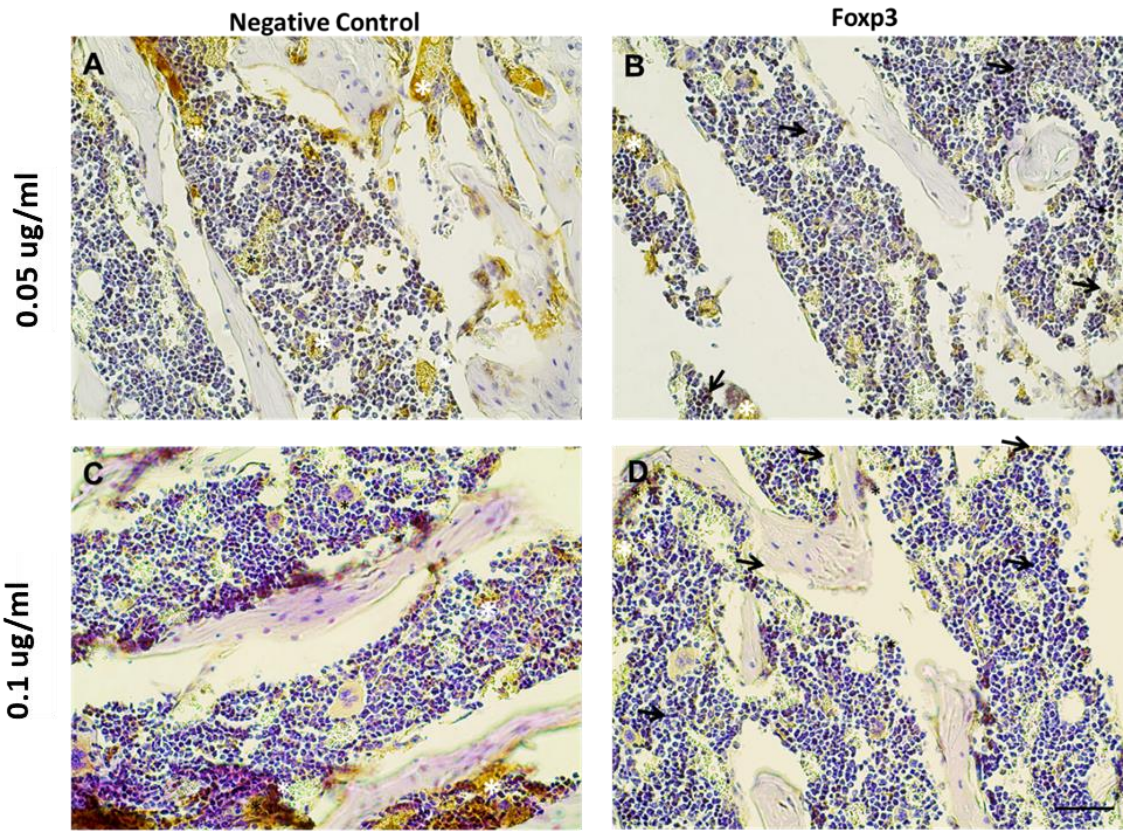

Figure 5.5 Optimisation of antibody concentration for anti-mouse Foxp3 staining. Mice tibia sections stained with anti-mouse Foxp3 0.05ug/ml (A) and 0.1ug/ml (D) concentration. Negative control PBS without IgG control (A and C). Scale bar 100 µm

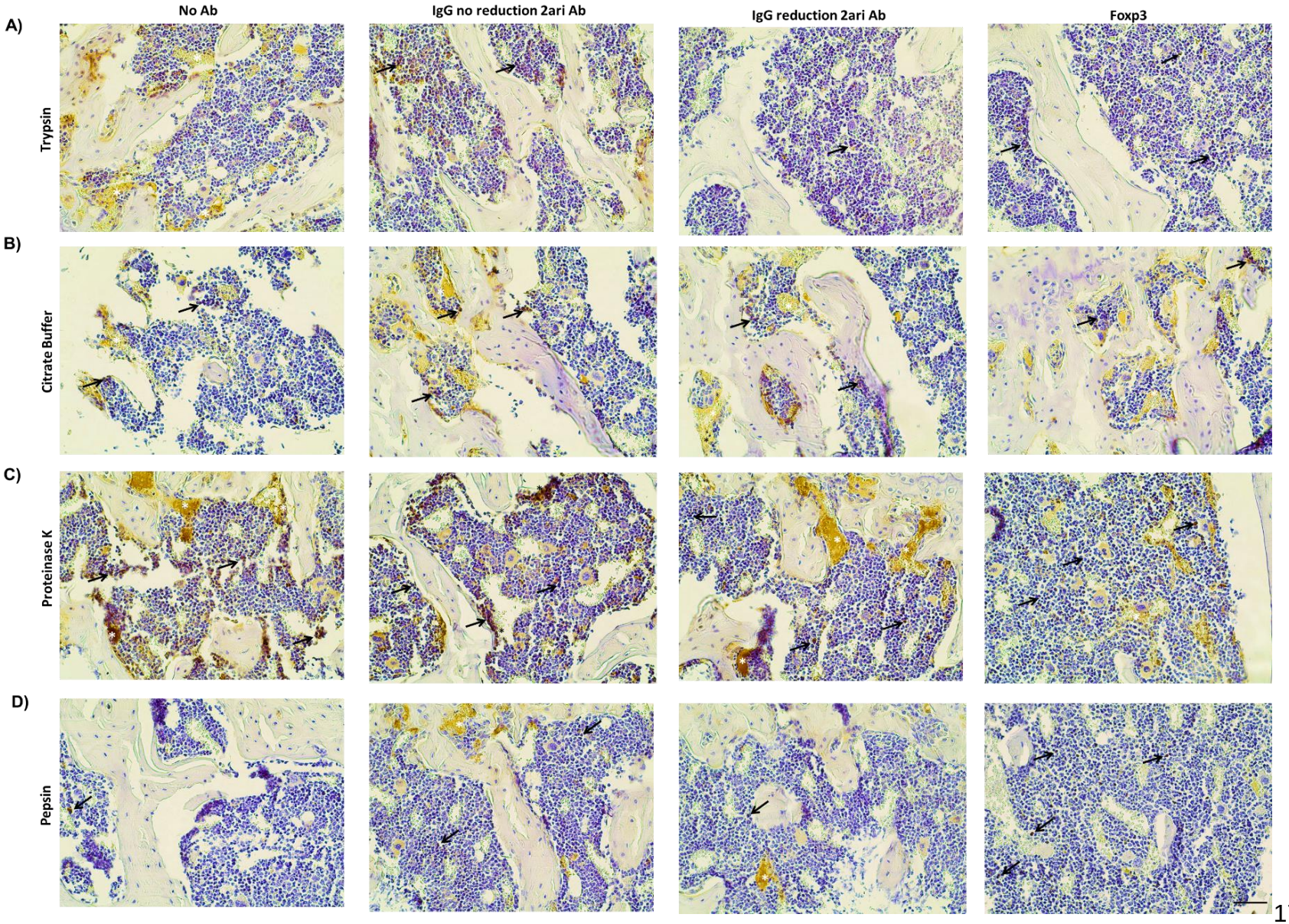
No Ab
IgG no reduction 2ari Ab
IgG reduction 2ari Ab
Foxp3
A)
Trypsin
B)
Citrate Buffer
C)
Proteinase K
D)
Pepsin

Figure 5.6 Optimisation of antigen retrieval method and secondary antibody concentration for anti-mouse Foxp3 staining. Mice tibia sections were stained with anti-mouse Foxp3. Trypsin, citrate buffer, proteinase K and pepsin were used as antigen retrieval buffers. Negative control staining was without IgG, with IgG and with IgG plus a reduction of the secondary antibody. Arrows point Tregs (Foxp3+). Scale bar 10µm

5.5.4. Subchondral bone localisation of Tregs in healthy ageing and osteoarthritis mice

The expression of Foxp3 in tibial subchondral bone on DMM-operated mice and aged-WT mice was assessed by immunostaining.

Presence of Tregs in the bone marrow can be seen in the DMM-model (**Figure 5.7 A-D**) and in the aged model (**Figure 5.8 A and B**) in both medial and lateral aspects of the tibial section. Tregs presence was higher in the DMM-model when compared to the non-operated mice in both sides medial and lateral. In the medial side thhe average for DMM-model was 10% cells while the average for non-operated 4% cells; P=0.02, On the other side, in the lateral side, the average for DMM-model 9.5% cells and the average for non-operated was 4.7% cells; however, in this case the difference was not significant P=0.66. There is no significance difference when comparing medial and lateral side on DMM-model, P=0.22 and there is significant difference in between the medial side for the DMM-operated compared to the lateral side of the non-operated, possible as a consequence of DMM-operated effects increasing the OARSI score.

An increase in Tregs localising on the tibial area can be seen with ageing, when compared to the previous described DMM-model young mice (**Figure 5.7**). Presence of Tregs in the aged mice was 35% of cells in the lateral side and 27% of cells in medial side without showing significance difference amongst the sides (**Figure 5.8 C**).

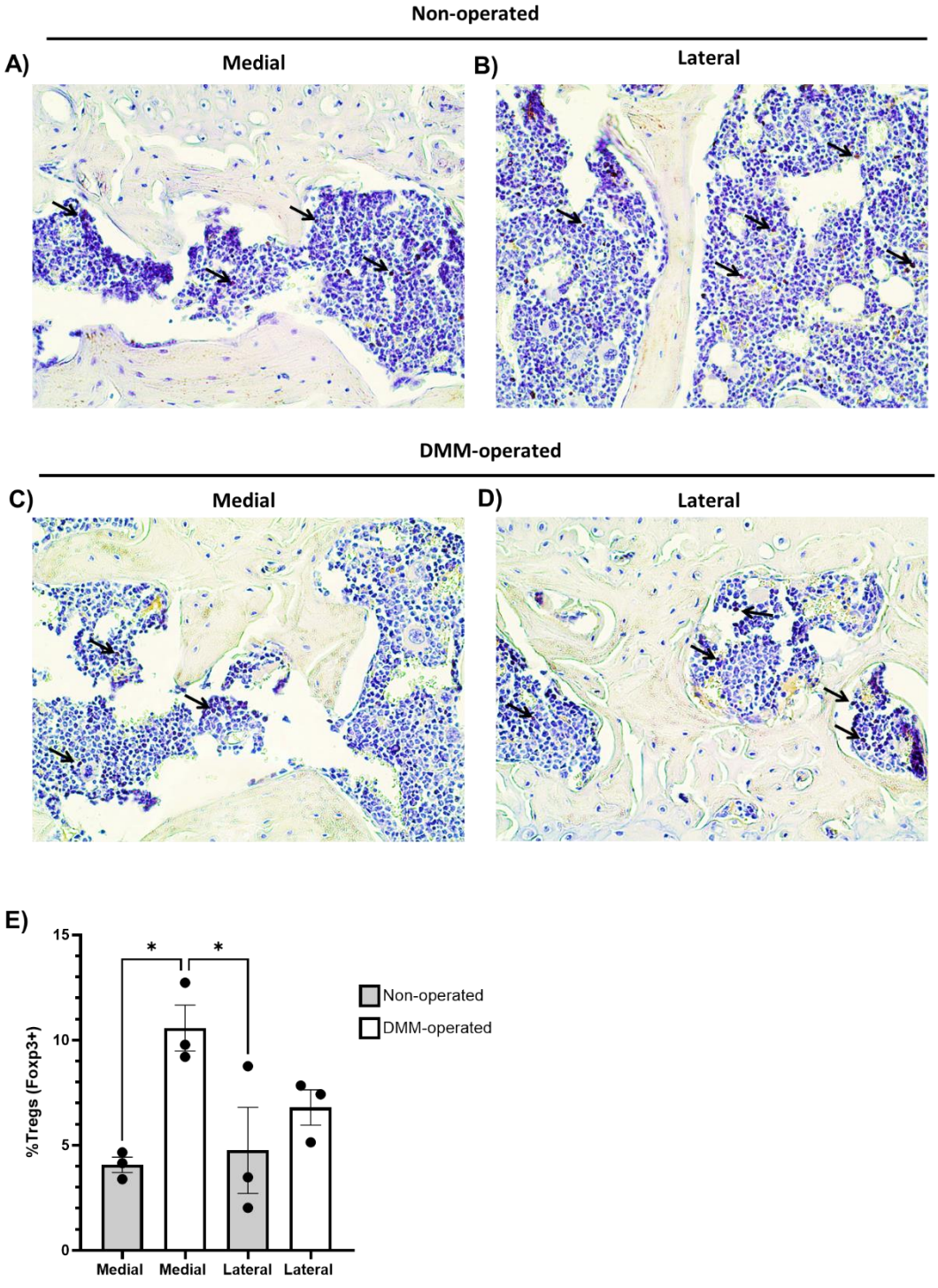
Non-operated
A)
Medial
B)
Lateral
DMM-operated
C)
Medial
D)
Lateral
E)
%Tregs (Foxp3+)
15
10
5
0
*
*
Non-operated
DMM-operated
Medial
Medial
Lateral
Lateral

Figure 5.7 Spatial localisation of Tregs in subchondral bone in DMM operated osteoarthritis mice. Mice tibia sections were stained with anti-mouse Foxp3. Negative control with IgG plus a 30% reduction of the secondary antibody. Images representative of different mice (N=3). Arrows point Tregs (Foxp3+). Scale bar 100 µm. Data shows mean with SEM and was analysed using Ordinary one-way ANOVA and Tukey's multiple comparisons *<0.05.

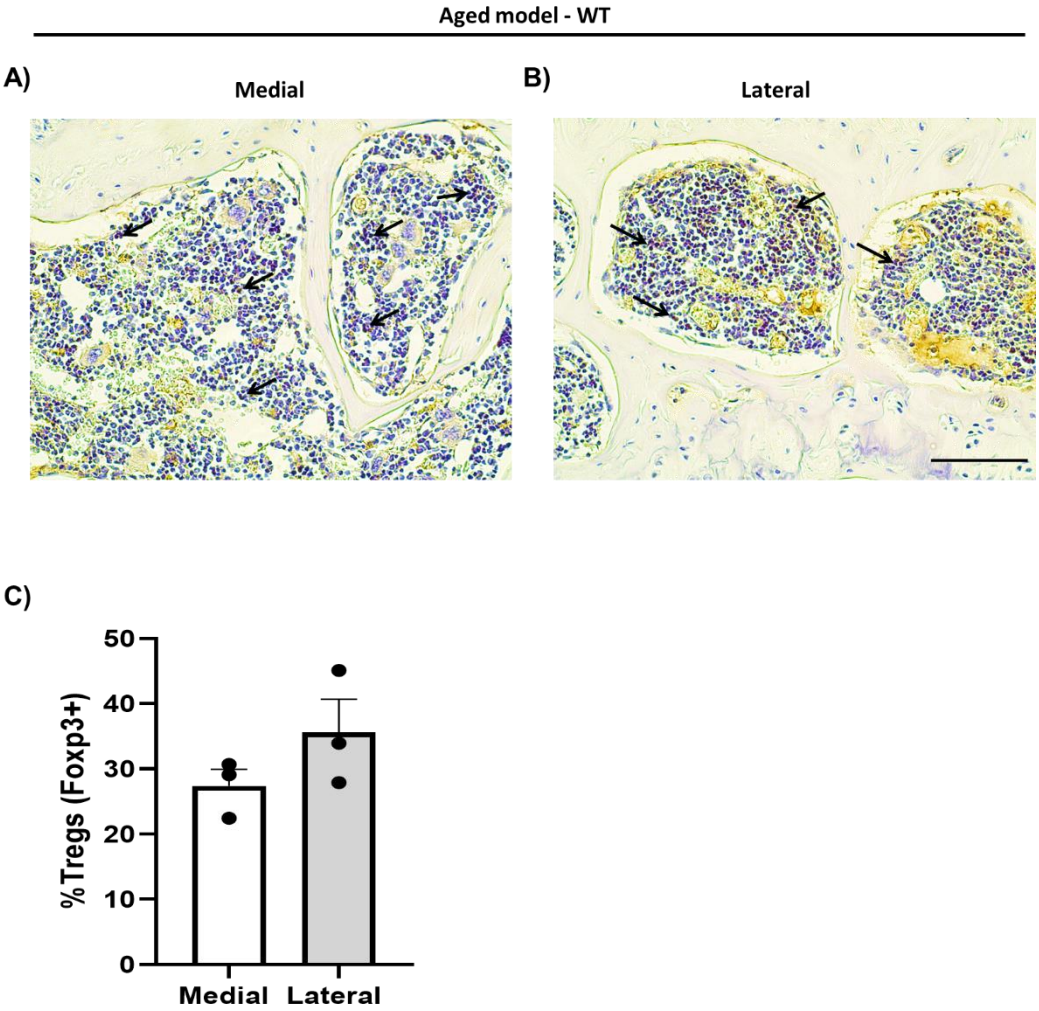

Figure 5.8 Spatial localisation of Tregs in subchondral bone in aged-WT mice. Mice tibia sections were stained with anti-mouse Foxp3 (A and B) Negative control staining was with IgG plus a 30% reduction of the secondary antibody. No significant difference was found in the % f Tregs between medial and lateral site (C). Images representative of different mice (N=3). Arrows point Tregs (Foxp3+). Scale bar 100 µm. Data shows mean with SEM and was analysed using paired T-test *<0.05.

5.5.5. Trabecular bone localisation of Tregs in healthy ageing and osteoarthritis mice

Furthermore, the presence of Tregs was analysed in the tibial bone sections in the trabecular bone beneath the growth plate in DMM-operated mice (**Figure 5.9 A-D**) and aged-WT mice (**Figure 5.10 A and B**).

Overall, no significant difference was found in between DMM-operated samples when compared with the non-operated control for either the medial or the lateral side of the tibia. In the DMM-operated mice the average of Tregs was 15% of total cells when compared to the average of 9% of Tregs of total cells in the medial non-operated control. Moreover, when looking at the lateral side of the tibia, the presence of Tregs was 15% of total cells in non-operated mice and 17% in DMM-operated mice (**Figure 5.9 E**).

Subsequently, Treg presence can be seen in the trabecular bone area of the tibial sections in the aged-WT mice (**Figure 5.10 A and B)**. Presence of Tregs in the aged mice was 23.05% of cells in the lateral side and 20.32% of cells in medial side without showing significance difference amongst the sides (**Figure 5.10 C**).

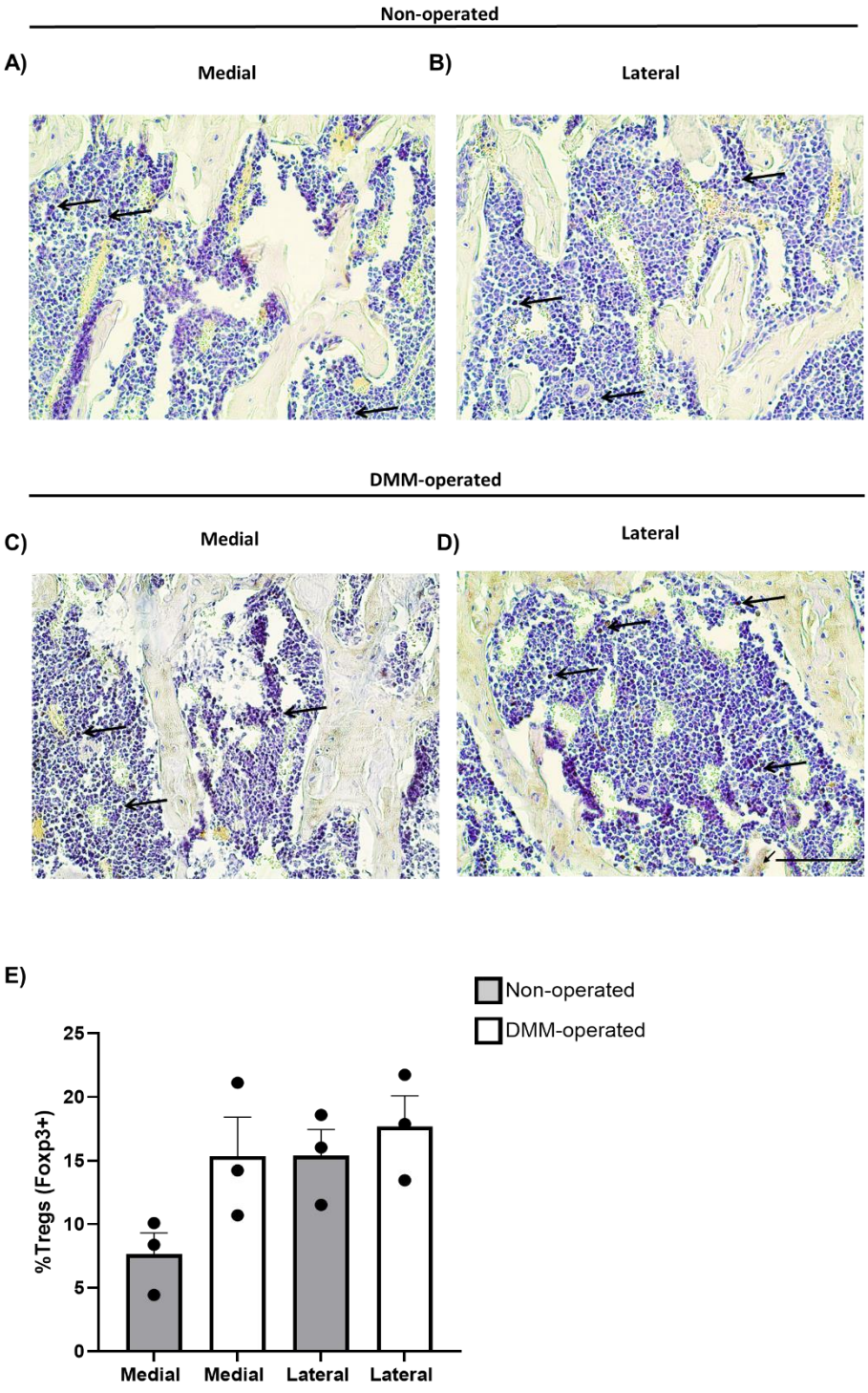
Non-operated
A)
Medial
B)
Lateral
DMM-operated
C)
Medial
D)
Lateral
E)
Non-operated
DMM-operated
%Tregs (Foxp3+)
25
20
15
10
5
0
Medial
Medial
Lateral
Lateral

Figure 5.9 Spatial localisation of Tregs in trabecular bone in DMM operated osteoarthritis mice. Mice tibia sections were stained with anti-mouse Foxp3 in non-operated medial site (A) and lateral site (B) and in DMM-operated medial site (C) and lateral site (D). Negative control staining was with IgG plus a 30% reduction of the secondary antibody. Images representative of different mice (N=3). Arrows point Tregs (Foxp3+). +). Scale bar 100 µm. Data shows mean with SEM and was analysed using Ordinary one-way ANOVA and Tukey's multiple comparisons *<0.05.

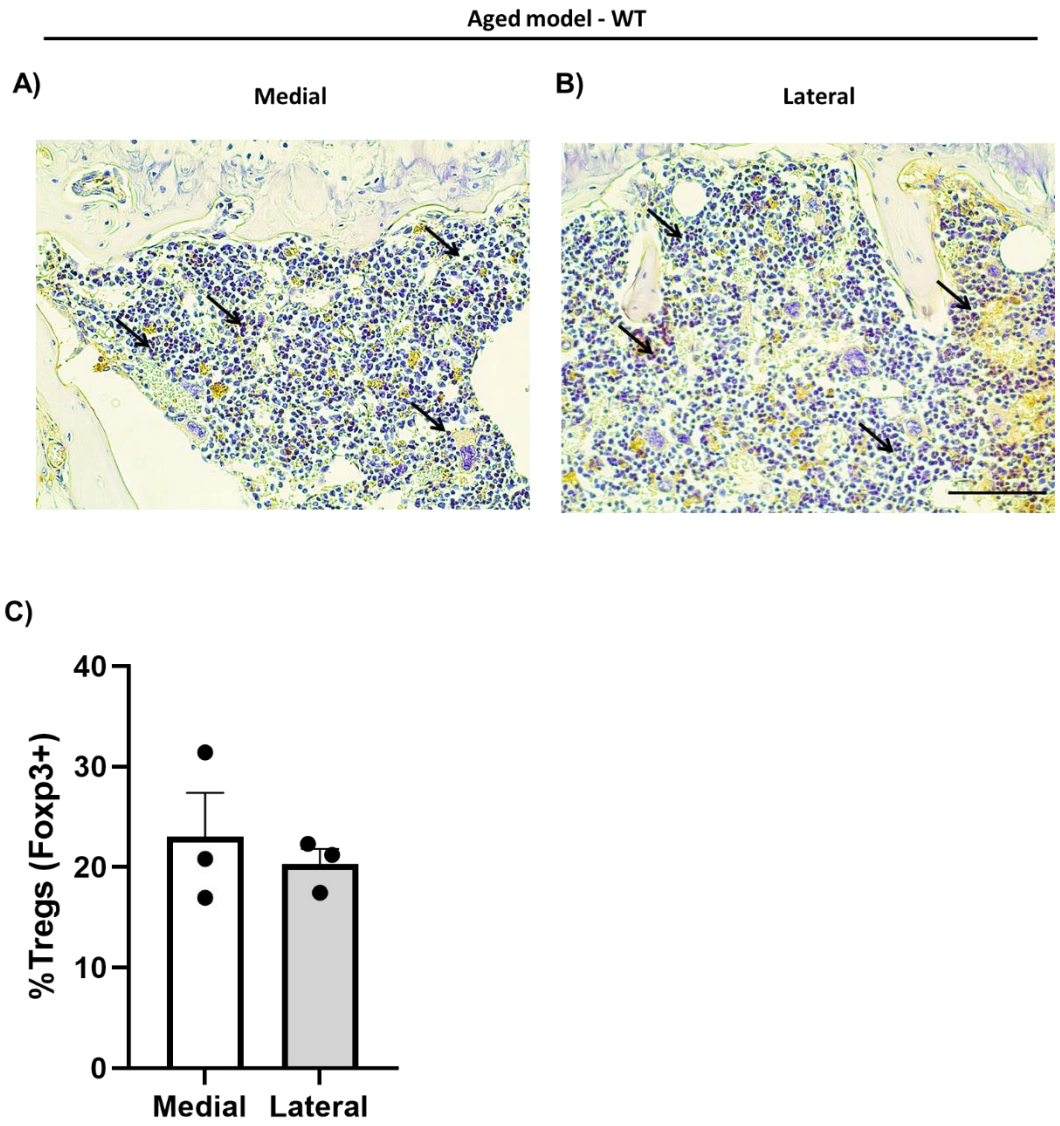

Figure 5.10 Spatial localisation of Tregs in trabecular bone in aged-WT mice. Mice tibia sections were stained with anti-mouse Foxp3 in aged-WT mice in medial site (A) and lateral site (B). Negative control staining was with IgG plus a 30% reduction of the secondary antibody. No significant difference was found in the % f Tregs between medial and lateral site (C). Images representative of different mice (N=3). Arrows point Tregs (Foxp3+). Scale bar 100 µm. Data shows mean with SEM and was analysed using paired T-test *<0.05.

5.5.6. Subchondral bone localisation of osteoclasts in healthy aged and osteoarthritis mice

Spatial localisation of osteoclasts was analysed in the tibial bone sections in the subchondral bone in DMM-operated mice (**Figure 5.11**) and aged-WT mice (**Figure 5.12**). The lack of presence of OC in the subchondral bone of healthy young and DMM model made the quantification not possible. However, we can conclude that there was no invasion of osteoclasts present in the subchondral bone area (**Figure 5.11 A-D**).

Additionally, the spatial localisation of osteoclast was analysed in aged-WT mice in the subchondral bone. There was no significant difference in osteoclast presence in neither the subchondral bone when comparing medial and lateral side of the tibia. In the subchondral bone the average of osteoclast number per bone surface was 0.05% in the medial side and 0.08% in the lateral side of the knee (**Figure 5.12 C**).

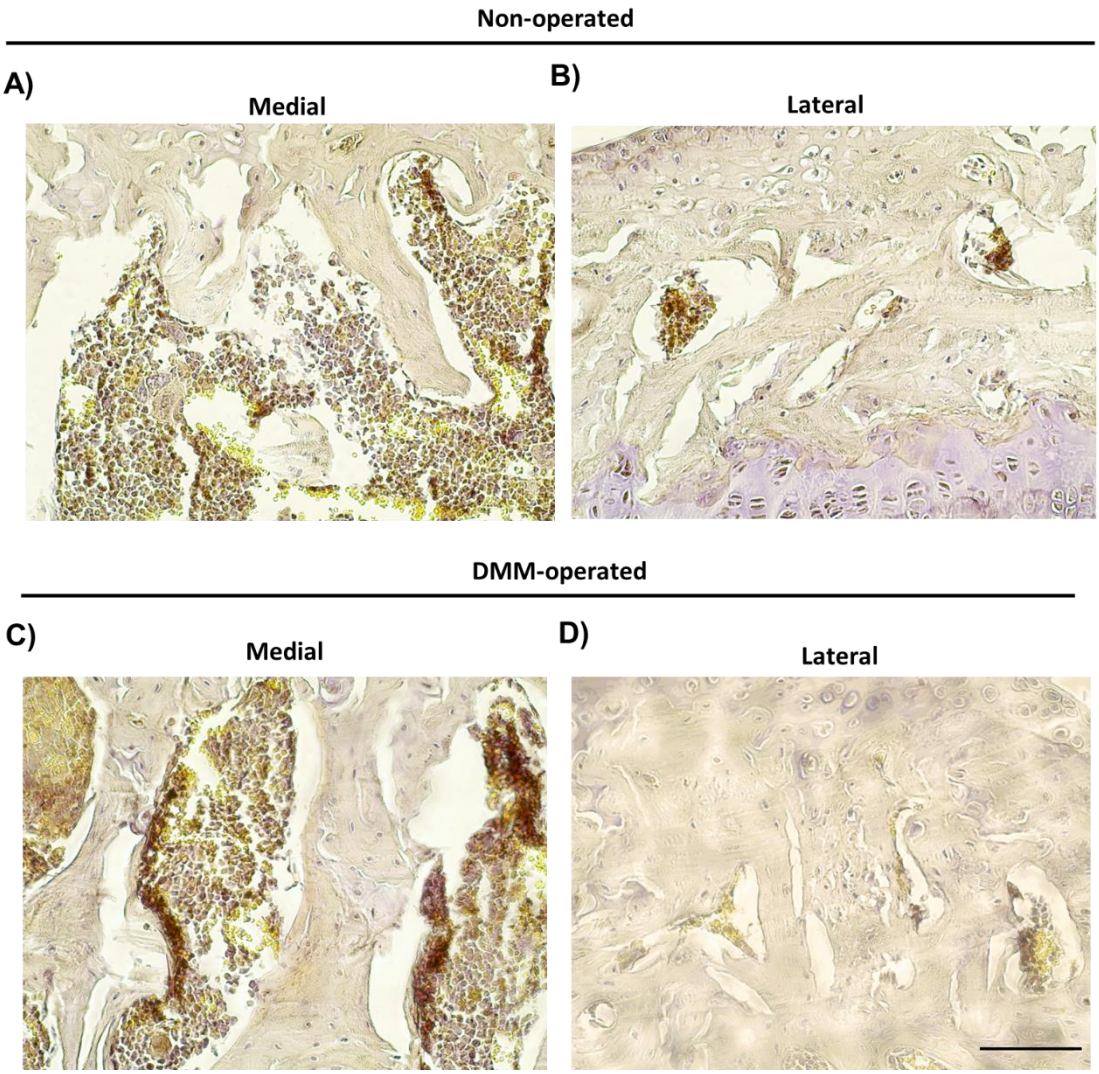

Figure 5.11 Spatial localisation of OC in trabecular bone in DMM-operated mice. Mice tibia sections were analysed with TRAP kit for OC in non-operated medial site (A) and lateral site (B) and in DMM-operated medial site (C) and lateral site (D), no TRAP positive cells were detected. Images representative of different mice (N=3). Scale bar 100µm.

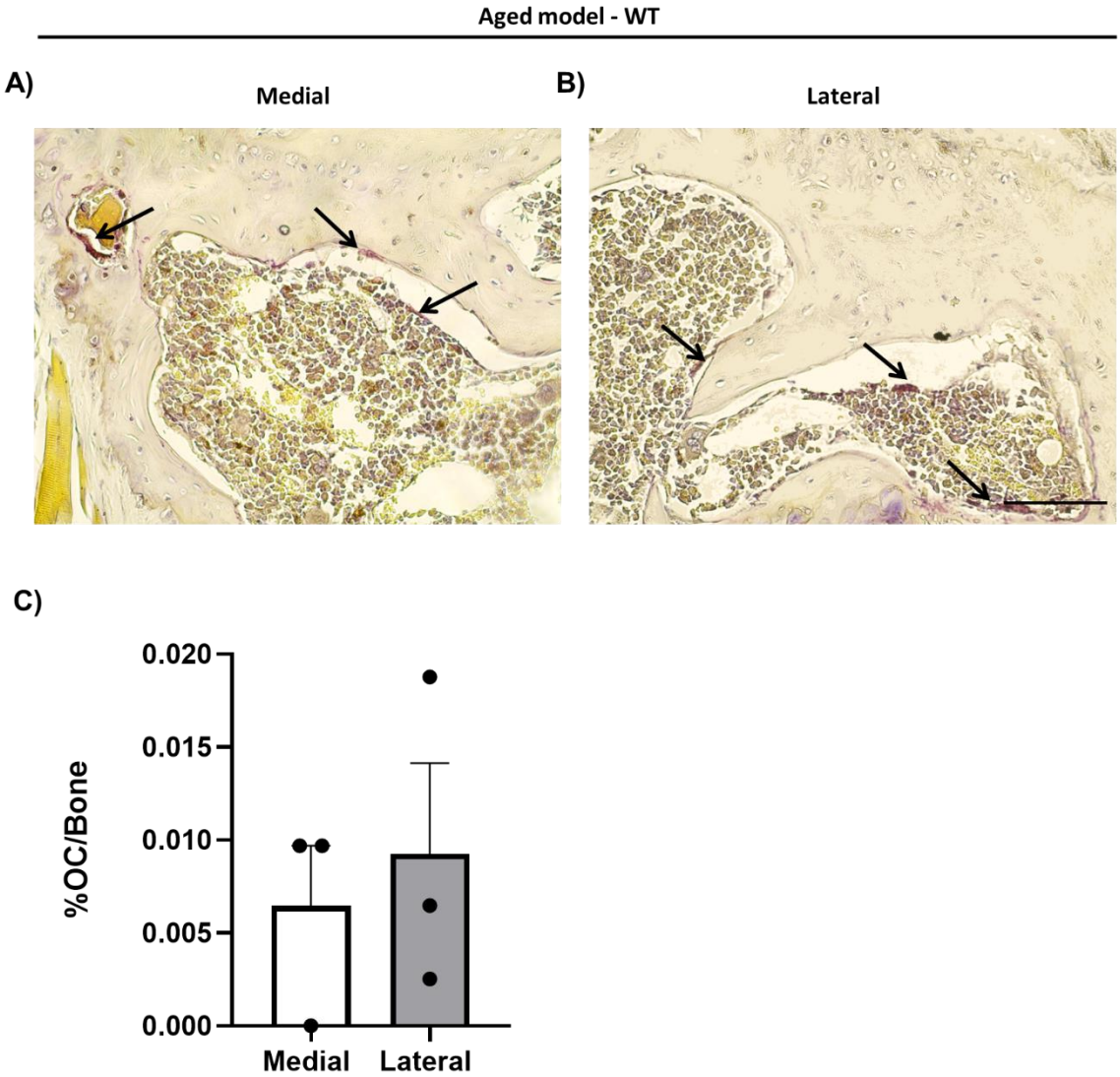

Figure 5.12 Spatial localisation of OC in subchondral bone in aged-WT mice. Mice tibia sections were stained with TRAP kit for OC in non-operated medial site (A) and lateral site (B). No significant difference was found in the number of osteoclasts between medial and lateral site (C). Images representative of different mice (N=3). Arrows point to positive TRAP-reactive OC. Scale bar 100 µm. Data shows mean with SEM and was analysed using t-student test p*<0.05.

5.5.7. Trabecular bone localisation of osteoclasts in healthy aged and osteoarthritis mice

Presence of osteoclasts was observed in DMM-operated mice (**Figure 13 A-D**) and aged-WT mice (**Figure 14 A-B**) in the trabecular bone underneath the growth plate of the tibia. In the trabecular bone there was a significant increase of osteoclast in the DMM-model medial side of the knee when compared with the control sample (average of 0.04% in the DMM- operated model and 0.01% in the non-operated control; P=0.02). Moreover, there was no significant difference in the number of osteoclasts in the lateral side of the knee when comparing DMM-operated model and non-operated control (average of 0.005% in the non-operated control and 0.025% in the DMM-operated) (**Figure 5.13 E**).

Moreover, the spatial localisation of osteoclast was analysed in aged-WT mice in the trabecular bone. There was no significant difference in osteoclast presence in either the trabecular bone when comparing medial and lateral side of the tibia. In the trabecular bone, the average of osteoclast number per bone surface was 0.014% in the medial side and 0.010% in the lateral side of the knee (**Figure 5.14 C**).

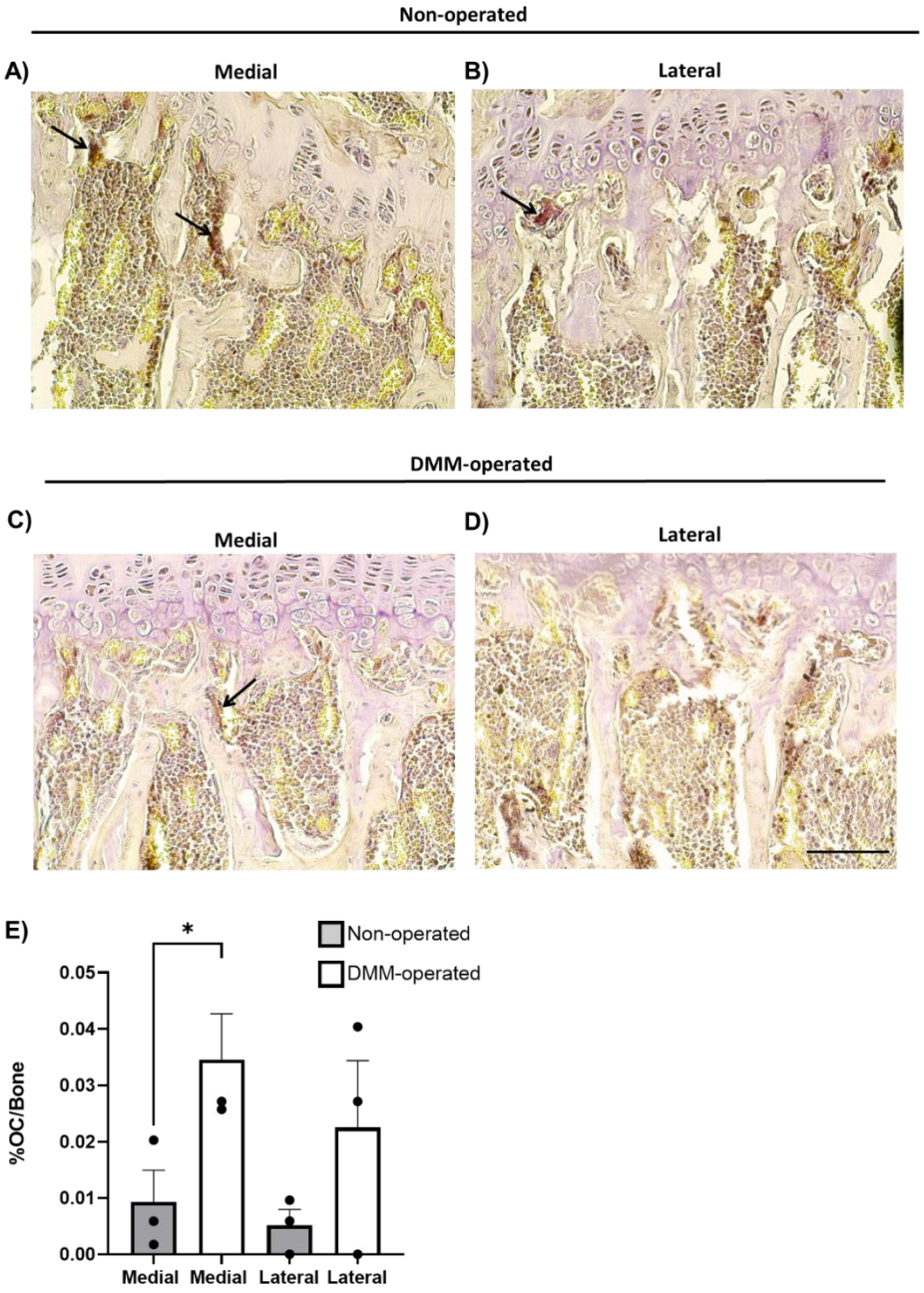

Figure 5.13 Spatial localisation of OC in trabecular bone in DMM-operated mice. Mice tibia sections were stained with TRAP kit for OC OC in non-operated medial site (A) and lateral site (B) and in DMM-operated medial site (C) and lateral site (D). Significant difference was found between non-operated and DMM-operated in the medial site and no significant difference was found in lateral site (E). Images representative of different mice (N=3). Arrows point to positive TRAP-stained OC. Scale bar 100µm. Data shows mean with SEM and was analysed using one way- ANOVA p*<0.05.

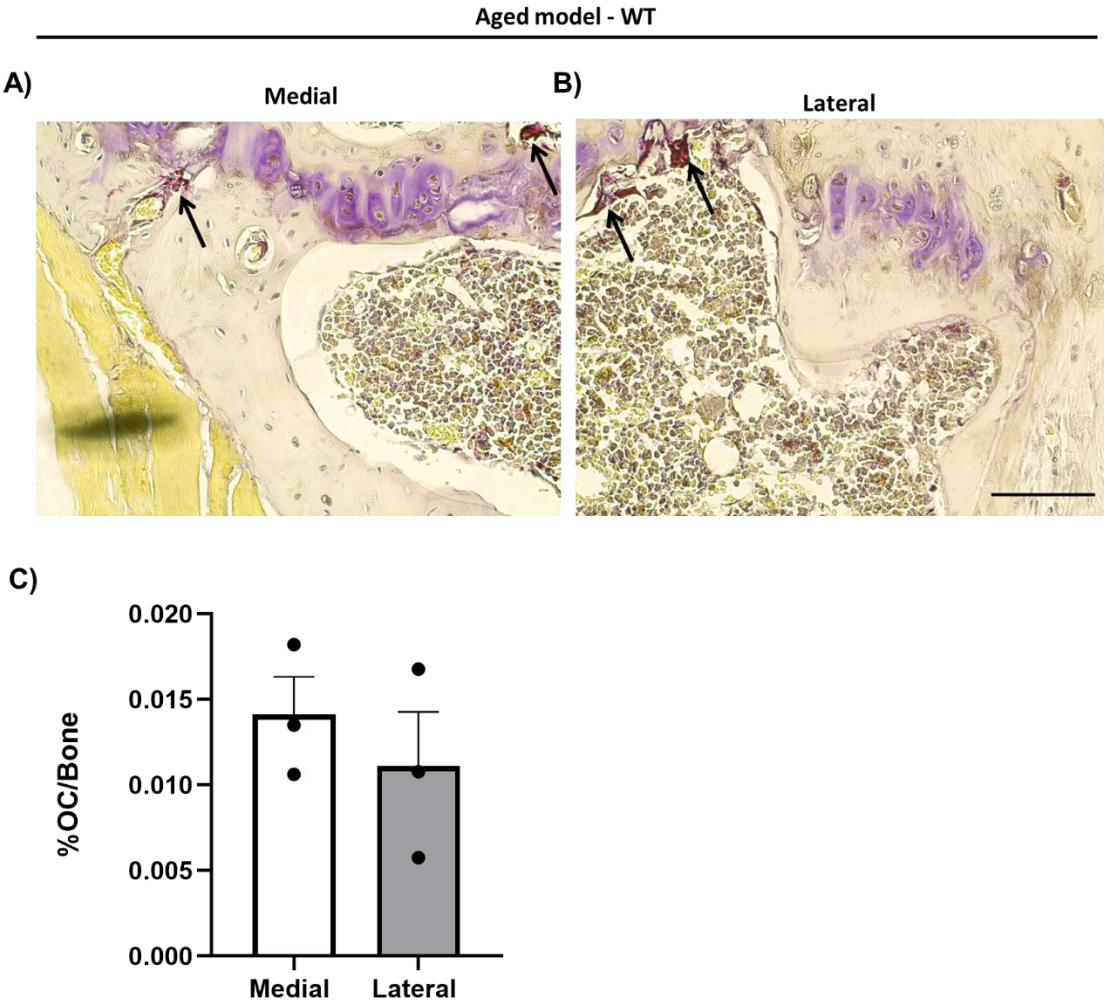

Figure 5.14 Spatial localisation of osteoclast in trabecular bone in aged-WT mice. Mice tibia sections were stained with TRAP kit for OC in non-operated medial site (A) and lateral site (B). No significant difference was found in the number of osteoclasts between medial and lateral site (C). Images are representative of different mice (N=3). Arrows point to positive TRAP-stained OC. Scale bar 100µm.

5.6. Discussion

Tregs play a role adaptive immune response by maintaining peripheral tolerance, protecting the host from autoimmunity, and limiting inflammatory diseases (Dias et al., 2017). Additionally, Tregs have also been shown to play an important role during bone remodelling regulation (Bozec & Zaiss, 2017). One of the proposed mechanisms by which Tregs can regulate the bone remodelling process is by inhibiting osteoclast formation, hence, creating an imbalance in between the bone breakdown and bone formation by suppressing the formation of osteoclasts and the resorption of bone (Bozec & Zaiss, 2017). The data presented in Chapters 3 and 4 of this thesis support such a role.

Tregs in homeostatic condition are found in the bone marrow therefore, they are found in close in proximity with osteoclasts on the surface of the bone (Camacho et al., 2020). However, the data in this chapter showed that the expression and spatial localisation of Tregs and osteoclast within the subchondral bone and the trabecular bone in healthy, osteoarthritic mice (DMM-operated), and aged-WT mice varies amongst the conditions. Together this suggests that changes in Treg expression and localisation may play a role during diseases progression and healthy ageing.

I initially aimed to determine the spatial localisation of Tregs in human samples obtained from patients undergoing knee replacement surgery to determine the role Tregs presence may play during osteoarthritic bone changes. However, histological analyses showed that those samples were lacking bone marrow, which

is key to the study since bone marrow is the tissue where Tregs can be found in the knee osteochondral unit. This, together with further limitations based on lab access and obtaining clinical samples encountered during the COVID-19 pandemic caused us to continue using murine samples instead. However, changing the study to murine samples opened further opportunities since those are naturally smaller than human making it possible to analyse changes in the whole joint.

This study confirmed in the DMM mouse, an increase of Treg expression in the subchondral bone area of the knee joint during osteoarthritis development. Tregs localisation increased significantly in the medial side of mice presenting an osteoarthritic phenotype (DMM-operated) as in comparison to the medial side of non-operated mice (**Figure 5.7**). No significant changes in the lateral side were found in either the DMM-operated mice or the control non-operated. This is a consequence of the DMM surgery applied since it is the site of surgery (the medial side) that will develop osteoarthritis (Glasson et al., 2007) and consequently were this project hypothesized that Tregs will migrate towards.

Changes in the Treg localisation of Tregs in the subchondral bone caused by healthy ageing may also be related with the natural development of osteoarthritis caused by ageing. In this study the mice model used C57/BL6 has been shown to naturally develop osteoarthritis with ageing, around 23 months of age, evidenced as increased articular cartilage chondrocyte cell death and decreased articular cartilage thickness (McNulty et al., 2012).

During the natural process of murine ageing, the Treg population was not significantly different in the medial compartment compared to the lateral compartment of the knee in the subchondral bone (**Figure 5.8**). However, when comparing the total number of Tregs found in subchondral bone young mice (either non operated or DMM-operated) and the total number of Tregs in aged-WT, there was a significant increase in the aged-WT mice, hence suggesting Tregs play a role during healthy ageing and therefore the expression of this will be expected to increase at later stages of osteoarthritis, since aged-WT mice presented early stage signs of osteoarthritis development based on the OARSI scoring as a consequence of natural developing of the disease. This data relates to previous data on Chapter 3, where there was a decrease in the number of Tregs in the healthy aged population in the peripheral blood. This may be due to the higher migration towards the site of bone damage.

Furthermore, the data found in this Chapter indicated no significant changes in Treg expression during surgical or ageing-related osteoarthritis development in the trabecular bone. mice when comparing with the non-operated mice. Changes in the Treg population may occur in the trabecular bone as a consequence of developing osteoarthritis with subchondral bone alteration being the predominant feature of induced osteoarthritis by DMM surgery, which caused lesions that are primarily located on the central weight bearing areas of tibial plateau (Nagira et al., 2020).

In order to understand the correlation of Treg numbers migrating towards the site of injury and the number of osteoclasts, this Chapter further looked to quantify osteoclast numbers in these murine models during osteoarthritis development.

The data presented in this chapter also showed that presence of osteoclast in the subchondral bone may be correlated to the stage of disease development and progressive with ageing. No presence of osteoclast was seen in the subchondral bone of young mice in either DMM-operated or non-operated control **Figure 5.11**) which may be due to the natural low bone resorption in the subchondral bone area and the natural localisation of osteoclasts into areas with a high level of resorption under healthy conditions (Zhu et al., 2021). DMM-operated mice presented a low grade of osteoarthritis (**Figure 5.3**), indicating that subchondral bone changes related to osteoarthritis progression, such as an imbalance in bone remodelling, are still not found based on the OARSI scoring. Furthermore, given the fact that the gifted murine samples used during the study, were mounted at 8-weeks post-surgery, based on the needs of other studies. This can have an effect in the osteoarthritic stage of the samples, being those at a very early stage of developing osteoarthritis. Most osteoarthritis studies on DMM-operated perform surgery 2-3 months old mice and further analyses are carried out 1-2 months later, given that while 2 months mice are skeletal considered matured adults, their skeletal tissue undergoes significant changes well past 2 months (Fang et al., 2018b).

Moreover, osteoclast numbers in the subchondral bone increased with ageing as well as with an increase of the osteoarthritis severity, as a consequence of natural disease development. Aged-WT mice presented a higher level of osteoarthritis based on the OARSI score (**Figure 5.4)** and consequently appear to have diseases

characteristic of late osteoarthritis phenotype, which can also correlate with high resorption produced by osteoclast (Bertuglia et al., 2016). Aged-WT mice presented higher levels of osteoclasts in the medial side of the tibiae when comparing it to the lateral side (**Figure 5.11**). Fang H. et al; showed invasion of osteoclasts into the subchondral bone during osteoarthritis development after 5 weeks post DMM-surgery (Fang et al., 2018a). However, in comparison to our study the mice used (C57/Bl6 wild type, DMM surgery at 8 weeks old) presented a higher OA score based on the OARSI method, and therefore suggests more advanced OA. This together, suggested that osteoclast invasion and a higher presence of those into the subchondral bone areas may be related with the disease's progression and natural ageing changes in bone remodelling.

The trabecular bone is a highly active area of the bone and bone remodelling occurs at a high rate under healthy conditions, given that it is located under the growth plate (Eriksen, 1986). This study also demonstrated that when compared to the levels found in subchondral bone, the expression of osteoclast was higher in the trabecular bone in both the surgical and ageing models of osteoarthritis. Thus, suggesting that the presence of osteoclast in the trabecular bone may not be representative of osteoarthritis progression and instead a natural effect of healthy bone growth.

The increase of Tregs and osteoclast expression during osteoarthritis development, in combination with previous literature that showed an interaction between both cell types, suggests that Tregs may play a role in osteoarthritis development by regulating osteoclast function. One possible effect of Tregs on

osteoclast is Tregs suppressing osteoclast function, which has been previously shown in Chapter 4 in accordance with previous studies showing osteoclast function suppression by Tregs, however, the specific mechanism of action remains unknown. There is still no clear evidence if Treg invasion during osteoarthritis development is a cause or a consequence of the diseases and further experiments focusing on the role Tregs play on osteoclast are essential to understand when and how Tregs affect osteoarthritis progression.

In conclusion, this Chapter showed that Tregs presence increases in the affected subchondral bone during osteoarthritis development and that Tregs levels will naturally increase with disease progression and during ageing. Moreover, it can also be concluded that trabecular bone invasion of Tregs during osteoarthritis development induced by DMM surgery could not be seen and this may be due to the experimental limitations and when surgery was induced as previously discussed. On the other hand, aged-WT mice showed a higher presence of Tregs in the trabecular bone in comparison with young-WT mice. (Stoop et al., 1999).

Osteoclast number was not affected by osteoarthritis development as no osteoclast were found in the subchondral bone during early osteoarthritis stages of the diseases. This study showed a higher presence of osteoclast in the subchondral bone in the aged-WT models. It can be assumed that osteoclast presence in the subchondral bone is related to osteoarthritis development and specific of disease progression.

Altogether the data in this Chapter supported the hypothesis from this thesis that Tregs and osteoclast have the potential to interact in the bone since higher levels of osteoclasts were found in the subchondral bone at the same time as higher presence of Tregs. This may suggest that higher osteoclasts numbers related to bone remodelling imbalance triggers recruitment of Tregs to suppress osteoclast function (as suggested in Chapter 4). Further work to understand the mechanism of action and the recruitment of Tregs towards the site of bone damage is key to develop specific Treg-osteoclast targeted therapies for bone damage diseases such as osteoarthritis.

Chapter 6

Final Discussion

6.1. General Discussion

Osteoarthritis is a bone disease characterised by articular cartilage degradation, increased bone remodelling and synovial inflammation. This study focused on the bone changes occurring during healthy ageing linked to bone resorption caused by osteoclasts, with the aim of informing on osteoarthritis. Osteoclasts have been a target for therapy in various bone diseases where increased high osteoclast activity is causing imbalanced bone remodelling. This thesis proposed a new therapeutic approach for the regulation of osteoclast function based on the emerging evidence suggesting the role of Tregs in bone mass regulation by suppressing osteoclast formation and function.

This thesis focused on identifying peripheral blood Treg migration towards the site of bone remodelling, by studying the differences in potential bone homing Tregs in healthy young and healthy ageing individuals. It is envisaged that this second population could be used as an age-matched control for further studies using osteoarthritis patients.

Additionally, the interaction of Tregs and osteoclast was analysed in *in vitro* studies to assess the efficacy of suppression of osteoclast function by Tregs. Finally, the

spatial localisation of both Tregs and osteoclasts was studied *in vivo* in osteoarthritic mouse models to corroborate the possibility of Tregs and osteoclast interaction in a physiological environment.

In Chapter 3, total Tregs (CD4+Foxp3+) and putative bone homing Tregs, identified by the expression of CCR4+ or CXCR4+, were quantified in the peripheral blood of healthy aged participants and healthy young controls. Identifying changes in the Treg population in blood during ageing will allow a further understanding of the population of Tregs migrating towards sites of bone injury. Hence, identifying that niche population of Tregs that present the ability to migrate towards bone may of interest in understanding ageing-related bone changes. This project originally hypothesized to find a higher level of Tregs in peripheral blood of the aged population as a consequence of an imbalance in bone turnover (**Figure 1.9**). However, in this study a decrease was observed in the population of Tregs ($CD4^+Foxp3^+$) in the healthy aged population when compared to the healthy young control. Changes in Treg populations during ageing can be due to sex (Huang et al., 2021; Robinson et al., 2022). However, data in chapter 3 showed no significant difference when comparing the Treg ($CD4^+Foxp3^+$) levels between male and female group, although it's probable that this is as a consequence of the small sample population – as the study was not designed to detect differences between sex. The higher predisposition of females to develop bone diseases could be related to the lower levels of Tregs in healthy individuals, which would act on regulating bone remodelling by suppressing osteoclasts by either having a direct impact on osteoclastogenesis interacting with macrophages (osteoclasts precursors) or by acting as an immune-response regulator interacting with other immune cells as described in section 3.6 Chapter 3 discussion.

This Chapter also looked to understand if a higher level of bone homing Tregs could be identified in the populations suffering from an imbalance in bone turnover (aged population) (Figure 1.9). Potential bone Tregs expressing the chemokine CXCR4, known to be the bone marrow Tregs marker, showed no significance difference between groups, healthy young and heathy aged. However, bone Tregs (CD4+Foxp3+CXCR4+) showed to be higher in numbers in the female group. Overall, females presented a lower population of Tregs (CD4+Foxp3+) but amongst those, the population of bone Tregs (CD4+Foxp3+CXCR4+) is higher than in the male group. This may be a consequence of hormonal sex differences caused by ageing and related to bone remodelling.

After menopause, bone remodelling in females is disrupted caused by impaired osteoclast function (Karlamangla et al., 2018). Females have a higher risk of developing bone diseases such osteoarthritis and osteoporosis (Afshan et al., 2012). Finding higher levels of bone Tregs that may present the ability to migrate towards bone injury sites may be related to the higher prevalence of developing bone diseases since bone breakdown may trigger recruitment of Tregs towards the site of bone damage. An increase in bone Treg population may also be an evolutionary resource where females have naturally a higher presence of bone Tregs given the higher incidence of suffering bone diseases. Further studies focusing on the function and migration ability of those Tregs is needed.

On the other hand, CCR4 expression on Tregs (CD4+Foxp3+CCR4+) did not show any changes related to either age or sex. This study identified CCR4 as a possible

marker to identify bone Tregs given the implication of CCR4 in Tregs to produce Treg migration towards sites of inflammatory bone response. Studies in mice have shown the correlation between CCR4 and bone loss during an inflammatory response (Araujo-Pires et al., 2015), however further studies need to be done in human to understand the anti-inflammatory function in bone tissue.

Data in this Chapter did not show any difference amongst groups since CCR4 would be related with Treg migration towards bone during an inflammatory condition. The participants who took place in the study were healthy. In the original study planning where osteoarthritis participants were accounted to take part in the study, it was hypothesised to find significant differences in the CD4+Foxp3+CCR4+ given the inflammatory bone condition related to osteoarthritis. However, further studies to understand CD4+Foxp3+ CCR4+ migration towards site of bone injury is still required.

Finally, Chapter 3 studied the Tregs effects on bone healing by measuring the correlation of Treg numbers in peripheral blood and CTX-1 levels in serum (as a marker of bone breakdown). This study hypothesised that higher levels of serum CTX-1 will be found in the aged population as a result of higher bone turnover and higher presence of Tregs in peripheral blood will be linked to lower CTX-1 levels in serum as a result of Tregs regulation of bone damage (**Figure 1.9**).

Data in this Chapter showed no significant difference in CTX-1 levels in serum between age groups (healthy young and healthy aged) or sex groups (female and male). This may be due to the small sample population. However, this study has no further information regarding the menopausal status of the participants, and this

could not be further analysed. This is not conclusive data give the sample size population but points a good direction for further investigation.

The focus of this study was to correlate levels of bone breakdown with Tregs presence in peripheral blood since higher levels of Tregs in peripheral blood are linked to lower CTX-1 levels in serum. However, data from this study is not conclusive given the limitations with the sample population. Further studies designed and powered to address sex-differences in Tregs, and bone would help understanding the effects of Tregs in bone breakdown and how age and gender may play a role in.

In order to understand the possible Treg function of suppressing osteoclast function in this thesis I have validated an *in vitro* method of osteoclast culture using monocytes (osteoclasts precursors) from whole blood and stimulated osteoclast formation with RANKL and MCSF (Agrawal et al., 2012). Co-culture of osteoclasts and Tregs requires Treg isolation from PBMCs.

This study hypothesised that healthy aged participants would have a lower osteoclasts function when compared to healthy young participants as a consequence of age-related bone remodelling imbalance (**Figure 1.9**). Osteoclastogenesis in the absence of Tregs was studied in this chapter to understand ageing changes that naturally occur through the process. Chapter 4 showed that the number of osteoclasts formed was not affected by age. However, the osteoclast activity was affected since aged participants presented a lower level of osteoclast resorption. Moreover, sex identification of the samples proved that the

female group shows less functional osteoclasts proven demonstrated by a lower level of resorption. As discussed in the previous section 4.6, Chapter 4 discussion, oestrogen decrease during menopause may be the key player in reducing osteoclast function since oestrogen inhibits osteoclast activity by inhibiting RANKL mediated osteoclast formation as well as increasing osteoclast apoptosis (Khosla et al., 2012). However, data in Chapter 4 showed a decrease in osteoclast activity, which in this case may suggest oestrogen levels in aged population, this was not controlled in the experiment since menopausal state or menopausal medication information was not obtained.

The data found here correlates with previous studies showing reduced bone regeneration and healing capacity observed in aged individuals (Becerikli et al., 2017).It is also possible that lower osteoclasts activity is related to sex differences more than age causes and that the reduction of osteoclast activity seen in the aged participants may be related to the sex of the participants, however, further investigations regarding the effects of sex on osteoclast activity are required in order to take final conclusions (Choi et al., 2021).

Co-culture of Tregs and osteoclasts, using cells derived from donors of different age and sex, showed no significant impact in the number of osteoclasts formed from precursors. However, Tregs do have an impact in osteoclast's activity as hypothesised in Chapter 1 (**Figure 1.9**). Data presented in Chapter 4 showed a decrease in osteoclast function when co-culture with Tregs in young and aged participants. Higher suppression of osteoclast activity was seen in the aged group as well as in the female group when compared to the young group and male group respectively. This may be related to the previous results found where aged and

female osteoclasts naturally have a reduced activity regardless of the presence of Tregs.

To conclude if the reduction seen is caused by Tregs suppression ability or is due to the natural osteoclast activity decrease, Chapter 4 presented a series of experiments co-culturing Tregs and osteoclasts in a combination of age groups (by culturing autologous Tregs and osteoclasts or mix matching young and aged Tregs and osteoclasts). The data found corroborated that in fact the changes seen in osteoclasts was caused by a higher suppression activity (answering the hypothesis stated in Chapter 1 (**Figure 1.9**) from aged Tregs as opposite of Tregs from different donors co-cultured with osteoclasts.

Chapter 4 concluded that Tregs do interact with osteoclast and produce an impact on osteoclast activity by suppressing osteoclast resorption. Also, aged differences can be corroborated since a natural decrease in osteoclast activity occurs with ageing and aged Tregs presented a higher suppressive activity.

In the final chapter, the spatial localisation of Tregs in bone was explored to identify the possible proximity with osteoclasts in the bone environment during osteoarthritis development and healthy ageing. Identifying changes in the localisation and numbers of Tregs in bone during osteoarthritis development is critical to understand their interaction with osteoclasts and their role in regulating bone remodelling.

This chapter showed an increased Treg presence in the subchondral bone (medial side, DMM-operated) during osteoarthritis development as well as during healthy

ageing when compared to the healthy control, which by natural ageing progression also presented a high level of natural osteoarthritis development. On the other hand, no changes were found regarding the number of Tregs in the trabecular bone. Only modest increases in Treg numbers were found in the trabecular bone in the ageing model, which are caused due to natural ageing progression and are not disease dependent (Stoop et al., 1999).

Altogether these findings correlate with osteoarthritis development changes which are first seen in the subchondral bone area of the joint. Recent evidence suggested that the thickening can start before changes in the articular cartilage degradation, an indication that subchondral bone plays a role in the development of osteoarthritis (G. Li et al., 2013a). These findings suggest that changes in the Treg expression and localisation can indeed be related with osteoarthritis development since a higher presence of Tregs were found in the presence of osteoarthritis damage in the bone.

This chapter also explored the presence and distribution of osteoclast in bone during osteoarthritis development with the purpose of study further correlation with the previous seen increased Tregs in bone during osteoarthritis diagnosis. The data found during this study showed that the presence of osteoclasts, in the subchondral bone was correlated with the disease development and progression. Higher levels of osteoclast indicated an increase in bone breakdown caused because of an imbalance in bone remodelling typical of osteoarthritis progression. Even though, the experimental design limitations faced during the study did not allow any osteoclast identification in the DMM-operated mice (given that the time post-surgery

was not enough for osteoarthritis changes to develop and be seen in histological analyses), an increase in osteoclast presence was detected in the healthy aged WT-mice. The increase in osteoarthritis in the healthy aged WT-mice are directly related with ageing changes, but also disease progression since those mice naturally developed a higher level of osteoarthritis than the DMM-operated mice. These findings suggested that an increase in osteoarthritis severity, and consequently higher bone breakdown produced by osteoclast, is also correlated with a higher presence of osteoclast in the subchondral bone. This could therefore explain the characteristic changes in osteoarthritis progression, related to higher bone resorption, imbalanced from bone formation causing impaired bone remodelling. Consequently, higher levels of bone remodelling caused by an increase in the osteoclast presence can be leading to a higher recruitment of Tregs to the site of bone damage since as shown in previous chapters, *in vitro* cultured Tregs suppress osteoclast function. Hence, identifying the mechanism of Treg migration towards the site of bone damage is critical for developing osteoarthritis specific treatment based on suppression of osteoclast function.

This chapter also explored the presence of osteoclast in the trabecular bone area, this showed higher levels of osteoclast in DMM-operated mice, but no change was found in the healthy aged WT-mice. These findings are not related with the disease's development progression since the trabecular bone is an active site of bone remodelling (Francisco J.A. De Paula, n.d.), hence a high presence of osteoclast in trabecular bone could be expected. However, the samples used for this study all presented early stages of disease development and it is likely that late stages of osteoarthritis disease may affect the trabecular bone, after complete

degeneration of the subchondral bone, therefore a higher level of osteoclast may be found in the trabecular bone.

The work presented in chapter 5, details the potential of Treg and osteoclast interaction (previously shown *in vitro*) in a more physiological representative model *in vivo*. Even though this study did not explore the direct interaction between osteoclast and Tregs, the increase of both populations during osteoarthritis progression indicates a correlation with bone breakdown damage, caused by osteoclast. Hence the recruitment of Tregs to the site of bone damage, potentially to suppress osteoclast function as shown in vitro studies carried out in Chapter 3. However, it is important to remark that Treg invasion to the site of bone damage during osteoarthritis development is still not clear to be a cause or a consequence of osteoarthritis development and understanding what leads to Treg migration towards damaged sites and how the interact with osteoclast is vital to identify possible therapies targeting osteoclast function for osteoarthritis development.

Treg cell therapy

Using Tregs therapy for autoimmunity and transplantation is emerging due to their antigen-directed immunosuppressive properties potential to mediate infectious tolerance. Treg cell therapy has shown therapeutic potential in preclinical models and in patients suffering from autoimmune disease (type 1 diabetes) or undergoing solid organ transplantation.

Initial efforts in the clinical use of Tregs have focuses on generating protocols for Treg isolation, expansion, and enrichment. The near future looks promising to obtain more definitive answers regarding their clinical efficacy. The advantages of

using Tregs therapeutic include the low number of cells needed because of the targeted and effective immune regulation and a lower risk of immunosuppressive side effects and enhanced safety due to the high Treg stability.

6.2. Directions for future research

The results presented in this thesis have identified a potential therapeutic use of Tregs, targeting osteoclast's activity in bone diseases such as osteoarthritis. Tregs have been proven to suppress osteoclast resorption *in vitro,* and this function has been proven to be age dependent. Moreover, Tregs have been shown to accumulate in the site of bone damage and number of Tregs positively correlates with an increase in osteoclast activity. However, further work is needed to further elucidate the mechanisms of Treg migration towards the site of bone damage and the interaction with osteoclasts.

Certainly, the examination of Tregs and osteoclasts interaction during osteoarthritis pathology will be of great interest in furthering our understanding of the role of Tregs during bone remodelling imbalance. During this PhD, access to osteoarthritis samples was unavailable given the COVID-19 restrictions. Hence it will be of great benefit for the field to carry out further *in vitro* co-culture of Tregs and osteoclasts to gain a better understanding of changes that may occur in both populations of cells during osteoarthritis pathology.

Further work focusing on the mechanism of Treg suppression will also be beneficial to understand the specific pathway of Treg action in suppressing osteoclast function. Understanding if the effects of Tregs on osteoclast function are altered

depending on the disease stage and/or during healthy ageing will be vital to develop a targeted therapy based on suppressing osteoclasts function by Tregs during disease development. Understanding the diseases level is complicated since most times diagnosis comes at a late stage of the diseases. Murine experiments, assessing different stages of the diseases to then obtain Tregs and osteoclasts to co-culture could be an initial plan.

Previous studies have looked at serum presence of cytokines involved in Treg regulation and activity in osteoclastogenesis during Gorham-Stout disease (GSD, increased bone erosion). High levels of IL-6 and reduction of TGFβ1 were found in GSD patients (Rossi et al., 2021), understanding these same markers during osteoarthritis development will help understanding the role of Tregs in the disease development. Another useful information will be to look at the osteoclast aspect, understanding the expression of genes involved in the acidification and resorption such as CLC7, CTSK and MMPs.

Previous studies have also demonstrated the implications of Tregs in osteoclasts by having an effect on the osteoclast sealing zone (Dohnke et al., 2022). Further studies looking at in vitro cultures of Tregs and osteoclasts focusing on the sealing zone formation will give an indication about Tregs mechanisms of action and how this may be playing a role during osteoclast formation and hence having a further effect on osteoclasts function. Sealing zone formation can be easily detected under confocal microscope staining for Actin, assessing the samples at different days of culture will allow to observe the formation of the sealing zone.

Moreover, understanding the migration of Tregs towards the site of bone injury will also be beneficial to further develop specific therapies using Tregs to target osteoclast function related to the precise mode of action. In order to do that, further characterisation of bone homing marker CXCR4 during osteoarthritis development at different stages will need to be analysed. This could be done by a literature search of bone migration markers and assessing their expression on Tregs by flow cytometry. Further investigation of the signals released by the bone to trigger migration towards the

site of injury will also help understanding how the different stages of bone damage may influence migration of Tregs towards the site of injury.

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Appendix

1. Participant's information

Sample ID	Method	Sex	Age (years)	Figure used
P1	fresh blood	male	28	figure 3.3, figure 3.4, figure 3.5, figure 3.6, figure 3.7
P2	buffy coat	male	29	figure 3.3, figure 3.4, figure 3.5, figure 3.6, figure 3.7
P3	fresh blood	female	40	figure 3.3, figure 3.4, figure 3.5, figure 3.6, figure 3.7
P4	fresh blood	male	25	figure 3.3, figure 3.4, figure 3.5, figure 3.6, figure 3.7
P5	buffy coat	male	52	figure 3.3, figure 3.4, figure 3.5, figure 3.6, figure 3.7
P6	buffy coat	female	70	figure 3.3, figure 3.4, figure 3.5, figure 3.6, figure 3.7
P7	buffy coat	female	63	figure 3.3, figure 3.4, figure 3.5, figure 3.6, figure 3.7
P8	buffy coat	female	70	figure 3.3, figure 3.4, figure 3.5, figure 3.6, figure 3.7
P9	buffy coat	female	66	figure 3.3, figure 3.4, figure 3.5, figure 3.6, figure 3.7, figure 4.11, figure 4.12, figure 4.13, figure 4.14
P10	buffy coat	female	74	figure 4.11, figure 4.12,figure 4.13, figure 4.14
P11	fresh blood	male	34	figure 4.11, figure 4.12,figure 4.13, figure 4.14
P12	buffy coat	male	47	figure 4.11, figure 4.12,figure 4.13, figure 4.14
P13	buffy coat	female	28	figure 3.3, figure 3.4, figure 3.5, figure 3.6, figure 3.7, figure 4.11, figure 4.12, figure 4.13, figure 4.14
P14	buffy coat	female	56	figure 3.3, figure 3.4, figure 3.5, figure 3.6, figure 3.7,
P15	fresh blood	male	35	figure 3.3, figure 3.4, figure 3.5, figure 3.6, figure 3.7
P16	fresh blood	male	35	figure 3.3, figure 3.4, figure 3.5, figure 3.6, figure 3.7

P17	buffy coat	male	63	figure 3.3, figure 3.4, figure 3.5, figure 3.6, figure 3.7, figure 4.11, figure 4.12, figure 4.13, figure 4.14, figure 4.15
P18	fresh blood	female	33	figure 3.3, figure 3.4, figure 3.5, figure 3.6, figure 3.7,
P19	buffy coat	female	60	figure 3.3, figure 3.4, figure 3.5, figure 3.6, figure 3.7
P20	fresh blood	male	28	figure 3.3, figure 3.4, figure 3.5, figure 3.6, figure 3.7
P21	buffy coat	male	57	figure 3.3, figure 3.4, figure 3.5, figure 3.6, figure 3.7
P22	fresh blood	female	23	figure 3.3, figure 3.4, figure 3.5, figure 3.6, figure 3.7, figure 4.11, figure 4.12, figure 4.13, figure 4.14, figure 4.15
P23	buffy coat	female	57	figure 3.3, figure 3.4, figure 3.5, figure 3.6, figure 3.7
P24	fresh blood	female	41	figure 4.11, figure 4.12,figure 4.13, figure 4.14, figure 4.15
P25	buffy coat	female	57	figure 3.3, figure 3.4, figure 3.5, figure 3.6, figure 3.7,
P26	fresh blood	male	30	figure 3.3, figure 3.4, figure 3.5, figure 3.6, figure 3.7,
P27	buffy coat	male	56	figure 4.11, figure 4.12,figure 4.13, figure 4.14, figure 4.15
P28	fresh blood	female	30	figure 3.3, figure 3.4, figure 3.5, figure 3.6, figure 3.7
P29	fresh blood	male	20	figure 3.3, figure 3.4, figure 3.5, figure 3.6, figure 3.7
P30	fresh blood	male	52	figure 3.3, figure 3.4, figure 3.5, figure 3.6, figure 3.7
P31	fresh blood	male	29	figure 3.3, figure 3.4, figure 3.5, figure 3.6, figure 3.7
P32	fresh blood	female	42	figure 3.3, figure 3.4, figure 3.5, figure 3.6, figure 3.7
P33-56	fresh blood	12 male, 12 female	unknown	figure 3.8
P57	fresh blood	female	45	figure 3.9
P58	fresh blood	male	36	figure 3.9
P59	fresh blood	male	52	figure 3.9
P60	fresh blood	female	33	figure 3.9

P61	fresh blood	male	28	figure 3.9
P62	fresh blood	male	30	figure 3.9
P63	fresh blood	female	57	figure 3.9, figure 3.10
P64	fresh blood	female	30	figure 3.9, figure 3.10
P65	fresh blood	male	35	figure 3.9, figure 3.10
P66	fresh blood	female	33	figure 3.9, figure 3.10
P67	fresh blood	male	19	figure 3.9, figure 3.10
P68	fresh blood	male	34	figure 3.9, figure 3.10
P69	fresh blood	male	28	figure 3.9, figure 3.10

2. Edinburgh Napier University ethics

You must check **either Box A or Box B** below and provide all relevant information in support of your application in the Details of Project section. If you answered **NO** to any of **questions 1-10**, or **YES** to any of **questions 11-15** (with a shaded background), then you **must** check Box B.

DETAILS OF PROJECT

Background information *(300 words maximum; references should be cited and listed)*

Osteoarthritis is the most common musculoskeletal condition among older people, affecting 3.8% of the global population (Palazzo et al. 2016). Osteoarthritis is a degenerative joint disorder, the most affected joints being the hands, knee and hips, causing joint debilitation. The aetiopathogenesis of osteoarthritis encompasses alterations in the articular cartilage, subchondral bone, ligaments, capsule and synovial membrane and for this reason, it is considered a disease of the whole joint (Martel-Pelletier et al. 2016). Despite the huge socioeconomic burden, there are currently no effective disease-modifying treatment options for osteoarthritis (Sun et al. 2017) and patients largely rely on the use of symptom-modifying therapies, such as pain-modifying drugs or total joint replacement (Little & Hunter 2013). A better understanding of osteoarthritis pathogenesis is therefore needed in order to identify novel therapeutic targets and to help reduce the burden of this globally-relevant disease (Sun et al. 2017).

Osteoarthritis historically has been considered a "wear and tear" disease and therefore commonly described as non-inflammatory (Berenbaum 2013). Despite this, inflammation is increasingly recognised to be involved in the developments of osteoarthritis. This has determined osteoarthritis as a more complex disease in which a number of inflammatory mediators are present in cartilage, bone and synovium (Haseeb & Haqqi 2013). This suggests that the aforementioned inflammatory factors may play a key role in the development of osteoarthritis by contributing to cartilage destruction, synovitis, aberrant bone remodelling and joint effusion (Bonnet & Walsh 2005).

Regulatory T cells (Tregs) are a subpopulation of T helper cells important for their role in the immune system maintaining peripheral tolerance, protecting the individual from autoimmunity, and limiting chronic inflammatory diseases (Dias et al. 2017). Evidences suggest that Tregs may play an important role in bone-related diseases through regulating bone remodelling. Specifically, Tregs inhibit the differentiation of osteoclasts (bone cells, which absorb bone tissue during growth and healing) (Bozec & Zaiss 2017). Thereby, inhibiting bone resorption which would lead to bone accumulation characteristic in osteoarthritis progression Accumulation of Tregs in the affected joints of osteoarthritis patients has been shown, suggesting then that their presence may be related with the disease development (Moradi et al. 2014). However the precise role of Tregs in osteoarthritis remains unclear, so a full characterisation of the Treg population in healthy and osteoarthritic patients is essential.

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Palazzo, C. et al., 2016. Risk factors and burden of osteoarthritis. *Annals of Physical and Rehabilitation Medicine*, 59(3), pp.134–138.

Sun, M.M.G., Beier, F. & Pest, M.A., 2017. Recent developments in emerging therapeutic targets of osteoarthritis. *Current Opinion in Rheumatology*, 29(1), pp.96–102.

1. Aims & research questions

In order to improve health and wellbeing of those who suffer from osteoarthritis a better understanding of the effects of Tregs in osteoarthritis bone pathology is needed. As the precise role that Tregs play in osteoarthritis remains unclear the aim of this project is to explore Treg function and role in bone health in osteoarthritis with the main purpose of identifying if Tregs offer a potential new therapeutic strategy to enhance regeneration in osteoarthritis. This will be done by studying the Treg population in osteoarthritis patients and will examine the hypothesis that: Tregs inhibit osteoclast formation.

Aims
Aim 1: Characterise Tregs in the peripheral blood of healthy donors.
Aim 2: Examine the ability of Tregs from healthy donors to suppress osteoclastogenesis in vitro

Note that this application is only made for the healthy control group as a separate application would be submitted for osteoarthritic patients, which, as they involve NHS permission will address this aspect also.

2. Participants

- ***Number & nature of sample:*** 14 participants aged over 50. All sample numbers/biological replicates for each set of experiments to be performed using human samples are based on power analyses informed by Cribbs et al. 2014 where they studied Treg population in rheumatoid arthritis, another form of arthritis, as no previous studies on Treg population in Osteoarthritis have been done before. Cribbs et al obtained a mean of 4.6 +- 0.9% Treg population in healthy controls compared with rheumatoid arthritis patients where 6.15 +- 2.4% was found. Therefore, for a $\alpha 0.05$ and a power of 0.80 we will use n=14 per group to ensure we detect statistically meaningful differences.

  Cribbs, A.P. et al., 2014. Treg cell function in rheumatoid arthritis is compromised by CTLA-4 promoter methylation resulting in a failure to activate the indoleamine 2,3-dioxygenase pathway. *Arthritis and Rheumatology*, 66(9), pp.2344–2354.

- ***Inclusion/exclusion criteria:***
  Completion of forms required attached in Appendix.

  Inclusion criteria: Healthy participants, age over 50, with no clinically diagnosed osteoarthritis by GP and no evidence of knee pain.
  Exclusion criteria: Clinically diagnosed with osteoarthritis or any injury to knee, or knee pain. Any patient suffering from autoimmune disease or transplanted organ. No patient under immunomodulatory treatment (e.g. cortico steroids, non-steroidal anti-inflammatory, ibuprofen) at the time of the study.

- ***Recruitment of participants, including details of formal permissions from another organisation (where appropriate):*** Recruitment of participants will be done on a voluntary basis with no coercion. Posters will be places around university and community areas and shared on social media as well.
  .
- ***Details of any relationship with participants which may affect the research:*** No existing relationships between researchers and participants out with research/study basis.

3. Outline of methods & measurements *(approx. 500 words)*

Healthy participants will be recruited via posters and contact the researcher via email (provided on poster). A detailed explanation of the study will be sent to those who show interest to volunteer as a participant. Before commencing any sample collection, participants will fill a questionnaire to ensure suitability and to obtain written consent , this will be anonymised so, the further analyses can be linked to the specific blood sample but not to the individual . Participants will be informed that they can withdraw from the study at any stage without a reason. Researcher and participant will agree on a day for the participant to visit the university and the below procedure will take part.
A fully qualified phlebotomist will extract up to 160ml of blood from the cephalic vein by venipuncture. Sample will be collected in an appropriate blood sample collection vecutainer. PBMCs (fraction of blood containing T cells and monocytes to be studied in aims 1 and 2) will be isolated from whole blood on the same sample collection day and will be analysed on the same day so storage of sample is not required. PBMCs will be purified using density gradient centrifugation. Cell populations will be measured/monitored using specific fluorochrome conjugated antibody.

Aim 1: Characterise Tregs in the peripheral blood of osteoarthritic patients and healthy donors.
Treg characterisation will be completed in order to identify the Treg population using flow cytometry. This will enable me to quantify the Tregs in the peripheral blood of healthy controls (n=14). First and in order to determine Treg population, flow cytometry analysis of bulk Tregs will be done based protein markers of Tregs (Foxp3 expression). Further readouts of bone homing receptors, activation status and functional mechanism will also be analysed by flow cytometry.
Further, correlation of the sub-populations determined by flow cytometry will be done using ELISA analysis of serum markers of osteoclastogenesis.

Aim 2: Examine the ability of Tregs from osteoarthritis patients and healthy donors to suppress osteoclastogenesis in vitro
Osteoclasts will be differentiated in vitro from monocytes (purified from the previously mentioned PBMC fraction) using recombinant growth factors (MCS-F and RANKL). Osteoclast function will be monitored by measuring the resorption of bone-like matrix (dentine) in vitro along with gene and protein expression of factors associated with osteoclast function. Treg populations from healthy controls and OA patients, as defined in aim 1, will be sorted and used and co-cultured with autologous osteoclast cultures to determine impact on osteoclast function.

4. Risks to participants' and researcher's safety & wellbeing

Participants: Due to the need of using venipuncture technique to obtain the blood sample, patients will experience some discomfort during blood extraction as per the puncture. Venipuncture will be done by a fully trained phlebotomist, reducing the possible distress and the very low risk of infection. This will be done at Edinburgh Napier University, participants will be informed of the procedure before starting and they can also stay in the lab after blood extraction until they feel comfortable to leave.

5. Consent and participant information arrangements, debriefing, withdrawal from the study
Participant will be required to fill in a questionnaire to ensure suitability and to obtain written consent. Participants will also be informed of the protocols and aims of the study as well as, their right to withdraw the study at any time without reason. Participants will be informed that under consent they may be contacted again to be a donor more than once, only applicable if sample provides relevant data, it will be made clear that they are under no obligation to provide additional samples.

6. Anonymity and confidentiality

Participants will fill in the questionnaire, and this will be anonymised to ensure that no personal information is used. Any further study publication will be done anonymously.

7. Data protection arrangements

Participant's data will be always stored on password-protected devices and will not be shared with any outsider to the study.

8. Ethical considerations raised by the project and how you intend to deal with them

Ethical considerations in this study relay on personal information and the risks of venipuncture. Aforementioned risks will be controlled and minimised by properly informing participants of all the steps through the study. Data provided by the participant will be stored under secured-devices ensuring that this data cannot be used to identify the individual. Sample collection will be done by fully qualified staff. Participants will never be hold against their will, so participating in the study is completely volunteer and participants can withdraw at any time without reason. All research staff will be friendly with the participants to guarantee their comfort during any process through the study.

DECLARATION

There is an obligation on the researcher to bring to the attention of the School Research Integrity Committee any issues with ethical implications not clearly covered by this application form

Appendix 1. Participant's questionnaire

Edinburgh Napier UNIVERSITY

PARTICIPANT'S QUESTIONNAIRE

This is a strictly private confidential document

Please read and answer the following questions carefully and truthfully. Information will be treated with the strictest confidence.

Name:

Date of Birth:

Email*:

Questions Yes/No

1. Have you ever been diagnosed with Osteoarthritis by your GP? YES/NO
2. Do you suffer from any type of knee injury/pain YES/NO
3. Have you ever had an organ transplant? YES/NO
4. Have you ever been diagnosed with an autoimmunity disease? YES/NO
5. Are you currently being treated with any immunomodulatory drugs (e.g. Corticoid steroids, non-steroidal anti-inflammatory, ibuprofen) YES/NO
6. If required by the study, would you be happy to be contacted to a repeat blood donation (by agreeing to be contacted you are under no obligation to provide an additional blood sample)? YES/NO

Declaration

I have read and understood all of the questions above thereby all my answers have been answered to the best of my knowledge and truthfully. I understand that this information is confidential and so it will be treated with the strictest confidence.

Name of participant	
Signature	
Date	

Name of researcher	
Signature	
Date	

*Providing the email is not mandatory, only for those participants who would agree to be contacted back for a repeat blood donation or further project information

Appendix 2. Debrief Sheet

DEBRIEF SHEET

The research team would like to thank you for volunteering for this study. Your participation has contributed towards the understanding of immune regulatory cells (Tregs) role in bone health and in osteoarthritis. As well as, understanding/developing future therapeutic interventions to enhance regeneration in osteoarthritis.

In order to achieve this, blood extraction from participant is required. Venipuncture (standard blood extraction, as carried out during routine blood sampling) will be done by a fully trained phlebotomist and under appropriate conditions. This will be carried out Edinburgh Napier University, the procedure will be explained by the trained phlebotomist prior to commencing and participants will be encourage to remain seated after extraction until they feel comfortable to leave.

Any pain or discomfort due to puncture will be mild and temporary, it will usually subside within minutes and not longer than hours. Applying pressure at the puncture site will reduce the risk of bruising. If at any point, on leaving the lab session, participant feels unwell we recommend you consult your GP. In case of emergency circumstances call NHS 24 or 999 and request an ambulance.

Contact details of the researcher:

Name: Raquel Lopera Burgueno
Email: [redacted]

Contact details of the project supervisor:

Name: Graham Wright
Email. [redacted]

Appendix 3. Edinburgh Napier University Research Consent Form

RESEARCH COSENT FORM

Edinburgh Napier UNIVERSITY

Regulatory T-cells: a new therapeutic strategy for osteoarthritis?

Edinburgh Napier University requires that all people participating in research studies give their written consent to do so. Please read the following study detailed points and consider that signing below will confirm your consent to take part in the study.

1. I freely and voluntarily consent to take part in the research project on the topic **Regulatory T-cells: a new therapeutic strategy for osteoarthritis?** Which Raquel Lopera-Burgueno, PhD student will conduct.

2. The goal of this study is to explore the role and function of Tregs in bone health and in osteoarthritis in order to identify whether Tregs offer a potential new therapeutic strategy to enhance regeneration in osteoarthritis.

3. My responsibilities as a participant will be to fill in all the ethical required documents truthfully before assisting the lab for blood extraction. This will be done in a unique session, where up to 160ml of blood will be taken by a qualified phlebotomist.

4. I also understand that all the data obtained from my samples will be anonymised. No linkage with the results and my name will be made in any further report where this data may be used.

5. I also understand that I will be able to withdraw the study at any time during the study period and without explanation with no negative consequences towards my participation. However if the data obtained has been already used for publications, due to its untraceable state, it will not be possible to remove it.

6. At any point of the study I will have the opportunity to ask questions regarding any procedure and this will be answer upon to my satisfaction before continuing with any further step. In addition to this, if I do not wish to answer any particular question I am free to decline.

7. Finally, I have read and understood the above and I freely give my consent to take part in this study.

Name of participant	
Signature	
Date	

Name of researcher	
Signature	
Date	

Appendix 4. Participant information sheet

Edinburgh Napier UNIVERSITY

Regulatory T cells: a new therapeutic strategy for osteoarthritis?

This is what will happen during the research study:

1. Participant will freely contact the researcher after recruitment poster interest. And so, more information about the study can be sent upon request. As well as all the ethical forms to complete in order to take part in the study as a participant.

2. After all the ethical forms have been approved, researcher and participant will agree a date for the lab session. This will be a unique session where 160ml of blood will be taken out by a qualified phlebotomist.

3. If required by the study, participants who previously agreed could be contacted to a repeat blood donation (by agreeing to be contacted you are under no obligation to provide an additional blood sample)

4. After the study has concluded, and only if the participant agreed to be contacted back, a short summary with the project outcome will be sent to the participant.

Appendix 5. Recruitment poster

Edinburgh Napier UNIVERSITY

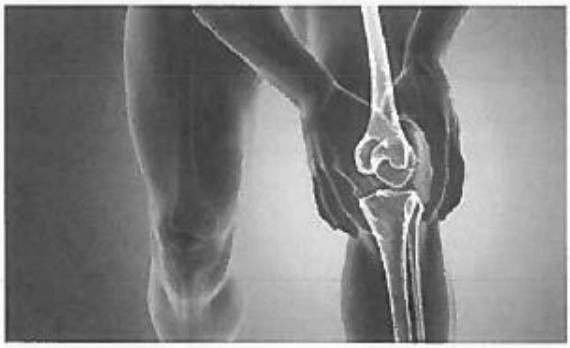
PARTICIPANTS NEEDED

Who can participate?

✓ **Over 50 years old**

✓ **No evidence of knee osteoarthritis or knee pain**

We are looking at possible new therapeutic strategies to enhance regeneration in osteoarthritis



Participants will contribute to the study with a one-time blood donation

To take part or for more information, please contact Raquel Lopera, PhD student.

[redacted]

3. Scottish National Blood Transfusion ethics contract

CONTRACT 20-06

Schedule Part 1

Product Description

Buffy Coats x 20
Plasma samples x 20

Requested by:

Miss Raquel Loper-Burgueño,
Edinburgh Napier University

Location
SNBTS
Jack Copeland Centre
52 Research Avenue North
Heriot-Watt Research Park
Edinburgh
EH14 4BE

Buffy Coats:
Edinburgh Component [redacted]
Fiona McGowan – [redacted]
Magdalena Buska [redacted]

Plasma Samples:
Testing – [redacted]
Jane Stephenson - [redacted]

It is agreed that the University shall make contact with the Location to arrange supply.

This Agreement shall be governed by and construed in accordance with accordance of law of Scotland the parties herby submit to the exclusive jurisdiction of the Scottish Courts. If you wish to receive the Products on the foregoing basis please sign and return the letter to; Business Development, Scottish National Blood Transfusion Service, The Jack Copland Centre, 52 Research Avenue North, Heriot-Watt Research Park, Edinburgh, EH14 4BE or pdf [redacted]

Yours faithfully

[redacted]

Dr David Colligan
For and on behalf of SNBTS

For and on behalf of the University I acknowledge and agree the terms of the foregoing letter.

[redacted] ..... Signed by

..... Print Name

..... Date

I acknowledge the terms of this letter including but not limited to clauses 9, 10 and 19.

Signed: [redacted] Date: 26 November 2020

[redacted]
PROFESSOR PETER G BARLOW
HEAD OF RESEARCH
SCHOOL OF APPLIED SCIENCES

This is the Schedule referred to in the foregoing Agreement between SNBTS and The University

This is the Schedule referred to in the foregoing Agreement between SNBTS and The University

Schedule Part 1

Product Description

25 buffy coats, 30 serum samples,

Requested by:

Norman Turner

Location
SNBTS
The Jack Copland Centre
52 Research Avenue North
Edinburgh
EH14 4BE

Buffy coats can be obtained from
Fiona Rooney – [redacted]
Magdalena Busk

Serum samples please contact Jane Stephenson – [redacted]

It is agreed that the University shall make contact with the Location to arrange collection.

Please quote the submission reference number 21~05 on all orders and communications as failure to do so will result in SNBTS being unable to supply.

This Agreement shall be governed by and construed in accordance with accordance of law of Scotland the parties herby submit to the exclusive jurisdiction of the Scottish Courts. If you wish to receive the Products on the foregoing basis please sign and return the letter to; Business Development, Scottish National Blood Transfusion Service, The Jack Copland Centre, 52 Research Avenue North, Heriot-Watt Research Park, Edinburgh, EH14 4BE or pdf [redacted]

Yours faithfully

[redacted]

Dr David Colligan
For and on behalf of SNBTS

For and on behalf of The University I acknowledge and agree the terms of the foregoing letter.

[redacted] Signed by

Norman Turner ..... Print Name

25/06/2021 ..... Date

I acknowledge the terms of this letter including but not limited to clauses 9, 10 and 19.

Signed: [redacted] Date: 25/06/2021

Norman Turner