Phenotypic Characterization of Peripheral Osteoclast Precursors their Lineage Relation to Macrophages and Dendritic Cells and their Population Dynamics Influenced by Parathyroid Hormone and Inflammatory Signals.

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Osteoclasts are unique multinuclear cells that are highly specialized for resorbing bone tissue and therefore play crucial roles in bone remodeling. The identification of bona fide populations of osteoclast precursors (OCPs) is crucial to understand their biology in homeostatic and pathological conditions. We identified a bone marrow precursor with high osteoclastogenic activity (Jacquin et al., 2006) and extended these findings to test their clonogenic potential and bone resorptive capacity at the single cell level. We also identified the phenotype of peripheral blood and spleen OCPs. In chapter III, we showed that bone marrow and peripheral OCPs gave rise to macrophages and dendritic cells (in addition to osteoclasts) when stimulated with differential cytokines, suggesting a common developmental pathway among these cells.

We developed a fluorescent reporter mouse model to study osteoclast migration and differentiation in vivo. Transplant studies showed that bone marrow OCPs home back to the bone marrow and migrated to the spleen while circulating in peripheral blood. Interestingly, spleen precursors were able to migrate to bone marrow and also engrafted in the spleen. In addition, transferred OCPs differentiated to fluorescent osteoclasts.
associated to bone surfaces. These experiments suggest that the bone marrow and spleen precursors are developmentally related.

Osteoclasts are critically involved in skeletal function such as bone remodeling, fracture repair, and in pathological bone resorption associated with inflammatory conditions. We challenged mice with LPS and found that peripheral OCPs increased their number and resorptive capacity. In addition, we studied how chronic inflammation modulated OCPs in arthritic (hTNF) mice, and found that peripheral precursors were increased in number. OCPs derived from CathepsinK-Cre-tdTomatoFP recipient mice and transferred to hTNF, engrafted to localize sites of inflammation and differentiated to RFP+ osteoclasts associated to bone surfaces in diarthrodial joints. These experiments suggest that inflammatory signals modulate osteoclast precursor migration, distribution, resorptive capacity and differentiation.

Finally, we studied the effect of parathyroid hormone (PTH) on osteoclast precursor populations in the bone marrow. We found that intermittent administration of PTH to mice increased the frequency of these populations in the bone marrow and increased their ability to form osteoclasts \textit{in vitro}.
Phenotypic Characterization of Peripheral Osteoclast Precursors their Lineage Relation to Macrophages and Dendritic Cells and their Population Dynamics Influenced by Parathyroid Hormone and Inflammatory Signals.

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Phenotypic Characterization of Peripheral Osteoclast Precursors their Lineage Relation to Macrophages and Dendritic Cells and their Population Dynamics Influenced by Parathyroid Hormone and Inflammatory Signals.

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CHAPTER I

Introduction

1. Osteoclasts and bone remodeling.

Osteoclasts are unique large multinuclear and highly specialized bone resorbing cells of hematopoietic origin (Walker, 1973). The process of bone resorption is the hallmark of osteoclast activity. Bone resorption is a complex process that requires the degradation of the inorganic (predominantly hydroxyapatite) and organic (predominantly type I collagen) components of bone tissue. First, osteoclasts firmly attach to the mineralized matrix and polarize their plasma membrane towards bone, forming the apical membrane domain or “ruffled border” that concentrates the necessary elements to degrade bone, and the basolateral membrane domain, which is localized in the opposite direction from the bone surface. The attachment of osteoclasts to bone creates an isolated microenvironment, known as the sealing zone, which is an organized structure surrounding the ruffled border and formed by densely packed actin-rich rings, known as podosomes that also confers motility to osteoclasts allowing them to migrate along the surface of the bone. (Raisz 1999; Teitelbaum 2000; Bruzzaniti and Baron, 2006; Pfaff and Jurdic, 2001; Destaing et al., 2003). The interactions of osteoclasts with bone tissue are also mediated by integrins, being αvβ3 the most abundantly expressed. These molecules confer firm adhesion to mineralized matrix and participate in osteoclast migration and signaling. Experimental evidence
has shown that knock out mice lacking the \( \beta_3 \) subunit of integrins are osteopetrotic and their osteoclasts failed to migrate and form the proper structures of the apical membrane domain (McHugh et al., 2000; Feng et al., 2001).

Second, after adhesion to the bone surface, bone degradation occurs next. Osteoclasts are equipped with the machinery that is necessary for bone demineralization. Among these are the ion transporter system, including \( \text{H}^+ \) adenosine triphosphatase (\( \text{H}^+ \text{ATPase} \)) and chlorine channels that release \( \text{H}^+ \) and \( \text{Cl}^- \) ions, respectively, which ultimately create an acid microenvironment that dissolves hydroxyapatite (Mori et al., 2002; Schlesinger et al., 1997). In addition, osteoclasts also release proteolytic enzymes such as Cathepsin K, matrix metalloproteinases (e.g., MMP-9) and TRAcP (tartrate-resistant acid phosphatase) for type I collagen degradation. TRAcP has been extensively used to identify osteoclast in vivo and in vitro. Finally, these events lead to the formation of a resorptive lacuna that is eventually replaced with new bone produced by osteoblasts. These are bone-forming cells of mesenchymal origin derived from osteoprogenitors that differentiate into osteocytes and ultimately generate mineralized matrix (Ducy et al., 2000). Consequently, osteoclasts are critical elements in bone remodeling, which is a metabolic process regulated by the balance between bone resorption and bone formation (mediated by osteoblasts); which is necessary to maintain bone homeostasis. Figure 1 shows a schematic representation of osteoclastic bone resorption and the molecules involved in bone degradation.

In addition to bone homeostasis, osteoclasts are also important in skeletal development and fracture repair, two processes in which bone remodeling is active and critical for the development of adult vertebrates and bone health.
Skeletogenesis, which occurs during embryogenesis and postnatal development, is a process in which cartilage is gradually replaced by bone to allow the growth of long bones until adulthood (Mackie et al., 2008). Most bones are formed through a process called endochondral ossification that involves the interplay of several cellular subsets. Chondrocytes produce cartilage and undergo hypertrophy, a process required for their maturation and terminal differentiation. The enlargement of these cells and the matrix they produce serves as supportive tissue for the initial steps of skeletal development (Mackie et al., 2008; Mackie et al., 2011). Blood vessel formation, induced by local factors like VEGF (vascular endothelial growth factor), surrounds the ossification site and brings hematopoietic progenitors from peripheral tissues that will eventually constitute bone marrow populations, as seen in adult vertebrates. Bone-resorbing osteoclasts derive from these progenitors and are fundamental to establishing the primary center of ossification, by removing the initially formed cartilage matrix that is eventually replaced by osteoblasts-produced bone (Mackie et al., 2008; Horton et al., 2009).

These events are critical for the elongation and ossification of bones necessary for the proper formation of the skeletal structure as bone growth occurs. Here, osteoclasts activity is also crucial for the formation of the bone marrow cavity that serves as a niche to maintain hematopoietic stem cells pluripotency and self-renewal, and for hematopoiesis to occur throughout the life of an individual.

Interestingly, in adult vertebrates the healing process of fracture repair recapitulates the process of endochondral ossification, in which cartilage is first produced to form a callus (the tissue that surrounds the fractured site of the bone). Next, cartilage and bone are remodeled by osteoclasts, to restore the original bone structure and then replaced by new bone (Shapiro et al., 2008; Al-Aql et al., 2008). The events occurring in fracture repair are therefore of relevance to
study skeletal development and bone remodeling, and indeed animal models of fractures are used by researchers to understand these mechanisms.

Bone remodeling, skeletal development and fracture repair highlight the importance of osteoclasts for bone homeostasis and health, however, aberrant signaling in the regulation of osteoclast activity has detrimental effects like those observed in pathological bone resorption associated with inflammatory conditions, e.g., rheumatoid arthritis (Raisz, 1999; Teitelbaum, 2000; Lorenzo et al., 2008). Figure 2 shows a schematic representation of osteoclast development and the process of bone remodeling.

2. **The ontogeny of osteoclasts**

The origin of osteoclasts and their precursor cells has been widely studied for many years. Initial studies by Walker, reported almost 40 years ago, demonstrated that osteoclast are derived from hematopoietic progenitors. These studies showed that osteopetrosis in microphthalmic (mi/mi) mice, which is caused by a defect in the microphthalmia transcription factor (MITF) that regulates osteoclasts fusion, was reversed when parabiosis (a surgical procedure to join the vascular system of two different mice) is made with a normal littermate (Walker, 1975a). This indicated that functional non-osteopetrotic hematopoietic precursors that circulate in the bloodstream are sufficient to rescue the disease. In addition, this was confirmed in transplant experiments of bone marrow and spleen cells from normal mice into osteopetrotic mice (Walker, 1975b). Interestingly, spleen cells derived from the mutant mice and transferred into normal recipient mice caused the normal mice to become osteopetrotic (Walker, 1975c). Clinically, bone marrow transplants in humans have also shown that osteopetrosis can be cured using this procedure (Coccia et al., 1980).
Once the hematopoietic origin of osteoclasts was established, the search for osteoclast precursors has been proposed as a way to study their precise phenotype and lineage, in mice and humans. Several laboratories made early contributions to the identification of hematopoietic cell populations that contain the potential of generating osteoclasts in vitro (Kukita and Roodman, 1989; Fujikawa et al., 1996; Quinn et al., 1996; Tsurukai et al., 1998). Later on, the use of FACS (fluorescence-activated cell sorting) technology has allowed a better identification of the cell surface phenotype of osteoclast precursors, allowing the isolation and characterization of such populations.

Early experiments performed by Muguruma and Lee identified a progenitor population in murine bone marrow with the potential to generate TRAcP+ osteoclasts in vitro when co-cultured with stromal cells. This population was negative for the B cell marker (B220/CD45R), granulocytes (Gr-1), macrophages (CD11b), and erythrocytes (Ter-119) but was positive for CD117 (c-kit, the stem cell factor receptor) (Muguruma et al., 1998). However, this population was not homogenous because they also generated other hematopoietic cells (i.e., granulocytes, macrophages and erythrocytes). Similarly, Arai et al. reported a population in the bone marrow that expressed CD117, low levels of CD11b, high levels of CD115 (c-fms, the M-CSF receptor) and generated osteoclasts in vitro with high efficiency, however this population also generated macrophages (Arai et al., 1999). More recently, Jacquin et al reported that multiple populations of osteoclast progenitors are present within the murine bone marrow. Between them, the highest potential to generate osteoclasts in vitro was contained in cells that are B220/CD45R/CD3-CD11b-CD115+CD117+ (also called “Population IV” or PIV). In addition, cells that are intermediate (Population V) or negative (population VI) for CD117 also generated osteoclasts, but in lower frequencies (Jacquin et al., 2006). Interestingly, the authors showed that PIV
differentiates into PV and PVI after 2 days of stimulation with M-CSF (macrophage-colony stimulating factor) and RANKL (receptor activator of NF-kB ligand, a tumor necrosis factor family member) but PV or PVI did not give rise to PIV. These experiments suggest a possible developmental relationship of PIV, being less mature than PV or PVI.

The evidence discussed above demonstrates that osteoclasts are derived from myeloid/monocytic progenitors and are generated through a progression that involves the fusion of mononuclear precursor cells into mature multinuclear bone-resorbing cells. The generation of multinuclear osteoclasts can be recapitulated in \textit{in vitro} cultures if osteoclast precursors are stimulated with soluble M-CSF and RANKL, and indeed this protocol constitutes an important assay to study osteoclast development and differentiation (Suda et al., 1999; Teitelbaum, 2000). Before the identification of RANKL, most studies were performed in the presence of stromal tissue and osteoblastic cells and cultures were also stimulated with parathyroid hormone (PTH) or 1,25 dihydroxyvitamin D3, the active form of vitamin D3. Our studies on osteoclastogenesis \textit{in vitro} are performed with highly purified and defined populations of osteoclast precursors stimulated with M-CSF and RANKL in the absence of stromal tissue or osteoblast-derived cell cultures.

\textbf{3. Regulation of osteoclast activity}

The development of osteoclasts is tightly regulated by cell interactions with osteoblastic cells and stromal tissue. Crucial for the development of functional osteoclasts are signals mediated by c-fms/CD115 (the M-CSF receptor) and RANK (receptor activator of NF-kB) both of which are expressed on the surface of osteoclast precursor cells (OCP) (Arai F., et al 1999; Suda et al., 1999) and are activated by binding of M-CSF (macrophage colony stimulating
factor) and RANKL (receptor activator of NF-kB ligand). M-CSF is secreted by stromal/osteoblastic cells and interacts with c-fms (M-CSF receptor) expressed on OCPs activating both the PI3K/Akt pathway and the Ras/Raf/MEK/Erk pathway which converge to activate Cyclin D, a protein that regulates cell cycle progression, therefore promoting proliferation and survival of OCPs (Ross and Teitelbaum 2005; Ross 2006). RANK ligand is expressed by stromal/osteoblastic cells and interacts with RANK expressed on OCPs (Fuller et al., 1998). The major adaptor molecule linking RANK to osteoclastogenesis is TRAF6 (TNFR-Associated Factor). This pathway controls the differentiation of osteoclasts through NF-κB and MAP kinase pathways that result in the activation of c-Fos and c-Jun (AP-1) and NFATc1 transcription factors for the expression of genes involved in the fusion (DC-STAMP and ATPv6d02), attachment of osteoclast to bone surfaces (β3-integrin) and bone resorptive capacity (cathepsin K, TRAcP, MMP-9, V-ATPase and carbonic anhydrase II) (Koga et al., 2004; Kadono et al., 2005).

Mice deficient in any of these signaling molecules are osteopetrotic due to impaired osteoclast function. For instance, RANK and RANKL knock-out mice are born with defects in hematopoiesis and bone abnormalities (osteopetrosis, increased bone mass, fragile bones and failure to erupt teeth) highlighting the importance of this pathway for proper osteoclasts development and functional activity (Dougall et al., 1999; Kong et al., 1999). Similarly, spontaneous mutations of the M-CSF gene in mice (like those seen in the op/op mouse) resulted in reduced number of osteoclasts due to compromised proliferation and further commitment of myeloid precursor cells to osteoclast lineage (Wiktor-Jedrzejczak et al., 1990). Important for the generation of multinucleated osteoclasts is the activation of fusion molecules. Among these, DC-STAMP (dendritic cell-specific transmembrane protein) is one of the crucial molecules involved
in osteoclastogenesis (Eleveld-Trancikova et al., 2005; Kukita et al., 2004). Mice lacking DC-STAMP are osteopetrotic, suggesting that multinucleation is necessary for the generation of functional resorbing osteoclasts (Yagi et al., 2005). These studies corroborated that M-CSF and RANKL signaling are essential and sufficient for the proliferation of mononuclear osteoclast precursors and terminal differentiation to mature multinuclear bone resorbing cells.

In addition to these pathways, other signals act synergistically to promote osteoclast activity. These include the immunoreceptor tyrosine-based activation motif (ITAM), which are intracellular adaptor proteins. Among these are: 1) DNAX-activating protein 12 (DAP12) which associates with the receptor TREM-2 (Triggering Receptors Expressed on Myeloid cells-2) and SIRPβ1 (Signal Regulatory Protein β1), and 2) FcRγ (Fc Receptor-γ-chain) which interacts with PIR-A (Paired Immunoglobulin-like Receptor A) and OSCAR (Osteoclast-Associated Receptor). Adaptor molecules DAP12 and FcRγ signal downstream of their receptors to activate Syk kinase and PLCγ2 which initiates calcium oscillations that leads to the upregulation of NFATc1 and CREB transcription factors that amplify the response for survival and differentiation of osteoclasts (Mocsai et al., 2004; Kim et al., 2005). The lack of these signaling adaptor molecules (DAP-12 and FcRγ) also resulted in an osteopetrotic phenotype in mice due to a reduced number of multinucleated osteoclasts and consequently reduced resorptive capacity (Humphrey et al., 2004; Mocsai et al., 2004; Koga et al., 2004).

A negative regulator of osteoclastogenesis is osteoprotegerin (OPG), which is a TNFR superfamily member that lacks the transmembrane domain and is produced by stromal/osteoblastic cells as a soluble decoy receptor for RANKL, and consequently prevents osteoclast differentiation. OPG is then a crucial factor that regulates bone remodeling. In support
of this, mice lacking OPG have increased number of osteoclasts and consequently develop osteoporosis (Simonet et al., 1997; Yasuda et al., 1998).

4. Osteoclasts and their lineage relation to monocytes

Studies on osteoclast development and other myeloid derived cells have led to the hypothesis that osteoclast progenitors share a common precursor with macrophages and dendritic cells. Most of these studies have been based on phenotypic similarities between these types of cells, identified through immunoreactivities with panels of monoclonal antibodies recognizing cell surface epitopes. Cells have been then isolated and their developmental potential assessed in \textit{in vitro} cultures, in the presence of differential cytokines. Osteoclasts and macrophages depend on M-CSF for their proliferation and differentiation. The fact that mice deficient in M-CSF (op/op mice) showed a complex phenotype that involves severe osteopetrosis (due to defects in osteoclasts development) and are deficient in the mononuclear phagocyte system particularly in macrophages supports the hypothesis of a common precursor for both lineages (Wiktor-Jedrzejczak et al., 1990; Dai et al., 2002). In addition, Tondravi et al., reported that in PU.1 deficient mice the development of osteoclasts and macrophages was arrested (Tondravi et al., 1997). Miyamoto et al. identified a myeloid precursor cell that expressed CD115 and differentiated to osteoclast when cultured in the presence of M-CSF and RANKL and to dendritic cells when cultured in the presence of GM-CSF and RANKL (Miyamoto et al., 2001). Further experiments by Servet-Delprat reported that a Flt-3 ligand-dependent macrophage precursor cells could give rise to osteoclast, dendritic cells and microglia, \textit{in vitro} (Servet-Delprat et al., 2002). It has also been shown that dendritic cells can trans-differentiate to osteoclasts, at least under certain inflammatory conditions (Rivollier et al., 2004).
All these data combined strongly support the hypothesis that osteoclast precursors and monocytes belong to the same lineage and might indeed exists a common precursor for all three mature monocyctic lineages: osteoclasts, macrophages and dendritic cells. Monocytes are a heterogeneous population of hematopoietic mononuclear cells that develop in the bone marrow from myeloid progenitors and consist of several functional subsets (Geissmann et al., 2008; Auffray et al., 2009). Monocytes belong to the mononuclear phagocyte system and have the ability to generate some tissue macrophages and dendritic cells, and maintain these populations in peripheral tissues during homeostasis and inflammation (Auffray et al., 2009; Geissmann et al. 2010). Macrophages and dendritic cells play important roles in the activation and development of immune responses against pathogenic threats (Gordon and Taylor, 2005; Randolph et al., 2008; Martinez et al., 2009; Biswas et al., 2010; Hashimoto et al., 2011). However the lineage relationship between macrophages, dendritic cells and osteoclasts have not yet been strictly established.

We hypothesize that osteoclasts are derived from a common bone marrow osteoclasts/macrophage/dendritic cell progenitor (OcMDC). This progenitor has the ability to migrate from the bone marrow and populate peripheral tissues with macrophage and dendritic cell populations and in the context of the bone marrow generate osteoclasts under the influence of the bone microenvironment.

Our working hypothesis focuses on the study of the origin, migration and differentiation of bone marrow and peripheral myeloid derived progenitors with osteoclastogenic potential. We hypothesize that circulating and resident peripheral tissue osteoclast precursors that originate in the bone marrow should still retain the potential to become osteoclasts when stimulated with M-CSF and RANKL in vitro and potentially serve as a tool to identify osteoclasts progenitor
populations in peripheral tissues. Using flow cytometry it is possible to isolate circulating and resident peripheral myeloid precursors and use the *in vitro* osteoclastogenic potential of these cells to identify osteoclast progenitor populations in different compartments of the body. Figure 3 shows our current working hypothesis for the developmental relationships between bone marrow and circulating osteoclasts precursors.

The understanding of osteoclast development, migration and differentiation has a profound impact in human disease because these cells are involved in the pathogenesis of inflammatory disease, as in the case of rheumatoid arthritis, and microbial infections such as bacteriosis and parasitosis (Strauss-Ayali et al., 2007; Serbina et al., 2008; Evans et al., 2009; Sponaas et al., 2009). Therefore, identification and characterization of bona fide populations of osteoclasts precursors should provide critical information for the understanding of the pathophysiology bone loss associated to inflammation and infections.

5. **Osteoclasts are regulated by parathyroid hormone**

Parathyroid hormone (PTH) is a protein of 84 amino acids that is produced by the parathyroid glands in response to low levels of circulating calcium (Ca$^{2+}$). PTH is the major systemic regulator of calcium homeostasis. It activates bone resorption that results in the release of calcium (as a product of bone degradation) from bone to the circulation. In addition, PTH acts on kidney to decrease Ca$^{2+}$ excretion and in the small intestine to increase absorption of dietary calcium, a process that depends on the active metabolite of vitamin D (calcitriol). These mechanisms are necessary to maintain Ca$^{2+}$ plasma levels in the blood (Brown and MacLeod, 2001).
PTH acts directly on cells expressing PTH receptors. For instance, binding of PTH-PTH receptors on osteoblasts increases their number in vivo and in vitro, and stimulates expression of RANKL on the surface of these cells (Juppner et al., 1991; Lee and Lorenzo, 1999; Calvi et al., 2003). Importantly, PTH inhibits the expression of OPG in osteoblasts. Therefore, the effects of PTH on osteoclastogenesis are regulated by at least two mechanisms, the induction of RANKL expression and the repression of OPG production (Huang et al., 2004). These mechanisms regulate bone remodeling and are therapeutic targets in bone disease. But what are the mechanisms of PTH-induced osteoclastogenesis?

Osteoclast precursor cells, which lack PTH receptors, respond to the increased levels of RANKL and differentiate into mature osteoclasts. Therefore, PTH effects on osteoclastogenesis are considered to be indirect mediated through osteoblast/stromal cell interactions with osteoclast precursor cells (Rouleau et al., 1988). These events highlight the importance of the tightly regulated interactions between osteoblast and osteoclast precursors that maintain bone homeostasis.

Due to the well-known actions of PTH on bone remodeling, a recombinant form of this hormone administered once a day (intermittent) is the basis for the treatment of osteoporosis in order to increase bone mass in patients and the only FDA-approved therapy for this condition in humans. However, the administration of PTH has both catabolic and anabolic effects on bone, depending on the dose and frequency of administration. A catabolic effect, or loss in bone mass, can be achieved by continuous administration of the hormone (Frolic et al., 2003). The anabolic effects of PTH could be useful for modulating bone remodeling in other conditions, and experiments in rodents have shown that PTH could improve the process of bone healing in
fracture repair (Andreassen et al., 1999). For this reason, PTH administration has been proposed for the treatment of fractures in humans (Barnes et al., 2008).

Besides its effects in bone remodeling, PTH also influences the stability of hematopoietic progenitors, particularly of hematopoietic stem cells (HSC), that are found in close proximity to osteoblastic lineage cells located on the edosteum (Calvi et al., 2003). It has been proposed that osteoblasts regulate HSC through cell interactions mediated by cell surface molecules. Some of these molecules activate important signaling pathways that maintain the number, pluripotency and self-renewal capability of HSC. These signaling pathways have been widely studied, among them are: Jag1-Notch signaling known to maintain the long-term repopulating activity and quiescence of HSC; N-cadherin interactions that provide cell adhesion and Angiopoietin1-Tie1 that supports HSC number (Adams and Scadden., 2006; Mercier et al., 2012). It has been reported that PTH regulates the expression of such molecules acting directly on osteoblastic cells and stromal tissue leading to the modulation of HSC activity (Adams and Scadden., 2006). In addition, daily doses of PTH have been reported to increase the number of HSC (Lin-CD117+Sca-1+) in the bone marrow of mice after endogenous administration for 6 to 8 weeks (Calvi et al., 2003). Others have reported that pre-treatment with intermittent administration of PTH for 5 weeks follow by 5 days of G-CSF (granulocyte-colony stimulating factor) mobilizes HSC from the bone marrow to the blood stream (Brunner et al., 2008).

These studies are important to design better protocols to harvest hematopoietic progenitors in donors to benefit bone marrow transplantation recipients. Also, PTH administration to mice undergoing bone marrow transplantation had improved the outcome of engraftment with 100% survival compared to mock injected mice (Adams et al., 2007). As a
result from all these studies, PTH is currently under consideration as an alternative approach to facilitate stem cell transplantation in humans (Ballen et al., 2007).

6. Osteoclasts, microbial infections and disease.

6.1. Inflammatory arthritis.

Osteoclasts play important roles in the pathogenesis of inflammatory arthritis, osteoporosis, osteopetrosis, Paget’s disease, pathological fractures, periodontal disease, and bacterial infections, among others (Lorenzo et al., 2008; Jones et al., 2011; Novack et al., 2008; Shapiro, 2008; Roodman and Windle, 2005; Erhlich and Roodman, 2005). However, the contribution of osteoclast precursor activation, migration, differentiation and activity in these conditions are not completely understood.

Among these pathologies, rheumatoid arthritis (RA) highlights the role of osteoclast as the main player on the bone and cartilage destruction leading to impaired joint function and consequent disability in humans. Rheumatoid arthritis, affecting approximately 1% of the population worldwide (Woolf and Pfleger, 2003), is an autoimmune disease characterized by chronic inflammation and hyperplasia of the synovial membrane, pannus formation and mononuclear infiltrates leading to destruction of bone and cartilage with eventual development of ankylosis, primarily affecting multiple peripheral diarthrodial joints (Firestein, 2003; McInnes and Schett, 2007; Woolf and Pfleger, 2003). The production of autoantibodies to the Fc portion of IgG immunoglobulins (rheumatoid factor) and against citrullinated peptides (anti-citrullinated protein antibody, ACPA), which occurs in early stages of the disease and prior to the development of the clinical phenotype, is another important characteristic of RA. In fact, these
autoantibodies are useful markers for the diagnosis of the disease (Walker et al., 1986; Van Venrooij et al., 2008).

The pathogenesis of RA is the product of a complex interplay between different compartments including: hematopoietic cells (T cell subtypes, B cells, dendritic cells, macrophages, monocytes, osteoclasts), mesenchymal cells (osteoblasts, fibroblast-like synoviocytes) and release of cytokines (TNFα, IL-1, IL-6, IL-17, IL-33, GM-CSF, M-CSF, RANKL), (Firestein, 2003; McInnes and Schett, 2007). The later are of great interest due to the potential to serve as therapeutic targets, particularly tumor necrosis factor (TNF), which is a known inflammatory cytokine crucial for the development of RA. Indeed, successful therapeutic interventions with TNF blockers to prevent bone and cartilage erosion, has lead to extensive use of these drugs (Infliximab, Etanercept, Adalimumab, Golimumab, Certolizumab pegol) (McInnes and Schett, 2011; Firestein, 2003).

TNFα is a pleiotropic cytokine produced by macrophages, monocytes, neutrophils, synovial fibroblasts and T cells, which induces activation, proliferation, differentiation and apoptosis of multiple cell subsets. TNF effects are mediated through TNF receptor 1 and TNF receptor 2. TNFR1 is expressed in most nucleated cells and is activated by binding of the soluble and membrane-bound form of TNF leading to the activation of the pro-inflammatory and pro-apoptotic pathways. On the other hand, TNFR2 expression is restricted to endothelial cells and some immune cells, and in contrast to TNFR1, TNFR2 activates a non-canonical pathway of NF-κB activation, however less is known about the effects of TNFR2 activation (Bluml et al., 2012). In RA, TNF primarily exerts its detrimental effects through TNFR1. Recently, as an alternative approach to anti-TNF therapy, a drug targeting the TNFR1 has been developed (Kontermann et al., 2008; Zettlitz et al., 2010). Indeed, a recent report showed that an antagonist of TNFR1
reduced arthritic symptoms in a Collagen Induced Arthritis (CIA) mouse model (Shibata et al., 2009). TNF and TNF receptors are highly expressed in the synovium of RA patients and are known to influence, at the transcriptional and post transductional levels, the production of other inflammatory cytokines like IL-1, IL-6, IL-17 and GM-CSF, promoting inflammatory cell recruitment and local cell differentiation in several cellular subsets involved in the maintenance of the chronicity of the disease (McInnes and Schett, 2007; Di Giovine et al., 1988; Firestein et al., 1990).

Among other pleiotropic effects, TNF is well know to promote bone resorption in inflammatory arthritis by inducing osteoclastogenesis in the local microenvironment of the inflamed joints (Nakashima and Takayanagi, 2009). In addition, it has been reported that TNF activates osteoblast/stromal cells and fibroblast to increase RANKL expression that in turn induces osteoclasts differentiation. Suggesting a synergistic effect of TNF and RANKL to promote bone erosion. It has also been shown that TNF acts directly on osteoclast precursor cells to promote osteoclastogenesis (Azuma et al., 2000; Kim et al., 2005; Kobayashi et al., 2000). These facts also highlight the relevance of osteoclast precursors, which are actively recruited toward sites of inflammation and eventually are induced (by local cytokines) to differentiate into osteoclasts that contribute to bone loss. The precise mechanisms initiating and maintaining the chronicity of inflammatory arthritis, and the signals and receptors involved in osteoclast precursor migration, differentiation and activity are still not completely understood and are current focus of extensive investigation.

Multiple animal models have been generated to study the mechanisms of arthritis. These models mimic several of the events occurring in RA however no single animal model completely represents the overall human disease. These mouse models have been widely used and
characterized, among them are: the collagen-induced arthritis (CIA) model, antibody-induced arthritis (AIA) and TNF-transgenic mice (Luross and Williams, 2001; Van der Berg, 2009; Bevaart et al., 2010). The later, is a model in which the human Tumor Necrosis Factor (hTNF) is overexpressed in mice. These mice were developed by microinjection of hTNF/β-globin fusion construct into mouse zygotes, resulting in the insertion of 5 copies of the human TNF gene (line 197) leading to the overexpression of TNF resulting in the development of arthritis. The features of arthritis development in these mice share many of the characteristics observed in the human disease. The onset of the disease occurs as early as 4 to 5 weeks, and progresses to severe polyarthritis at 8 to 10 weeks (Keffer et al., 1991). As in most mouse models of arthritis, anti-TNF monoclonal antibody administration suppresses the development of arthritis, although does not offer a cure. We have used hTNF mice to test the dynamics, migration and osteoclastogenic potential of hematopoietic populations in inflammatory arthritis.

6.2. Bacterial infections and osteolysis

Bacterial infections are associated with an inflammatory process and when present in the context of bone they result in localized osteolysis: periodontal disease, osteomyelitis and arthritis (Nair, 1996). Several hypotheses have emerged on the activation of osteoclastogenesis by bacterial components. Lipopolysaccharide (LPS) is believed to be one of the major bacterial mediators of osteoclast activation and osteolysis (Abu-Amer et al., 1997). In vitro studies have shown that in RAW 264.7, a murine macrophage cell line, LPS induced osteoclast formation with increased bone resorptive activity (Islam et al., 2007). Studies in vivo have shown that administration of LPS in mice induced osteoclast precursor differentiation leading to an increase in bone resorption (Sakuma et al., 2000; Zhuang 2007).
LPS is the major structural component of the outer membrane of gram-negative bacteria. The structure of LPS consists of 3 components: 1) a hydrophobic domain known as lipid A (endotoxin), which is a highly conserved glucosamine-based phospholipid that contains the inflammatory effects of LPS, 2) an oligosaccharide as the core region and 3) a distal polysaccharide known as the O-antigen, a component that varies among bacteria and does not trigger an immune response on its own (Raetz and Whitfield, 2002; Beutler and Rietschel, 2003).

In humans and mice, LPS is recognized by receptors of the innate immune system. These receptors, known as Toll-like receptors, are homologs of receptors initially identified in Drosophila (Lemaitre, 2004). In general, TLRs recognize highly conserved regions of pathogen molecules (known as pathogen-associated molecular patterns or PAMPs) that function as ligands to activate the immune response (Medzhitov R, 2001). Therefore, TLR have been classified as part of a family of pathogen-recognition receptors (PRR). In particular, LPS is recognized by TLR4 that is expressed in the cell surface of immune cells in the host. TLR4 requires the complex of several extra- and intra-cellular accessory molecules to initiate signaling pathways necessary to trigger an immune response (Medzhitov R, 2001).

TLR4 contains a conserved sequence of molecules known as LRRs (leucin-rich repeats) that are involved in the recognition of PAMPs, a transmembrane domain, and a cytoplasmic Toll/interleukin-1 receptor (TIR) domain responsible for intracellular signaling. Engagement of LPS results in the activation of transcription factors (NF-kB and AP-1) and expression of inflammatory cytokines (Akira and Takeda, 2004).

In an infected host, LPS in the circulation binds to LBP (LPS binding protein) a plasma protein constitutively produced in the liver and in greater amounts after gram-negative bacterial infections. The major role of the soluble complex of LPS-LBP is to transfer LPS to CD14, a LPS
high-affinity co-receptor that is anchored to the cell surface of hematopoietic cells. CD14 is particularly expressed on macrophages as the first line of defense. Another component of the LPS recognition complex is MD-2 (myeloid differentiation factor-2) a small protein associated with the ectodomain of TLR4 that also functions as a bridge between LPS and TLR4. These accessory molecules are required to be assembled for the LPS-induced activation of TLR4 that ultimately results in the production of pro-inflammatory cytokines (Akira and Takeda, 2004; Dobrovolskaia and Vogel, 2002).

Once the TLR4 complex has been activated, the intracellular domain of TLR4 interacts with a variety of adapter proteins classified in two major signaling pathways. First, myeloid differentiation primary response protein 88 (MyD88) dependent, which is a pathway that requires the recruitment of TIRAP (TIR domain-containing adapter protein) to the intracellular domain of TLR4, and then complex with MyD88 to initiate a series of signaling cascades for the activation of the NF-κB and MAP kinase (AP-1 transcription factor) family members that ultimately result in the gene transcription and protein production of inflammatory cytokines, like IL-6 and TNFα. A second pathway is MyD88 independent, and differs from MyD88-dependent pathway in that it utilizes other adapter proteins to signal. It requires Trif (TIR domain-containing adapter inducing IFNβ) and TRAM (Trif-related adapter molecule) to complex to the intracellular TIR domain of TLR4 resulting in the activation of IRF3, NF-κB and AP-1 for the production of Type I IFN. This pathway has been shown to be active in MyD88-deficient mice and also showed a delay in the activation of NF-κB and MAP kinases pathways in response to LPS, suggesting a time difference in the production of inflammatory cytokines and type I IFN (Akira and Takeda, 2004; Dobrovolskaia and Vogel, 2002, Kawai and Akira, 2006; Lee et al., 2012).
As described above, TLR4 activation by LPS results in the production of TNF\(\alpha\), one of the inflammatory cytokines required for the initiation of the immune response against gram-negative bacterial infections, as already mentioned, TNF\(\alpha\) is also a cytokine with implications in the development of inflammatory arthritis. Therefore, hypotheses have emerged on the role of bacterial infections and inflammatory cytokine production for the activation of osteoclast precursors as mediators of osteolysis in disease (Mormann et al., 2008; Itoh et al., 2003; Zou and Bar-Shavit, 2002). Due to the correlation between bacterial infections and inflammatory arthritis as a risk factor for developing RA and the crucial role of osteoclasts in bone loss, we have also investigated whether LPS could modulate the osteoclastogenic potential of specific populations of osteoclasts precursors.

In conclusion, all the previous published work discussed above indicates that osteoclasts are crucial elements in skeletal development, bone homeostasis and bone repair. They are essential for developing vertebrates and for maintaining the proper structure of bone that ultimately account for skeletal health. However, inherited defects in osteoclast function or aberrant signals that favors exacerbated resorptive activity of osteoclasts, results in the development of pathological states. Therefore, understanding the ontogeny, migration and differentiation of osteoclast precursors as well as the mechanisms that trigger bone resorption are of great importance for the development of therapeutic approaches that could allow us to modulate osteoclast activity to prevent bone loss in disease. For these reasons, my current research focused on 1) the identification and characterization of bona fide populations of osteoclast precursors in multiple tissues, 2) their localization and migratory properties, 3) the hormonal and inflammatory signals that modulate their activity and 4) their ability to resorb bone under normal and pathological conditions.
7. Figures

Figure 1. Schematic representation of bone resorption. Osteoclasts attached to bone tissue and degrading the mineralized matrix, formed by osteoblast, by secreting several factors including: proton pumps, cathepsin K, matrix metalloproteinases (MMPs) and TRAcP (tartrate-resistant acid phosphatase).
Figure 2. Schematic representation of the process of bone remodeling. Osteoclasts formed from the fusion of mononuclear myeloid precursors and attach to bone degrading its surface and moving along bone tissue. Osteoclasts form a lacuna at site of bone resorption, which is eventually replaced by new bone made by osteoblast, that originate from mesenchymal precursors. These events maintain the homeostasis of bone tissue in adult vertebrates.
Figure 3. Working hypothesis. These studies focus on the generation, migration and differentiation of bone marrow and peripheral osteoclast precursors during homeostasis and inflammation. We hypothesize that osteoclasts are derived from a common precursor that also generates macrophages and dendritic cells. These precursors have the ability to migrate and populate peripheral tissues and are influenced by inflammatory and hormonal signals to modulate their activity.
CHAPTER II

Materials and Methods

1. Animal subjects

All C57BL/6 male and female mice were purchased from Charles River Laboratories (Wilmington, MA). Transgenic mice pOBCol3.6 GFP (sapphire) and pOBCol3.6 CyanFP were a kind gift from Dr. David Rowe (UCHC Farmington, CT, USA). Human TNF transgenic mice were a kind gift from Dr. Georg Schett (University of Erlangen–Nuremberg, Erlangen, Germany). Cathepsin K-Cre mice and ROSA26-CAG-LoxP-tdTomato reporter mice were crossed to generate ctsk<sup>cre<sup>+/−</sup></sup>;tdTomatoFP mice as described in Chapter IV. All the animals were housed in sterile microisolators and given water and rodent chow <i>ad libitum</i>. Animals were housed in the Center for Laboratory Animal Care at the University of Connecticut Health Center. All animals were used according to protocols approved by the Animal Care Committee of the University of Connecticut Health Center.

2. Antibodies and Flow Cytometry

All of the antibodies used for cell surface phenotypic analyses and sorting of mouse hematopoietic progenitors from bone marrow, spleen and peripheral blood are commercially available. These include: anti-B cell lineage antibody (mAb): anti-CD45R/B220 (RA3-6B2); anti-CD19 (1D3); anti-T cell lineage mAb: anti-CD3 (145-2C11); anti-CD4 (GK1.5) and anti-
CD8 (53.6.7); anti-NK cell mAb: NK1.1 (PK136); anti-erythroid progenitor monoclonal mAb (Ter119); anti-monocyte/macrophage antibodies: anti-CD11b/Mac-1 (M1/70), anti-F4/80 (BM8); anti-dendritic cell mAb: anti-CD11c (N418); anti-granulocyte mAb: anti-Gr-1 (RB6-8C5); anti-progenitor cell antibodies: anti-Ly-6A/E Sca-1 (E13.161.7), anti-ckit/CD117 (2B8), anti-c-fms/CD115 (AFS98), anti-CD135/Fit-3 (A2F10); anti-IL-7Ra/CD127 (A7R34); anti-MHC and co-stimulatory molecules: anti-MHCI/H-2Kb (AF6-88.5), anti-MHCII/I-A/I-E (M5/114.15.2), anti-CD86/B7-2 (GL1), anti-CD80/B7-1 (16-10A1), anti-CD40 (1C10); anti-chemokine receptor antibodies: anti-CCR2 (475301), anti-CCR5/CD195 (C34-3448), anti-CCR7/CD197 (4B12), anti-adhesion molecules mAb: anti-CD62L/L-selectin (MEL-14), anti-Ly6C (AL-21), anti-Ly6G (1A8).

All these antibodies were obtained directly conjugated to fluorochromes or biotinylated from e-Biosciences (San Diego, CA, USA), BioLegend (San Diego, CA, USA), Pharmingen/BD Bioscience (San Diego, CA, USA) or R&D System (Minneapolis, MN, USA). All antibodies were optimized, in terms of their concentration and titration. Labeling of cells for flow cytometric analysis or cell sorting was performed by standard staining procedures wherein directly conjugated or biotinylated mAb plus second step reagents were sequentially added to the cell preparation of interest. All antibodies were titrated for optimal dilutions. All stainings were done on ice and dead cells were identified and excluded by their ability to incorporate propidium iodide (PI). Flow cytometry analysis and sorting was performed in a BD-FACS Aria (BD Biosciences. San Jose, CA, USA) equipped with five lasers and 18 fluorescence detectors. All the data was analyzed using FlowJo software (Tree Star Inc., Ashland, OR).
3. Isolation of Hematopoietic Precursors

For the isolation of hematopoietic precursors, bone marrow cells were harvested for single cell suspensions by flushing femurs and tibias with staining medium (Hank’s balanced salt solution (HBSS); 10 mM HEPES; 2% newborn calf serum) into 15 mL tubes using a 21-gauge needle. Cells were centrifuged (1500 rpm for 5 min at 4°C), the supernatant removed and the pellet containing leukocytes and red blood cells (RBC) lysed by hypotonic shock with 1 ml of red blood cell lysing buffer (Sigma, St Louis, MO), incubated for 5 min and then washed with staining medium and centrifuged again. The final pellet was resuspended in 10 ml of staining medium and filtered through a 100 µm nytex mesh. Live and dead cells were counted in a hemocytometer by trypan blue exclusion.

Spleens were removed from mice and gently dissociated using the frosted ends of two microscope slides in 10 mL of staining media in a Petri dish. Cells were then transferred to a 15 ml tube, centrifuge, the supernatant removed and red blood cells lysed, washed, centrifuge and resuspended in staining medium and counted.

Peripheral blood was collected from the tail vein of mice in a 5 ml tube, containing 500 µl of 5 mM EDTA in PBS. In order to separate peripheral blood mononuclear cells, two ml of 2% dextran was added and incubated at 37°C for 30-45 minutes. The cloudy upper phase was transferred to a 5 ml tube and centrifuged (1500 rpm for 5 min at 4°C), the supernatant removed and the pellet resuspended in 1 ml of RBC lysing buffer.

For analysis and sorting of hematopoietic progenitors, cells were stained with a mix of antibodies in staining media and incubated on ice for 45 minutes, followed by washing in staining medium. As a second step, only used for biotinylated antibodies, cells were stained with streptavidin coupled to the fluorochromes of interest on ice for 45 minutes. Finally, cells were
resuspended in staining medium containing 1μg/ml of PI. For osteoclast precursors, cells were stained with a mix of antibodies containing anti-CD3, anti-B220/CD45R, anti-NK1.1 coupled to APC Alexa750 (or APCeFluor780) and biotinylated anti-CD115. These cells and antibodies were incubated for 45 min, washed with staining medium and centrifuged. The pellet was then resuspended in a cocktail of antibodies including anti-CD11b coupled to Pacific Blue (or eFluor450); anti-CD117 coupled to APC and streptavidin coupled to PE (for biotinylated antibodies). Finally, cells were resuspended in staining medium containing 1 mg/ml of PI. For further analysis of individual OCP populations, the combination of fluorochromes was optimized to include as many antibodies as possible for each individual analysis.

For analyses of early hematopoietic progenitors, cells suspensions were stained with a cocktail of biotinylated antibodies against mature hematopoietic markers. These included: lymphoid markers CD3, CD4, CD8, CD45R/B220, NK1.1; myeloid markers CD11b, and Gr-1 and the erythroid marker Ter119. After incubation and washing, cells were subsequently stained with streptavidin coupled to PE-Texas Red, anti-CD117 coupled to APC, anti-Sca1 coupled to FITC, anti-CD135 coupled to PE and anti-CD127 coupled to Pacific Blue (or eFluor450). Cells were then resuspended in staining medium containing PI and analysis for flow cytometry.

For FACS analysis of sorted cells cultured with cytokines, media was removed and washed 2X with PBS and incubated for 15 min at 37°C in 0.05% Trypsin in 0.53 mM EDTA (Invitrogen) to dissociate the cells from the culture plates. Cells were then transferred to a 1.5 mL tube and centrifuged (1500 rpm for 5 min at 4°C) and stained with antibodies as described above.
4. Single cell sorting

Single cell sorting of isolated myeloid progenitor populations was performed using an Automated Cell Deposition Unit (ACDU) installed in the BD-FACS Aria cell sorter. Cells were plated at one cell per well into 96 well tissue culture plates. Single cells were cultured in conditions allowing their differentiation to macrophages, dendritic cells and osteoclasts, as described below.

5. Osteoclast progenitor cultures and quantification

Sorted OCP cells were centrifuged and resuspended in MEMα (GIBCO BRL, Carlsbad, CA) with 10% fetal bovine serum (FBS), penicillin, streptomycin, recombinant mouse M-CSF (R&D Systems, Minneapolis, MN) and RANKL, both at 30ng/mL. The cells were plated in five replicates per mouse in 96 well plates for each experimental group and incubated at 37°C with 5% CO₂ for as long as required per individual experiment. Fresh medium was changed every 48 hours. For single cell cultures, individual cells were first expanded with M-CSF (30 ng/ml) for 18 days then media was removed and changed to media containing M-CSF and RANKL (both at 30 ng/ml). Osteoclast formation was evaluated after an additional 5 to 7 days in culture.

To quantify TRAcP+ multinuclear and mononuclear cells in vitro, cells were fixed with 2.5% glutaraldehyde in PBS for 30 minutes and stained for TRAcP using the Leukocyte Acid Phosphatase kit (Sigma) according to the manufacturer’s instructions. Osteoclasts were identified as TRAcP+ cells with more than 3 nuclei. TRAcP+ mononuclear cells were also counted.

6. Bone resorption assay

Sorted osteoclast progenitor populations were tested for their ability to resorb bone.
Osteoclast precursors at a density of 1,000 cells were seeded in triplicates on bovine cortical bone slices in 96 well plates containing MEMα (GIBCO BRL) with 10% fetal bovine serum (FBS), penicillin, streptomycin, recombinant mouse M-CSF (R&D systems) and RANKL (both at 30ng/mL) for 12 days. As mentioned above, for single sorted cells, individual cells were first expanded with M-CSF followed by M-CSF and RANKL (both at 30ng/mL) and osteoclast formation was evaluated after 5 to 7 days. The bone slices were then fixed with 2.5% glutaraldehyde in PBS, stained for TRAcP and visualized by microscopy to assure that osteoclasts have formed on the bone slices. To visualize resorption pits, bones were sonicated in a 0.25M NH₄OH solution (2x ~30sec to 1 min) to remove osteoclasts and resorption pits were then stained with 1% toluidine blue (Sigma) in 1% sodium borate (Sigma) for 0.5 to 1 minute and washed with dH₂O. To evaluate the ability of osteoclasts to resorb bone we measured the area of individual pits of each group using the Via-160 video image measurement system (Boeckeler Instruments, Tucson, AZ). Data were analyzed and compared between groups as needed.

7. Osteoclasts area measurements

The size of osteoclasts grown on tissue culture dishes was measured as the area of multinuclear TRAcP+ osteoclasts from vehicle and treated groups and evaluated using the Via-160 video image measurement system (Boeckeler Instruments).

8. Phagocytosis assay

Single cells were cultured for 14 days with M-CSF (30 ng/ml) and tested for their ability to phagocytose pHrodo E. coli bacterial particles (Invitrogen. Carlsbad, CA). These particles
contain rodamine, which is colorless at neutral pH and activated at the low pH inside vesicles of phagocytic cells. Cells derived from bone marrow progenitors or control RAW 264.7 cells were pre-treated for 4 hr at 37°C with DMSO (10μM) or Cytochalasin D (10μM) then washed 2X with PBS and cultured with Rodo E. coli bacterial particles for 1 hr at 37°C in media containing MEMα 10% FBS and pen/strep. Wells containing cells were washed 2X with PBS and phagocytosed particles were visualized by fluorescent microscopy, using a Zeiss Imager Z1 microscope (Carl Zeiss, Thornwood, NY) and detected using a TRITC (red) filter (Chroma Technology, Bellows Falls, VT).

9. Antigen presentation assay

Single cells were cultured for 8 days with GM-CSF (R&D Systems, Minneapolis, MN) and then media changed to media containing GM-CSF and IL-4 (R&D Systems, Minneapolis, MN) for an additional 4 to 6 days. All cytokines were used at a concentration of 30 ng/ml. Cells were then tested for their ability to process and present antigen to T cells, leading to their activation. Dendritic cells derived from osteoclast precursors (20,000 cells per well) or dendritic cell line DC2.4 (100,000 cells per well) were co-cultured with B3Z T cells. This T cell line recognizes the ovalbumin peptide SIINFEKL in the context of H-2Kb and expresses β-galactosidase upon activation by cognate antigen presenting cells 14. Ovalbumin (OVA, 300μg/mL) or SIINFEKL peptide (SHL8, 300μg/mL) was added to the cultures and incubated for 24 hr. Cells were then incubated with the galactoside analog chlorophenol red-β-D-galactopyranoside (CPRG) for 12 hours and supernatants were tested for β-galactosidase activity, through a colorimetric assay.
10. LPS administration

Mice 8 to 10 weeks old were injected i.p. with LPS 055-B5 (Sigma-Aldrich, L-2880) at a dose of 10 mg/kg/mouse and their organs harvested for FACS analysis of hematopoietic precursors and sorted for osteoclast cell cultures at different time points. Control mouse groups received one i.p., injection of PBS.

11. Parathyroid hormone (bPTH) administration

Mice were injected subcutaneously with bovine PTH (1-34) (Bachem America, Torrance, CA) at a dose of 80 µg/kg/day for 7 or 14 days. Control mice received subcutaneous injections of PBS.

12. Bisphosphonate treatment

Mice were in 4 groups, 1) vehicle; 2) PTH; 3) Alendronate; 4) PTH + Alendronate. 1) Vehicle mice were pre-treated with 1 injection containing PBS and 0.1% BSA (Alendronate vehicle), seven days later vehicle mice were treated with daily injections containing PBS, 0.1% BSA and 1mM HCl (PTH vehicle) for 14 days. 2) For this PTH group, mice were treated with daily injections of bovine PTH 1-34 (80 µg/kg) for 14 days. 3) Mice were pre-treated with 1 injection of Alendronate (100 µg/kg) followed by 2 more injections, once weekly, 7 days after and 14 days after pre-treatment. 4) Mice were pre-treated with 1 injection of Alendronate. Seven days later, mice were treated with bovine PTH 1-34 (80µg/kg) for 14 days. In addition, two more injections of Alendronate were given, as described in 3). Bone marrow and spleens from all 4 groups were harvested the following day after the last PTH injection, processed as indicated above, and stained with antibodies for hematopoietic progenitors. Samples were analyzed by
flow cytometry, as described above. The levels of carboxy-terminal collagen crosslinks (CTX) were measured using the RatLaps EIA (#AC-06F1 Immunodiagnostic Systems, Scottsdale, AZ).

13. Competitive reconstitution of osteoclast precursors

C57BL/6 CD45.1 mice were used as recipients while CD45.2 mice were used as donors. CD45.1 mice were lethally irradiated with 975 rads. Dose was split in two: 1) 475 rads were given and 4 hours later 2) 500 rads were given. Six hours later, mice were injected i.p. with 250 µL of anesthetic (Ketamine/Xylazine) and cells in 100 µL of sterile PBS were transferred by retro-orbital injection using a 0.5 mL BD insulin syringe with 29 G x 1/2 in. Number of transferred cells depend on individual experiments and vary from $5 \times 10^3$ to $1 \times 10^5$ cells per mouse. For these experiments, hematopoietic stem cells and osteoclast precursors were co-injected in mice. Anesthesia was prepared as indicated using the following formula:

| Preparation of anesthetic: to 4.4 mL of sterile PBS add the following: |
|-----------------|--------------|----------------------------------|

<table>
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<th>Drug</th>
<th>Volume</th>
<th>Specifications</th>
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<td>Ketaset (ketamine HCL) 100mg/mL (10 mL/bottle). Lot#: 440782. NDC 0856-2013-01.</td>
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<tr>
<td>Xylazine</td>
<td>0.1 mL</td>
<td>TranquiVed (Xylazine hidrochloride) 100mg/mL (50mL bottle). Lot#: LA06308. NDC 50989-234-11</td>
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Experimental mouse groups were as follow:

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<th>Radio-protective source (CD45.1)</th>
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<td>Non-irradiated</td>
<td>1) Total BM or SP WT CD45.2 (2x10^6) 2) Total BM or SP WT CD45.2 CathepsinK-Cre-TFP (2x10^6)</td>
<td>None</td>
</tr>
<tr>
<td>No.</td>
<td>Condition</td>
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| 2   | Non-irradiated | 1) FACS-sorted LKS WT CD45.2 (5K)  
2) FACS-sorted LKS WT CD45.2 CathepsinK-Cre-TFP (5K)                                                                                     |
| 3   | Non-irradiated | 1) FACS-sorted BM PIV WT CD45.2 (100K)  
2) FACS-sorted BM PIV WT CD45.2 CathepsinK-Cre-TFP (100K)                                                                                   |
| 4   | Irradiated     | 1) Total BM or SP WT CD45.2 (2x10⁶)  
2) Total BM or SP WT CD45.2 CathepsinK-Cre-TFP (2x10⁶)                                                                                        |
| 5   | Irradiated     | FACS-sorted LKS WT CD45.2 (5K)                                                                                                            |
| 6   | Irradiated     | FACS-sorted LKS WT CD45.2 CathepsinK-Cre-TFP (5K)                                                                                          |
| 7   | Irradiated     | FACS-sorted BM PIV WT CD45.2 (100K)                                                                                                        |
| 8   | Irradiated     | FACS-sorted BM PIV WT CD45.2 CathepsinK-Cre-TFP (100K)                                                                                     |
| 9   | Irradiated     | FACS-sorted BM TN/CD115+ WT CD45.2 (100K)                                                                                                   |
| 10  | Irradiated     | FACS-sorted BM TN/CD115+ WT CD45.2 CathepsinK-Cre-TFP (100K)                                                                                |
| 11  | Irradiated     | FACS-sorted SP OCP WT CD45.2 (100K)                                                                                                        |
| 12  | Irradiated     | FACS-sorted SP OCP WT CD45.2 CathepsinK-Cre-TFP (100K)                                                                                     |

LKS (Lin⁻c-Kit⁻Sca-1⁺)  
BM PIV (B220⁻CD3⁻CD11blowCD115⁻CD117⁻)  
BM TN/CD115+ (B220⁻CD3⁻CD11blowCD115—=PIV+PV+PVI)  
SP OCP (B220⁻CD3⁻NK1.1⁺CD11bhighCD115⁻)

Competitive reconstitution experiments were performed as follows: BM PIV (B220⁻CD3⁻CD11blowCD115⁺CD117⁻) or BM TN/CD115+ (PIV+PV+PVI), or Spleen osteoclast precursors (B220⁻CD3⁻NK1.1⁺CD11bhighCD115⁻) were isolated to high purity by flow cytometry (BD-FACS Aria; BD Biosciences, San Jose, CA, USA) from donor CD45.2 mice (or CD45.2 CathepsinK-Cre-TFP mice), as indicated above. Then cells were co-transferred with a radio protective source of Hematopoietic Stem Cells (Lin⁻c-Kit⁺Sca-1⁺, LKS) derived from donor WT CD45.1 mice.

Controls included transfer of total BM cells, total SP cells, sorted-LKS, sorted BM OCPs or sorted-SP OCPs from CD45.2 or CathepsinK-Cre-TFP transgenic mice into non-irradiated
CD45.1 recipient mice and total BM cells, total SP cells or sorted-LKS from transgenic mice into irradiated CD45.1 recipient mice.

All recipient mice transplanted were euthanized at the indicated time points and bone marrow, spleen and peripheral blood were harvested (as indicated above) to test engraftment hematopoietic tissues. Engraftment was assessed by 1) FACS analysis (recipient CD45.1 versus donor CD45.2 congenic markers), 2) microscopy of histological frozen sections of femur to identify osteoclast associated with bone surfaces (identified by tdTomato-Fluorescent Protein, DAPI and TRAcP) and 3) osteoclast cultures (M-CSF + RANKL) of total cells derived from BM, SP and PBL to identify tdTFP positive forming osteoclasts.

14. Fracture procedure in mice

To study migration and differentiation of osteoclast precursors in models of active bone remodeling we performed fractures as follow. Immediately prior to the procedure, all mice were subcutaneously injected with 0.2 mg/kg buprenorphine (Reckitt Benckiser Pharmaceuticals, Richmond, VA) and then every 12 hours for two days to control pain. Mice were then anesthetized with 1-3% isoflurane administered through inhalation for 5 minutes at a volume of 2 ml/min using an isoflurane vaporizer (oxygen was used as a carrier) and given during the fracture procedure. The left knee was shaven and prepared using sterile surgical techniques. A 25-gauge needle was used to perforate the articular surface of the femoral condyle through the skin. This initial perforation was then used to insert a precut stainless steel 316LVM wire (0.010” OD: Small Parts, Mirama, FL) into the medullary canal to the proximal end of the femur. The remaining end of the stylus was cut with a wire cutter and covered under the skin. A mid-diaphyseal fracture was then created using a 3-point bending system adapted from Bonnarens
and Einhorn (Bonnarens and Einhorn, 1984; Einhorn, 1998; Maturano et al., 2008). Fractures were radiographed by the Faxitron x-ray system (Faxitron, X-ray, Wheeling, IL) under anesthesia to monitor pin fixation, fracture site and ongoing callus formation post fracture.

For adoptive transfers, mice were first injected i.p. with 250 µL of anesthetic (Ketamine/Xylazine) and recipient-derived cells in 100 µL of sterile PBS were transferred by retro-orbital injection. Two sets of experiments were performed using cells derived from bone marrow or spleen CathepsinK-Cre-tdTomato transgenic mice: 1) total bone marrow or spleen and 2) FACS-sorted bone marrow or spleen OCPs, were transferred at day 0 (the day when the fracture was performed) then at day 7 after the fracture. Mice were then euthanized and bone marrow, spleen and peripheral blood were harvested and organs processed (as indicated above) to assess engraftment by 1) histological frozen sections of femur to identify osteoclasts associated with bone and cartilage tissue at the fracture site of the callus (identified by tdTomato-Fluorescent Protein, DAPI and TRAcP) and 2) osteoclast cultures (M-CSF + RANKL) of total cells derived from bone marrow, spleen and peripheral blood to identify tdTFP positive forming osteoclasts.

To prepare femurs for histology, first the skin and muscle were carefully removed. Second, the pin was carefully pulled from the fractured femur by gently but firmly holding the proximal end of the femur, without damaging the callus. Samples were then prepared for frozen sections.
15. Cells transfers to human TNF mice and fractured mice

Bone marrow, spleen or peripheral blood cells derived from CathepsinK-Cre-tdTomatoFP transgenic mice were isolated and FACS-sorted to transfer (as described above) into hTNF transgenic mice at different time points. Engraftment of cells was then assessed by 1) histological preparations of femur and 2) osteoclast cultures of cells derived from BM, SP and PBL of hTNF mice to test whether RFP+ osteoclast engrafted in central and peripheral organs.

16. Histological preparation of fluorescent samples

Mice were euthanized and femurs prepared for histology (the skin and muscle were carefully removed). The samples were then placed into tissue cassettes and fixed in 4% paraformaldehyde and pH adjusted with NaOH (pH 7.4) for 3 days at 4°C with gentle rocking. The samples were then decalcified in a 14% EDTA solution (pH 7.1) at 4°C for 5 days. The 14% EDTA solution was changed every 24 h. To prepare the samples for embedding, the bones were soaked in 30% sucrose in PBS at 4°C for 24 h. Femurs were then placed in disposable histology molds (Tissue Tek Cryomolds, 25x20x5mm) and embedded in OCT compound (Thermo Shandon, Pittsburgh, PA) and placed in dry ice to freeze the samples and stored at -20°C. Frozen sections were then transferred to a cryostat and 8 μm frozen sections were cut using the CryoJane method using a Leica CM1900 Cryostat. The sections were collected with CryoJane tape and then transferred to glass slides. The slides were let to dry at 4°C in the dark and then rehydrated in PBS for 10 min and covered with coverslips and visualized by fluorescence microscopy.
17. Hematoxylin and TRAcP stains.

After fluorescence evaluation by microscopy, histological preparations were stained with hematoxylin. Cover slips were removed from sections and washed in PBS for 10 min, let dried and stained with Hematoxylin for 2 min then gently rinsed in PBS. These steps were repeated if stains were faint. Samples were then stained for TRAcP (prepared as indicated above) by submerging the glass slides in the solution for approximately 10 min or until TRAcP positive cells were detected. Samples were then covered with coverslips and scanned using the AxioVision software.

18. Microscopy

For fluorescent microscopy we used a Zeiss Imager Z1 microscope (Carl Zeiss, Thornwood, NY) and specific fluorescence detected using filters for GFP, TRITC (red for tdTomatoFP), sapphire and a dual filter for TRITC/Sapphire (Chroma Technology, Bellows Falls, VT). DAPI was used to identify nuclei in cells and used in combination with fluorescent frozen sections. Scanning of histological preparations was performed using the AxioVision software.

19. Statistical analysis

All data was analyzed using GraphPad Prism 5 software using unpaired Student’s t test.
CHAPTER III

Identification of a common precursor for osteoclasts, macrophages and dendritic cells

1. Introduction

Monocytes are a heterogeneous population of mononuclear cells that develop in the bone marrow from hematopoietic myeloid progenitors (Auffray et al., 2009a; Geissmann et al., 2010; Robbins et al., 2010). Once developed, they can differentiate in the bone marrow and/or migrate via the bloodstream to peripheral organs where they further mature to maintain a pool of tissue-associated macrophages and dendritic cells (DCs), both of which play crucial roles in the maintenance of tissue homeostasis and immune functions (Chan et al., 2007; Geissmann et al., 2008; Hume, 2006).

The concept of a common progenitor for macrophages and DCs was initially proposed after taking into consideration the shared functional characteristics between these cell types. Macrophages are tissue scavenging phagocytic cells that internalize cell debris and bacteria. They are also potent inducers of inflammatory mediators. DCs, although phenotypically and morphologically distinct, are also phagocytic cells with the ability to induce inflammatory responses and they are the quintessential professional antigen-presenting cells that are crucial for the initiation of the adaptive immune responses.

Recently, several publications have identified and characterized a bone marrow common progenitor cell for these two functionally related cell types. Using flow cytometry combined with
in vitro and in vivo differentiation assays, it was reported that cells with the phenotype Lin-CD115+ CD135+ CX3CR1+ correspond to an isolatable progenitor cell with the ability to generate macrophages and DCs (Geissmann et al., 2010; Geissmann et al., 2008; Chow et al., 2011).

Osteoclasts (OCs) are unique bone-resorbing multinuclear cells, which are critically involved in skeletal function such as bone remodeling, fracture repair and pathological bone resorption associated with inflammatory conditions (Lorenzo et al., 2008). Osteoclasts are derived from myeloid progenitors through signals mediated by c-fms/CD115 and RANK (Arai et al., 1999; Suda et al., 1999). The dependence of osteoclastogenesis on signaling through c-fms/CD115 indicates that they also belong to the monocyte lineage. In addition, because both cytokine pathways are important for DC development, a common origin of osteoclasts and DCs has been proposed (Arai et al., 1999). Miyamoto et al isolated osteoclast precursor cells bearing the phenotype CD117+ CD115+ RANK- from murine bone marrow and tested their ability to differentiate into osteoclasts and dendritic cells in vitro (Miyamoto et al., 2001). They showed that DCs and osteoclasts could share a common progenitor. Given these findings, it is likely, that a common monocyte progenitor exists for macrophages, DCs, and osteoclasts.

Searching for osteoclast-committed progenitors in the bone marrow we identified a population of cells with high osteoclastogenic potential (Jacquin et al., 2006). This population is characterized by the phenotype: B220−CD3−CD11b<sup>low</sup> CD115<sup>+</sup> CD117<sup>+</sup>, accounts for 0.1-0.3% of total nucleated cells, and has the ability to generate mature bone-resorbing osteoclasts when cultured in the presence of M-CSF and RANKL. In the present work, we evaluated this population for its ability to also generate macrophages and DCs, both at a population and at a clonal level. We report that at a clonal level, this population is able to generate mature bone-
resorbing osteoclasts, phagocytic macrophages and antigen-presenting dendritic cells in vitro, indicating that there exists a common developmental pathway for all these cell types.

In addition to bone marrow, cells with osteoclastogenic potential exist in blood and peripheral hematopoietic organs (Xing et al., 2005). The functional meaning of these populations and/or their relationship with osteoclastogenic bone marrow progenitors is not well established. It has been proposed that these cells correspond to peripheral progenitors that are able to progress into multiple terminally differentiated monocyte cells, and that under pathological conditions, they could contribute to osteoclastogenesis with a direct impact on extra medullary osteolysis. Because several research groups have revealed a considerable heterogeneity within monocyte populations in the periphery and the association of cells within the various types of monocytic cells is not well defined (Auffray et al., 2009a), we thought it would be important to characterize any preferential populations with osteoclastogenic potential, and determine if these could also generate other types or terminally differentiated monocyte lineage cells. Using similar approaches to the ones utilized for the characterization and isolation of bone marrow osteoclast progenitors, we have identified populations with high osteoclastogenic potential in peripheral blood and spleen. As observed in the bone marrow populations, these peripheral populations contain common progenitors for osteoclast, macrophages and dendritic cells. These progenitors share phenotypic markers with the bone marrow progenitor, but differ in their expression of CD11b. Both spleen and peripheral blood monocyte progenitors share the phenotype: B220−CD3−NK1.1−CD11b+Ly-6C^{hi}CD115+CCL2^{hi}CX3CR1^+. 
2. Results

2.1. Identification of a clonal population of bone marrow osteoclast progenitors

Our previous work has described that osteoclast progenitor activity in the bone marrow is contained in three discrete populations (populations IV, V, and VI; Figures 1A) (Jacquin et al., 2006). From them, population IV, bearing the phenotype B220-CD3-CD11b\textsuperscript{low}CD115\textsuperscript{+}CD117\textsuperscript{+}, contains the highest \textit{in vitro} osteoclastogenic potential. As a progression from those studies, we evaluated the clonal ability of each of these populations to generate osteoclasts \textit{in vitro}.

We sorted individually each one of the three populations and subsequently plated them at a single cell per well in 96 well plates, using the single cell deposition unit available in our cell-sorting instrument. Our initial sorts reached over 95% of purity. This efficiency coupled to the subsequent cloning (Supplemental Figure 1A), equivalent to a second sort, ensures 100% purity. After plating, single cells were expanded for 18 days in M-CSF (30 ng/ml) and the growth efficiency was evaluated by visual observation of expanded cells in each well. Subsequently we replaced the media to one containing M-CSF and RANKL (both at 30 ng/ml) and cultured the expanded cells for an additional 5 to 7 days to stimulate the formation of multinucleated TRAcP\textsuperscript{+} osteoclasts.

To evaluate cloning efficiency, we quantified the percent of wells generating colonies (more than 5 cells per well) at the end of the experiment. This analysis rendered cloning efficiencies of 97%, 77% and 62% for populations IV, V, and VI respectively (Figure 1B).

More significantly, we found that the TRAcP\textsuperscript{+} cell formation efficiency of the three populations (defined as the number of colonies with TRAcP\textsuperscript{+} cells at the end of the experiment divided by the total number of plated wells) was equivalent to the cloning efficiency of the
populations. For population IV, 92% of the clones formed TRAcP+ cells while for population V and population VI these values were 55% and 28%, respectively (Figure 1B). Importantly, when single cells were sorted onto bovine cortical bone slices, and induced towards osteoclastogenesis as described, cells from population IV were able to form mature bone-resorbing osteoclasts as assessed by TRAcP staining and toluidine blue staining-pit formation (Figure 1C).

In summary, this data showed that population IV is a homogeneous population of osteoclast progenitors with the ability to generate bona fide osteoclasts at a clonal level. Interestingly, as depicted in Figure 1D, the general phenotypic characteristics of population IV match the phenotype of a population described by Fogg et al., as a clonogenic precursor for macrophages and DCs (Fogg et al., 2006). Thus, using similar strategies, we investigated if population IV also has the ability to generate cells with characteristics of macrophages and DCs.

2.2. Differentiation of bone marrow osteoclast progenitors into functional macrophage- and dendritic- like cells in vitro

To test if population IV could correspond to a common progenitor for osteoclasts, macrophages and dendritic cells, we cultured single cells in 96 well plates in the presence of cytokines favoring either the differentiation of monocyte progenitors into macrophage or DC-like cells. For macrophage cultures, single cells were cultured for 14 days in the presence of M-CSF (30 ng/ml). For dendritic cell cultures, single cells were initially expanded in media supplemented with GM-CSF (30 ng/ml) for 8 days, followed by media containing GM-CSF plus IL-4 (both at 30 ng/ml) for 4 additional days. For both conditions, we calculated the cloning efficiency, evaluated morphological characteristics and defined cell surface phenotypes.
Phagocytic properties were tested for cells that were cultured to become macrophages while cells that were cultured to become dendritic cells were tested for their ability to present antigen.

When cultures were initiated as single cells, the cloning efficiency of population IV to generate either macrophages or dendritic cells *in vitro* was 94% and 100% when cultures were initiated at 5 cells/well. Characteristic morphological features observed for single colonies are shown in Figure 2A. Phenotypic analysis of the cultures at the conclusion of the experiment showed the upregulation of mature monocyte markers. In addition, each condition resulted in the differential expression of a number of lineage specific markers. For example, cultures progressing towards macrophages showed preferential expression of CD11b and F4-80, while cultures progressing in DC polarizing conditions showed preferential expression of CD11c and MHC class II (Figure 2B).

We also isolated bone marrow population IV cells from CX3CR1 GFP transgenic mice, a reporter system in which GFP (green fluorescent protein) was inserted into the *Cx3cr1* locus by homologous recombination. Results from these experiments showed that over 99.5% of these cells express CX3CR1 (data not shown), and the expression of CX3CR1 was maintained in cells after being differentiated into either macrophage-like or dendritic-like cells.

To test if the monocyte common progenitor generated functionally active macrophages and dendritic cells, we evaluated their ability to phagocytose or present antigens, respectively (Figure 3). For phagocytosis assays, we used commercially available bacterial particles conjugated with rhodamine, a fluorochrome that fluoresces at low pH (i.e. inside macrophage phagocytic vesicles) but not at neutral pH. We incubated *in vitro*-differentiated macrophages, derived from single cell sorts, with these particles and found that cells that were differentiated towards macrophages were able to ingest bacteria particles and activate the red fluorochrome at
low pH inside vesicles. In contrast, cells treated with cytochalasin D, which interferes with microtubule polymerization and prevents phagocytosis, showed reduced phagocytic activity (Figure 3A). RAW 264.6 cells, used as positive control, showed equivalent results.

Dendritic cells are professional antigen-presenting cells with the ability to intake and process antigens and present them on their cell surface to T cells in the context of MHC class molecules. To test antigen presentation in DCs elicited from cloned single cell cultures, we incubated them with B3Z T cells, which is a modified T cell clone that recognizes OVA and has the ability to express β-galactosidase upon activation by cognate antigen (Shastri and Gonzalez, 1993). Experiments were performed in the presence of ovalbumin or the SIINFEKL peptide, which is specific for the T cell receptor on B3Z T cells. We tested the ability of in vitro-differentiated, single cell-derived DC cultures to activate T cells in the context of MHC class I molecules. The read-out for this experiment is a colorimetric assay that measures the activity of β-galactosidase. We found that with either ovalbumin or SIINFEKL peptide, B3Z T cells were activated to levels that were significantly above control values when they were incubated with cloned population IV cells that were driven towards DCs (Figure 3B).

These results directly demonstrated a common developmental pathway for osteoclasts, macrophages and dendritic cells through a bone marrow common progenitor with the phenotype B220<sup>−</sup>CD3<sup>−</sup>CD11b<sup>−/low</sup>CD115<sup>+</sup>CD117<sup>+</sup>CX3CR1<sup>+</sup>. Significantly, this phenotype is similar to the common progenitor for macrophages and dendritic cells that was reported by Fogg et al., 2006.

2.3. Phenotypic characterization of peripheral osteoclast progenitors.

Cells with osteoclastogenic potential exist in the periphery (Xing et al., 2005). However, the function and ultimate lineage fates of these putative peripheral osteoclast progenitors are not
well established. It has also recently been appreciated that peripheral monocyte populations are widely heterogeneous and contain cells with multiple related phenotypes and functional abilities (Auffray et al., 2009; Geissmann et al., 2010). Therefore, we attempted to define where within this heterogeneity does the ability to generate osteoclasts reside. Using a strategy that was similar to the one we previously utilized to identify murine bone marrow osteoclast progenitor, we fractionated murine spleen and peripheral blood cells and tested their in vitro osteoclastogenic potential.

In spleen, we found that cells that were negative for lymphoid markers (LYM) identifying T cells, B cells and Natural Killer cells (CD3, B220/CD45R and NK1.1) and positive for CD11b generated a higher number of multinuclear TRAcP⁺ OCLs than did the CD11b negative fraction (113±6 vs 9±7 p=0.0001, when 20,000 cells were plated under osteoclastogenic conditions). This population could be further dissected by its expression of Ly6C and CD115. We found that the population bearing the phenotype LYM CD11b⁺ Ly6Chi CD115⁺ contained the highest potential to form OCLs in vitro (Figure 4). This phenotype is consistent with reported characteristics associated to peripheral circulating monocytes (Geissmann et al., 2003; Ziegler-Heitbrock et al., 2010). This population also was negative for the granulocyte marker Ly6G, expressed intermediate levels of CD117, and evaluation of cells from CX3C1R1-GFP mice showed that it was positive for CX3CR1 (data not shown).

When we tested the cloning and precursor frequency of this progenitor population by single cell plating, we found it to have a low colony formation efficiency compared with the progenitor populations from bone marrow, since 100% efficiency was reached only when 50 or more cells were plated per well (Supplemental Figure 2). This result indicates that this
population is still heterogeneous or its progression in culture is dependent on density requirements.

We next evaluated these splenic osteoclastogenic progenitors for their ability to generate macrophages and dendritic cells in vitro. As shown in Figure 5, we sorted the above described splenic monocyte progenitors, incubate them with M-CSF or GM-CSF plus IL-4 and tested their expression of surface markers that characterize macrophages or DCs. Spleen monocyte progenitors cultured with M-CSF for 8 days generate a population expressing high levels of CD11b and F4/80, low levels of CD11c and low levels of MHC class II. In contrast, when cultured with GM-CSF plus IL-4, the developed population expressed high levels of CD11c, low levels of CD11b and F4/80 and high levels of MHC class II. As observed in bone marrow progenitors, both in vitro generated cell types still retained the expression of CX3CR1 (data not shown).

Interestingly, we identified an osteoclastogenic progenitor with similar phenotypic characteristics to the spleen progenitor in peripheral blood (Figure 6). This progenitor was also Ly6G− Ly6Chigh and expressed high levels of CD115. Also, expansion and differentiation potential in limiting numbers showed equivalent results to those observed for the progenitor populations isolated from spleen (Supplemental Figure 2).

In summary, these data indicate that there exists a phenotypically similar population of cells with high osteoclastogenic potential in spleen and peripheral blood. Their phenotype is indicative of a cell with characteristics of inflammatory monocytes (Supplemental Figure 3 and Supplemental Table 1) (Yona and Jung, 2010). These cells are homogeneous in their expression of multiple cell surface markers and, although not strictly proven at a clonal level, cells bearing
this phenotype also had the ability to generate macrophage like and dendritic-like cells with high efficiency.

3. Discussion

We have previously identified a bone marrow progenitor able to generate osteoclasts in vitro with high efficiency (Jacquin et al., 2006). This progenitor, characterized by the cell surface expression phenotype: B220− CD3−CD11b−/low CD115+ CD117+, shares characteristics with monocyte progenitors and for that reason we tested its ability to differentiate into functional osteoclasts, dendritic cells and macrophages. Published studies of monocyte progenitors have focused mostly on their bipotential ability to generate macrophages and DCs, while analyses of osteoclastogenesis have not been considered (Fogg et al., 2006; Auffray et al., 2009b). We report here that this progenitor population can generate mature functional cells of the three types at the single cell level. Hence, this result demonstrates the existence of an isolatable common monocyte progenitor for osteoclasts, macrophages and DCs (OcMDC).

In addition to the studies identifying common progenitors for macrophages and DCs (Geissmann et al., 2010; Geissmann et al., 2008), an independent study has reported a related population to the one we have identified, with the ability to serve as precursors of osteoclasts and DCs (Barrow et al., 2011). We think that the population characterized in this report is not fundamentally different from the published ones, although detailed phenotypic analysis indicated some minor discrepancies. For example, Auffray et al. reported that the common macrophage/DC precursor falls within a population expressing CD135 (Auffray et al., 2009b). Our analyses of CD135 expression showed that population IV can be dissected into two discrete populations. However, we found that both populations had the ability to generate osteoclasts to a
similar extent (data not shown). We did not perform separate assays for macrophages and DCs from each isolated population, but considering the cloning efficiency observed for macrophage and DC formation, it is clear that, at least in vitro, the CD135 marker does not segregate lineage potential within our monocyte precursor population.

We have also identified and characterized monocyte progenitors in spleen and peripheral blood that can efficiently form osteoclasts in vitro. Like the bone marrow progenitors, these cells also express CD115 and CX3CR1, but in contrast, the peripheral osteoclastogenic activity was contained within the CD11b positive population. The overall phenotypic characteristics of these populations are consistent with cells belonging to a peripheral inflammatory type monocyte population. Comparing cloning frequency with osteoclast precursor frequency, we can conclude that the osteoclastogenic potential of clones expanded in culture is as high as the ones observed in bone marrow. These populations also have the capacity to generate macrophages and DCs in vitro. Compared with the bone marrow counterpart, these peripheral osteoclast precursor populations showed a lower frequency for colony formation, which implies that they are still heterogeneous and that some of their components cannot be expanded in vitro. Alternatively, these cells could be more dependent on density for their progression in culture.

Different groups have found that the peripheral monocyte populations are heterogeneous and that such heterogeneity could be explained by an inherent plasticity of these populations which allows them to generate different types of macrophages, depending on differential microenvironmental cues (Gordon and Taylor, 2005; Mosser and Edward, 2008; Swirski et al., 2011; Willenborg et al., 2012).

The osteoclastogenic potential of peripheral monocyte progenitors is intriguing as their functional meaning is not evident. Especially, considering that the main inducing cytokines for
osteoclastogenesis, M-CSF and RANKL, are expressed to various degrees in periphery without formation of osteoclasts at these sites.

One possibility is that monocyte progenitors circulate to the periphery for additional maturation and then migrate back to bone where they are induced to terminally differentiate into osteoclasts because of a relatively unique osteoclastogenic microenvironment, which amplifies the response to M-CSF and RANKL. Specifically, recent work demonstrating that collagen proteins are ligands for OSCAR (Barrow et al., 2011), a receptor expressed on osteoclasts that appears to amplify RANKL signaling (Koga et al., 2004; Mocsai et al., 2004), may help explain the unique osteoclastogenic inducing properties of the bone microenvironment. Alternatively, monocyte peripheral progenitors could maintain their osteoclastogenic potential, independent of the bone marrow counterparts, and upon migration to sites of bone remodeling, be induced to form osteoclasts.

In a physiological or tissue repair scenario, inflammatory signals provided by microfractures, which need continuous remodeling for their resolution, could serve as the driving force to attract monocyte progenitors. Furthermore, these peripheral progenitors could be attracted during the generation of a fracture repair callus, serving as the main source of osteoclasts at this site (Fazzalari, 2011; Schindeler et al., 2008). Finally, in pathological inflammatory conditions, these progenitors may be important/crucial contributors to pathologic osteolytic processes as it occurs in rheumatoid arthritis or periodontal disease (Graves, 1999; Schett and Teitelbaum, 2009).

Finally, we propose that circulating monocyte progenitors are derived from a common bone marrow osteoclasts/macrophage/dendritic cell progenitor (OcMDC), which we have now characterized at a clonal level. However, the lineage relationship between the bone marrow and
peripheral monocyte progenitors is yet to be defined. One requirement to study this aspect is the availability of bona fide visual reporters that allow the detection of terminally differentiated cells in vivo. Until recently, there existed less than ideal systems for doing this with osteoclasts. However, we have found that some forms of the collagen type I promoter can drive the expression of fluorescent reporter proteins in terminally differentiated osteoclasts in vitro and in vivo (Boban et al., 2006). More recently, the cathepsin K promoter has been proposed and validated for its expression in terminally differentiated osteoclasts (Nakamura et al., 2007; Sanchez-Fernandez et al., 2012). Using these systems we are planning to study migration patterns of our various progenitors in vivo to more clearly define the exact phenotype of osteoclast progenitor cells that circulate, home to bone and differentiate into mature multinucleated resorbing cells in vivo during normal remodeling or in disease states.
4. Figures

Figure 4. Single cell cloning and phenotypic analysis of bone marrow osteoclast progenitors. A) Flow cytometry plots showing the dissection of bone marrow populations containing osteoclast progenitor activity. Population IV (B220−CD3−CD11blowCD115+CD117+) contains the highest potential to generate osteoclasts in vitro. B) Evaluation of colony formation and osteoclast formation efficiency of populations IV, V and VI after single cell cloning by FACS. PIV contains the highest cloning efficiency (PIV=96.3 ± 0.9 versus PV=74.8 ± 1.3, p=0.0001, and PIV versus PVI=60±0.8, p=0.0001) and TRAcP+ cell formation efficiency (PIV=91.5 ± 0.7 versus PV=53.3 ± 1.2, p=0.0001, and PIV versus PVI=26.8 ± 1.0, p=0.0001) when compared to PV and PVI. Data represents the mean±SEM of 4 independent experiments. C) Pictures showing colonies of PIV from single cells after expansion with M-CSF for 14 days (left panel) and subsequently cultured with RANKL and M-CSF for 5 days to generate TRAcP+ osteoclasts (middle). Right panel shows bone resorption pits generated by osteoclasts generated from PIV after single cell cloning. D) Flow cytometric analysis of freshly isolated bone marrow PIV showing the expression of surface markers (histograms) common to monocyte populations.
D

Myeloid markers

Chemokine receptors and adhesion molecules

MHC and costimulatory molecules

PIV: B220<sup>+</sup>CD3<sup>+</sup>CD11b<sub>low</sub>CD117<sup>+</sup>CD115<sup>+</sup>
Figure 5. Evidence of a common bone marrow monocyte progenitor for osteoclast, macrophages and dendritic cells in vitro. A) Single cell cloning of BM-PIV cultured with M-CSF for 14 days or with GM-CSF for 8 days and IL-4 for 4 additional days in 96 U-bottom well plates. Microphotographs represent cells derived from a single colony of macrophage-like cells (top) or dendritic-like cells (bottom). Bar graphs on right indicate colony formation efficiency of single cells (93.5 ± 1.92) cultured with M-CSF (top) and single cells (93.5 ± 1.92) cultured with GM-CSF and IL-4 (bottom). Plating 5 cells per well resulted in a 100% cloning efficiency and no statistically significant differences were found. Data represents the average ± SD of 4 independent experiments. B) Comparative phenotypic analysis of PIV with cells derived from cultures under macrophage inducing (M-CSF) and DC inducing (GM-CSF+IL4) conditions. Macrophage-like cells expressed the phenotype CD11b+ CD11c- F4.80+ MHC II-, while DC-like cells expressed the phenotype CD11b+ CD11c+ F4.80- MHC II+. 
Figure 6. Bone marrow-derived monocyte progenitors generate functional macrophage- and dendritic-like cells in vitro. A) Phagocytosis assay. BM-PIV single cells were cultured with M-CSF for 14 days in 96 U-bottom well plates (top row) and were tested for their ability to phagocytose *E. coli* bacterial particles (pH rodo, invitrogen). BM-PIV macrophages or RAW 264.7 cells (control) were pre-treated with Cytochalasin D (10µM for 4 hrs) and then cultured with *E. coli* particles (0.1µg/µL for 1hr) as indicated in upper panel. BM-PIV induced macrophages phagocytosed *E. coli* particles similarly to RAW 264.7 cells and Cytochalasin D prevented the phagocytosis of *E. coli* particles (middle and bottom row, respectively). Imaging was performed using a Zeiss Imager Z1 microscope (Carl Zeiss, Thornwood, NY) and detection of fluorescence using a TRITC (red) filter (Chroma Technology, Bellows Falls, VT). B) Antigen presentation assay. BM-PIV single cells were cultured with GM-CSF for 8 days and IL-4 for 4 additional days in 96 U-bottom well plates. BM-PIV induced DCs or DC2.4 control DC line, were co-cultured with B3Z T cells. Ovalbumin or SIINFEKL peptide was added to the cultures and incubated for 24 hr. Cells were then incubated with CPRG for 12 hours and supernatants were tested for B-galactosidase activity, measured by absorbance. Bar graphs represent one experiment of 2 independent experiments (top: control DC2.4 cells, bottom: BM-PIV DCs). Data represents the mean ± SEM of 1 out of 2 independent experiments. Statistical significant differences were found in OVA and SIINFEKL compared to controls, *** p≤0.005.
Figure 7. Identification and phenotypic characterization of spleen monocyte progenitors with osteoclastogenic activity. A) Phenotypic dissection of spleen monocytes by flow cytometry. Osteoclastogenic precursor candidates were identified based on the lack of expression of CD3, CD45R/B220, NK1.1 (LYM) and high expression of CD11b. Four populations contained among these cells were identified in the context of cell surface expression of Ly6C and CD115. B) In vitro osteoclastogenic assays. The four populations identified in A, were sorted and plated in 96-well plates with M-CSF (30ng/mL) and RANKL (30ng/mL) at the indicated density, cells were fed every 48 hrs. TRAcP assays were performed at different time points and positive cells were counted. Left graph shows multinuclear TRAcP+ osteoclasts (3 or more nuclei per cell) and right graph shows mononuclear TRAcP+ cells. Data represents the mean ± SEM of 1 out of 3 independent experiments. Statistical significant differences were found (LYM-CD11b-Ly-6C<sup>high</sup>CD115<sup>high</sup> vs LYM-CD11b-Ly-6C<sup>low</sup>CD115<sup>low</sup> at day 6, day 7, day 8 and day 9) * p≤0.05, ** p≤0.005, *** p≤0.0005.
Figure 8. Evidence of a common splenic monocyte progenitor for osteoclast, macrophages and dendritic cells in vitro. A) Cells with the phenotype LYM- CD11b+ Ly6C+ CD115+ were sorted to homogeneity and plated at a density of 5,000 cells in GM-CSF (30ng/mL) plus IL-4 (30ng/mL) for 8 days, M-CSF (30ng/mL) for 8 days or M-CSF (30ng/mL) plus RANKL (30ng/mL) for 8 days in 96 well plates. B) Images of cells developed at the end of the culture period. DC- and macrophage-like cells are visualized by phase contrast (right and middle) and osteoclasts are visualized by light microscopy after TRAPc staining (right). C) After culture, cells were detached from 96 well plates and analyzed for their cell surface expression of signature cell surface markers for DC and macrophages. Cells cultured with GM-CSF plus IL-4 (top row, histograms) show preferential expression of cell surface molecules related to DCs (CD11c+MHCII+F480 CD11b\textsuperscript{low}), and cells cultured with M-CSF (bottom row) show preferential expression of cell surface markers related to macrophages (CD11b\textsuperscript{+}F480\textsuperscript{+}CD11c\textsuperscript{low}MHCII).

A  Spleen Monocytes

B220\textsuperscript{-}/CD3\textsuperscript{-}/NK1.1\textsuperscript{-}  B220\textsuperscript{-}/CD3\textsuperscript{-}/NK1.1\textsuperscript{-}

Ly6C  Ly6C

CD115 (c-fms)  CD115 (c-fms)

GM-CSF + IL-4  M-CSF  M-CSF + RANKL

B Dendritic Cells  Macrophages  Osteoclasts

BF  BF  TRAep

40X  40X  70X

C

% of Max

CD11b  CD11c  F4/80  MHCII

Macrophages (M-CSF)  Dendritic cells (GM-CSF + IL-4)
Figure 9. Identification and phenotypic characterization of peripheral blood monocyte progenitors common to osteoclasts, macrophages and dendritic cells. A) Phenotypic dissection of peripheral monocytes by flow cytometry. As observed in spleen, osteoclastogenic precursor candidates were identified based on the lack of expression of CD3, CD45R/B220, NK1.1 (LYM) and high expression of CD11b. Four populations contained among these cells were segregated in the context of cell surface expression of Ly6C and CD115. B) In vitro osteoclastogenic assays. The four populations identified in A, were sorted and plated in 96-well plates with M-CSF and RANKL at the indicated density; cells were fed every 48 hrs. TRAcP assays were performed at different time points. Left graph shows multinuclear TRAcP+ cells and right graph shows mononuclear TRAcP+ cells. Data represents the mean±SEM of 1 out of 3 independent experiments. Statistical significant differences were found (LYM CD11b*Ly-6C<sup>high</sup>CD115<sup>high</sup> vs LYM CD11b*Ly-6C<sup>high</sup>CD115<sup>low</sup> at day 6, day 7 and day 8) * p≤0.05, ** p≤0.005.
Figure 10. Fluorescence-activating cell sorting and monocyte functional assays.
A) Shows an example of bone marrow monocyte progenitors isolated by FACS to an initial purity of approximately 97%. A second sort was necessary to reach 100% purity. Single cell sorting of populations PIV, PV and PVI were performed to test the differentiation and functionality of macrophages, dendritic cells and osteoclasts. B) Peripheral monocytes were purified using the same approach shown in A). The FACS dot plots shown are of peripheral blood monocytes. Single cell sorting for both spleen and peripheral blood monocytes was performed only to test the ability of these cells to differentiate to macrophages, dendritic cells and osteoclasts, functional assays required a higher number of cells.

A Bone Marrow Monocyte Progenitor

B Spleen or Peripheral Blood Monocyte Progenitor
Figure 11. Single cell sorting of peripheral tissue monocyte progenitors.
A) Spleen or B) peripheral blood monocyte progenitors were single cell sorted by FACS to a density of 1, 5, 50 or 100 cells per well, and tested for their ability to differentiate to macrophages, dendritic cells and osteoclasts. Single cell sorts of 1, 5, 50 or 100 cells per well of spleen monocytes were cultured for 13 days with M-CSF for macrophages (left bar graph), 13 days with M-CSF and 7 additional days with both M-CSF and RANKL for osteoclasts (middle bar graph) or 13 days with GM-CSF and 7 additional days with both GM-CSF and IL-4 for dendritic cells (right graph). For peripheral blood monocytes, single cell sorts of 1, 5, 50 or 100 cells per well were cultured for 15 days with M-CSF for macrophages (left bar graph), 15 days with M-CSF and 5 additional days with both M-CSF and RANKL for osteoclasts (middle bar graph) or 14 days with GM-CSF and 6 additional days with both GM-CSF and IL-4 for dendritic cells (right graph).
Figure 12. CCR2 expression on bone marrow and peripheral tissue monocyte progenitors. 
A) Bone marrow monocyte progenitors express low levels of the chemokine receptor CCR2. B) Spleen and C) peripheral blood Ly-6C\textsuperscript{high} and Ly-6C\textsuperscript{low} monocyte progenitors differentially express CCR2. Ly-6C\textsuperscript{high} monocytes express high levels of CCR2 that correspond to inflammatory monocytes while Ly-6C\textsuperscript{low} monocytes express low levels of CCR2 and correspond to non-inflammatory monocytes.
Table 1. Phenotypic comparison of bone marrow and peripheral tissue monocyte progenitors.
Comparison of cell surface markers expression of freshly isolated monocyte progenitors derived from bone marrow, spleen or peripheral blood. Differences found among progenitors are highlighted.

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<tr>
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Bone marrow monocyte: B220-CD3-CD11bloCD117highCD115high
Spleen monocyte: LYM-CD11bhighLy-6ChighCD115high
Peripheral blood monocyte: LYM-CD11bhighLy-6ChighCD115high
CHAPTER IV

In vivo migration of osteoclast precursors in transplantation and fracture repair.

1. Introduction

For decades, cells of hematopoietic origin have been widely studied in order to understand their development, migration and function. Most of the in vivo studies are based on transplantation settings using lethally irradiated recipients to assess the ability of donor cells to engraft and reconstitute tissues (Kondo et al., 1997; Spangrude et al., 1988; Akashi et al., 2000; Fogg et al., 2006; Auffray et al., 2009). Another widely used model is the generation of fractures in mice to study the differentiation and activity of osteoclasts in situ during active bone remodeling in localized sites of inflammation. The later will be particularly important to study the mechanisms of tissue remodeling, since osteoclast precursors (as discussed in chapter I) have shown the potential to give rise to macrophages, scavenger cells known to participate in tissue repair, and dendritic cells. However, studies on the in vivo migration and dynamics of osteoclast precursors during bone marrow reconstitution and the in situ differentiation and interactions of these populations with other cell types in fracture healing, are limited.

For these reasons, the use of alternative approaches and the combination of several techniques should give us greater advantages to study multiple parameters in a desire population
of cells. Recent advances in several scientific fields including molecular biology and genetics, have allowed scientists to engineer a variety of transgenic mouse models carrying fluorescent reporters driven by specific promoters (expressed in a particular tissue of interest), with the purpose of identifying various cell populations based on the expression of fluorescent proteins (Hume et al., 2011). This technology is one of the most useful approaches to study the distribution, migration, differentiation and functions of different type of cells and the organization of cell populations within tissues, in the context of the mouse anatomy. Our main interest is the use of these approaches to study osteoclast precursor populations and their progeny in specific compartments of the body. In particular, we are interested to take advantage of transgenic mice that express fluorescent proteins under the control of specific promoters expressed in osteoclasts, macrophages or dendritic cells. We aim to isolate osteoclast progenitors and test their ability to engraft, migrate and establish tissue-associated cell populations in lethally irradiated recipients and in mouse models of fracture repair. These experiments should allow us to explore the dynamics of myeloid precursor cells and their development into mature functional subsets by identifying mature resorbing osteoclast associated to bone tissue, or macrophages and dendritic cells in hematopoietic and lymphoid tissues.

It has been proposed that bone marrow contains a population of osteoclast progenitors that exit this compartment to circulate via the bloodstream and then associate to peripheral tissues while still retaining the potential to become osteoclasts, \textit{in vitro}. In humans, evidence had demonstrated that an osteoclast precursor circulates in the blood stream and might be contained within peripheral reservoirs (Fujikawa et al., 1996). In support of this hypothesis, our work discussed in chapter II aimed to identify, characterize and isolate cells with osteoclast activity from bone marrow, spleen and peripheral blood and demonstrated that myeloid precursor
populations with a defined phenotype differentiated clonally into macrophages, dendritic cells and osteoclasts. However, some of the current questions in bone biology, focus on the relevance of peripheral osteoclast precursors and their role in homeostatic conditions, inflammation, tissue repair and disease. Therefore, the generation of labeling systems to track osteoclast precursors migration, localization and activity during homeostatic conditions, and the use of these systems in combination with mouse models of disease, in which osteoclast activity is compromised, could help us define the necessary conditions that modulate osteoclast precursors activity. These studies will be important for us to understand the biology of osteoclast under certain conditions and to identify potential targets to modulate osteoclast activity.

2. Results

2.1. Generation of a mouse fluorescent reporter system to label osteoclasts

Several mouse reporter lines have been used to identify resorbing osteoclasts in vitro and in vivo. However, these fluorescent reporters are not specific for osteoclasts and generally labeled multiple populations of cells. In addition many of previous experiments have not been performed on specific populations of precursors cells, which complicate the identification of target cells (Chiu et al 2004; Hume, 2011). The need to use more specific promoters for driving the expression of fluorescent proteins to study osteoclasts differentiation, led us to the development of a new osteoclasts labeling system from existing transgenic mouse models. These mice are generated crossing Cathepsin K-Cre mice with ROSA26-CAG-LoxP-tdTomato mice (Nakamura et al., 2007; Madisen et al., 2010). The progeny is heterozygous mice that express a variant of Red Fluorescent Protein (tdTomato) under the control of cathepsin K promoter, which is highly express in mature resorbing osteoclasts. Although other cells have shown to express
low levels of cathepsin K, for example: macrophages (Herroon et al., 2012), we can easily discriminate osteoclasts from those and corroborate their origin by TRAcP staining and DAPI since multinucleation is a characteristic of osteoclasts. Figure 1 shows an image of osteoclast cultures using cells derived from ctsk<sup>cre/+</sup>;tdTomatoFP mice. Note that only cells cultured with soluble M-CSF and RANKL expressed tdTomatoFP and were identified as osteoclasts, but cells culture with M-CSF alone does not generate cells expressing tdTomatoFP.

These experiments showed that this transgenic fluorescent mouse model could be useful to study osteoclasts differentiation <em>in vitro</em> and promise a new tool to investigate migration, localization and differentiation <em>in vivo</em>. Indeed, further experiments we recently performed have demonstrated the efficiency of purified myeloid precursor cells derived from cathepsinK-tdTomato fluorescent reporter mice and transferred into non-fluorescent recipient mice, to identify <em>in vivo</em> multinuclear osteoclasts associated to bone tissue.

**2.2. Bone marrow and peripheral osteoclast precursors engrafted in lethally irradiated recipients and developed into mature resorbing osteoclast.**

To study the <em>in vivo</em> migration and differentiation of osteoclasts we have performed competitive reconstitution experiments using transgenic mice expressing fluorescent proteins controlled by different promoter. First, we used a currently available mouse model that was generated to study osteoblast (not osteoclasts) development but showed to be useful to identified GFP multinuclear osteoclast <em>in vitro</em> and <em>in vivo</em>. These model express GFP under the control of a 3.6 kb fragment of the rat type I collagen promoter (pOBCol3.6 GFP mice) and is useful to study mesenchymal progenitors differentiation, however, histological preparations identified GFP osteoclasts associated to bone tissue (Boban et al., 2006). Further experiments showed that HSC
transplantation resulted in engraftment and differentiation of donor cell into GFP osteoclasts. Here, we used pOBCol3.6 GFP mice to isolate from them specific populations of osteoclast precursors and asked whether this model could be useful to study osteoclast migration and differentiation in vivo. For these experiments we transferred osteoclast precursors (B220–CD3–
CD11b<sub>low</sub>CD115<sup>+</sup>CD117<sup>+</sup>) from the BM of pOBCol3.6GFP CD45.2 donor mice into lethally irradiated C57BL/6 CD45.1 (WT) recipient mice. All irradiated mice were co-transferred with a radio protective source of HSC (Lin<sup>c</sup>-Kit<sup>+</sup>Sca-1<sup>+</sup>, LKS) derived from WT mice. Our experimental controls included transfer of OCP from the BM of pOBCol3.6GFP mice into non-irradiated WT mice and LKS from pOBCol3.6GFP mice into irradiated WT recipient mice (figure 2, experimental design).

Engraftment was assessed by histology and flow cytometry and showed that WT irradiated recipients (receiving BM OCP from pOBCol3.6GFP donor mice) had engrafted in the BM and spleen (1 and 0.2% of total CD45.2, respectively) as shown by FACS analysis 10 days after adoptive transfers were performed. Few donor cells were detected in peripheral blood of recipient mice (figure 3) In addition, frozen histological sections of femurs showed fluorescent OCLs associated to endosteal bone surfaces of irradiated but not of non-irradiated mice (figure 4 and data not shown). We have also transferred splenic OCPs (LYM<sup>+</sup>CD11b<sup>+</sup>CD115<sup>+</sup>) from pOBCol3.6GFP reporter mice into irradiated WT CD45.1 mice and found engraftment of CD45.2 donor cells in the BM and spleen 10 days after transfers (FACS) (figure 3).

Due to the immediate availability of pOBCol3.6 GFP mice, these experiments were performed to obtain preliminary data and set the necessary conditions in competitive reconstitution experiments to facilitate further experiments. However, we aim to use more specific promoters to identify osteoclasts in vivo.
For these experiments we used ctsk\textsuperscript{cre/+};tdTomatoFP mice, this system has greater osteoclast-specificity than pOBCol3.6-GFP mice. Similar to the experiments described above, using FACS we isolated OCPs from the BM and spleen of CD45.2 donors and transferred them into lethally irradiated CD45.1 recipients. Recipients were assessed for engraftment of donor cells by 1) FACS analysis (CD45.1 and CD45.2 congenic markers) and 2) histology of frozen sections of femur to identify osteoclast associated to bone tissue (Jiang et al., 2005). Control subjects included transfer of OCP from the BM of ctsk\textsuperscript{cre/+};tdTomatoFP mice into non-irradiated WT mice and LKS from ctsk\textsuperscript{cre/+};tdTomatoFP mice into irradiated WT recipient mice (figure 2, experimental design). As expected, we identified engraftment of CD45.2 donor cells in bone marrow, spleen and peripheral blood by flow cytometry, 15 days after transfers (figure 5). In addition we identified mature resorbing CatK-tdTFP multinuclear osteoclast associated to bone tissue in recipient mice transferred with both bone marrow precursors (figure 6A and 6B) and spleen precursors (figure 7). We performed TRAcP staining to corroborate the definitive presence of osteoclasts and DAPI for multinucleation.

In summary, this work demonstrated that ctsk\textsuperscript{cre/+};tdTomatoFP reporter mice are a useful fluorescent mouse model to study the migration and development of OCP \textit{in vivo}. In addition, we showed that bone marrow- and spleen-derived OCP transplanted into lethally irradiated mice have the ability to home to the bone marrow, circulate in the blood stream and established peripheral populations in the spleen after lethal irradiation. These experiments suggest a common developmental pathway of osteoclast precursors that, in adult mice, originate in the bone marrow and exit via the blood stream and circulate to peripheral tissues to become tissue-associated cells, at least in the spleen. However, further experiments are necessary to confirm this hypothesis.
2.3. *Fracture healing, a mouse model to study osteoclasts biology*

Fracture repair, a process mimicking endochondral ossification during skeletal development of growing mammals, is a specialized form of wound healing in which cartilage and bone is generated rapidly. Fracture healing occurs in response to damage or break of bone tissue and triggers a series of complex but well-orchestrated cellular and molecular events resulting in the restoration of the proper architecture of bone structure and skeletal function. The healing process is generally divided into three stages: inflammation, repair and remodeling.

The fracture of a long bone results in the disruption of soft tissue, bone marrow integrity and vascularity which initiates local inflammation with infiltration of inflammatory cells (monocytes, granulocytes, macrophages) and production of a variety of cytokines (IL-1, IL-6, TNFa) and growth factors including BMPs (bone morphogenetic proteins), TGF-b (transforming growth factor-b), M-CSF, VEGF (vascular endothelial growth factor), PDGF (platelet-derived growth factor) and FGF (fibroblast growth factor). The inflammatory process is therefore necessary to recruit cellular components for tissue remodeling, to control possible infections and to induce the proliferation of hematopoietic and mesenchymal cells that ultimately supply the demands of new tissue regeneration including cartilage and bone (Schindeler et al., 2008; Little et al., 2007; Keramaris et al., 2008).

As described before, fractures heal through a mechanism of endochondral ossification (Chapter I). During this stage a cartilaginous template is formed, by chondrocytes, to provide support to the fractured bone and prevent further mechanical stress. Fibroblasts also contribute to the generation of fibrous tissue and along with chondrocytes form an isolated environment of supporting soft tissue known as “callus”. The formation of cartilage precedes the generation of new bone tissue. Tissue damage induced-production of osteogenic factors (namely BMPs)
mediate the proliferation of osteoblast and consequently new bone tissue is been actively generated. At this stage, the initial soft callus (fibrocartilage) is replaced then by hard callus. Interestingly, at this stage osteoblasts produce woven bone, which is indeed the same type of bone produced during fetal development. Woven bone is a less organized assembly of collagen fibers, which is later replaced by highly organized and compacted layers of collagen fibers, known as lamellar bone. During the process of callus formation, bone tissue remodeling is occurring simultaneously and osteoclast activity is high and gradually increased. Osteoclasts mediate bone remodeling in areas of new bone formation, leading to the restoration of the original bone structure (Bastian et al., 2011; Barnes et al., 2008). Therefore fracture repair is a suitable model to study migration of osteoclast precursors and their differentiation to resorbing osteoclasts during active bone remodeling.

We hypothesize that during fracture healing, peripheral osteoclast precursors are preferentially been recruited to localized inflammatory sites to readily support the demands of sustain bone remodeling during fracture repair. However, we think that bone marrow-derived local precursors also contribute, to some extent, to the pool of osteoclast precursor. These studies will be important to understand the biology of osteoclast and their contribution to bone repair during pathological and non-pathological conditions, and might also contribute to the development of therapies to enhance bone repair that could be beneficial to treat impaired bone healing conditions in humans (advanced age, osteoporosis, corticosteroid treatment).
2.4. Engraftment and differentiation of osteoclasts in a murine model of fracture repair

Using CathepsinK-tdTomatoFP mice, we isolated bone marrow cells and then transferred them into fracture mice to test the migration, localization and differentiation of osteoclast precursors to the fractured site. Briefly, 6 to 8 weeks old mice were anesthetized and femoral fractures were then performed on the right hind limb, as previously described (Kaback et al., 2008; Kaback et al., 2008; Naik et al., 2009) and mice were transferred with cells derived from those fluorescent reporter mice and assessed for engraftment of donor cells. For these experiment, total bone marrow (2x10^6 cells/mouse) cells isolated from Cathepsin-K-tdTomato mice were transferred by retro-orbital injection in 100 microL of sterile PBS per mouse at day 0 (the day of the fracture) and at day 7 after the fracture. Engraftment was evaluated by histology of frozen sections and cell culture of osteoclast, 14 days after the fracture (figure 8, experimental design). To monitor the site of the fracture, callus formation and healing of fracture, mice were radiographed using a Faxitron x-ray system (Faxitron, X-ray, Wheeling, IL), figure 9 shows a radiograph of a femoral fracture properly performed.

Results of histological preparations showed engraftment of donor osteoclast precursors that have differentiated in vivo into mature resorbing osteoclast identified by a cluster of red fluorescence cells and localized within the fracture callus in areas of active bone remodeling (figure 10). In addition, we also tested whether donor cells have migrated and engrafted to other tissues, therefore we isolated total bone marrow and spleen cells and cultured them in vitro in 96-well plates at a density of 1x10^5 cells/well with M-CSF and RANKL for 8 days. Culture plates were then exposed to fluorescence using a Zeiss microscope and RFP+ osteoclast forming in cultured were detected using a TRITC (red) filter (data not sown). These results indicate that
bone marrow contains a fraction of osteoclast precursors able to home back to the bone marrow at the site of fracture healing (perhaps driven by the inflammation process itself) and differentiate to osteoclasts in active sites of bone resorption. Interestingly, engraftment of cells occurs in peripheral tissues, like the spleen, suggesting that under these inflammatory conditions a fraction of myeloid precursors is exposed to the necessary signals that direct them to establish tissue-associated populations. Further experiments are necessary to test whether defined populations of osteoclasts precursors are those that migrate to the fracture site and differentiate \textit{in situ}.

3. Discussion

Our results in Chapter III showed the identification of a common monocyte precursor able to differentiate to macrophages, dendritic cells and osteoclast. These precursors were found in the bone marrow and peripheral hematopoietic tissues in adult mice and our experiments suggested a developmental relationship among these precursors, however that hypothesis has not yet been tested. Therefore in Chapter IV we aimed to study the migratory properties and the developmental relationship among osteoclast precursors from bone marrow and periphery and whether they are able to differentiate into mature resorbing osteoclast \textit{in vivo}. First we generated a mouse fluorescent reporter line by crossing CathepsinK-Cre mice with ROSA-td-tomato fluorescent protein reporter mice that will serve as a source to isolate precursor cells, transfer those to non-fluorescent recipient mice and allow us to track osteoclast \textit{in vivo}. Osteoclasts express high levels of cathepsin K and therefore red fluorescent protein that can be easily identify by microscopy. To our knowledge there are no reports in the literature of the use of these
reporter mouse lines and therefore we offer a new alternative strategy to study osteoclast biology in vitro and in vivo.

As shown in figures 3 to 7, we performed a series of experiments to isolate osteoclast precursors derived from fluorescent reporter mice and adoptively transferred those into lethally irradiated recipient mice to study osteoclast development. We found that bone marrow precursors engrafted in the bone marrow but also repopulated spleen precursors and continue to circulate in peripheral blood at least for 15 days after been transplanted. In addition, transfers using spleen osteoclast precursors showed engraftment of these in the bone marrow of recipient mice as well, but also migrated to peripheral tissues and were found in the spleen and peripheral blood. These precursors seem to proliferate (for at least 15 days) and still preserved their osteoclastogenic potential since we were able to detect red fluorescent multinuclear osteoclast associated to endosteal surfaces in recipient mice for both bone marrow or spleen donor derived precursors. These experiments showed that osteoclasts precursor cells that develop in the bone marrow, and give rise to osteoclast, could also migrate via peripheral blood and associate to hematopoietic tissues and maintain these peripheral tissue myeloid precursor populations that could eventually give rise to resident macrophages and dendritic cells. Interestingly, spleen osteoclast precursors also migrated to and engrafted in the bone marrow and differentiated into multinuclear functional osteoclast under these conditions. But, why do spleen resident myeloid cells have osteoclastogenic potential? What signals are required for those precursors to leave peripheral tissues and migrate to bone marrow to differentiate to osteoclast? These are some of the questions that remained unanswered in osteoclast biology.

These are also some of our current interests and we aim to understand the need of having a reservoir of myeloid precursor cells that develop to osteoclast when expose to the proper
environment, and whether these populations are important during inflammatory conditions. Therefore we tested the ability of bone marrow osteoclast precursor cells derived from fluorescent reporter mice to migrate to sites of inflammation. For these experiments we performed fractures in the femurs of adult mice, which offers a model of disease-free inflammation combined with active bone remodeling suitable for these studies. We found that bone marrow cells transferred by retro-orbital injection to fractured mice indeed migrated to the fracture site and participated in the bone remodeling process. We identified osteoclast as a cluster of red fluorescent cells in the callus of fractured femurs. These preliminary results demonstrated the efficiency to combine fluorescent reporter lines in models of inflammation and bone remodeling to study osteoclast biology. Therefore, in Chapter V we will further study the dynamics of osteoclast precursor populations in inflammatory conditions.

Another hypothesis that is not completely understood relies on osteoclast precursors and their developmental relationship to macrophages and dendritic cells. Our in vitro experiments have suggested that osteoclast precursors might share a common developmental pathway with macrophages and dendritic cells. Although we did not test this hypothesis, we have proposed the use of fluorescent reporter mouse models in transplantation settings to test this hypothesis in vivo. Therefore, future experiments will focus on competitive reconstitution transplantation in mice to study the in vivo differentiation of osteoclasts precursors into macrophages and dendritic cells.

In the case of macrophages, we will isolate bone marrow and peripheral precursors from mice that express tdTomato Fluorescent Protein under the control of Lysozyme M promoter (LysM) that is specifically expressed in myeloid cells, and its expression preferentially up-regulated in macrophages (Hume, 2011). These mice will be generated crossing LysM-Cre mice
with ROSA26-CAG-LoxP-tdTomato mice (Hume, 2011; Madisen et al., 2010). Osteoclast precursors will be isolated using FACS and cells will be transferred into irradiated recipient WT mice, as described previously. Then hematopoietic and non-hematopoietic tissues will be isolated for histological analysis to determine their localization and possible functions.

For **dendritic cells**, myeloid progenitors will be isolated from mice expressing mCherry Fluorescent Protein under the control of CD11c promoter that is expressed in dendritic cells (Hume, 2011; Khanna et al., 2010). This model has been widely used to study dendritic cell migration and development. Similar to the experiments described above, we will perform transplants in a competitive reconstitution setting. The engraftment of donor cells will be assessed by 1) Flow cytometric analysis of various tissues and 2) histology of frozen hematopoietic and lymphoid tissues (Jiang et al., 2005; Khanna et al., 2010).

Currently, these experiments are under development and will allow us to identify fluorescent macrophages or dendritic cells, derived from osteoclast precursors, to characterize their mature populations and their localization, and possibly their functions among tissues.
4. Figures

Figure 13. *In vitro* osteoclastogenic assays using cells derived from CathepsinK-Cre-tdTomatoFP mice. Bone marrow cells were isolated and plated at a density of $1 \times 10^5$ cells per well in the presence of M-CSF and RANKL. Osteoclasts were identified and expressed RFP+ only when exposed to RANKL. Cultures with M-CSF only do not generate OCLs and are RFP negative.

CathepsinK-tdTomatoFP
($cts^{\text{cre/+}};\text{CAG tdTomatoFP}$)

M-CSF

M-CSF+RANKL

Total Bone Marrow cell cultures
(5 days in the presence of cytokines)
Figure 14. Experimental design of adoptive transfers in a competitive reconstitution mouse model. Cell transfers were performed using 2 lines of fluorescent reporter mice: 1) CathepsinK-tdTomato and 2) pOBCol3.6GFP. For each experiment WT CD45.1 mice were used as recipient subjects and to isolate HSC CD45.1+ for radioprotection. After cell transfers, mice were housed for 10 or 15 days then euthanized and their organs removed to perform flow cytometry analysis, histology and cell cultures to assess engraftment of donor cells.

1) CathepsinK-tdTomatoFP
2) pOBCol3.6GFP

C57BL/6 (CD45.2) → Bone marrow HSC (5x10^3 cells), or
   2) BMOC-PIV (1.0x10^5 cells), or
   3) SP OCP (1.0x10^6 cells)

Donor

975 Rads

10-15 days

Recipient C57BL/6 (CD45.1) → 1) FACS
                              2) Histology
Figure 15. *In vivo* engraftment of bone marrow- and spleen-derived osteoclast progenitors in adoptive transfer mouse model using pOBCol3.6 GFP transgenic mice. We FACS-sorted and transferred 1x10^5 OCP derived from BM or SP of CD45.2 pOBCol3.6GFP transgenic mice into CD45.1 lethally irradiated mice and evaluated tissue engraftment 10 days after by FACS analysis of bone marrow, spleen and peripheral blood of mice. In mice that received 1x10^5 SP OCP cells, engraftment was detected in bone marrow and spleen and a small percentage in peripheral blood. Experimental controls were 1) non-irradiated mice transplanted with CD45.2 BM-PIV or SP OCP, 2) non-irradiated mice transplanted with CD45.2 HSC and 4) Irradiated mice transplanted with CD45.2 HSC derived from pOBCol3.6GFP transgenic mice. All irradiated donor mice (CD45.1) were co-injected with a radio-protective source of 5,000 CD45.1 HSC. All FACS pseudocolor plots were first gated on leukocytes and PI negative cells. Percentages represent total CD45.2 expression. Data shown is representative of 2 independent experiments with a total of 5 mice per experiment.
Figure 16. *In vivo* osteoclast differentiation in competitive reconstitution experiments, using pOBCol3.6 GFP transgenic mice. Histological preparations of frozen femurs were processed using the CryoJane method and sectioned with a Leica CM1900 Cryostat. Femur sections of 8µM depth were then scanned using AxioVision software to identify GFP+ osteoclasts. On the right 20X magnification images of indicated areas showing GFP+ osteoclasts. For microscopy we used a Zeiss Imager Z1 microscope (Carl Zeiss, Thornwood, NY) and detected using GFP (green) filter (Chroma Technology, Bellows Falls, VT). Samples are from transplants of BM osteoclast precursors into irradiated recipients.
Figure 17. *In vivo* engraftment of bone marrow- and spleen-derived osteoclast progenitors in adoptive transfer model using CathepsinK-Cre-tdTomatoFP transgenic mice. We FACS-sorted and transferred $1 \times 10^5$ OCP derived from BM or SP of CD45.2 CathepsinK-Cre-tdTomatoFP transgenic mice into CD45.1 lethally irradiated mice and evaluated tissue engraftment 15 days after by FACS analysis of bone marrow, spleen and peripheral blood of mice. Experimental controls were 1) non-irradiated mice transplanted with CD45.2 BM-PIV or SP OCP, 2) non-irradiated mice transplanted with CD45.2 HSC and 4) Irradiated mice transplanted with CD45.2 HSC derived from CathepsinK-Cre-tdTomatoFP. All irradiated donor mice (CD45.1) were co-injected with a radio-protective source of 5,000 CD45.1 HSC. All FACS pseudocolor plots were first gated on leukocytes and PI negative cells. Percentages represent total CD45.2 expression. Data shown is representative of 2 independent experiments with a total of 5 mice per experiment.
Figure 18. *In vivo* differentiation of bone marrow-derived osteoclast precursors into multinuclear TRAcP+ osteoclasts in competitive reconstitution experiments.

Histological preparations of frozen femurs were processed using the CryoJane method and section with a Leica CM1900 Cryostat. Femur sections of 8 microM depth were then scanned using AxioVision software to identify RFP+ osteoclasts. A) The dotted square on the left indicates the scanned anatomical site of the femur. The middle panel shows 10X magnification of the indicated section exposed to red fluorescence. In bright red osteoclasts were found associated with bone surfaces. Images shown are from one recipient mouse and fluorescent patterns are representative of 5 mice.
B) Panels show 20X magnification images for detection of RFP+ osteoclast that overlap 100% with TRAcP staining and were multinuclear as shown by DAPI. For microscopy we used a Zeiss Imager Z1 microscope (Carl Zeiss, Thornwood, NY) and detected using a TRITC (red) filter and DAPI (blue) filter (Chroma Technology, Bellows Falls, VT). Samples are from transplants of BM osteoclast precursors into irradiated recipients. Images shown are from one recipient mouse and fluorescent patterns are representative of 5 mice.
**Figure 19. In vivo differentiation of spleen-derived osteoclast precursors into multinuclear TRAcP+ osteoclasts in competitive reconstitution experiments.**

Histological preparations of frozen femurs were processed using the CryoJane method and section with a Leica CM1900 Cryostat. Femur sections of 8μm in depth were then scanned using AxioVision software to identify RFP+ osteoclasts. A) The dotted square on the left indicates the scanned portion of the femur. The middle panel shows 10X magnification of the indicated section scanned with the red filter. On the right 20X magnification images showing RFP+ multinuclear osteoclasts, corroborated with TRAcP and DAPI stains. For microscopy we used a Zeiss Imager Z1 microscope (Carl Zeiss, Thornwood, NY) and detected using a TRITC (red) and DAPI (blue) filters (Chroma Technology, Bellows Falls, VT). Samples are from transplants of SP osteoclast precursors into irradiated recipients.
Figure 20. Experimental design for generation of fractured mice and cell transfer procedure. Fractures were performed on the right femur of 8 weeks old mice and cells derived from CathepsinK-tdTomato donor mice were transfer at the indicated time points. Mice were euthanized 14 days after the fracture and femurs were prepared for histological analysis and spleens were harvested for cell cultures, as described in methods.
Figure 21. Monitoring of fracture procedure by X-ray. Fractures in mice were corroborated by radiography using the Faxitron x-ray system (Wheeling, IL) under anesthesia, immediately after the procedure to monitor pin fixation as well as on going callus formation (not shown).
Figure 22. *In vivo* engraftment and differentiation of bone marrow cells into osteoclast in a fracture model using CathepsinK-Cre-tdTomatoFP transgenic mice. Mice were fractured and total bone marrow cells were transfer (as indicated in figure 8) and evaluated by histology (as described in methods) for engraftment and differentiation of cells into osteoclast *in situ* at the fracture site. Fluorescent microscopy of 8 microM sections showed a cluster of RFP+ osteoclast localized at sites of active bone resorption.
CHAPTER V

Studies on the dynamics of osteoclast precursor populations influenced by inflammation

1. Introduction

1.1. Lipopolysaccharide and osteoclasts

Monocytes, macrophages and dendritic cells respond to pathogen insult by expanding their effector populations and secreting factors to eliminate infections (Akira et al., 2004). As described above, lipopolysaccharide (LPS), which is a major constituent of Gram-negative bacterial cell walls, binds to a complex of TLR4 receptor and CD14 co-receptor both of which are expressed in monocytes. Therefore, monocytes respond to LPS during bacterial infections or to exogenous administration of LPS in mice (Akira et al., 2004).

Osteoclast precursors are phenotypically similar to monocytes and also known to be influenced by bacterial components during infections. In addition, it has been reported that LPS is the major mediator of osteoclast activation. OCPs also express TLR4 and CD14 in their cell membrane and therefore they are thought to respond to LPS signaling. However, less is known about the effects of LPS and how the signals involved in the activation of these populations mediate bone loss (Chapter I).

In general, we want to understand the mechanisms of action of PAMPs, particularly LPS, in osteoclast precursors and their response to these inflammatory stimuli. Our hypothesis is that
LPS treatment activates osteoclast precursors by modulating their frequency, number, distribution and osteoclastogenic potential.

As previously discussed, during gram-negative bacterial infections LPS induces the production of TNFα which is a pro-inflammatory cytokine that is also involved in osteoclast activation, and this might be one of the mechanisms by which LPS induces osteolysis. Interestingly, TNFα is also implicated in inflammatory arthritis and consequently it has been proposed that bacterial infections that mediate osteolysis might also be triggers for such conditions in susceptible individuals. However, these mechanisms are still under investigation. Here we investigated the role of LPS administration in mice in the activation of osteoclast precursor populations. In addition, we studied the dynamics of these populations in inflammatory arthritis, using a mouse model of rheumatoid arthritis in which human TNFα is overexpressed.

1.2. Inflammatory arthritis in human TNF transgenic mice

Osteoclasts are also involved in inflammatory disease associated to bone loss. It is known that during inflammatory arthritis myeloid cells migrate to inflammatory sites in arthritic mice and contribute to the pathology of the disease by generating active bone-resorbing osteoclasts. It is also known that bone resorption is increased at localized sites of inflammation in diarthrodial joints and might almost exclusively be attributed to osteoclast activity (Xing and Schwarz, 2005; Schett and Teiltelbaum, 2009; Okamoto et al., 2011). However, the origin and phenotype of those precursors is under investigation and a current subject of extensive research. We have recently identified populations of osteoclasts in hematopoietic tissues with high clonal efficiency, showed their migratory properties and found a possible correlation with bone marrow and peripheral precursors in vivo, under homeostatic conditions (Chapters III and IV). However, the
dynamics of these populations and their osteoclastogenic potential influenced by inflammatory signals in mouse models of disease, particularly in arthritis, remain to be explored. Therefore, we hypothesized that circulating precursors migrate to inflammatory sites in diartrodial joints of transgenic mice overexpressing human Tumor Necrosis Factor (hTNF) and are activated by the inflammatory milieu to become resorbing osteoclasts when proper signaling favors osteoclastogenesis, and contribute to the chronic loss of bone tissue.

The human Tumor Necrosis Factor (hTNF) transgenic mouse is a model of rheumatoid arthritis. These mice were developed by microinjection of hTNF/β-globin fusion construct into mouse zygotes, resulting in the insertion of 5 copies of the human TNF gene (line 197) leading to the overexpression of TNF and resulting in the development of arthritis. The features of arthritis in these mice share many of the characteristics observed in human rheumatoid arthritis primarily affecting diarthrodial joints (e.g., the knee joint): inflammatory cell infiltration to synovium, hyperplasia of synovial lining cells, pannus formation and articular cartilage and bone tissue erosion with eventually development of ankylosis and osteoporosis (Keffer et al., 1991; Firestein 2003). In hTNF transgenic mice the onset of the disease occurs as early as 4 to 5 weeks, and progress to severe polyarthritis at 8 to 10 weeks (Keffer et al., 1991). As in most mouse models of arthritis, anti-TNF monoclonal antibody administration suppresses the development of arthritis, although does not offer a cure. We have used hTNF transgenic mice in combination with CathepsinK-Cre-TdTomatoFP reporter mice to test the dynamics, migration and osteoclastogenic potential of hematopoietic populations in inflammatory arthritis.
2. Results

2.1. The effect of Lipopolysaccharide on osteoclast precursor populations

Using flow cytometry we identified the cell surface expression of Pattern Recognition Receptors (PRRs) known to be involved in the recognition of LPS in osteoclast precursors. We found that all our previously identified precursor populations (including bone marrow, spleen and peripheral blood) express intermediate to high levels of both TLR4 and its co-receptor CD14 (figure 1). Interestingly, sorted bone marrow and spleen CD14+ cells cultured in vitro with M-CSF and RANKL contained a population with osteoclastogenic potential, as these cells formed multinuclear TRAcP+ cells. In contrast, CD14 negative cells did not generate TRAcP+ osteoclasts (figure 2). These observations prompted us to study the in vivo dynamics and osteoclastogenic potential of osteoclast precursors in mice challenged with LPS.

In these experiments, wild type 8 to 10 week old mice were injected intraperitoneally (IP) with LPS 055-B5 (Sigma-Aldrich, L-2880) to establish a LPS dose-response and to study the dynamics of osteoclasts over time. We isolated bone marrow, spleen and peripheral blood leukocytes and analyzed the changes in distribution and phenotype of hematopoietic populations. In the LPS treated group, we found a decrease in the total bone marrow cellularity by approximately 25% but no changes occurred in spleen total cell numbers (figure 3). Early adult hematopoietic progenitor number (LKS, Lin^c-kit^Sca-1^) in the bone marrow was decreased by approximately 60% but interestingly in the spleen we found that LKS populations increased in number by 8 fold, suggesting that these precursors had migrated from bone marrow to peripheral tissues or alternatively that extramedullary hematopoiesis occurred (figure 3). Unexpectedly, we observed a dramatic increase in the percentages and numbers of osteoclasts precursors in the
bone marrow, which showed an opposite effect to changes observed in early hematopoietic precursors (figure 4A). This data suggested a specific expansion of precursors committed to the macrophage/osteoclast lineage. Similar to the bone marrow, spleen osteoclast precursor populations increased 10-fold compared to wild type controls (figure 4B). Interestingly, the in vitro osteoclastogenic potential of spleen, but not of bone marrow, precursors resulted in a 7-fold increase in the number of TRAcP+ multinuclear osteoclasts formed in cultures stimulated with M-CSF and RANKL (figure 4B and 4A, respectively). These experiments showed that LPS administration increased OCPs numbers in the bone marrow and periphery, and suggest that LPS primed OCPs through TLR4/CD14 activation and, in turn, these primed OCP to become more susceptible to M-CSF and RANKL induced osteoclastogenesis in vitro.

**2.2. Overexpression of human TNF in transgenic mice modulates osteoclast precursors**

We have characterized the phenotype and distribution of osteoclast precursors and other hematopoietic populations in hTNF transgenic mice that develop spontaneous inflammatory arthritis around 8 weeks of age, with symptoms and features that are similar to the ones observed in humans, including diarthrodial joint inflammation and cell infiltration (Keffer et al., 1991; Li and Schwarz, 2003). Using flow cytometry we isolated bone marrow, spleen and peripheral blood from 8 weeks old male and female WT and hTNF mice and compared the cell phenotype and distribution. We found that bone marrow osteoclast precursors in both WT and in hTNF transgenic male mice, defined as CD3−B220−CD11blowCD115+CD117high (population IV), -CD117int (population V) and -CD117low (population VI) were no different in their percentages and absolute numbers. In female mice, percentages of these populations were similar however
absolute numbers were slightly increased in the hTNF transgenic and significant differences were found only in OCP population VI (figure 5). Interestingly, in peripheral blood and spleen we found increased percentages and absolute numbers of osteoclast precursor populations. Peripheral populations analyzed were those previously identified to generate osteoclasts in vitro, and defined by the lack of lymphoid markers (B220, CD3 and NK1.1), high expression of Ly-6C (higher osteoclastogenic potential) and low expression of Ly-6C (lower osteoclastogenic potential). Further dissection of CD115hiCCR2hi cells, contain within Ly-6C populations were also analyzed. The majority of the populations showed a 2- to 4-fold increase in these cells in hTNF mice compared to WT. Figure 6 shows spleen OCPs analysis of male mice and figure 7 of female mice. Peripheral blood precursor frequencies are shown in figure 8.

Our results suggest that in hTNF transgenic mice the overexpression of TNF influence peripheral osteoclast precursors to proliferate preferentially over bone marrow populations, and these cells might be directly involved in the migration to localized sites of inflammation in the joints and then differentiate to resorbing osteoclasts contributing to bone loss. Whether TNF directly influences the migration and osteoclastogenic potential of precursor or indirectly, by triggering the production of other cytokines and chemokines, remains to be known. However, some reports have shown that TNF itself can induce or enhance osteoclastogenesis (Chapter I).

2.3. Human TNF transgenic mice: a model to study osteoclast migration and differentiation

Our recent development of a fluorescent transgenic mouse model to label osteoclasts has demonstrated to be useful tool to study osteoclast migration and differentiation in situ, as our previous transplant and fracture experiments showed. Here, we tested the potential of osteoclast
precursors derived from CathepsinK-Cre-TdTomatoFP reporter mice to migrate and differentiate in the context of inflammatory arthritis using hTNF transgenic mice as recipients.

For these experiments we isolated total bone marrow (2x10^6) cells derived from CathepsinK-Cre-tdTFP mice and transferred them by retro-orbital injection to hTNF transgenic mice. We performed 2 injections: the first injection was at 8 weeks of age and the second injection at 9 weeks of age. To assess engraftment, at 10 weeks of age, we isolated the hind limbs of hTNF and WT mice and prepared for histology, as described in methods. We carefully preserved the knee joint and prepared sections of 8microM of thickness using a cryostat, and fluorescent microscopes to visualize engraftment of fluorescent osteoclasts associated to bone tissue. At the knee junction, we found RFP+ osteoclasts in areas proximal to subchondral bone (just below articular cartilage) and also associated with trabecular bone (figure 9). Regardless, damage to the bone tissue structure was evident and although we did not perform measurements to quantify these observations, several reports have previously defined such changes in bone tissue abnormalities (Chapter I).

Our previous fracture experiments detected engraftment of donor osteoclast precursors in the spleen of recipient fractured mice, an interesting observation since no other stimulus except the fracture itself showed to be sufficient to induce the establishment of tissue-associated populations. We reasoned that in other conditions of chronic inflammation, like those observed in arthritis, might induce osteoclast precursors to associate to peripheral tissues. Therefore, we evaluated whether donor CathepsinK-Cre-tdTFP cells transferred to hTNF mice engrafted in the spleen of recipient mice. For these experiments we isolated and processed spleen cells and cultured 1x10^5 total spleen cells per well (approximately 2,500 OCP) in 96-well plates with M-CSF and RANKL for 8 days. We found RFP+ osteoclasts in cultures as shown in figure 1.
Although these cells are not abundant, these results indicate that overexpression of TNF stimulate osteoclast precursor populations to migrate to peripheral tissues and become resident cells.

Future experiments will focus to evaluate whether inflammatory conditions in these mice are sufficient to support sustained proliferation of transferred donor cells and whether the osteoclastogenic potential of these cells remain and how they contribute to bone loss.

3. Discussion

Pathogen-associated molecular patterns (PAMPs) found in microorganisms have been studied for years and are known to interact and activate receptors expressed in the cell surface of hematopoietic cells and trigger an immune response in the host that ultimately results in the elimination of infectious agents. In chapter III we showed that osteoclast precursors express markers related to monocytes, macrophages and dendritic cells. Here we show that bone marrow and peripheral OCPs express TLR4 and CD14 in their cell surface suggesting that these precursors might respond to pathogenic insult. In addition, we showed that CD14+ cells generated multinuclear osteoclasts in in vitro cultures when stimulated with M-CSF and RANKL.

These results prompted us to study the effect of LPS on the dynamics and osteoclastogenic potential of these populations. Therefore we performed experiments in mice challenged with LPS. Systemic administration of LPS to mice altered the bone marrow microenvironment by decreasing BM cellularity. This reflected a decreased in the percentage and number of early hematopoietic precursors (LKS) as well. However, the opposite effect was seen in the spleen in which LPS induced an increase in the percentage and number of LKS without
altering the cellularity of the tissue, and therefore suggesting two possibilities: 1) a release of BM precursors to peripheral tissues with migration to the spleen or 2) LPS directly induced extramedullary hematopoiesis in the spleen by acting on hematopoietic precursors.

Interestingly, LPS did not affect the osteoclastogenic potential of bone marrow OCPs in vitro, but spleen OCPs did increase their osteoclastogenic potential suggesting that LPS preferentially modulates peripheral OCPs activity, perhaps by activating intracellular signaling pathways that lead to inflammatory responses. These pathways might be activated through TLR-4/CD14 signaling since OCPs express these receptors that are known to respond to LPS. Another possibility is that additional pathways are activated on OCPs causing a synergistic effect that makes OCPs to proliferate and increase their responsiveness to osteoclastogenic cytokines. These data suggest that inflammatory process during bacterial infections modulate osteoclastogenesis and consequently bone remodeling and might help explaining mechanisms of bone erosion during bacterial infections. Future experiments will focus on the in vivo potential of peripheral precursors to contribute to bone loss during infections.

Another model of inflammation in which osteoclast activity is increased and contributes to the chronicity of a pathological process is rheumatoid arthritis. Transgenic mice that overexpress human TNF spontaneously develop inflammatory arthritis and have been widely used to study pathogenic mechanisms. We used this mouse model to test whether our fluorescent reporter mouse lines were useful to study migration and differentiation of osteoclast precursors in inflammatory conditions. Adoptive transfers of bone marrow cells showed engraftment of donor osteoclast precursors in recipient mice, identified by red fluorescence and their association with bone surfaces in proximity to articular cartilage. The particular localization of donor-derived osteoclasts in subchondral bone suggests that the inflammatory milieu activates OCPs to
migrate and target diarthrodial joints. One possibility is that the upregulation or activation of chemokine receptors (CCR2, CX3CR1 and others) induced the migration of OCPs to localized sites of inflammation. CCR2 is known to be associated with migration of inflammatory cells (monocytes and neutrophils) to sites of infection or inflammation, but less is known about CX3CR1 in infections and arthritis.

In this context, is possible that local bone marrow OCPs migrate and differentiate to resorbing osteoclasts in sites of inflammation, however we did not detect changes in percentages and numbers of bone marrow OCPs. In contrast, peripheral blood and spleen OCPs were increased in numbers suggesting a preferential expansion and activation of these precursors that might induce their migration to peripheral joints and differentiation to osteoclasts that participate in bone erosion.

In addition, we found engraftment of OCPs in the spleen, as shown by in vitro osteoclast cultures that detected donor-derived RFP+ osteoclasts. These results suggest that stimuli triggered by the inflammatory milieu are sufficient to induce activation, migration and differentiation of osteoclast precursors in arthritis. These preliminary data showed the potential of fluorescent reporter mice to study osteoclast activity in disease models of inflammation. Future experiments will focus on studies of the mechanisms of migration of osteoclast precursors and bone loss in arthritic animals.

Identifying signaling pathways and the genes they activate, chemokine receptors and how they induce mobilization of OCPs populations, and the cytokines and factors that activate osteoclasts differentiation and induce bone resorption could help us to understand the mechanisms by which bacterial infection components and inflammatory signals during disease
modulate osteoclast activity and enhance bone resorption for the development of alternative therapeutic strategies to prevent inflammation and bone loss.
4. Figures

Figure 23. Bone marrow and peripheral osteoclast precursors express CD14 and TLR4 on their cell surface. A) FACS analysis of bone marrow populations PIV, PV and PVI. Histograms on the right show expression of CD14 and TLR4 for each individual population. All 3 OCP populations express these markers. B) FACS analysis of circulating and spleen OCPs for CD14 and TLR4. Control samples (tinted histogram) are unstained cells showing a negative peak for fluorescence.
Figure 24. Bone marrow and spleen CD14+ myeloid precursors contained high osteoclastogenic activity. Bone marrow and spleen cells were stained with CD14 antibody and populations negative and positive for CD14 were FACS-sorted and plated at a density of 10,000 cells per well in M-CSF and RANKL. Top left bar graphs show BM mononuclear TRAcP+ cells (CD14- 92±6 vs. CD14+ 72±4, p=0.08) and top right show multinuclear TRAcP+ OCLs (CD14- 30±5 vs. CD14+ 290±18, p=0.0001). Bottom left bar graphs show spleen mononuclear TRAcP+ cells (CD14- 64±6 vs. CD14+ 109±14, p=0.01) and bottom right show multinuclear TRAcP+ OCLs (CD14- 6±1 vs. CD14+ 181±5, p=0.0001). Cultures were stained for TRAcP after culturing for 5 to 8 days.
Figure 25. LPS administration modulates early hematopoietic precursors. Mice 8 to 10 weeks old were injected i.p. with LPS 055-B5 (Sigma-Aldrich, L-2880) at a dose of 10 mg/kg/mouse or PBS and their organs harvested 7 days later for FACS analysis and to isolate hematopoietic precursors to measure osteoclast activity. A) Bar graphs showing bone marrow and B) spleen total tissue cellularity and, percentage and number of LKS (Lin^c-kit^Sca-1^+^) hematopoietic precursors in those tissues. *** p<0.0005.
Figure 26. LPS administration increases osteoclast precursor number and modulates their osteoclastogenic potential. Mice 8 to 10 weeks old were i.p., injected once with LPS 055-B5 (Sigma-Aldrich, L-2880) at a dose of 10 mg/kg/mouse or PBS and their organs harvested 7 days later for FACS analysis and sorting of osteoclast precursors to measure osteoclast activity. A) Bar graphs showing bone marrow and B) spleen osteoclast precursor percentage and absolute numbers. The bar graphs on the right for A) and B) show cell cultures of sorted OCPs plated at a density of 1,000 and 5,000 cells per well in 96 well plates in 5 replicates for each individual mouse, respectively. Data shows mean values of one experiment with 3 to 5 mice for each experimental group. ** p<0.005 and *** p<0.0005.
Figure 27. Cell frequency and number of bone marrow osteoclast precursors in Human TNF transgenic mice. Eight weeks old mice were euthanized and bone marrow harvested to analyze OCPs populations by flow cytometry. Bar graphs represent mean values of percentages (left) and numbers (right) of BM OCPs populations (PIV, PV and PVI as described before) in male (top) and female (bottom) mice. * p<0.005.
Figure 28. Cell frequency and number of spleen osteoclast precursors in Human TNF transgenic male mice. Eight weeks old male mice were euthanized and spleen harvested to analyze OCPs populations by flow cytometry. Bar graphs represent mean values of percentages (left) and absolute numbers (right) of spleen OCP populations gated on B220^CD3^-NK1.1^-CD11b^+ and analyzed separately for Ly6-C^high (top graphs) and Ly6-C^low (bottom graphs). Populations gated on Ly-6C were subsequently gated on CD115 and CCR2. * p<0.05, ** p<0.005 and *** p<0.0005.
Figure 29. Cell frequency and number of spleen osteoclast precursors in Human TNF transgenic female mice. Eight weeks old female mice were euthanized and spleen harvested to analyze OCPs populations by flow cytometry. Bar graphs represent mean values of percentages (left) and absolute numbers (right) of spleen OCP populations gated on B220^-CD3^-NK1.1^-CD11b^+ and analyzed separately for Ly6-C^{high} (top graphs) and Ly6-C^{low} (bottom graphs). Populations gated on Ly-6C were subsequently gated on CD115 and CCR2. * p<0.05, ** p<0.005 and *** p<0.0005.
Figure 30. Cell frequency and number of peripheral blood osteoclast precursors in Human TNF transgenic mice. Peripheral blood was withdrawal from the tail vein of eight weeks old mice and processed as described in methods. Cells were then analyzed by flow cytometry for circulating OCPs populations. Bar graphs represent mean values of percentages of male (left) and female (right) mice and for Ly6-C\textsubscript{high} (top) and Ly6-C\textsubscript{low} (bottom) populations (gated first on B220\textsuperscript{-}CD\textsubscript{3}\textsuperscript{-}NK1.1\textsuperscript{-}CD11b\textsuperscript{+}). Populations gated on Ly-6C were subsequently gated on CD115 and CCR2. * p<0.05, ** p<0.005 and *** p<0.0005.
Figure 31. *In vivo* engraftment and differentiation of bone marrow cells into osteoclast in a mouse model of arthritis. Total bone marrow (2x10^6 cells per mouse) derived from CathepsinK-Cre-tdTomatoFP mice was transferred by retro-orbital injections to hTNF mice. Two injections were given: 1) at 8 weeks of age, 2) at 9 weeks of age, and mice were euthanized at 10 weeks of age. Histological preparations of frozen femurs were processed using the CryoJane method and section with a Leica CM1900 Cryostat. Femur sections of 8microM depth were then scanned using AxioVision software to identify RFP+ osteoclasts localized in endosteal areas in close proximity to the knee junction (white arrows on the right). For microscopy we used a Zeiss Imager Z1 and a TRITC (red) filter.
Figure 32. *In vivo* engraftment and differentiation of bone marrow cells into osteoclast in a mouse model of arthritis. hTNF mice transferred with bone marrow cells (as in figure 9) derived from CathepsinK-Cre-tdTFP mice were euthanized and their spleens were processed as described in methods. Total cells were then cultured *in vitro* at a density of $1 \times 10^5$ cells per well in 96 well plates with M-CSF and RANKL for 8 days. Osteoclasts formed in culture were exposed to red fluorescence to identify those expressing RFP and derived from donor CathepsinK-Cre-tdTFP mice. Fluorescent pictures show osteoclast in red (left), in bright field (right) and an overlap of both (bottom).
CHAPTER VI

Parathyroid Hormone Regulates the Distribution and Osteoclastogenic Potential of Hematopoietic Progenitors in the Bone Marrow.

1. Introduction

Parathyroid hormone is the major systemic regulator of calcium homeostasis and bone metabolism. PTH is known to maintain calcium homeostasis in bone, kidney and indirectly in the gastrointestinal tract. It is also known that PTH regulates phosphorus metabolism. Interestingly, the in vivo effect of PTH is dependent on the dose and frequency of administration. Continuous administration of PTH induces catabolic effects on bone, while intermittent PTH administration exerts anabolic effects (Schiller et al., 1999; Mohan et al., 2000; Frolic et al., 2003; Koh et al., 2005; Wang et al., 2005; Li et al., 2007; Pettway et al., 2008; De Freitas et al., 2009). Currently, for human therapy, one daily injection of human PTH (1-34) is a Food and Drug Administration approved treatment for osteoporosis in the United States.

Parathyroid hormone activates cells bearing the PTH/PTH related protein (PTHrP) receptor by direct engagement of these molecules (Juppmner et al., 1991). The PTH/PTHrP receptor is present on osteoblasts and other mesenchymal-derived cells in the bone tissue microenvironment. In contrast, most of the literature agrees that osteoclasts and their precursor
cells lack high-affinity PTH receptors (Teitelbaum et al., 2000; Fuller et al., 1998; Zaidi, 2007; Lupp et al., 2010). Therefore, it has been proposed that the effects of PTH on osteoclasts are indirectly driven by osteoblasts/stromal tissue (Zhao et al., 1999). In addition, PTH upregulates the Receptor Activator of NF-κB Ligand (RANKL) gene expression and protein production on osteoblasts/stromal cells and therefore, can indirectly activate osteoclast precursors to develop into mature resorbing osteoclasts (Lee and Lorenzo, 1999). Indeed, the identification of RANK/RANKL interactions as the main signal regulating osteoclastogenesis, originates from *in vitro* studies of osteoclast differentiation, in which hematopoietic cells and bone marrow stromal cells were co-cultured in the presence of parathyroid hormone (PTH) or 1α, 25 (OH)2-vitamin D3 (Suda et al., 1999; Lorenzo et al., 2008). These studies also indicated that osteoclastogenesis was dependent on cell-to-cell interactions between myeloid progenitors and osteoblasts or other cells of mesenchymal origin.

However, most studies have been focused on the effects of PTH on osteoblasts and recent published literature has shown that PTH could also modulate hematopoietic parameters (Adams et al., 2007), but direct studies of the effects of PTH on osteoclast progenitor populations and osteoclast development have not been done in a comprehensive fashion. This is important, as a complete understanding on how PTH modulates different cell types in the bone microenvironment is crucial for developing new strategies to treat osteoporosis and other conditions that require new bone formation, like bone fractures, as well as designing stem cell base therapies, i.e., hematopoietic stem cell transplantation, mobilization and harvesting of hematopoietic progenitors (Adams et al., 2007; Rodan and Martin, 2000; Neer et al., 2001; Cheng et al., 2009).
We have identified a discrete population of cells containing most of the *in vitro* osteoclast precursor (OCP) activity in murine bone marrow (Jacquin et al., 2006). In the current studies we examined if these populations of OCPs were affected by *in vivo* administration of PTH to mice. In addition, we examined the effects of PTH on the distribution of early adult hematopoietic progenitors in the murine bone marrow and spleen since PTH is known to modulate early hematopoiesis *in vivo* (Calvi et al., 2003).

2. Results

2.1. The effect of PTH (1-34) on osteoclast progenitor populations in the bone marrow.

We have previously identified three OCP populations in murine bone marrow with high osteoclastogenic potential (Jacquin et al., 2006). As depicted in Figs 1A and 1B, these populations share the phenotype CD3−CD45R−CD11b<sup>low</sup>CD115<sup>+</sup> and are further separated by their reactivity against CD117 as: CD117<sup>high</sup> (population IV), CD117<sup>int</sup> (population V) and CD117<sup>low</sup> (population VI).

In the current study, we evaluated the effect of intermittent PTH administration on osteoclast progenitor (OCP) populations measuring the distribution of populations IV to VI at day 7 and day 14 after treatment. As shown in Figures 1C and 1D, at day 7 of PTH treatment, the osteoclast progenitor populations in the bone marrow showed a significant increase in the percentages of PIV (vehicle=0.1890±0.0342 vs. PTH=0.3400±0.0583, p=0.042) and PV at day 7 (vehicle =0.3090±0.0258 vs. PTH=0.4064±0.0269 p=0.018). No changes were observed in the percentages of population VI. In contrast, PTH decreased the absolute numbers of PVI at day 7.
(vehicle=2.7x10^6±5.1x10^5 vs. PTH=1.5x10^6±2.4x10^5, p=0.047). Interestingly, there was no effect of PTH on the percentage or absolute number of osteoclast progenitors in mice at day 14 (Figs. 1E and 1F). Hence, intermittent PTH administration induced a transient modulation of osteoclast progenitor populations.

2.2. Administration of parathyroid hormone increases the in vitro osteoclastogenic potential of bone marrow OCP populations in C57BL/6 mice.

We next examined the in vitro osteoclastogenic potential of osteoclast progenitors from C57BL/6 mice treated with daily injections of bPTH (1-34) for 7 or 14 days. As an osteoclast progenitor population we isolated cells with the phenotype of CD3-CD45R-CD11b^{low}CD115^{+} (TN/CD115^{+}) from bone marrow. This population contains the majority of the in vitro osteoclastogenic activity in bone marrow. Populations containing over 99.1% purified cells were cultured with M-CSF and RANKL in 96 well plates at a density of 1,000 cells per well. The formation of osteoclast like cells (OCL) was evaluated at days 4, 5 and 6 of culture. Cells were fixed, stained for TRAcP and multinuclear vs. mononuclear TRAcP^{+} cells were counted. We observed that 7 days of bPTH treatment increased in vitro osteoclastogenesis by approximately 1.4 to 2.0 fold, compared to cells from vehicle-treated mice, depending on how long the cells were cultured in vitro (figure 2). In these time course experiments a significant increase was observed in multinuclear TRAcP^{+} osteoclasts in the PTH group at day 4 (vehicle=86±9 vs. PTH=118±11, p=0.042), day 5 (vehicle=112±17 vs. PTH=192±13, p=0.002) and day 6 of culture (vehicle=89±12 vs. PTH=142±5, p=0.001). In addition, there was a trend toward a decrease in the number of mononuclear TRAcP^{+} osteoclasts in the PTH group at day 5 (vehicle=136±14 vs. PTH=103±11, p=0.086). The decrease in mononuclear osteoclasts may be related to the higher
rate of multinuclear osteoclast formation in the PTH group. As observed when analyzing the distribution of OCPs, we found no effect of 14 days of PTH treatment on the number of osteoclasts, which formed in cultures from bone marrow OCP cells that were treated with M-CSF and RANKL. Therefore, the increase in osteoclastogenic potential from these progenitors was transient and could be related to the transient increase in certain progenitor populations.

2.3. **In vitro TRAcP+ osteoclasts from the PTH treated mice are larger in size than vehicle treated mice.**

Initial evaluation of OCL generated *in vitro* from PTH treated animals suggested that they generated larger multinucleated cells. Therefore, we measured the size of multinucleated OCL. FACS sorted osteoclast progenitors (TN/CD115+) from mice that were vehicle-treated or PTH-treated for 7 or 14 days were cultured with M-CSF and RANKL. At day 5 of culture we performed TRAcP assays and measured the area of individual osteoclasts from both groups. As shown in Figure 3, we found that osteoclasts from the PTH-treated group were larger than those from the vehicle-treated group. Seven days of *in vivo* PTH treatment increased the size of osteoclasts significantly compared to the vehicle-treated group (vehicle=0.0895±0.0082 mm² vs. PTH=0.1408±0.0115 mm², p=0.0003). Interestingly, cultures of cells treated with PTH for 14 days also had a significant increased in the size of osteoclasts compared to the vehicle-treated group (vehicle=0.1074±0.012 mm² vs. PTH=0.1519±0.012 mm², p=0.0089), despite the lack of an effect at this time point of PTH treatment on osteoclast progenitors or the number of osteoclasts that formed in the cultures.
2.4. Osteoclasts from PTH treated mice formed larger resorption pits than vehicle treated mice.

To evaluate whether PTH treatment affected resorption activity, we plated purified osteoclast progenitors (TN/CD115+) from vehicle- or PTH-treated mice, onto bovine cortical bone slices in the presence of M-CSF and RANKL. Twelve days after seeding, bone slices were stained for TRAcP to identify osteoclast. Bone slices were sonicated and then stained with toluidine blue to identify resorption pits. We measured the area of individual bone resorption pits from each group as shown in Figure 4. We observed that in vitro osteoclasts derived from mice treated with PTH for 7 or 14 days formed larger resorption pits than those from vehicle treated mice (7 days: vehicle=0.000618±0.000079 mm² vs. PTH 0.001542±0.00019 mm², p=0.01; 14 days: vehicle=0.000681±0.00020 mm² vs. PTH 0.001934±0.00043 mm², p=0.03).

2.5. Parathyroid hormone affects the dynamics of early hematopoietic progenitors in the bone marrow and periphery.

Several recent reports have shown that exogenous PTH treatment could significantly modify early hematopoietic parameters. These include increases in the number of early hematopoietic progenitors in the bone marrow (Calvi et al., 2003), and a potentiating effect on cytokines that induce their egress into the periphery. This has led to the proposal that PTH treatment could be used as a more effective way to mobilize progenitors, facilitating their harvest from blood for hematopoietic stem cell based therapies (Adams et al., 2007). To test whether these effects were also evident in our experiments, we examined the effects of PTH treatment in vivo at days 7 and 14 on the percentages and absolute numbers of early hematopoietic precursors, assessed by the phenotype Lin−CD117+Sca1+ (LKS). As depicted in Figure 5, we found a
statistically significant increase in the percentages of LKS in the bone marrow at 7 days (Control 0.57±0.051 vs. PTH 0.7924±0.062, p=0.01) and 14 days (Control 0.397 ± 0.023 vs. PTH 0.588 ± 0.059, p=0.004) of treatment. However, when the absolute number of these cells was analyzed, we found a significant increase at day 14 (control= 2.26x10^5±2.2x10^4 vs. PTH= 3.10x10^5±3.1x10^4 p=0.04), but not at day 7 of treatment (control= 3.34x10^5±3.8x10^4 vs. PTH= 3.83x10^5±3.5x10^4 p=0.35).

The LKS population is heterogeneous and includes early self-renewing hematopoietic stem cells with the ability to reconstitute the hematopoietic system of lethally irradiated recipients in a long-term fashion. To test if this population (LT-HSC) was affected by the PTH treatment we evaluated the LKS fraction that was negative for the CD135 molecule (Lin^-CD117^-Sca-1^-CD135^-) (Spangrude et al., 1988; Uchida et al., 1992; Christensen and Weissman, 2001). The distribution of this population followed the same changes observed for the LKS population with an almost significant increase in its percentage at day 7 (Control 0.201±0.031 vs. PTH 0.280±0.024, p=0.051) and no significant changes in absolute number. In contrast, at day 14 of PTH treatment there was an increase in both the percentage (Control 0.084±0.008 vs. PTH 0.144±0.022, p=0.02) and absolute number (control= 4.7x10^5±4.91x10^3 vs. PTH 7.2x10^4±1.01x10^4, p=0.03) of LT-HSCs.

We also evaluated the effect of PTH treatment on the distribution of common lymphoid progenitors (CLP), with the phenotype Lin^-CD117^-CD127^- (Kondo et al., 1997), and found no differences between vehicle- and PTH-treated mice either at 7 or 14 days.

Because of the reported ability of PTH to mobilize early hematopoietic progenitors, we evaluated the presence of LKS cells in periphery. Seven days after treatment there were no changes in the distribution of LKS cells in the spleen of PTH-treated animals compared to
vehicle-treated controls. In contrast at day 14, PTH treated animals showed a significant increase in the percentage (control 0.037±0.008 vs. PTH= 0.072±0.014, p=0.03) and absolute number (control 3.81x10^4±7.3x10^3 vs. PTH 7.53x10^4±1.31x10^5 p=0.02) of LKS cells in the spleen.

2.6. Osteoclast activity is not responsible for the effects of PTH treatment on early hematopoietic progenitors.

Alendronate is a second-generation nitrogen-containing bisphosphonate used for the treatment of osteoporosis. As an antiresorptive (antianabolic) agent, it is known to inhibit the enzyme farnesyl pyrophosphate synthase (FPPS), inducing apoptosis in osteoclasts, and consequently suppressing osteoclast-mediated bone resorption (Van Beek et al., 1997; Luckman et al., 1998; Graham and Russel, 2007; Amelio et al., 2010). Because we observed a significant increased in the percentage and number of LKS in the bone marrow and spleen (see figure 5), and an increased in the activity of osteoclasts \textit{in vitro} (figures 3 and 4) with 14 days of PTH treatment, we asked whether osteoclasts activity in the bone marrow affects hematopoietic progenitors or if this effect was attributed to PTH effects independent of osteoclast activity. Therefore, we treated mice with vehicle, PTH alone, alendronate or PTH in combination with alendronate for a 14-day period (figure 6, experimental design).

As shown previously, in the bone marrow, the PTH treatment induced a modest increase in the number of early hematopoietic progenitors (LKS cells) from 3.65x10^5±2.15x10^4 for vehicle to 4.61x10^5±3.07x10^4 for PTH treatment (p=0.04). The same trend, although non-significant, was observed when vehicle (3.65x10^5±2.15x10^4) was compared with mice treated with PTH plus alendronate (4.24x10^5±8.26x10^4). In the spleen, as expected, there was evidence of a significant egress of LKS cells when vehicle-treated mice were compared with PTH-treated
groups (1.88x10^4±3.31x10^3 for vehicle versus 7.19x10^4±7.53x10^3 for PTH treatment, p=0.003). This effect was maintained on the co-treatment of PTH with alendronate (1.88x10^4±3.31x10^3 for vehicle versus 5.95x10^4±5.58x10^3 for PTH with alendronate, p=0.002), see figure 7.

To test the effectiveness of alendronate for limiting osteoclast resorption, we measured the levels of serum CTX (carboxy-terminal collagen crosslinks) in the different groups of mice and found increased levels in PTH-treated mice when compared with vehicle-treated mice (53.2±6.8 ng/mL versus 34.7±2.2 ng/mL, p<0.05), see figure 7. This increased activity was not observed on the co-treatment of mice with PTH plus alendronate (33.0±3.2 ng/mL). These results indicate that alendronate treatment limited osteoclastogenic activity without altering the egress of early hematopoiesis progenitors to the periphery.

3. Discussion

Parathyroid hormone exerts different effects on bone in vivo depending on the method of treatment. Continuous administration results in a net increase in bone resorption. In contrast, when given intermittently (i.e. daily injections), PTH induces a net increase in bone formation. However, the mechanisms underlying the paradoxical effects of PTH remain unknown. Most studies of the molecular and cellular mechanisms of the effects of PTH on bone have focused on osteoblastic lineage cells. It remains unclear whether the main effects are due to its ability to activate osteoblast precursor cells or quiescent osteoblastic cells. It also may inhibit apoptosis in mature osteoblasts, which might prolong the anabolic effects of PTH (Schiller et al., 1999; Mohan et al., 2000; Frolic et al., 2003; Koh et al., 2005; Wang et al., 2005; Li et al., 2007; Pettway et al., 2008; De Freitas et al., 2009; Jilka et al., 2009). However, to fully appreciate the responses of bone to PTH, it is also necessary to understand how PTH administration affects
other type of cells in the bone microenvironment that are implicated in bone turnover. Osteoclasts are another potential target of PTH action since the physiologic activities of osteoblasts and osteoclasts are tightly coupled.

To date no studies have reported effects of PTH treatment on specific osteoclast progenitor populations. Here, we demonstrate that daily administration of PTH to mice significantly increased the percentages of specific populations of osteoclast progenitors compared to vehicle treated mice. It appears that these effects are a transient response to PTH since the number of osteoclasts progenitors was increased at 7 days of treatment but not at 14 days. Moreover, we think that this effect might be more specific for less mature populations of osteoclast precursors since we only observed an increase in the percentages of population IV and V. From previous studies it is known that PTH induces a transient increase in the expression of RANKL on the surface of osteoblastic cells when administered on a daily basis (Neer et al., 2001; Cheng et al., 2009). These effects on osteoblastic cells might help to explain the increase that we observed in osteoclast precursor cells after seven days of treatment (the acute response) and the return to normal levels at day 14. In addition, our in vitro osteoclastogenesis results found that the number of multinuclear TRAcP+ osteoclasts in cultures of cells from PTH-treated mice was significantly increased after 7 days of treatment but not after 14 days. These in vitro results correlate with our studies in vivo demonstrating that daily PTH administration increased the percentages of less mature OCP populations at day 7 but not at day 14. Our previous work demonstrated that these less mature cells are more efficient at forming osteoclasts in vitro (Jacquin et al., 2006).

Interestingly, we also observed that PTH treatment for both 7 and 14 days increased the size of osteoclasts that formed in cultures of OCP cells (TN/CD115+). In addition, these cells
were more active at resorbing bone since they formed larger resorption pits compared to the osteoclasts from vehicle-treated mice. The ability of PTH treatment in vivo to enhance subsequent RANKL and M-CSF stimulated osteoclast formation and bone resorption in vitro does not appear dependent on changes in the percentages and/or absolute number of osteoclast precursor population IV, V and VI in bone marrow since they occurred in OCP cells from mice treated with PTH for 14 days, whereas PTH had no effect on the distribution of these populations. Rather, a more likely explanation is that intermittent in vivo PTH primed the cells and induced sustained changes in the OCP cells, possibly by an epigenetic mechanism. This in turn, caused them to be more susceptible to M-CSF and RANKL stimulation. We are currently investigating the mechanisms by which these sustained changes may be occurring and attempting to identify the cells that respond and contribute to the overall in vivo effects of PTH on OCP progenitor populations.

In addition to our findings described above, we also studied the effects of daily administration of PTH for 7 and 14 days on early hematopoietic stem cells (LKS), long term self-renewing HSCs (LT-HSCs) and common lymphoid progenitors (CLPs). The effects of PTH on hematopoietic progenitors from bone marrow and peripheral blood have been previously described (Adams et al., 2007; Brunner et al., 2008). However, to our knowledge the current study is the first to report the effect of PTH on cells in the spleen. We observed a significant increase in the percentage but not on the absolute numbers of LKS and LT-HSC cells at day 7 of PTH treatment. We found no changes in the percentage or absolute number of CLP in the bone marrow and LKS in the spleen. Interestingly, on day 14 the percentages and absolute number of LKS and LT-HSC in bone marrow and LKS in the spleen increased significantly. These results suggest that PTH affects the distribution of these early precursors, possibly by altering their
niche (Adams et al., 2007; Calvi et al., 2003; Zhang et al., 2003; Lo Celso et al., 2009). These alterations could be due to a direct effect of PTH on cells that support the stability of hematopoietic niches or, alternatively, as a product of increased osteoclastogenesis. This latter possibility has been documented in recent years in studies showing that osteoclast activity may play a role in the dynamics of hematopoietic niches (Kollet et al., 2007).

Because we observed that hematopoietic progenitors from bone marrow and spleen at 14 days of PTH treatment increased in percentage and number and because this correlated with an increase in the activity of osteoclasts (bone resorption assay) we tested the possibility that osteoclast activity in bone marrow might affect the distribution of hematopoietic progenitors in bone marrow and spleen. Even when alendronate efficiently blocked the increased in bone resorption induced by PTH treatment, we observed no significant differences in percentage or number of LKS cells from mice co-treated with alendronate (when compared to control) and no differences in LKS from mice co-treated with PTH and alendronate (when compared to PTH treatment alone). These results suggest that the activity of osteoclasts in the bone marrow microenvironment does not influence the dynamics of hematopoietic progenitors and is not responsible for the egress of hematopoietic progenitors to peripheral tissues. Thus, the increase we observed on hematopoietic progenitors in the bone marrow and spleen should be caused by a more direct effect of PTH on cells responsible for maintaining the stability of hematopoietic niches.

In conclusion, we propose that PTH by acting on cells of either osteoblastic lineage or other populations, indirectly affects specific populations of OCP by 1) transiently increasing their numbers in vivo, 2) priming them to become more responsive to M-CSF and RANKL and 3) affecting their proliferation and ability to fuse and form larger osteoclasts. In turn, these
changes caused by PTH produce osteoclasts that are more active at resorbing bone in vitro. PTH also increased the number of hematopoietic progenitors in bone marrow and spleen, an effect that is independent on the activity of osteoclasts. Insights into the cellular and molecular mechanisms of the anabolic effects of PTH on osteoclastogenesis and bone might help to develop and/or optimize therapeutic strategies to modulate bone remodeling and improve bone quality. In addition, the identification of bona fide osteoclast precursors in peripheral tissues is crucial to understand the distribution and mobilization of these populations within the body after PTH treatment.
4. Figures

Figure 33. The effect of PTH on osteoclast progenitors. A) FACS plots of TN fraction (CD45R\(^-\)/CD3\(^-\)/CD11b\(^{low}\)). B) Distribution of TN fraction into six populations (PI to PVI) in the context of CD115 and CD117 cell surface markers. C) Analysis of percentage and D) absolute number of osteoclast progenitors (PIV to PVI) after 7 daily injections with PTH. E) Analysis of percentage and F) absolute number of osteoclast progenitors (PIV to PVI) after 14 daily injections with PTH. The data represents the mean ± SEM values of four replicates per mouse of two independent experiments with a total of 11 mice for vehicle group (white bars) and 10 mice for PTH group (black bars). Statistically significant difference between vehicle and PTH groups (a) p≤0.05.
Figure 34. *In vitro osteoclastogenesis*. Osteoclast progenitors were FACS sorted and plated in 96 well plates at a density of 1000 cells per well, and TRAcP assays were performed at different time points, as indicated. The upper left panel represents the TRAcP+ multinuclear OCL and the upper right panel represents the TRAcP+ mononuclear cells at day 7 after PTH treatment. The lower left panel represents the TRAcP+ multinuclear OCL and the lower right panel the TRAcP+ mononuclear cells at day 14 of PTH treatment. The data represents the mean ± SEM values of four replicates per mouse of two independent experiments with a total of 8 mice for vehicle group and 10 mice for PTH group. Statistically significant difference between vehicle and PTH groups (a) p≤0.05, (c) p≤0.001.
Figure 35. Measurement of the size of multinuclear TRAcP+ OCL in vitro. A) Microphotographs of TRAcP+ multinuclear OCL from FACS sorted osteoclast progenitors (TN/CD115+) at day 5 of culture with M-CSF and RANKL derived from vehicle- or PTH-treated mice. B) Area of individual OCL measured from 7 days (left graph) or 14 days (right graph) of treatment with PTH. The data represents the mean values of individual OCL from vehicle group (white bar, n=255) and PTH group (black bar, n=258) at day seven and vehicle group (white bar, n=244) and PTH group (black bar, n=248) at day 14. The data represents the mean ± SEM values of four replicates per mouse of two independent experiments with a total of 8 mice for vehicle group and 10 mice for PTH group. Statistically significant difference between vehicle and PTH groups (c) p ≤0.001.
Figure 36. Bone resorption assay. Osteoclast progenitors were FACS sorted and plated onto bovine cortical bone slices in 96 well plates at a density of 1000 cells per well. TRAcP assays were performed at day 12 after culturing with M-CSF and RANKL to identify osteoclast on bone then resorption pits were stained with toluidine blue. A) Representative microphotograph of bone resorption pits from vehicle treated mice (left) and PTH treated mice (right). B) Measurement of individual resorption pit area (mm²) at day 7 (left) and 14 (right) of treatment. The data represents the mean ± SEM values of triplicates of bovine cortical bone slices per group from two independent experiments with a total of 6 mice for vehicle group and 6 mice for PTH group. Statistically significant difference between vehicle and PTH groups (a) p ≤ 0.05, (b) p ≤ 0.01.
Figure 37. PTH regulates hematopoietic stem cells in bone marrow and spleen. A) Representative dot plots of LKS (Lin\(^{-}\)CD117\(^{-}\)Sca-1\(^{+}\)) cells in bone marrow (upper panel) and spleen (lower panel) from vehicle- (left) and PTH-treated mice (right). All plots were gated on negative populations for lineage markers. B) Representative bar graphs of the percentage (left) and absolute numbers (right) of LKS, LT-HSC and CLP in the bone marrow at day 7 (upper graphs) and C) day 14 (lower graphs) with PTH treatment. D) Representative bar graphs of percentage (left) and absolute number of LKS in spleen at day 7 and E) day 14 with PTH treatment. The data represents the mean ± SEM values from four independent experiments with a total of 20 mice for vehicle group and 20 mice for PTH group. Statistically significant difference between vehicle and PTH groups (a) p ≤0.05, (b) p ≤0.01.
Figure 38. Experimental design used to test the effects of osteoclast activity on hematopoietic precursor activity.

**Experiment Design**

PTH treatment
(1 injection daily, 14 injections total, Day 7 to Day 20)

(1st PTH injection)

Day 0

Day 7

Day 14

Day 20

Day 21
(Take Down)

(1 injection at Day 0)

(1 injection weekly; 2 injections total, at Day 7 and at Day 14)

**Mouse Groups:**

1. Vehicle, n=4
2. PTH (14 days), n=4
3. Alendronate, n=4
4. PTH (14 days) + Alendronate, n=5

**Day of Injections:**

- Day 0
- Day 7
- Day 14
- Day 20
- Day 7 to Day 20 (PTH, daily)

**Treatments:**

- PTH (Bachem) = 80 μg/ Kg
- Alendronate (Sigma # A4978) = 100μg/Kg
- PTH Vehicle = PBS, 0.1% BSA, 1 mM HCL
- Alendronate Vehicle = PBS, 0.1% BSA

Vehicle group mice were pre-treated with Alendronate vehicle and then treated with PTH vehicle.
Figure 39. Osteoclast activity is not responsible for the effects of PTH treatment on early hematopoietic progenitors. Bar graphs (top) showing absolute numbers of cells in the spleen of mice treated as indicated. Table (bottom) shows CTX values for each individual experimental group. The data represents the mean ± SEM values of one experiment with a total of 5 mice per group. Statistical significant difference between vehicle and PTH groups was found, (*) $p \leq 0.05$. 

### LKS in spleen

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* $p < 0.05$

### CTX values

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* $p < 0.05$
CHAPTER VII

General Discussion

Osteoclasts are unique large multinuclear and highly specialized bone resorbing cells of hematopoietic origin (Walker, 1973). Osteoclasts derive from bone marrow myeloid/monocytic precursors that require signals mediated by c-fms and RANK for their proliferation, survival and differentiation. They are generated through a progression that involves the fusion of mononuclear precursor cells into mature multinuclear osteoclasts. The process of bone resorption is the hallmark of osteoclast activity. Therefore, they are critical elements in skeletal development, bone remodeling, fracture healing and pathological bone resorption. For these reasons, the identification of specific populations of osteoclast precursors is fundamental to understand their biology and their mechanisms of action in skeletal function and disease.

We have previously identified osteoclast precursors populations in the bone marrow (Jacquin et al., 2006) and performed experiments at the single cell level to demonstrate their clonogenic potential and bone resorptive capacity (Jacome-Galarza et al., 2013). A previous report in the literature indicated that osteoclast precursors differentiated to dendritic cells in addition to osteoclasts (Miyamoto et al., 2001). Another report showed that osteoclasts and macrophages derived from a similar precursor cell (Arai et al., 1998). However, the lineage relationship among these precursors has not been established. Further analysis of their phenotype showed that these precursors share common characteristics with monocytes (precursors of macrophages and dendritic cells), which prompted us to investigate whether our bone marrow
osteoclast precursor differentiated to all these three lineages. Our experiments in Chapter III demonstrate that defined populations of osteoclast precursors are common to osteoclasts, macrophages and dendritic cells (Jacome-Galarza et al., 2013).

Because osteoclast precursors have been found in peripheral tissues (Fujikawa et al., 1996) and monocytes are known to differentiate in the bone marrow and migrate to periphery (Geissmann et al., 2003; Geissmann et al., 2008), we asked whether we could find myeloid precursor cells in other tissues. We found these precursors in peripheral blood and spleen that were able to differentiated into osteoclasts, macrophages and dendritic cells, suggesting a developmental relationship with the bone marrow precursor. But questions about these precursors remain to be answered. Why circulating and peripheral tissue resident cells have osteoclastogenic potential? Why are they also common precursors to macrophages and dendritic cells?

One possibility is that osteoclast precursors that develop in the bone marrow need additional maturation steps in peripheral tissues before they migrate back to the bone marrow and differentiate to osteoclasts. A fraction of these precursors remain associated to peripheral tissues to maintain the pool of tissue resident macrophages and dendritic cells. In support of this hypothesis, it has been reported that monocytes shuttle back and forth from the bone marrow to peripheral tissues via the bloodstream, and that Ly-6C<sup>hi</sup> monocytes become Ly-6C<sup>low</sup> monocytes in the bone marrow even after they have migrated to peripheral tissues (Geissmann et al., 2010; Shi and Pamer 2011). The differences in Ly-6C expression determine the different functional subsets: Ly-6C<sup>hi</sup> monocytes differentiate into inflammatory dendritic cells (TipDCs) and inflammatory macrophages (M1 type), while Ly-6C<sup>low</sup> monocytes differentiate into “alternatively activated macrophages” (M2 type) involved in tissue repair. Our defined
populations of osteoclast precursors in the bone marrow and periphery express high levels of Ly-6C but a fraction of them express low levels of Ly-6C, which also differentiate to osteoclast but less efficiently.

Another possibility is that Ly-6C$_{hi}$ OCPs and Ly-6C$_{low}$ OCPs give rise to different types of osteoclasts that differ in their activity. For example, it has been shown that osteoclasts in calvaria differ from those in long bones because they used different enzymes to degrade bone. Calvarial osteoclasts depend on the expression of cathepsin B, cathepsin K and MMPs for bone resorption, while osteoclasts from long bones required the expression of cathepsin B and cathepsin K to degrade bone, and do not depend on MMPs for their activity (Everts et al., 1999). However, it is currently unknown whether osteoclasts from different anatomical locations derived from the same precursor or from different precursors. If this is true, then it is possible that Ly-6C expression determine the type of osteoclasts that will be generated. Further experiments are required to confirm these hypotheses and will focus to test whether Ly-6C$_{hi}$ precursors directly differentiate to osteoclasts or become Ly-6C$_{low}$ precursors before they terminally differentiate, and also whether they differentiate to osteoclasts regardless of Ly-6C expression and give rise to different osteoclast subsets.

These hypotheses also bring more questions about the origin and functions of osteoclasts. Would the calvaria osteoclast efficiently resorb bone when in the context of long bone tissue microenvironments? Are osteoclasts the same regardless of their localization? Or, do they just simply differentially express resorptive factors depending on the bone surface they associate with? Are the osteoclasts that participate in fracture repair and arthritis different from those involved in bone remodeling?
Our experiments in chapter IV focused on the migration of osteoclast precursors to study the lineage relationship among bone marrow and peripheral precursors. We performed competitive reconstitution experiments in mice and transferred bone marrow or peripheral osteoclast precursors to lethally irradiated mice. Bone marrow derived precursors engrafted in the bone marrow, peripheral blood and spleen. Also, transferred spleen precursors engrafted in these same tissues. These experiments showed that bone marrow precursors repopulated peripheral tissues and that the spleen precursors had the ability to migrate to the bone marrow, suggesting a developmental relationship among bone marrow and peripheral osteoclast precursors. Then we studied whether these precursors also differentiated to osteoclast in vivo.

We first developed a new fluorescent transgenic mouse model to identify mature multinuclear osteoclasts in vivo. We generated these mice by crossing CathepsinK-Cre mice with ROSA-td-tomato fluorescent protein reporter mice. The offspring are mice that express RFP in cells that express cathepsin K (a characteristic of osteoclasts). This reporter mouse line served as a source of osteoclast precursor cells for transfer to non-fluorescent mice to track fluorescent osteoclast in vivo. Here, we showed that transferred bone marrow or peripheral osteoclast precursors derived from CatK-Cre-TdTomatoFP mice migrated to the bone marrow and differentiated to mature resorbing osteoclasts, as assessed by histological preparations of frozen femurs showing that osteoclasts expressed cathepsin K (RFP), were multinuclear (DAPI) and were found associated to bone tissue (TRAcP). These experiments showed that bone marrow and spleen precursors have the ability to differentiate to osteoclast in vivo, and suggest that bone marrow precursors give rise to spleen precursors that under certain conditions migrate back to the bone marrow to become osteoclasts. These observations support at least two hypotheses: 1) osteoclast precursors require additional maturation steps in peripheral tissues before terminal
differentiation in bone or 2) only when required, peripheral precursors migrate to bone and become osteoclasts influenced by the bone microenvironment.

Therefore, we studied osteoclast migration and development in the context of conditions that modulate their activity. Because osteoclasts are important in fracture healing, a process in which bone remodeling is active and critical for bone health, our studies in Chapter IV showed that we could used models of fracture repair in combination with our newly developed fluorescent transgenic mouse model to study the ability of osteoclast precursor cells to migrate to the fracture site and participate in bone remodeling in vivo. Histological preparations of fractured femurs showed engraftment of donor cells at the fractured site and the presence of RFP osteoclasts on bone surfaces in active sites of bone remodeling within the fracture callus.

Further experiments are necessary to understand the role of osteoclast precursors in homeostasis, and the signals required for their migration, localization, activity and the conditions necessary for them to preferentially differentiate into one type of cell or the other. For these reasons we studied osteoclast precursor migration, development and function during inflammation.

Signals derived from the inflammatory processes induce myeloid precursor cells to migrate and differentiate into inflammatory dendritic cells and macrophages. Similarly, osteoclast precursors are influenced by inflammatory conditions, as it occurs in infections and rheumatoid arthritis. However, less is known about the pathological mechanisms of disease that modulate osteoclast activity.

Pathogen-associated molecular patterns (PAMPs) found in microorganisms have been studied for years and are known to interact and activate receptors expressed on the cell surface of hematopoietic cells and trigger an immune response in the host that ultimately result in the
elimination of infectious agents. In chapter V we showed that bone marrow and peripheral OCPs expressed TLR4 and CD14 on their cell surface, suggesting that these precursors might respond to pathogenic insult. These results prompted us to study the effect of LPS on the dynamics, distribution and osteoclastogenic potential of osteoclast precursor populations. We performed experiments in mice and challenged them with LPS. In the bone marrow, LPS administration induced an increase in the percentage and number of osteoclast precursors without altering their osteoclastogenic potential. In the spleen similar results were observed, however, the osteoclastogenic potential of these cells were dramatically increased, suggesting that LPS primed OCPs were more responsive to M-CSF and RANKL in vitro. These data suggest that the inflammatory process during bacterial infections can modulate osteoclastogenesis and consequently bone remodeling.

However, the fact that LPS did not influence the osteoclastogenic potential of bone marrow precursors could be explained by differences in the response to acute versus chronic disease. It is possible that in order to increase bone resorption and bone loss constant exposure to inflammation is required. For example, periodontal disease results from persistent microbial infections of the periodontium (the tissue that surrounds and supports teeth) leading to progressive bone loss. LPS is known to be involved in chronic periodontitis and in osteoclast activation that regulates alveolar bone destruction in teeth (Nagasawa et al., 2000). In our model of LPS challenge it is possible that continuous administration of LPS is necessary to increase the osteoclastogenic potential of bone marrow precursors. Alternatively, is possible that LPS activates peripheral osteoclast precursors to proliferate and migrate to bone tissue (this could explain the increase in number of bone marrow precursors) but chronic exposure to LPS (perhaps in bone) is required to amplify the response and increase the osteoclastogenic potential of bone.
marrow precursors. Further dose-dependent and time-dependent experiments will help us to understand the *in vivo* potential of bone marrow and peripheral precursors that contribute to bone loss during infections.

Another model of inflammation in which osteoclast activity is increased and contributes to the chronicity of the disease is rheumatoid arthritis. Transgenic mice that overexpress human TNF spontaneously develop inflammatory arthritis and have been widely used to study pathogenic mechanisms of arthritis. In these mice, peripheral blood- and spleen-derived osteoclast precursors increased in percentage and number when compared to non-arthritic mice. Interestingly, no changes on these populations were observed in the bone marrow. These observations suggest that the chronic inflammatory process modulates peripheral precursor frequencies and perhaps these precursors are the pool of inflammatory cells that migrate to peripheral joints and contribute to bone loss when differentiated to osteoclasts.

In order to test this hypothesis we took advantage of the cathepsin K fluorescent transgenic mouse model and study the migration and differentiation of osteoclasts in the context of inflammatory arthritis. We isolated total bone marrow cells derived from CathepsinK-Cre-TdTomato mice and transferred them into hTNF transgenic mice. These experiments showed engraftment of osteoclasts in femurs of recipient mice identified by red fluorescence and associated to subchondral bone, in proximity to articular cartilage. We isolated the spleens of recipient hTNF mice and cultured cells in the presence of M-CSF and RANKL to generate osteoclasts *in vitro* and found that these cultures contained a fraction of the transferred bone marrow cells (derived from CathepsinK-Cre-TdTomato mice) that generated RFP+ osteoclasts. These experiments provided a new reporter transgenic mouse model to study osteoclast differentiation in disease models of arthritis and suggest that inflammatory signals in arthritis are
sufficient to induce activation, migration and differentiation of osteoclast precursors in arthritic joints and migration to other peripheral tissues.

Although we did not provide mechanisms to explain these observations, we hypothesize that osteoclast precursors contribute to arthritis by two possibly mechanisms: 1) activation of local bone marrow precursors (by the inflammatory milieu) that induce their migration to the joints and differentiation to osteoclasts and 2) circulating precursors are activated in peripheral tissues and recruited to arthritic joints by signals derived from the inflammatory process occurring at sites of joint inflammation. These mechanisms might also be occurring simultaneously.

Understanding the mechanisms by which bacterial infections components and inflammatory signals during disease modulate osteoclast activity and enhance bone resorption could provide help in the development of alternative therapeutic strategies to prevent bone loss.

We hypothesize that the chemokine receptor CCR2 is involved in the migration of osteoclast precursors from the bone marrow to peripheral tissues and is particularly important in inflammation. This receptor is known to be required for monocytes to migrate to sites of infection and inflammation (Serbina et al., 2008; Geissmann et al., 2008; Sponaas et al., 2009; Shi and Pamer 2011). We are currently generating transgenic mice that lack the CCR2 chemokine receptor and that simultaneously express red fluorescent protein in cells expressing cathepsin K, which will allow us to track in vivo fluorescent osteoclast that derived from precursors that lack CCR2 receptors.

Finally, because parathyroid hormone (PTH) is known to regulate bone resorption, our studies in chapter VI focused on the effect of PTH on osteoclast precursor populations. PTH is secreted by the parathyroid glands in response to low levels of calcium. PTH acts on
stromal/osteoblastic cells in bone and indirectly activates bone resorption that results in the degradation of bone tissue and the release of calcium. This process regulates calcium metabolism. But how does PTH regulate osteoclast activity? Although these mechanisms are not completely understood, our experiments provided evidence of possible mechanisms. First, 7 days of PTH administration to mice increased the percentages of OCPs in the bone marrow and increased their osteoclastogenic potential in vitro. In contrast, these effects were not seen at 14 days of PTH treatment. However, at both time points (7 and 14 days) in vitro cultures of OCPs (stimulated with M-CSF and RANKL) demonstrated that the size of multinuclear osteoclasts and their resorptive capacity increased. Since it is known that PTH increases the expression of both M-CSF and RANKL on stromal/osteoblastic cells, a possible mechanism is that OCPs responded to the increased levels of these cytokines that resulted in the amplification of signaling pathways involved in the proliferation and survival of osteoclast precursors and their differentiation into larger and more resorptive osteoclasts. This effect seemed to persist even when the proliferation of osteoclast precursors was not seen at 14 days. Perhaps the stronger signaling response occurred due to an increased in the production of adaptor molecules (TRAF6, ITAM adaptors) and transcription factors (NK-kB, NFATc1, CREB) that more actively expressed osteoclastogenic genes. Indeed, it has been shown that overexpression of TRAF6 adaptor molecule lead to a more pronounced osteoclastogenesis (Kadono et al., 2005). This might explain the sustained changes observed in their resorptive capacity.

PTH is also known to increase the number of osteoblast and inhibit OPG expression. Therefore, it is possible that osteoclast precursors proliferated more due to the decreased in inhibitory signals in addition to the increase in osteoclastogenic cytokines. Also, it is possible that their rate of fusion also increased, which resulted in the generation of larger osteoclasts.
(which requires more fusion events per osteoclasts) and this might explain that at 14 days the percentages of precursor cells did not change, however osteoclasts were larger and more active.

Figure 40 shows our working model on osteoclast development, migration and differentiation in homeostasis and inflammation: 1) a defined population of osteoclast precursors in the bone marrow give rise to osteoclast, macrophages and dendritic cells and 2) were able to development into functional resorbing, phagocytic and antigen presenting cells \textit{in vitro}. 3) Fluorescent mouse models allowed us to study osteoclast precursors differentiation in bone tissue and their migration to other hematopoietic tissues. This led us to the identification of peripheral precursors with osteoclastogenic potential that also differentiated to macrophages and dendritic cells. Additional fluorescent mouse models (CD11c mCherry and LysM-Cre-tdTomato) should help us to establish the subsets of macrophages and dendritic cells that osteoclast precursors differentiate to and their localization in peripheral tissues. 4) Inflammation in arthritis influenced the dynamics of peripheral precursors and induced their migration to arthritic joints and their differentiation into osteoclasts. 5) LPS also modulated peripheral precursors and increased their osteoclastogenic potential. 6) As our model of sterile inflammation we used fracture healing in mice to study osteoclast migration and differentiation. The mechanisms involved in activation (cytokines), migration (chemokine receptors) and differentiation in both inflammation and pathologies are the focus of future investigation, and all the described models should be useful. We finally showed that 7) parathyroid hormone regulated bone marrow precursor by increasing their proliferation and inducing phenotypic and functional changes in osteoclasts. These showed that osteoclastogenesis is modulated in a differential fashion when driven by inflammatory versus hormonal signals.
In conclusion, we reported for the first time a common myeloid precursor in the bone marrow, spleen and peripheral blood that differentiates to osteoclast, macrophages and dendritic cells. We developed a new fluorescent transgenic mouse model to study osteoclast migration and differentiation that helped us to establish developmental relationships among osteoclast precursors and study osteoclasts in the context of fracture healing and inflammatory conditions. We also investigated the osteoclastogenic potential and the dynamics of osteoclast precursor populations during LPS challenge, chronic inflammation in rheumatoid arthritis and parathyroid hormone regulation and found that osteoclast activity is preferentially modulated by different inflammatory signals. These studies are important to identify equivalent populations in humans, study their correlation with pathologies associated with abnormal immune function and bone resorption, and understand mechanisms of disease in order to modulate their activity and develop therapeutic strategies to prevent bone loss associated to inflammation.
Figure 40. Working model of osteoclast development and signals that affect osteoclast activity. Signals derived from parathyroid hormone regulate osteoclast activity in bone. Inflammatory signals derived from LPS preferentially target peripheral precursors. In inflammatory arthritis influences osteoclast from either bone marrow or periphery and induce bone erosion. Fracture repair exclusively occur ion bone however both bone marrow and peripheral precursors might participate in the healing process.
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