The Impact of Myeloid Lineage Cells on Skeletal Homeostasis and Repair

by

Megan N. Michalski

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Doctoral Committee:

Professor Laurie K. McCauley, Chair
Assistant Professor Rogerio Castilho
Professor Jeffrey L. Curtis
Professor Renny T. Franceschi

Megan N. Michalski

mmichals@umich.edu

ORCID iD: 0000-0003-2253-3919

DEDICATION

To my supportive family and friends: my parents Michael and Michelle Michalski, my sister Merry Michalski, my fiancé Bryan Wazbinski, my best friend Nithya Nagaraj-Baker and my furry companion Walter.

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ABBREVIATIONS

BMP, bone morphogenetic protein; c-Fms, colony-stimulating factor-1 receptor; CLOD, clodronate-loaded liposome treated; CSF-1, colony-stimulating factor-1; GM-CSF, granulocyte-macrophage colony-stimulating factor; HSCs, hematopoietic stem cells; IL, interleukin; iPTH, intermittent parathyroid hormone treatment; LPS, lipopolysaccharides; MAFIA, macrophage Fas-Induced apoptosis; M-CSF, macrophage colony stimulating factor, MFG-E8, milk fat globule-epidermal growth factor 8; MSC, mesenchymal stem cell; OSM, oncostatin M; ONJ, osteonecrosis of the jaw; PS, phosphatidylserine; RANKL, receptor activator of nuclear factor kappa-B ligand; TGF-β, transforming growth factor beta, TNF-α, tumor necrosis factor alpha; TRAP, tartrate resistant acid phosphatase

ABSTRACT

Bone is in a constant state of remodeling, a process which was once attributed solely to osteoblasts and osteoclasts. Decades of research has identified many other populations of cells in the bone that participate and mediate skeletal homeostasis. Recently, osteal macrophages emerged as vital participants in skeletal remodeling and osseous repair. The exact mechanistic roles of these tissue-resident macrophages are currently unknown. Macrophages are highly plastic in response to their micro-environment and are typically classified as being pro- or anti-inflammatory (pro-resolving) in nature. Given that inflammatory states result in decreased bone mass, proinflammatory macrophages may be negative regulators of bone turnover. Pro-resolving macrophages have been shown to release anabolic factors and present a potential target for therapeutic intervention in inflammation-induced bone loss and fracture healing. To better understand the role of macrophages in bone, an *in vitro* approach was used to study mechanisms of bone marrow macrophages. Additionally, macrophage functions *in vivo* were assessed in normal bone turnover and in oral osseous wound healing.

The process of apoptotic cell clearance, termed efferocytosis, is mediated by pro-resolving macrophages and may contribute to steady-state bone turnover as well as fracture healing. This process of efferocytosis by bone marrow macrophages was investigated *in vitro*. Interleukin-10, a pro-resolving cytokine, enhanced bone marrow macrophage efferocytosis of apoptotic bone marrow stromal cells (apBMSCs) in a phospho-STAT3 dependent manner. Additionally, macrophages engulfing apBMSCs secreted a unique profile of cytokines compared to macrophages engulfing apoptotic neutrophils. Macrophages displayed increased secretion of

monocyte chemotactic protein 1/chemokine (C-C motif) ligand 2 (MCP-1/CCL2) and transforming growth factor beta 1 (TGF-β1) in response to apBMSCs. This secreted protein profile suggests efferocytosis of apoptotic bone cells signals to recruit new osteoblast progenitors to repopulate apoptotic cells and initiate a new round of bone formation.

Milk fat globule-EGF factor 8 (MFG-E8) is a secreted glycoprotein that facilitates the process of efferocytosis by acting as a bridge between apoptotic cells and phagocytes. The role of MFG-E8 in bone turnover was investigated. Mice deficient in MFG-E8 displayed decreased bone with age which correlated with an increased inflammatory phenotype and accumulations of apoptotic cells in the spleen. Increased inflammation leads to increased osteoclast differentiation and activation, resulting in decreased bone mass. MFG-E8 deficient mice displayed increased osteoclast numbers per bone surface. Intermittent daily parathyroid hormone (iPTH) administration is a known anabolic bone agent yet its use in patients is currently limited to cases of severe osteoporosis. MFG-E8 deficient mice responded to iPTH administration with a greater anabolic response than WT control mice. This suggests iPTH treatment may be beneficial in patients with inflammation-induced bone loss.

Ineffective oral wound healing is detrimental to patients' oral health related quality of life. Delineating the cellular mechanisms involved in optimal healing will elicit better approaches to treating patients with compromised healing. To study the role of phagocytic myeloid cells on oral wound healing, phagocytes were depleted with clodronate-loaded liposomes at the time of tooth extraction in mice. Depletion of macrophages and osteoclasts via clodronate treatment increased extraction socket bone fill 14 days after surgery, suggesting a crucial balance of macrophages and osteoclasts is necessary to promote effective healing.

A better understanding of the exact mechanisms by which macrophages mediate bone homeostasis and healing will lead to an expansion of pharmacologic targets for the treatment of osteoporosis and inflammation-induced bone loss.

CHAPTER 1

INTRODUCTION

Myeloid cells in bone

The skeleton provides structural support, is a reservoir of calcium, and supports the hematopoietic system. Given the importance of these functions, skeletal homeostasis is crucial to maintaining systemic homeostasis. The bone and bone marrow make up a highly dynamic organ, which is constantly in a state of turnover. Bone modeling and remodeling are dependent on traditional bone cells (osteoclasts, osteoblasts and osteocytes) as well as the hematopoietic cells of the bone marrow compartment which includes myeloid and lymphoid cells [1-3]. Cells of the myeloid lineage are important regulators of skeletal homeostasis and are the topic of this dissertation.

The common myeloid progenitor cells give rise to megakaryocytes, erythrocytes, mast cells and myeloblasts. Myeloblasts then become basophils, neutrophils, eosinophils, or monocytes. Cells originating from monocytes include tissue resident macrophages, macrophages of the immune response and dendritic cells. Macrophages, Greek for “big eaters,” are phagocytic cells with pro- and anti-inflammatory functions. They are recruited to sites of infections and inflammation and aid in the process of tissue repair. The bone and bone marrow consist of various macrophages occupying specific niches including: hematopoietic stem cell niche macrophages, erythroid island macrophages, osteal macrophages, and osteoclasts. The exact

mechanisms by which myeloid cells regulate bone turnover are elusive and are currently being investigated.

The work presented in this dissertation focuses on myeloid cell contributions to bone homeostasis and repair. Chapter 2 presents an in depth literature review on the role of macrophages in skeletal health with a focus on therapeutic options available and how they alter myeloid/macrophage populations in bone. In addition, Chapter 2 introduces a major function of macrophages: apoptotic cell clearance. This process has been termed efferocytosis, from the Latin root "efferre" meaning "to take to the grave" or "to bury" [4]. Efferocytosis is a highly regulated process and is crucial to maintaining tissue homeostasis. Accumulations of apoptotic cells lead to deleterious effects and are a hallmark of autoimmune diseases, such as systemic lupus erythematous. The role of macrophage efferocytosis in bone is underappreciated and is investigated in Chapters 3 and 4.

Macrophages act differently according to their environment. Tissue resident macrophages in the lung will see and respond to different stimuli than those in the liver, for example. Bone marrow macrophage efferocytosis of osteoblastic cells was investigated to determine what factors regulate efferocytosis in the bone and how the macrophages respond to those cues. It is hypothesized that due to the pro-resolving nature of the process of efferocytosis, macrophages may positively regulate bone turnover by engulfing apoptotic bone cells and releasing factors to help recruit a new set of progenitor populations. The link between macrophage efferocytosis and bone turnover is currently being investigated. Chapter 3 highlights a series of *in vitro* experiments which investigate mediators of efferocytosis as well as specific macrophage responses to apoptotic bone cell "meals." The findings of this chapter demonstrate that bone marrow derived macrophages secrete monocyte chemoattractant protein-1 (MCP-1) and

transforming growth factor beta 1 (TGF-β1) in response to apoptotic bone marrow stromal cells but not apoptotic neutrophils. MCP-1 and TGF-β1 are known monocyte and mesenchymal cell recruitment factors, respectively. Given the specificity of the secretion of these factors in response to engulfing apoptotic bone cells, these data suggest one function of macrophages in bone is to support the repopulation of the bone forming unit as cells undergo apoptosis. These findings contribute to the growing literature on the role of macrophages in bone.

As mentioned above, efferocytosis is a highly coordinated process. It requires the recognition of apoptotic cells by phagocytes, a rearrangement of cytoskeletal elements in the phagocytes to surround and engulf the apoptotic cell, and the processing of the dead cell internally [5-7]. Milk fat globule epidermal growth factor 8 (MFG-E8) is a secreted glycoprotein that binds to phosphatidylserine exposed on the outer membrane of cells undergoing cell death in addition to binding the $\alpha_v\beta_3/\alpha_v\beta_5$ integrin expressed on the macrophage [8]. MFG-E8 deficiency leads to various alterations in tissue homeostasis, healing, and inflammatory response [9-13]. **Table 1.1** details some of the phenotypes seen in mice lacking functional MFG-E8. In addition to the findings reported in the table, MFG-E8 has recently been identified as a positive regulator of bone turnover [14-16], but the mechanistic role, specifically regarding efferocytosis, has not been described. In chapter 4, the contributions of MFG-E8 to bone turnover were investigated in a mouse model of MFG-E8 deficiency. Mice with the deficiency had inflammatory-like phenotypes, increased accumulations of apoptotic cells in the spleen, and decreased bone with age. A known anabolic bone therapeutic agent (teriparatide, hPTH 1-34) was administered to MFG-E8 deficient mice to investigate the ability to rescue the phenotype. Interestingly, treatment with intermittent PTH caused an anabolic response in the MFG-E8 deficient mice that exceeded the anabolic changes seen in WT control mice. The data presented in chapter four expands our

understanding of factors that regulate bone turnover and gives further insight on the potential use of anabolic bone agents in inflammation-induced bone loss.

In addition to investigating macrophages in homeostatic bone turnover, the role of bone macrophages in healing was investigated. There are various tools to deplete different cell types and assess the resulting phenotype. Chapter 5 focuses on the results of depleting phagocytic cells using liposomes loaded with clodronate. When engulfed, liposomes containing clodronate are metabolized to a toxic byproduct which leads to death of the phagocyte. The effect of depleting phagocytic cells via clodronate-loaded liposomes on normal bone turnover was investigated. Additionally, the role of these phagocytes in facilitating oral osseous wound healing was investigated using a tooth extraction protocol. Understanding the contributions of specific cells to the healing process will aid in the design of targeted therapeutics for oral osseous wound healing.

The work presented in this dissertation highlights various models to study myeloid cell contributions to bone homeostasis and healing and provides a better understanding of the functional roles of these cells in bone.

Table 1.1. Reported phenotypes in MFG-E8 deficient mice.

Ref #	Year	Journal	Authors	Title	Tissue	Outcome	Phenotypes
8	2002	Nature	Hanayama et al.	Identification of a Factor that Links Apoptotic Cells to Phagocytes	Peritoneal	Efferocytosis	Treatment with MFG-E8 increased uptake of apThymocytes (ICAD-Sdm mice, dexamethasone induced)
							MFG-E8 secreted from activated macrophages binds to apoptotic cells and brings them to phagocytes for engulfment
9	2004	Science	Hanayama et al.	Autoimmune Disease and Impaired Uptake of Apoptotic Cells in MFG-E8–Deficient Mice	Spleen	Cell expression	MFG-E8 expressed in CD68+ cells, not F4/80+. In the spleen, these are the tingible body macrophages
					Lymph node	Cell expression	CD68+ cells in the lymph node expressed MFG-E8
					Peritoneal	Efferocytosis	Thioglycollate-elicited macrophages cultured with CAF-null apThymocytes for 1.5hrs. -/- macrophage phagocytic index was lower
							Efferocytosis of wild-type apThymocytes was also
							Phagocytosis of fluorescent microspheres was
					Spleen	Size	Splenomegaly in -/- mice
						Histology	enlarged white pulps, carried numerous germinal centers in -/-
						FACs	2-3 more lymphocyes in -/- spleen, but T:B ratio unchanged
					Kidney	Size	Glomerulonephritis in -/-
10	2005	Molecular Biology of the Cell	Atabai et al.	Mfge8 Is Critical for Mammary Gland Remodeling during Involution	Mammary Gland	Apo cell #	Mfge8 Mutants Have Increased Numbers of Apoptotic Cells in Involution
					Mammary Gland	Adipocytes	Mfge8 mutant mice have a delay in alveolar collapse and adipocyte repopulation during early involution
						Phagocytosis	Mfge8 mutants have impaired mammary gland epithelial cell phagocytosis in vivo and in vitro
						Clearance	Epithelial Cells from Mfge8 Mutant Mice Have Impaired Apoptotic Cell Clearance In Vitro

Table 1.1. continued

Ref #	Year	Journal	Authors	Title	Tissue	Outcome	Phenotypes
12	2007	PNAS	Nandrot et al.	Essential role for MFG-E8 as ligand for avb5 integrin in diurnal retinal phagocytosis	Retinal Pigment Epithelium (RPE)	Uptake	MFG-E8 +/+ RPE contained more phagosomes than -/-
					RPE	Binding	Phenotypically normal but have impaired photoreceptor outer segment fragment binding
					RPE	Binding and Uptake	Addition of 0.25uM MFG-E8 increased totel POS uptake (binding plus engulfment)
					FBS	Culture conditions	It is well established that FBS promotes engulfment of surface-bound POS by RPE in culture by activating MerTK (13, 14). Hence, lack of FBS practically eliminated POS internalization by all types of RPE tested (Fig. 4F). Taken together, these results indicate that MFG / RPE cells in culture have reduced binding activity toward isolated POS in the absence of
17	2009	Journal of Clinical Investigation	Atabai et al.	Mfge8 dimishes the severity of tissue fibrosis in mice by binding and targeting collagen for uptake by macrophages	Lung	Protein expression	Mfge8 is expressed throughout the lung and expression is increased after injury
					Lung	Efferocytosis	In vivo bolus of apo thymocytes administered, no difference in phagocytic index of -/- mice
					Lung	Fibrosis	Mfge8 deficiency results in exaggerated fibrotic response to bleomycin
					Lung	Collagen uptake	Mfge8 mediates collagen uptake in vitro (-/- alveolar macrophages had decreased uptake, rMfge8 tx increased uptake in +/+ and -/- alveolar macs)
					Lung	Collagen uptake	Mfge8 mediates collagen uptake in vivo (intratracheal injection - measure uptake, -/- had decreased collagen uptake)
11	2012	PNAS	Kudo et al.	Mfge8 suppresses airway hyperresponsiveness in asthma by regulating smooth muscle contraction	Lung		Mfge8-/- mice develop exaggerated airway hyperresponsiveness after ova sensitization
							Mfge8 regulates ASM calcium sensitivity by regulating RhoA induction and activity
							Mfge8 expression is decreased in airways of patients with asthma

Table 1.1. continued. References from table: [8-12, 14, 17-21]

Ref #	Year	Journal	Authors	Title	Tissue	Outcome	Phenotypes
14	2014	Journal of Immunology	Abe et al.	Regulation of Osteoclast Homeostasis and Inflammatory Bone Loss by MFG-E8	Osteoclast	Gene expression	MFG-E8 gene expression is increased in RANKL induced RAW264.7 cells
							MFG-E8 inhibits RANKL-induced differentiation of RAW264.7 OCLs
							MFG-E8 regulates OCL differentiation and function, -/- mice have had increased TRAP+ cells, increased resorptive pit area
							rMFG-E8 inhibits ligature-induced periodontal bone loss *in vivo*
18	2014	Nature Medicine	Khalifeh-Soltani et al.	Mfge8 promotes obesity by mediating the uptake of dietary fats and serum fatty acids	Adipocytes		Fatty acid uptake in -/- primary adipocytes is decreased
					Serum		Mfge8 mediates absorption of dietary fats (olive oil oral gavage!)
					Liver, Heart		Mfge8 mediates fatty acid clearance from serum and deposition in peripheral organs
							Mfge8 increases fatty acid uptake through PI3K
							Mfge8-/- mice are protected from diet-induced obesity
					Liver	Adipocytes	Increased adipocytes in -/- on normal diet in liver
					Liver	Triglycerides	Liver TG levels decreased in -/- mice on normal diet
19	2016	JCI Insight	Khalifeh-Soltani et al.	Mfge8 regulates enterocyte lipid storage by promoting enterocyte triglyceride hydrolase activity	Enterocytes	Triglyceride accumulation	MFG-E8-/- mice display increased TG accumulation after dietary fat challenge and high-fat diet
20	2016	Journal of Pediatric Surgery	Hansen et al.	Deficiency in milk fat globule-epidermal growth factor-factor 8 exacerbates organ injury and mortality in neonatal sepsis	Lungs, Intestine	Neonatal sepsis	MFG-E8-/- mice had increased inflammation, tissue injury, neutrophil infiltration in murine neonatal sepsis
21	2017	Gastroenterology	An et al.	Milk Fat Globule-EGF Factor 8, Secreted by Mesenchymal Stem Cells, Protects Against Liver Fibrosis in Mice.	HSC culture		MFG-E8 decreases HSC activitation by TGFβR1 down-regulation
					Liver	Liver fibrosis	MFG-E8 decreases fibrosis in liver

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CHAPTER 2

MACROPHAGES AND SKELETAL HEALTH

Introduction

The skeleton is a complex organ that provides structural support, protection of the body's internal organs, houses the hematopoietic system, and serves as a reservoir of calcium. Bone is in a constant state of turnover which is balanced by bone formation and resorption; processes which are controlled by the activity of bone resident cells: osteoblasts, osteocytes, and osteoclasts. The bone and bone marrow consist of several other cells that play crucial supportive roles in the process of homeostatic bone turnover including, but not limited to, T and B cells, mast cells, and monocytes/macrophages [1-5]. An imbalance in the bone remodeling process due to alterations in osteoblast or osteoclast activity can lead to elevated bone mass (increased osteoblast activity or decreased osteoclast activity) or decreased bone mass (decreased osteoblast activity or increased osteoclast activity).

Approximately 44 million Americans have low bone mass, placing them at a high risk of developing osteoporosis, a disease affecting over 10 million Americans [6]. Loss of bone mass leads to increased fracture risk, which has a high rate of morbidity and mortality in the elderly population [7]. Osteoporosis-related fractures often lead to hospitalizations and nursing home placement, decreasing the patient's quality of life and posing a large burden on healthcare systems. Bone loss leading to osteoporosis has been widely studied in the context of menopause and estrogen deficiency and is associated with an increase in osteoclast activity relative to

osteoblast activity [8]. Estrogen does not likely mediate osteoclast activity directly, however the withdrawal of estrogen results in the increased production of inflammatory cytokines [9]. These inflammatory mediators are major contributors in the increased osteoclast activity seen in menopause-associated bone loss. An early study by Horton et. al demonstrated that osteoclasts which were exposed to activated leukocyte cell supernatant increased osteoclast number and activity [10]. This finding led to a large body of research investigating various proinflammatory factors and their role in osteoclast stimulation. Some of these proinflammatory cytokines which have been shown to increase osteoclastic differentiation and activity include tumor necrosis factor alpha (TNF-α) [11-15], prostaglandins [16], interleukin-1 (IL-1) [11, 12, 17-20], IL-6 [21-23], IL-11[24, 25], IL-15 [26], and IL-17 [27]. The increase in osteoclast differentiation is in large part due to an increase in receptor activator of nuclear factor kappa-B ligand (RANKL) production in target cells. RANKL binds to RANK on pre-osteoclasts and induces differentiation. However, it has been shown that TNF-α can induce osteoclast differentiation when RANKL levels are lower than necessary for osteoclastogenesis [28].

Increased proinflammatory cytokine production is not only seen in during estrogen withdrawal but is also associated with various inflammatory diseases. Systemic and/or local bone loss is often seen in patients with inflammatory diseases [29, 30] including systemic lupus erythematous [31], rheumatoid arthritis [32-34], cystic fibrosis [35], chronic obstructive pulmonary disease [36], inflammatory bowel disease (IBD) [37, 38], and periodontal disease [39]. The inflammatory process is a complex response which is mediated by various cells of the innate and adaptive immune systems. The direct effect of inflammatory cytokines on osteoclast activity has been well studied, and the cells mediating these effects are becoming more appreciated for their roles in bone homeostasis.

The focus of this review is the role of immune cells, specifically monocyte/macrophages, on the maintenance of bone and their contributions to bone disease. Additionally, the therapeutic potential of targeting osteal macrophages in bone-related diseases will be highlighted.

Translating traditional macrophage actions to their roles in bone

Macrophages, Greek for "big eaters," were first described by Elie Metchnikoff over 100 years ago and are traditionally known for their phagocytic roles in inflammation and immunity [40]. They are a heterogeneous population of cells with multiple phenotypes whose function is based on surrounding environmental cues. These macrophage phenotypes, commonly referred to as polarizations, were once considered to be distinct populations which could be divided into M1 (classically activated) or M2 (alternatively activated) subsets [41, 42]. A large body of research has focused on defining these populations of cells, and it has become clear that macrophage polarization cannot simply be divided into two unique populations, but rather consists of a spectrum of phenotypes [43-45]. A collaboration between multiple groups has worked together to create a set of standards which outline the sources of macrophages, activators of macrophages, and defines various markers of macrophage activation [46]. Due to the heterogeneity of macrophages, the original definitions of these cells are no longer categorized as M1 or M2. Not only have subcategories been identified such as M2a, M2b, and M2c [47], but the original two designations are currently referred to as "M1-like" and "M2-like," due to the overlap of the expression of markers.

M1-like or classically-activated macrophages are defined for their role in mediating an inflammatory response. They polarize toward the M1 phenotype in response to inflammatory cytokines released from Th1 cells, such as IL-1 and IL-6. M2-like macrophages are present

during the resolution phase of inflammation and are responsible for anti-inflammatory cytokine production and enhanced clearance of apoptotic cells, termed efferocytosis [48-50]. Exposure to anti-inflammatory cytokines IL-4, IL-12 and IL-10 leads to M2-like macrophage polarization [51], and increased apoptotic cell clearance [52-54].

Tissue-resident macrophages are found in nearly all tissues other than hyaline cartilage, and play additional roles other than immunity and inflammation, including supporting tissue homeostasis, clearance of debris and tissue repair [55-58]. The differentiation and maintenance of tissue-resident macrophages is unique compared to that of adult hematopoietic cell renewal via hematopoietic stem cells (HSCs). It has recently been shown that many adult tissue-resident macrophages differentiate from a Tie2+ cellular pathway which leads to yolk sac-derived myeloid progenitors that develop prior to the appearance of HSCs [59, 60]. This distinction is important because the unique origin and differentiation of tissue macrophages has significant relevance to disease.

Tissue-resident macrophages perform specific functions based on the tissue in which they reside. For example, the lung consists of alveolar macrophages which survey for inhaled pathogens and regulate homeostasis of the tissue through surfactant clearance [61-63]. Kupffer cells in the liver participate in the clearance of aged erythrocytes [64]. **Figure 2.1A** depicts several of the tissue-resident macrophages. Of interest to this review, the bone and bone marrow microenvironment maintains several tissue-resident macrophage populations, each with distinct locations and functions.

The bone and bone marrow consist of three known distinct macrophage populations: bone marrow macrophages (erythroid island macrophages and hematopoietic stem cell macrophages), osteoclasts, and a recently defined population of macrophages termed osteal

macrophages or “osteomacs” [3] (**Figure 2.1B**). Historically, bone marrow macrophages have been studied in the context of erythropoiesis [65, 66] and hematopoietic stem cell niche maintenance [67, 68]. Osteoclasts have long been identified as the tissue-resident macrophages in bone, although they now share this designation with the other bone macrophage populations. Osteoclasts differentiate down the monocyte lineage, and fuse to become multinucleated tartrate-resistant acid phosphatase (TRAP)-positive cells. Osteal macrophages are a distinct subset of bone macrophages located in close proximity to the bone surface and are F4/80-positive (**Figure 2.2**) [69] and TRAP-negative [3]. These osteal macrophages were characterized and found frequently located next to active bone forming osteoblasts. The majority of osteoblasts on the inner surface of cortical bone are covered in F4/80+, CD68+, Mac-3+, TRAP– macrophages [70]. **Table 2.1** details the known markers and functions of the four macrophages present in bone. The specific myeloid progenitors and functional roles of the osteal macrophage are currently under investigation. The proximity to the bone forming unit suggests that they communicate with bone resident cells and play a supportive role in the bone remodeling process.

To investigate the functional role of osteal macrophages, several *in vitro* assays have identified a supportive role for macrophages in mediating bone formation. An early study by Champagne et al. [71] demonstrated that human and murine macrophages produce bone morphogenetic proteins (BMPs), specifically BMP-2 and BMP-6. Mesenchymal stem cells (MSCs) (osteoblast progenitor cells) grown in conditioned media from J774A.1 macrophage cells displayed increased osteoblast differentiation gene expression, and treatment of macrophages with anti-BMP-2 prevented the pro-osteogenic effect [71]. Primary cell cultures which are used to assess osteoblastogenesis and mineralization consist of a heterogeneous population of cells. To measure the contribution of macrophages in these cultures, primary

calvarial osteoblast cultures were sorted for macrophage markers and found to consist of 11% to 17% F4/80+ macrophages [3]. When macrophages were depleted from cultures using a magnetic sorting technique, mineralization and osteoblastic differentiation gene expression was significantly reduced [3]. Nicolaidou et al. [72] also found that monocytes/macrophages induce human MSC differentiation into osteoblasts and increase mineralization. Increased oncostatin M (OSM) production by monocytes led to upregulation of signal transducer and activator of transcription 3 (STAT3) in MSCs and enhanced differentiation. Neutralizing antibody to OSM decreased MSC differentiation into osteoblasts [72]. These findings were supported by a study showing that OSM produced by activated circulating CD14+ or bone marrow CD11b+ monocytes/macrophages induced osteoblast differentiation and matrix mineralization from human mesenchymal stem cells [73]. Treatment of MSCs with recombinant OSM also stimulated osteoblast differentiation [74].

These *in vitro* data support the hypothesis that macrophages are important in mediating osteoblastic differentiation and mineralization. *In vivo* macrophage ablation models further support these findings. The macrophage Fas-induced apoptosis (MAFIA) mouse model results in depletion of colony-stimulating factor-1 receptor (c-Fms) positive myeloid lineage cells upon administration of AP20187 [75, 76]. MAFIA mice administered AP20187 displayed markedly reduced osteoblast lining bone surfaces, decreased bone formation, and an overall reduction in bone volume [3, 77, 78]. Another method to deplete macrophages is the lysozyme-M (LysM) driven cre model. LysM is expressed in cells of the myeloid lineage [79] and when LysMcre mice were crossed with R26RDTA mice, LysM expressing cells including monocytes and macrophages were depleted [80]. Macrophage depletion using the LysMcre-DTA model led to decreased bone growth in young mice and osteoporosis in skeletally mature mice [80].

Given that osteoclasts and macrophages differentiate from the monocytic lineage, macrophage ablation experiments targeting either c-Fms or LysM should also affect osteoclasts. In the MAFIA mouse model using c-fms driven macrophage depletion, Cho et al. [78] demonstrated that the dosing regimen of AP20187 used successfully depleted macrophage populations without altering the number of osteoclasts per bone surface *in vivo.* Conversely, Vi et al. [80] demonstrated in the LysM model of macrophage depletion that osteoclasts were present in macrophage depleted mice, however they were reduced and less active. *In vitro* assays of osteoclast resorptive potential showed that osteoclasts from the LysM mice were still functional at resorbing bone. It is important, therefore, that models of macrophages depletion used to investigate bone phenotypes, should consider the potential osteoclast effects. Taken together, these studies show that macrophages are important and necessary contributors to the bone modeling and remodeling process. Their mechanistic roles are under ongoing investigation.

Macrophages are well known for their role as professional phagocytes. As mentioned previously, macrophages can be polarized based on the environment in which they reside. M1-like macrophages participate in the inflammatory response and are primed by proinflammatory cytokines. Proinflammatory cytokines enhance osteoclast differentiation and resorption by upregulation of RANKL, resulting in low bone mass phenotypes. M2-like macrophages, on the other hand, are considered resolution phase macrophages and participate in the clearance of apoptotic cells, termed efferocytosis, following an inflammatory milieu [50, 81-83]. Under normal conditions, approximately one million cells in the body become apoptotic each second, and the effective clearance of these apoptotic cells is crucial to prevent an abnormal inflammatory response or systemic autoimmunity [84-86]. The bone marrow is a complex organ that consists of millions of cells which undergo turnover daily, resulting in a large amount of

apoptotic cells to be cleared. To maintain homeostasis, these cells must be rapidly and effectively cleared. In regards to the bone forming unit cells, osteoblasts have three fates: they become bone-lining cells, osteocytes or undergo apoptosis. Of the osteoblasts initially at remodeling sites, more than 50% are thought to undergo apoptosis [87]. The role of pro-resolving macrophages and the process of efferocytosis have recently surfaced in bone, and studies investigating apoptotic cell clearance may lead to a better understanding of the functional role of osteal macrophages.

Macrophage polarization is of interest in the context of bone and may provide clues into the functional role macrophages play in skeletal homeostasis [88]. Macrophage expression of the osteoinductive factors BMP-2 and BMP-6 was reduced when macrophages were stimulated with lipopolysaccharide (LPS), a known proinflammatory M1 macrophage mediator [71]. Conditioned media from macrophages treated with LPS were unable to produce the stimulatory effect on MSC differentiation into osteoblasts [71]. This suggests that proinflammatory mediators may result in reduced bone phenotypes by altering macrophage expression of osteoinductive factors. Furthermore, conditioned media from macrophages treated with IL-4 and M-CSF (M2 activation) increased osteoblast maturation from MSCs, whereas macrophages treated with granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-gamma and LPS (M1 activation) did not [74]. These findings support alternatively activated macrophages as mediators of bone homeostasis.

The functional role of macrophages and the participation of efferocytosis in bone remodeling is further supported by macrophage ablation experiments. The depletion of myeloid lineage cells via the MAFIA mouse model resulted in decreased F4/80+ cells, CD68+ cells, and reduced bone mass [78]. In the same study, clodronate-loaded liposomes were administered to

mice to deplete mature macrophages with phagocytic capacity. This method of macrophage ablation also led to a decrease in F4/80+ cells, however, CD68+ and CD163+ cells were reciprocally increased as was bone mass. CD68+ and CD163+ cells are mature phagocytic macrophages that participate in the process of efferocytosis. It is hypothesized that these cells were increased after administration of the clodronate liposomes in a positive feedback mechanism [78]. To further support the hypothesis that the increase in CD68+ cells in the clodronate model also coincided with increased apoptotic cell clearance, TUNEL staining verified a reduction in apoptotic cells in the clodronate-treated mice compared to the MAFIA mouse model. Furthermore, clodronate-treated mice displayed increased whole bone marrow gene expression of M2-related genes and no change in M1-related genes [78]. Furthermore, the clodronate-liposome treatment resulted in increased expression of osteogenic genes including Wnt-10b and TGF-β1. The increased bone mass in the clodronate model of macrophage depletion suggests a correlation between the process of apoptotic cell clearance and bone turnover.

During programmed cell death, cells begin to expose phosphatidylserine (PS) on their outer membrane. Phagocytic cell receptors recognize PS on apoptotic cells which leads to signaling and initiates engulfment of the apoptotic cell [83, 89]. Milk fat globule-epidermal growth factor 8 (MFG-E8), also known as lactadherin, is a secreted protein which forms a bridge between PS on the apoptotic cell and the vitronectin receptor ($\alpha_v\beta_3$ integrin) on the phagocyte. MFG-E8 knockout mice displayed decreased bone mass and accelerated bone loss due to ovariectomy [90]. Cell autonomous studies revealed MFG-E8 knockout osteoblasts mice had decreased mineralization and knockout osteoclasts were more active. The phagocytic capacity of bone marrow macrophages were not assessed in this study but were shown to have decreased

phagocytosis by others [91]. These data suggest that MFG-E8 may have direct roles in osteoblast and osteoclast activity, but MFG-E8-mediated efferocytosis by macrophages may also contribute to the low bone mass phenotype seen in these mice. Further investigation into the direct effects of efferocytosis on bone turnover is important in better delineating the mechanistic roles of macrophages in mediating bone homeostasis.

Recent studies have begun to define the role macrophage-mediated clearance of apoptotic bone cells on bone turnover [54, 92]. Live cell imaging shows that bone marrow derived macrophages readily engulf apoptotic MC3T3 (osteoblastic) cells, resulting in complete clearance [92]. Similarly, bone marrow macrophages engulf apoptotic primary bone marrow stromal cells as shown using flow cytometric analysis in conjunction with ImageStream analysis (**Figure 2.3**). IL-10 was investigated as a mediator of apoptotic bone marrow stromal cell clearance [54]. Bone marrow derived macrophages primed with IL-10 displayed enhanced efferocytosis of apoptotic bone cells and mimicked an M2-like phenotype (CD206+). Following engulfment of apoptotic bone marrow stromal cells or MC3T3 cells, macrophages secreted the osteogenic molecule TGF-β1 and monocyte cell attractant chemokine (C-C motif) ligand 2 [54]. These factors were upregulated in comparison to the engulfment of apoptotic neutrophils, suggesting that efferocytosis of apoptotic bone cells leads to a distinct expression profile which may aid in the recruitment of progenitor cells to repopulate the dead/dying cell populations. The exact mechanistic role of osteal macrophages in supporting bone turnover is currently being investigated, and efferocytosis shows potential to positively regulate these processes.

Macrophages in bone disease

Tissue homeostasis requires a tightly organized system of various cell types working together to maintain balance. If the balance is disrupted, the progression of aberrant disease states may persist. While a direct link between macrophage function and bone-related diseases has not been thoroughly investigated, there are clear macrophage phenotypes which present in various disease states. As mentioned previously, proinflammatory mediators enhance osteoclastogenesis and activity in part due to upregulation of RANKL. Increased inflammatory cytokines have been associated with post-menopausal osteoporosis, yet the *in vivo* characterization of bone marrow or osteal macrophage phenotypes under these conditions is not known. It may be speculated that increased inflammatory cytokines encourages a shift in macrophage phenotypes toward M1-like. The exact role of macrophages in osteoporosis is as yet unknown.

During aging, repair processes, including fracture repair, often become less efficient [93, 94]. The cellular processes which are impaired in aging are under investigation. When bone marrow from 4 week old mice was transplanted into 12 month old mice, fracture healing was enhanced in the older mice [95]. The positive effects were attributed to the young inflammatory cells aiding in the repair processes. Additionally, parabiosis experiments showed that youthful circulating factors mediated repair processes in aged mice [96]. While the bone marrow transplants and parabiosis studies result in the exchange of a heterogeneous population of cells, macrophages may play a contributory role. In fact, in other tissues, macrophages have been shown to be less effective at tissue repair with age [97]. Aging also coincides with an increase in proinflammatory mediators, decrease in M2-like macrophages, and decrease in phagocytic capacity [98-103]

Rheumatoid arthritis displays local and systemic bone loss and is associated with joint destruction. The exact pathogenesis of rheumatoid arthritis is unknown, yet macrophage activation contributes greatly to its presentation. Macrophages have been shown to be an important source of proinflammatory cytokines such as IL-1β, IL-6, TNF-α and GM-CSF locally and systemically [104, 105]. The increased proinflammatory cytokines enhance osteoclast differentiation and activity via upregulation of RANKL, and leads to bone destruction. Macrophages and their byproducts have become candidate targets in the treatment of rheumatoid arthritis, and include anti-TNF antibodies and the inhibition of c-Fms [106]. However, it is proposed that specifically targeting proinflammatory M1-like macrophages may result in more positive results than depletion of all macrophages [107]. Additionally, mice deficient in MFG-E8, a protein that mediates apoptotic cell clearance, have exacerbated rheumatoid arthritis presentation [108]. While macrophages were not characterized in this study, the known role of MFG-E8 in M2-mediated clearance of apoptotic cells suggests that M2 macrophages may be important in preventing rheumatoid arthritis symptoms by aiding in inflammation resolution and debris clearance.

Osteonecrosis of the jaw (ONJ) presents as exposed necrotic bone in the oral cavity. ONJ is often associated with high-dose intravenous antiresorptive therapies used in patients with metastatic bone disease [109]. The exact etiology is not known for ONJ, but inflammatory macrophages have recently been shown to play a role [110]. Increased IL-17 correlated with an increase in M1 to M2 macrophage ratio in patients ONJ lesions. Adoptive transfer of M2 macrophages expanded *ex vivo* decreased ONJ severity, IL-17 production and M1 to M2 ratio in a mouse model for ONJ [110]. Neutralizing antibody to IL-17 also decreased the M1 to M2 ratio and disease prevalence in mice. These data suggest that M1 macrophages correlate with ONJ

disease presentation and severity, and targeting M1 macrophage activation by IL-17 may serve as a potential therapeutic for patient at risk for developing ONJ lesions.

Macrophages in bone repair

Macrophages function to maintain normal tissue homeostasis and play a crucial role in tissue damage repair. This is also true in osseous wound healing. Following a fracture, bone is regenerated to fill in the fracture space to restore form and function. After tissue injury, damaged tissue and apoptotic cells are abundant and must be cleared to allow for proper modeling of the site. Bone healing generally involves five stages: the inflammatory response, soft callus formation, hard callus formation, union, and bone remodeling (reviewed in [111]). The initial inflammatory response provides an influx of cells, including macrophages. Macrophages are important in the early stages of bone healing, yet their more crucial role has been shown to be during the subsequent anabolic steps in bone repair. The MAFIA mouse model was used to assess the macrophage contribution to healing via intramembranous ossification or endochondral ossification [112, 113]. In both models of repair, macrophage depletion at the time of injury resulted in substantially reduced collagen type-I deposition and bone mineralization leading to impaired bone repair. Vi et al. [80] utilized the macrophage depletion LysMcre-DTA model and similarly found that macrophage depletion significantly impaired tibial fracture healing. To better assess the role of macrophages in the later stages of fracture healing, the MAFIA mouse model was also utilized to deplete macrophages after the initial inflammatory events and prior to the anabolic phase. Interestingly, macrophage depletion during this phase also led to decreased healing [112, 113]. These data suggest that macrophages are important during various stages of bone repair and are necessary for proper healing. Additionally, this may implicate different

macrophage phenotypes during healing as the initial inflammatory macrophages may be distinct from those present during the anabolic phase. Indeed, inflammatory and osteal macrophages predominate in different locations within fracture sites, further supporting separate functional roles [112, 113]. Clearly, macrophages present a possible therapeutic target for enhancing fracture healing.

The mechanisms by which macrophages exert their positive effects on bone formation are not clearly understood. Guihard et al. [114] expanded on their *in vitro* findings that OSM produced by macrophages supports osteoblastic mineralization by investigating signaling during tibial injury healing. They found that OSM expression was increased during the inflammatory phase of healing and that macrophage depletion via the clodronate-loaded liposome model led to decreased OSM expression [114]. Furthermore, OSM and OSM receptor null mice had fewer osteoblasts and less bone formation within the injury site. These data support OSM as an important pro-anabolic molecule in the healing of bone and a potential target for fracture healing therapeutics. Macrophages can secrete other osteoinductive factors (TGF-β1, BMP-2, BMP-4, BMP-6), which have only begun to be explored in the context of macrophage-mediated osseous wound healing. Macrophages are implicated in the heterotopic ossification that occurs following tissue injury due to trauma and burns playing an important role as immune effector cells in ectopic bone formation [115]. One study demonstrated a role for macrophage derived BMP4 in a genetic mouse model where soft tissue injuries led to bone with marrow in extraskeletal tissues [116]. As macrophages are typically at the interface of the adaptive and innate immune systems and have both inflammatory and anti-inflammatory phenotypes, their ability to orchestrate osseous wound healing in various scenario requires further study.

Current osteoporosis therapies and how they affect macrophages

Antiresorptives are commonly used therapeutics to combat the bone loss associated with osteoporosis secondary to menopause or systemic inflammation as well as to treat metastatic bone diseases and hypercalcemia. Broadly, antiresorptives inhibit osteoclast activity, either through targeting key differentiation steps or preventing mechanisms which osteoclasts use to adhere to and resorb bone. The most common category of antiresorptives used clinically are the bisphosphonates.

Bisphosphonates attach to hydroxyapatite in bone and are incorporated into osseous surfaces. Depending on the structure of the bisphosphonate, as osteoclasts resorb bone that has incorporated bisphosphonates, the bisphosphonates inhibit osteoclast activity or induce apoptosis. Nitrogen-containing bisphosphonates inhibit farnesyl pyrophosphate synthase leading to impaired ability to adhere to bone and produce protons necessary for resorption [117, 118]. Simple bisphosphonates on the other hand do not contain nitrogen. These molecules are metabolized by osteoclasts to a toxic analogue of adenosine triphosphate (ATP) resulting in osteoclast apoptosis [119, 120]. The effects of bisphosphonates on osteoclasts have been widely studied, yet the effects on macrophages are just becoming apparent [121]. The phagocytic nature of macrophages makes them susceptible to uptake of bisphosphonates [122, 123]. Macrophages treated in culture with nitrogen-containing bisphosphonates versus simple bisphosphonates responded differently. Simple bisphosphonates reduced inflammatory cytokine (IL-1β, IL-6, nitric oxide) production by macrophages whereas nitrogen-containing bisphosphonates enhanced IL-1β and IL-6 production [124, 125]. These results indicate that bisphosphonates alter cytokine release profiles by macrophages and may therefore alter their interactions with bone cells. Bisphosphonates have also been shown to decrease osteoclastogenesis [126, 127]. Osteoclast

precursors are of the monocyte-macrophage lineage and may also be altered in the process of reduced osteoclastogenesis. The exact alterations in these cell populations are not appreciated, but further understanding may allow for better tailored osteoporosis therapies.

Denosumab is also an antiresorptive, yet its mechanism of action is different than bisphosphonates. Denosumab is a human monoclonal antibody that binds to RANKL which selectively inhibits osteoclastogenesis. It is FDA approved for the treatment of postmenopausal osteoporosis for women with high or increased fracture risk [128]. Due to its long half-life, administration of denosumab for the treatment of osteoporosis is once every six months, making it more manageable than daily or weekly bisphosphonates [129]. Monocytes and macrophages express RANK, the receptor for RANKL. Interestingly, studies have shown that denosumab does not alter the functions of monocytes [130, 131]. Additionally, denosumab was not shown to alter differentiation or viability of macrophages from monocytes [132]. Reports of severe infections as low incidence adverse events in cancer patients taking denosumab have not surfaced in osteoporosis patients, yet raise the potential for high doses impacting aspects of the host response [133]. While studies do not imply that denosumab changes macrophage differentiation, further exploration of macrophage responses to RANKL inhibitors seems prudent.

Odanacatib is a cathepsin K inhibitor, a key enzyme utilized by osteoclasts to break down bone. Recently, Merck discontinued its clinical trials of odanacatib due to stroke risk, however cathepsin K inhibition should not be ruled out as a target for treating reduced bone mass. Odanacatib has been shown to suppress inflammation and macrophage numbers in sites with increased inflammation such as periodontal and endodontic lesions [134, 135]. This result is an important finding for the potential treatment of inflammation-induced bone loss. The mechanism by which odanacatib alters macrophages is not clear. Given that macrophages are important cells

for the maintenance and turnover of bone, there is a need for further identification of the effects on macrophages by bone therapeutics. Off target effects of drugs may be due to macrophage responses versus traditional bone cell effects.

Antiresorptives are the most widely used therapeutic for the treatment of osteoporosis where they prevent further loss of bone in patients already exhibiting low bone mass. Due to the impact on reducing ongoing resorption, these agents provide increases in bone yet they do not stimulate bone formation. Teriparatide (hPTH 1-34) is the only anabolic osteoporosis therapeutic that has current US FDA approval. PTH-like analogs (ex. Abaloparatide) are currently under clinical investigation [136]. Parathyroid hormone (PTH), when pathologically elevated in the body or administered continuously, is catabolic in nature and acts as a signal to sequester calcium from the skeleton. However, if PTH is administered intermittently, an anabolic response leads to a net increase in bone [137, 138]. Teriparatide is currently only reserved for severe cases of osteoporosis due to its high cost, delivery method (injection), and black box warning attributed to the development of osteosarcomas in rats treated intermittently with PTH [139]. [139]. Any increase in risk of osteosarcoma in humans has not surfaced in the fourteen years it has been in clinical use [140, 141]. The exact mechanism of the anabolic response is still being investigated, and a better understanding of the effects of teriparatide will allow it to reach more patients with osteoporosis, to aid in the healing of fractures, and serve to treat patients with inflammatory diseases resulting in bone loss. Additionally, the delivery method is currently via subcutaneous injection and is administered systemically. Local delivery of teriparatide is under investigation for local bone regenerative application [142-144]. In rats, local intra-oral administration of PTH was as effective as subcutaneous injection in enhancing hard and soft tissue healing following tooth extraction [145]. Such application could have a positive impact in

conditions such as periodontal disease, bone defects or fractures where systemic administration has a demonstrated benefit [146, 147] but systemic application is unnecessary.

The role of macrophages has been outlined in the context of homeostatic bone turnover, disease and healing. Their role in mediating aspects of the PTH anabolic response is currently under investigation. Intermittent PTH treatment has been shown to be more effective when immune cells, such as macrophages, are present [78]. In a clinical trial investigating the effect of local teriparatide treatment of periodontal defects, disease sites displayed an anabolic response to teriparatide therapy, whereas non-diseased, non-inflamed sites showed no change in the same patient (although the study was not designed specifically to address this endpoint) [146]. Additionally, the anabolic response is more robust in sites of injury such as tibial fracture, tooth extraction sockets, and endodontic lesions versus non-wounded bone [148, 149]**.** These findings led to the hypothesis that cells mediating the inflammatory process may be supportive of the anabolic response of intermittent PTH treatment.

The role of macrophages in mediating the anabolic response to intermittent PTH comes from mouse models of macrophage ablation. Mice treated with intermittent PTH display increased bone mass and an increase in F4/80-positive cells lining the periosteal and endosteal bone surfaces [78]. In the same study, macrophages were ablated using the MAFIA mouse model and mice were treated with intermittent PTH. The anabolic response was lost in the mice with depleted macrophages. Interestingly, when macrophages were depleted using clodronate-loaded liposomes, the anabolic response was amplified. This led to an investigation of the subsets of macrophages which were altered in the two macrophage ablation models. CD68-positive macrophages are mature macrophages which demonstrate phagocytic capacity. In the MAFIA mouse model, all macrophages were depleted including CD68-positive macrophages.

Conversely, in the clodronate-loaded liposome model, CD68-positive macrophages were amplified, an increase which may reflect a compensatory feedback loop [78]. These observations not only implicate a role for macrophages in the anabolic response to intermittent PTH therapy, but also demonstrate that particular macrophage subsets are necessary for the PTH response. Further investigation of the clodronate model showed an increase in whole bone marrow osteogenic gene expression including Wnt-3a, Wnt-10b and TGF-β1, all of which were further increased with PTH treatment [78]. The source of the increase in gene expression is likely from a heterogeneous population of cells, but may be attributed to downstream effects of the increased CD-68+ cells and M2 macrophage upregulation. Studies investigating intermittent PTH treatment in non-lethally irradiated mice showed similar trends as the clodronate-loaded liposome model [150]. Non-lethal irradiation caused a decrease in marrow cells, however CD68+ cells were expanded. Mice treated with PTH following irradiation showed an increased anabolic response [150]. Due to the large amount of apoptosis that occurs following irradiation, efferocytosis machinery is likely altered to compensate for the influx and may lead to an upregulation of CD68+ phagocytic cells. This is consistent with the findings seen in the clodronate-loaded liposome model, and suggests that intermittent PTH therapy is enhanced in an environment in which apoptotic cell clearance is increased. As was seen in the clodronate model, TGF-β gene expression in the marrow was increased in the irradiated mice, a factor which may potentiate PTH effects [150]. Additionally, mechanical ablation of bone marrow in rats resulted in an increase in bone formation which was further increased with intermittent PTH therapy [151].

Further support of the hypothesis that the effects of intermittent PTH therapy are dependent on the macrophage actions comes from metabololipidomic profiling [92]. Intermittent

PTH increased proresolving mediators, including resolvin D1 and D2, in the bone marrow. Resolvins participate in the resolution phase of inflammation and mediate efferocytosis [152]. Human and mouse derived macrophages displayed increased engulfment of apoptotic osteoblasts when primed with resolvin D1 and D2 [92]. As macrophages do not have the PTH receptor, the mechanism by which PTH exerts its effects on macrophages is currently under investigation. PTH has been shown to have both pro- and anti-apoptotic effects on osteoblasts [153, 154]. In culture, early osteoblasts treated with PTH demonstrated decreased apoptosis, whereas mature osteoblasts treated with PTH displayed increased apoptosis. This led to the hypothesis that PTH promotes the apoptosis of less functional cells to promote reconstitution of the bone forming unit with younger, more functional osteoblasts. The increase in turnover of the osteoblast population may lead to the upregulation of alternatively activated M2-like macrophages and increased efferocytosis of apoptotic cells. The subsequent release of osteogenic factors leads to the recruitment of osteoblast precursors to facilitate the repopulation of the bone forming unit.

The exact mechanism by which PTH may regulate macrophages is unknown, as macrophages do not express the PTH receptor. Osteoblasts carry the PTH receptor and respond to PTH treatment. Osteoblasts treated with PTH show increased expression of macrophage responsive factors such as M-CSF [155], IL-6 [156], sIL-6R [157], and chemokine (C-C motif) ligand 2 [158]. In the presence of PTH, osteoblasts may secrete factors which promote macrophage differentiation and recruitment, leading to increased availability of macrophages to exert their pro-anabolic effects on bone.

Potential for targeted therapy that modulate macrophages to increase bone regeneration

Studies which have assessed the role of macrophages in the context of bone homeostasis have utilized models of macrophage ablation and characterized the aberrant effects. While these studies have shed light on the importance of this cell type on bone homeostasis, repair and anabolism, the ability to positively manipulate these cells to aid in bone regeneration is less appreciated. Clearly, targeting macrophages to assist in bone anabolism in cases of reduced bone mass or to aid in fracture repair shows promise in the field of bone biology. Colony-stimulating factor-1 (CSF-1), mediates myeloid to monocyte, macrophage, dendritic cell and osteoclast differentiation [159-161]. CSF-1 has been investigated for its potential application in tissue repair including fracture healing [159, 162]. When CSF-1 was administered to rabbits during femoral osteotomy healing, mineralized bone was significantly increased compared to control groups [162]. Additional experiments employing the use of macrophage CSF-1 demonstrated that bone mass and formation are increased with CSF-1 treatment [163, 164] and in a mouse model of osteoporosis, M-CSF prevents ovariectomy-induced bone loss [165]. Interestingly, recent studies have demonstrated that CSF-1 treatment increases osteal macrophages but not osteoclasts [112, 113]. This suggests that the ability to positively manipulate osteal macrophage numbers is a viable therapeutic target.

OSM released from macrophages has been shown to be anabolic in nature [72-74, 114, 166]. The therapeutic potential of this molecule has been explored, and could potentially serve to enhance osteoblastic differentiation and mineralization. An early experiment tested the potential of an adenoviral vector encoding murine OSM injected into knee joints to alter inflammation and stimulate bone formation [167]. OSM was shown to increase joint inflammation, but also displayed increased bone apposition at periosteal sites which correlated with increased

osteoblasts and decreased osteoclasts. Additionally, in lethally irradiated mice, OSM administration enhanced hematopoietic stem/progenitor cell recovery, suggesting OSM may serve to maintain the hematopoietic environment after injury. Like OSM, other factors that can be secreted by macrophages have been shown to be osteoinductive, including BMP-2 and could serve as potential therapies to enhance bone turnover.

The ability to directly target macrophages such that M1-like cells are decreased and M2-like cells are increased may facilitate the positive effects of macrophages on bone. Treating inflammation-induced bone loss has centered on targeting the production of Th1 by T cells to reduce inflammation and decrease osteoclast activity [168]. These therapies undoubtedly would have effects on macrophages as well. For example, Abatacept is a receptor construct that targets CD80/CD86 and is used as an anti-inflammatory to treat rheumatoid arthritis [169], and Tocilizumab is a humanized monoclonal antibody to IL-6R and is also used to treat rheumatoid arthritis as well as juvenile idiopathic arthritis [170]. The exact effects of these therapeutics to alter macrophages in respect to bone turnover are not appreciated, but a better understanding of these processes can help identify optimal treatment regimens.

In an interesting study of rheumatoid arthritis, injection of apoptotic thymocytes into the peritoneum reduced the presentation of rheumatoid arthritis in a streptococcal cell wall-induced rheumatoid arthritis model [171]. This was attributed to a decrease in TNF production by macrophages. This may also reflect an increase in resolution M2-like macrophages in response to the increased apoptotic cell insult. This further supports the idea that the process of efferocytosis results in favorable outcomes for bone in models of disease.

Other macrophage-targeted therapies under investigation

Altering macrophages to aid in the repair processes is not a new idea. In tissues other than bone, macrophages are potential targets for reducing symptoms of autoimmune diseases or enhancing tissue repair [172]. While many of these targeted therapies are still being investigated in animal models, clues from these studies in non-bone tissues may aid in the development of macrophage-targeted therapies to alter bone formation and repair. It is thought that an imbalance in M1/M2 macrophages that favors proinflammatory M1 cells is the basis for many diseases and issues with healing. Targeting macrophages to increase M2-like macrophages may serve to decrease any negative effects seen by increased M1-like cells. There are many drugs that are anti-inflammatory and could potentiate an M2 increase and a few will be reviewed here.

A well accepted paradigm in macrophage biology is IL-4 mediates macrophage phenotypic switch toward the alternatively activated M2-like state. In a mouse model of myocardial infarction, M2-like cells predominate after cardiac injury. When M2 macrophages were depleted, mice showed significantly worse prognoses when myocardial infarction was induced [173]. Administration of IL-4 increased M2-like macrophages in mice and increased prognosis of myocardial infarcted mice. Early studies showed that IL-4 inhibits resorption [174], yet the therapeutic potential of IL-4 in fracture repair or osteoporosis therapy has not been thoroughly investigated and may serve as a positive regulator of repair and regeneration.

Another example of an approach to target macrophage polarization is CD200. CD200 interaction with its receptor increases alternatively activated macrophages [175]. Treatment of macrophages with human CD200-Fc up-regulated expression levels of M2-like markers and suppressed M1-like markers. These increases coincided with increased TGF-β expression in macrophages, and decreased proinflammatory macrophages. Interestingly, CD200 has been

shown to play a role in bone turnover and CD200 signaling may be a future therapeutic target to promote M2-like macrophages to aid in tissue repair. Although promising, macrophage plasticity targets must be explored more thoroughly in in animal models prior to pre-clinical settings to understand the complexity of the potential human response.

Conclusions and Future Directions

The heterogeneity of macrophages, their varying functional roles, and plasticity make them difficult but exciting targets for therapeutic intervention. In the context of bone, certain macrophage subsets have been shown to mediate turnover and healing. However, it must be noted that most strategies to target macrophages in bone will likely have off-target effects on osteoclasts. This review has outlined the known roles of macrophages in bone, the potential mechanisms behind their pro-osteogenic effects and the possibility to target macrophages to aid in bone related disease treatment and fracture healing. It is promising that macrophage stimulating molecules in combination with current approved osteoporosis therapies could improve patient outcomes. Clearly, more in depth characterization of macrophages in bone must be completed to identify the best possible strategies to target macrophages to aid in bone repair and regeneration.

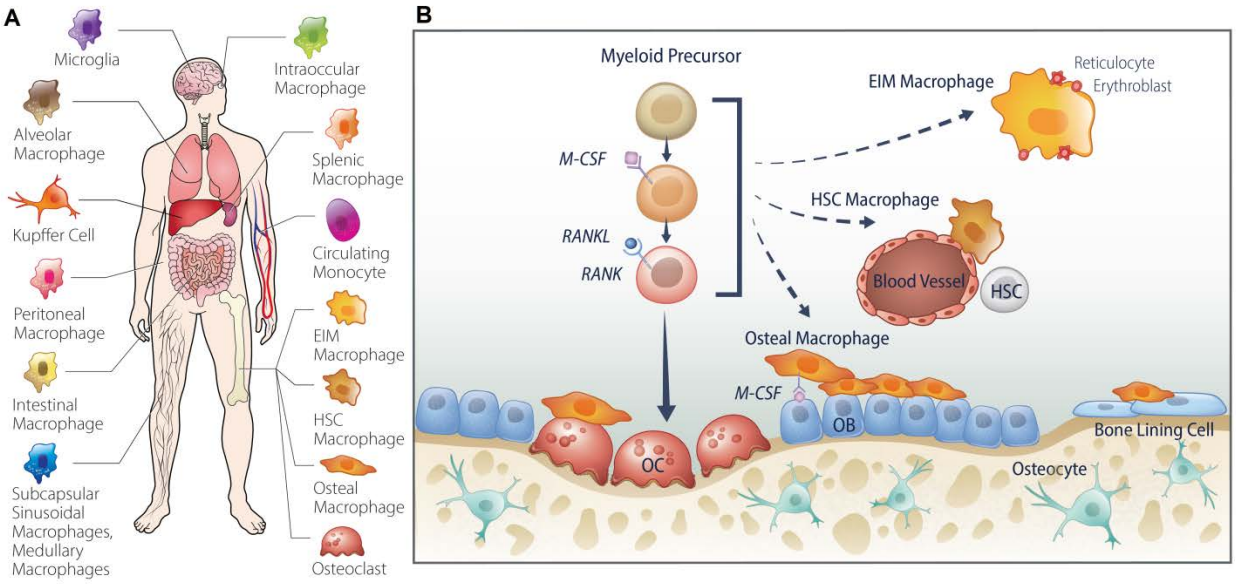

Figure 2.1. Tissue resident macrophages. (**A**) Macrophages are present in nearly all tissues in the body and perform different functional roles based on their location. (**B**) In the bone and bone marrow there consist several populations of macrophages. Osteoclasts are bone-resorbing cells which differentiate from monocyte precursors in the presence of M-CSF and RANKL. Erythroid island macrophages (EIM) are found interacting with and supporting erythroblasts during erythropoiesis. These macrophages are distinct from HSC niche macrophages which are found adjacent to blood vessels and support HSC self-renewal and cycling. Osteal macrophages are found adjacent to bone forming cells (osteoblasts), dormant bone-lining cells, and osteoclasts. The functional role of osteal macrophages is proposed to support bone formation through mechanisms currently under investigation.

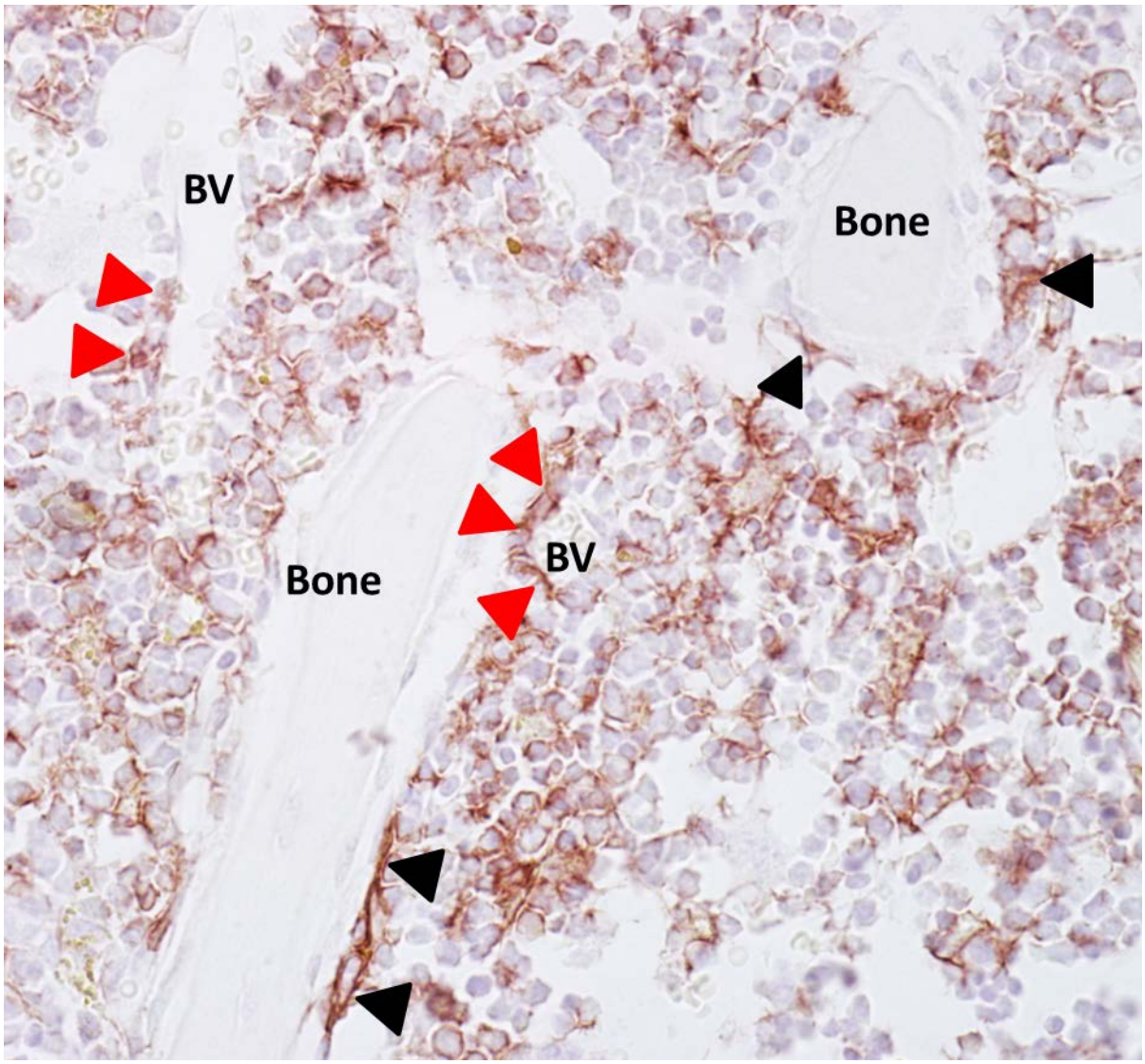

Figure 2.2. F4/80 positive cells are located throughout the marrow and intimately associated with bone surfaces. Tibiae from 22-week old C57BL/6 mice were paraffin embedded, sectioned and immunohistochemical stained (brown) for F4/80 and counterstained with hematoxylin (blue) as previously described [78]. Macrophages are located adjacent to bone surfaces (black arrowheads) and throughout the marrow space. F4/80+ cells are also associated with blood vessels (BV) and support hematopoiesis (red arrowheads).

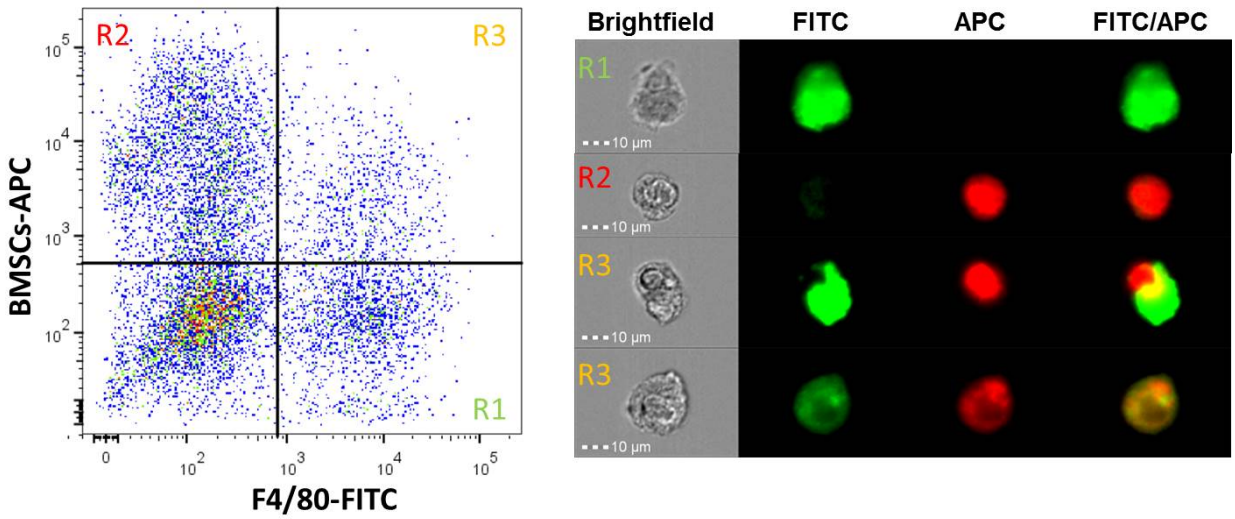

Figure 2.3. Internalization of apoptotic bone marrow stromal cells by macrophages. Bone marrow macrophages were stained for F4/80-FITC and apoptotic bone marrow stromal cells (BMSCs) stained with Cell Tracker Deep Red, co-cultured for 1hr and analyzed via flow cytometry. Representative fluorescence-activated cell sorting (FACs) dot plots (left) indicate macrophages alone (R1), apBMSCs alone (R2), or macrophages with internalized apoptotic BMSCs (R3). Representative photo from Image Stream which captures single cell images showing either single cells or engulfment (right). These images depict efferocytosis at two stages, recognition and internalization, and demonstrate that bone marrow macrophages in culture efficiently phagocytose apoptotic bone marrow stromal cells.

Table 2.1. Bone macrophage markers and roles.

Macrophage	Current Known Markers	Roles	References
Erythroblastic island macrophages (EIM)	CD11b F4/80 CD169 VCAM-1 ER-HR3 Ly6G TRAP (neg)	Support erythropoiesis	[66, 176]
HSC niche macrophages	CD11b F4/80 CD169 VCAM-1 CD234 Ly6G (neg) TRAP (neg)	Support and regulate HSC niche self-renewal and cycling	[68, 77, 177, 178]
Osteal macrophages	F4/80 CD115 CD68 Mac-3 TRAP (neg)	Support bone homeostasis Support fracture healing	[3, 113]
Osteoclasts	F4/80 (neg) TRAP (pos) Cathepsin K Calcitonin Receptor	Bone resorption Efferocytosis	[179, 180] [181]

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CHAPTER 3

MODULATION OF OSTEOBLASTIC CELL EFFEROCYTOSIS BY BONE MARROW MACROPHAGES

Abstract

Apoptosis occurs at an extraordinary rate in the human body and the effective clearance of dead cells (efferocytosis) is necessary to maintain homeostasis and promote healing, yet the contribution and impact of this process in bone is unclear. Bone formation requires that bone marrow stromal cells (BMSCs) differentiate into osteoblasts which direct matrix formation and either become osteocytes, bone lining cells, or undergo apoptosis. A series of experiments were performed to identify the regulators and consequences of macrophage efferocytosis of apoptotic BMSCs (apBMSCs). Bone marrow derived macrophages treated with the anti-inflammatory cytokine interleukin-10 (IL-10) exhibited increased efferocytosis of apBMSCs compared to vehicle treated macrophages. Additionally, IL-10 increased anti-inflammatory M2-like macrophages ($CD206^+$), and further enhanced efferocytosis within the $CD206^+$ population. Stattic, an inhibitor of STAT3 phosphorylation, reduced the IL-10-mediated shift in M2 macrophage polarization and diminished IL-10-directed efferocytosis of apBMSCs by macrophages implicating the STAT3 signaling pathway. Cell culture supernatants and RNA from macrophages co-cultured with apoptotic bone cells showed increased secretion of monocyte chemotactic protein 1/chemokine (C-C motif) ligand 2 (MCP-1/CCL2) and

transforming growth factor beta 1 (TGF-β1) and increased *ccl2* gene expression. In conclusion, IL-10 increases M2 macrophage polarization and enhances macrophage-mediated engulfment of apBMSCs in a STAT3 phosphorylation-dependent manner. After engulfment of apoptotic bone cells, macrophages secrete TGF-β1 and MCP-1/CCL2 both factors that fuel the remodeling process. A better understanding of the role of macrophage efferocytosis as it relates to normal and abnormal bone turnover will provide vital information for future therapeutic approaches to treat bone related diseases.

Introduction

The critical process of bone formation depends on the lifespan and activity of osteoblasts lining the bone surface. Three fates have been described for osteoblasts: they either become osteocytes embedded in mineralized matrix, bone-lining cells which form layers over bone surfaces and at remodeling sites, or they undergo programmed cell death (apoptosis). Of the osteoblasts initially at remodeling sites, 30-50% become osteocytes and bone-lining cells, which leaves a large percentage of cells thought to undergo apoptosis [1]. The course of events after osteoblast apoptosis has been under appreciated. This is in part due to the inability of assays to accurately detect and quantify apoptotic osteoblasts readily undergoing apoptosis. In normal physiology, cell death is followed by rapid and efficient removal of apoptotic cells by phagocytic cells, predominantly macrophages. This process of apoptotic cell recognition and clearance is termed efferocytosis [2].

Macrophages are immune cells known for their role in infection and inflammation. A focus on their role in bone has only recently emerged [3]. Macrophages are prominent players in bone homeostasis and are highly implicated in fracture healing [4-6]. They are often found at

sites of remodeling, and are intimately associated with bone forming osteoblastic cells. Additionally, macrophage numbers increase significantly in fracture sites, and bone repair is severely diminished after tibial injury in macrophage-ablated mouse models [5]. Apoptotic cells increase in sites of injury, and a crucial component of the healing process is the effective clearance of these cells by phagocytes, including macrophages. The recognition and subsequent efferocytosis of dead and dying cells leads to the secretion of anti-inflammatory cytokines such as TGF-β and IL-10 [7, 8], as well as osteoinductive factors including osteopontin, and BMP-2 [9-11]. The process of efferocytosis has been extensively studied in other tissues. Recently, macrophages were found to efferocytose apoptotic osteoblasts [12] yet the impact of efferocytosis in bone has not been clearly defined.

Macrophages have been shown to polarize into two populations: classically activated M1 macrophages and alternatively activated M2 macrophages. M2 macrophages are present during the resolution phase of inflammation and are responsible for anti-inflammatory cytokine production and enhanced efferocytosis [13, 14]. In the presence of the anti-inflammatory cytokines IL-4 and IL-10, macrophages polarize to M2-like macrophages [15], and increase apoptotic lymphocyte and apoptotic neutrophil clearance [16, 17]. IL-10 is an important bone cytokine which inhibits osteoclastogenesis [18] and exhibits a protective role in periodontal disease [19]. Furthermore, IL-10 deficient mice exhibit low bone mass phenotypes with reduced bone formation compared to wild-type controls [18, 20]. To our knowledge, the role of IL-10-induced efferocytosis in bone has not been investigated.

Additonally, it is unclear which factors regulate the clearance of apoptotic bone cells by bone resident macrophages and how macrophages respond to the englulfment of an apoptotic bone cell. The apoptotic cell identity can impact the response elicited by the phagocyte and the

response of a bone marrow macrophage to an apoptotic bone cell is of interest to the present work. This study aimed to determine factors and signaling which affect macrophage efferocytosis of apoptotic osteoblastic cells and changes in macrophage gene expression in response to these apoptotic cells.

Materials/Methods

Animals

All animals were maintained in accordance with institutional animal care and use guidelines, and experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Michigan. C57BL/6J (The Jackson Laboratory, Bar Harbor, ME) mice were used for *in vitro* experiments unless otherwise indicated. Human CD68-GFP reporter mice (hCD68-GFP) mice were generously provided by Dr. Ajay Chawla (University of California San Francisco). hCD68-GFP mice were generated by cloning a cDNA fragment encoding EGFP (from pEGFP-N1 vector, Invitrogen) into the 1265 vector containing human CD68 promoter (−2.9 kb). DNA was then excised from the vector and injected into mouse oocytes by pro-nuclear injection [21].

Cell Culture

Primary bone marrow cells were collected from 4-8 week old C57BL/6J or hCD68-GFP mice. Bone marrow-derived macrophages (BMMs) were differentiated *in vitro* from bone marrow flush in α-MEM medium (10% FBS, Pen/Strep, glutamine) with murine M-CSF (30ng/mL eBioscience) for 6 days. At day 7, macrophages were plated at 2.5 x 10^5 cells/well in 12-well plates (for efferocytosis assays) or 1.5 x 10^6 cells/well in 6-well plates (for protein/RNA). Bone

marrow stromal cells (BMSCs) were derived from bone marrow flush and cultured in α-MEM medium (20% FBS, Pen/Strep, glutamine) containing 10nM dexamethasone (Sigma) and used at passage 1-2. Bone marrow neutrophils were isolated as previously described [22]. Briefly, bone marrow was flushed from 8-12 week old C57BL/6J mice with RPMI supplemented with 10% FBS and 2 mM EDTA, red blood cells lysed using 0.2% NaCl, and neutrophils separated by density gradient centrifugation (Histopaque 1119 and Histopaque 1077). Neutrophils were harvested at the interface of the Histopaque 1119 and Histopaque 1077 layers and confirmed using FACs anaylsis for $CD11b^{+}Ly6G^{+}$ cells.

Macrophage treatment

All recombinant murine proteins were obtained from R&D Systems. BMMs were treated at day 8 with rmIL-10 (0.1-100ng/mL), rmCCL2/MCP-1 (10ng/mL), rmMFG-E8 (10ng/mL), sIL-6R (100ng/mL), or vehicle (0.5% BSA in 1X PBS) for 4-24 hours in low serum containing media (0.5% FBS). In a set of experiments BMMs were pre-treated with an inhibitor of pSTAT3 (Stattic, 6.25μM, CalBiochem) or DMSO (vehicle) 2 hours prior to rmIL-10 treatment.

Efferocytosis Assay

BMSCs were stained with CellTracker Deep Red (APC^{+}, Invitrogen) and exposed to UV light for 30 minutes to induce apoptosis. Apoptotic BMSCs (apBMSCs) were recovered for 2 hours at 37ºC, enumerated via trypan blue exclusion (confirming cell death), and added to BMM cultures at a 1:1 ratio for 0.5-6 hours. BMMs co-cultured with apBMSCs were harvested and stained with F4/80-FITC (Abd Serotec, CI:A3-1), fixed with 1% formalin and efferocytosis was assessed via flow cytometric (FACs) analyses (BD FACSAria^{TM} III) for double labeled cells ($FITC^{+}APC^{+}$)

reflecting engulfment. Macrophages cultured alone were harvested and stained with the following macrophage-specific antibodies: F4/80 FITC (CI:A3-1, BIORAD), CD86 PE (GL-1, Biolegend,) and CD206 PE (C068C2, Biolegend). Cells were fixed, permeabilized with Permeabilization Buffer (Biolegend), incubated with CD68 PE (Y1/82A, Biolegend) and assessed via FACs analyses.

Confocal microscopy

BMMs from hCD68-GFP mice were plated in 1.5-mm coverglass chambers (8×10^4 cells/well), treated for 24 hours with vehicle or rmIL-10 (10ng/mL) and apBMSCs (stained with CellTracker DeepRed) were added at a 1:1 ratio to BMMs for 1 to 24 hours and fixed with ice-cold methanol for 20 mins. Cells were then washed with PBS and covered with ProLong® Gold antifade reagent with DAPI (Life Technologies). Confocal microscopy images were analyzed using the Leica inverted SP5X confocal microscope system with two-photon film and Leica software (Leica Microsystems).

Mouse Inflammation Antibody Array

BMMs were plated into 6-well dishes (1.5×10^6 cells/well), then co-cultured with or without apBMSCs at a 1:1 ratio in α-MEM (0.25% FBS). Supernatants were collected after 18 hours and proteins were analyzed using the mouse inflammation antibody array C1 (catalog no. AAM-INF-1–8, RayBiotech, Inc.) per manufacturer's instructions.

TGF-β1 ELISA

Supernatants were collected from BMMs alone, BMMs/apBMSCs or BMMs/apNeutrophils after 18 hours of co-culture. TGF-β1 protein levels in culture supernatants were measured with the Quantikine mouse TGF-β1 ELISA (R&D systems) per manufacturer's instructions. Briefly, acid-activated supernatant samples, standards, and controls were added to anti-mouse TGF-β1 antibody pre-coated microplates and incubated at room temperature for 2 hours. Wells were washed and incubated with TGF-β1 conjugate for 2 hours, washed and substrate solution added for 30 minutes. Stop solution was added and A450 values (corrected with A570) were measured using an EZ Read 400 microplate reader (Biochrom).

QRT-PCR

Total RNA was isolated from BMMs alone, apBMSCs alone and BMM/apBMSC co-culture after 18 hours using the Qiagen RNeasy Mini Kit. Reverse transcription PCR was conducted, and the cDNA products were amplified and detected using TaqMan Universal PCR master mix (Applied Biosystems) and TaqMan probes, including mouse Ccl2 (Mm00441242_m1) and mouse Actb (Mm02619580_g1) as an endogenous control. Realtime PCR was analyzed on ABI PRISM 7700 (AppliedBiosystems).

Statistical Analyses

Statistical analyses were performed by unpaired Student's *t* test to compare two groups or ANOVA to compare three or more groups with a significance of $p < 0.05$. Data are presented as mean ± S.E.

Results

IL-10 Enhances Bone Marrow Derived Macrophage Engulfment of Apoptotic Bone Marrow Stromal Cells

To investigate the impact of IL-10 on macrophage-mediated efferocytosis of apoptotic bone cells, bone marrow macrophages (BMMs) were treated with IL-10 and efferocytosis of apoptotic bone marrow stromal cells (apBMSCs) was determined. BMMs (F4/80-FITC$^+$) were co-cultured with apBMSCs (DeepRed-APC$^+$) and double positive (FITC$^+$APC$^+$) cells were quantified using flow cytometric cell sorting, reflecting engulfment (**Figure 3.1A, left panel**). Engulfment was confirmed by ImageStream analysis which images individual cells in the flow stream to confirm the presence of fluorescent markers. Double-positive cells were confirmed to to be FITC$^+$-macrophages associated with APC$^+$-apBMSCs (**Figure 3.1A**). To assess the effect of various cytokines on efferocytosis, BMMs were treated with IL-10, CCL2, MFG-E8 and sIL-6R for 24 hours and efferocytosis of apBMSCs measured after 1 hour of co-culture. These cytokines were chosen based on their ability to regulate efferocytosis in non-skeletal tissues (IL-10 and MFG-E8) or important bone formation regulators (CCL2 and sIL-6R). IL-10 enhanced engulfment of apBMSCs by 22% and in a dose-dependent manner (**Figure 3.1B and C**), whereas MCP1, MFG-E8 and sIL-6R did not demonstrate significant changes in efferocytosis at that time point. To determine the optimum IL-10 treatment length, BMMs were treated for 4-48 hours with IL-10. BMMs treated for 24 hours with IL-10 displayed increased efferocytosis compared to shorter treatment times (**Figure 3.1D**). Macrophages treated with IL-10 for 24 hours showed enhanced efferocytosis when co-cultured for shorter durations (0.5-2 hours) compared to longer co-cultures (6 hours), suggesting IL-10 increased the rate of efferocytosis

(**Figure 3.1E**). IL-10 treatment of BMMs enhanced engulfment of apBMSCs in a time and dose-dependent manner.

IL-10 Polarizes Bone Marrow Derived Macrophages to M2-like Phenotype with Enhanced Efferocytic Capacity

Macrophages are described as classically (M1) or alternatively (M2) activated. M2 polarized macrophages are resolving cells with higher efferocytic capacity than M1 macrophages [13]. To determine the impact of IL-10 treatment on BMM phenotypes, equal numbers of BMMs were treated with IL-10 for 24 hours and assessed for M1 ($F4/80^+CD86^+$) and M2 ($F4/80^+CD206^+$) polarization. IL-10 did not affect cell number or viability (**Figure 3.2A**), mature ($CD68^+$) or M1 macrophages (**Figure 3.2B**). Treatment with IL-10 significantly increased M2 polarization of BMMs (**Figure 3.2C**). This effect of IL-10 is consistent with previous reports [23, 24], however, the impact of IL-10 within the M2 population is unclear. To investigate the role of IL-10 on altering efferocytosis within the $CD206^+$ population, efferocytosis of apBMSCs by $CD206^+$ cells was measured. IL-10 increased the percentage of $CD206^+$ cells that efferocytosed apBMSCs (**Figure 3.2D**), suggesting that IL-10 not only polarized BMMs to M2 macrophages, but also specifically enhanced their efferocytic ability.

IL-10 Increases Efferocytosis in a STAT3 Activation Dependent Manner

IL-10 signals through the JAK/STAT pathway and inhibition of JAK/STAT signaling has been shown to decrease efferocytosis [25]. To measure the effect of JAK/STAT pathway inhibition on IL-10 induced efferocytosis, BMMs were pre-treated with the phospho-STAT3 (pSTAT3) inhibitor Stattic prior to IL-10 treatment and efferocytosis measurement. BMMs were

isolated from mice which express GFP under the human CD68 promoter to visualize $CD68^+$ (mature phagocytic) cells (**Figure 3.3A**). Macrophages harvested from the hCD68-GFP mice displayed increased efferocytosis of apBMSCs following IL-10 treatment as measured by FACs analysis and confocal microscopy (**Figure 3.3B and C**). BMMs pre-treated for 2 hours with Stattic and subsequently treated with IL-10 had reduced efferocytosis of apBMSCs compared to DMSO control treated macrophages (**Figure 3.3D**). Macrophages pre-treated with Stattic followed by vehicle treatment also displayed decreased efferocytosis compared to controls suggesting some baseline efferocytosis in vehicle treated macrophages requires signaling through the JAK/STAT pathway, likely by other cytokine signaling. To investigate the role of JAK/STAT signaling in IL-10 induced M2 polarization, BMMs were treated with Stattic for 2 hours followed by IL-10 treatment and assessed for $CD206^+$ (M2 polarization). In vehicle treated BMMs, Stattic did not alter the M2 population; however, Stattic treatment prior to IL-10 treatment significantly reduced the IL-10 mediated M2 shift in macrophage polarization (**Figure 3.3E**) suggesting that IL-10 shifts macrophage polarization and enhances efferocytosis of apBMSCs via signaling through JAK/STAT pathway.

Macrophages Secrete MCP-1/CCL2 and TGF-β1 in Response to Apoptotic Bone Marrow Stromal Cells

Macrophages release factors when engulfing apoptotic cells that signal to surrounding cells [8, 26, 27]. To determine the response of macrophages to apBMSCs, BMMs were cultured alone or with apBMSCs for 18 hours and supernatants harvested for analysis of secreted proteins. Supernatants harvested from co-culture of BMMs with apBMSCs showed significantly increased secreted CCL2 levels compared to macrophages cultured alone (**Figure 3.4A**). RNA

harvested from macrophages co-cultured with apBMSCs also showed increased *ccl2* gene expression (**Figure 3.4B**) compared to BMMs alone or BMMs co-cultured with apoptotic neutrophils. In other tissues, TGF-β1 is an important anti-inflammatory cytokine released from macrophages after efferocytosis [8]. Supernatants from macrophages cultured with apBMSCs versus macrophages alone or versus macrophages cultured with apoptotic neutrophils displayed increased total secreted TGF-β1 levels (**Figure 3.4C**). Hence, in response to engulfment of apoptotic bone cells, bone marrow macrophages secrete the anti-inflammatory cytokine TGF-β1 and chemokine CCL2, which are both important factors related to bone homeostasis.

Discussion

Patients exhibiting increased inflammation associated with diseases such as rheumatoid arthritis and inflammatory bowel disease are often at a higher risk of generalized osteoporosis and increased fracture risk [28-30]. Chronic inflammation is associated with systemic overproduction of pro-inflammatory mediators, which are thought to play a role in decreasing bone formation [31]. Periodontal disease activates macrophages and triggers systemic inflammation of the vasculature and atherosclerotic signs [32]. Macrophages are immune cells which mediate pro-inflammatory or anti-inflammatory conditions yet little is known about their role in the basic cell biology of bone.

Macrophages have recently become of interest in the bone field due to their intimate association with the bone forming unit and contributions to osteoblast function [4-6]. It is hypothesized that the anti-inflammatory function of macrophages and the clearance of apoptotic cells may contribute to bone turnover and regeneration [12]. In humans, billions of cells die daily which is unappreciated by current methods used to detect apoptotic cells from *in vivo* tissue

samples. This is due to the rapid and effective clearance of apoptotic cells, termed efferocytosis [2]. Macrophages are vital mediators of cellular turnover, maintenance of extracellular matrix homeostasis and modulation and resolution of inflammation [33]. Understanding their mechanisms of action as they relate to bone remodeling provides a basis for the prevention and treatment of chronic inflammatory conditions.

Anti-inflammatory cytokines such as IL-4 and IL-10 released by immune cells have been shown to increase human peripheral blood macrophage engulfment of non-bone associated apoptotic cells [16, 17]. IL-10 contributes to bone turnover by regulating osteoclastogenesis and IL-10 deficient mice display osteopenia [20, 34], but the role of IL-10-associated efferocytosis by bone marrow macrophages is largely unknown. To study mechanisms of efferocytosis in bone marrow derived macrophages, a series of *in vitro* experiments were performed to determine how bone macrophages respond to apoptotic bone marrow stromal cells and what factors may influence the rate of engulfment. IL-10 treated macrophages displayed enhanced clearance of apoptotic bone marrow stromal cells. The effect of IL-10 on bone marrow macrophages was in part due to polarization of macrophages toward the pro-resolving M2 phenotype. While there was a significant increase in M2 macrophages, the increase was not the only contributing factor to an increase in efferocytosis. The $CD206^{+}$ M2 macrophages displayed increased efferocytosis of apoptotic bone cells after treatment with IL-10 compared to vehicle treated macrophages. This suggests that not only does IL-10 polarize macrophages to M2, but it also enhances the M2 macrophage efferocytic capacity.

The effect of IL-10 on efferocytosis of apoptotic lymphocytes and neutrophils has been measured, and downstream signaling of IL-10 has been identified [16, 17]. IL-10 signals through the JAK/STAT pathway, in which STAT3 is phosphorylated, dimerizes and translocates to the

nucleus to regulate transcription of various genes [35-37]. To validate the downstream events of IL-10 signaling, macrophages were treated with an inhibitor of STAT3 phosphorylation (Stattic) prior to IL-10 treatment. Efferocytosis of apoptotic bone cells was decreased with Stattic treatment, demonstrating that the IL-10 effect occurs in a pSTAT3-dependent manner. Previous studies have also indicated that inhibition of STAT3 phosphorlyation decreases MFG-E8 mediated phagocytosis of apoptotic mimicry beads [25].

Following IL-10 activation of macrophages and efferocytosis of apoptotic bone cells, proteins released by macrophages signal to surrounding cells. How macrophages respond to apoptotic bone cells may indicate what effects efferocytosis have on bone modeling and remodeling. The identity of the apoptotic cell, bone marrow stromal cells in this case, contributes to the profile of genes upregulated in macrophages partaking in efferocytosis. In the present study, bone marrow derived macrophages increased secretion of monocyte chemoattractant protein-1/CC chemokine ligand 2 (MCP-1/CCL2) after co-culture with apoptotic bone cells. CCL2 is associated with monocyte/osteoclast precursor recruitment and enhances osteoclastogenesis [38]. These data suggest that CCL2 secreted from macrophages engulfing apoptotic bone cells could aid in the recruitment of monocytes to enhance bone turnover by increasing osteoclasts and bone macrophages.

TGF-β1 was also found to be increased after efferocytosis of apBMSCs. Previous studies using human peripheral blood macrophages showed increased TGF-β1 levels following efferocytosis of apoptotic human derived neutrophils [8]. The release of TGF-β1 following apoptotic cell engulfment is often credited with an anti-inflammatory function. Interestingly, in the context of bone, TGF-β1 enhances the recruitment of mesenchymal stem cells which are precursors to osteoblastic cells [39, 40]. The secretion of TGF-β1 in response to apoptotic bone

cells may contribute to repopulating the bone forming units. Other osteogenic factors such as BMP-2 and osteopontin have been shown to be released from M2 macrophages, but it is unclear how the protein levels change during the process of efferocytosis [10, 11].

Macrophages are crucial regulators of bone turnover in steady state bone remodeling and wound healing [4, 5]. A better understanding of the role of these macrophages is important in developing ways to utilize macrophages in therapeutic interventions for osteoporosis, fracture healing, periodontal wound healing, and osteonecrosis. These data demonstrate one mechanism by which macrophages efferocytose apoptotic bone cells and their subsequent release of factors important in bone remodeling (**Figure 3.5**). Future work to determine the association of efferocytosis and bone turnover is important to better understand the mechanisms by which macrophages contribute to bone modeling and remodeling.

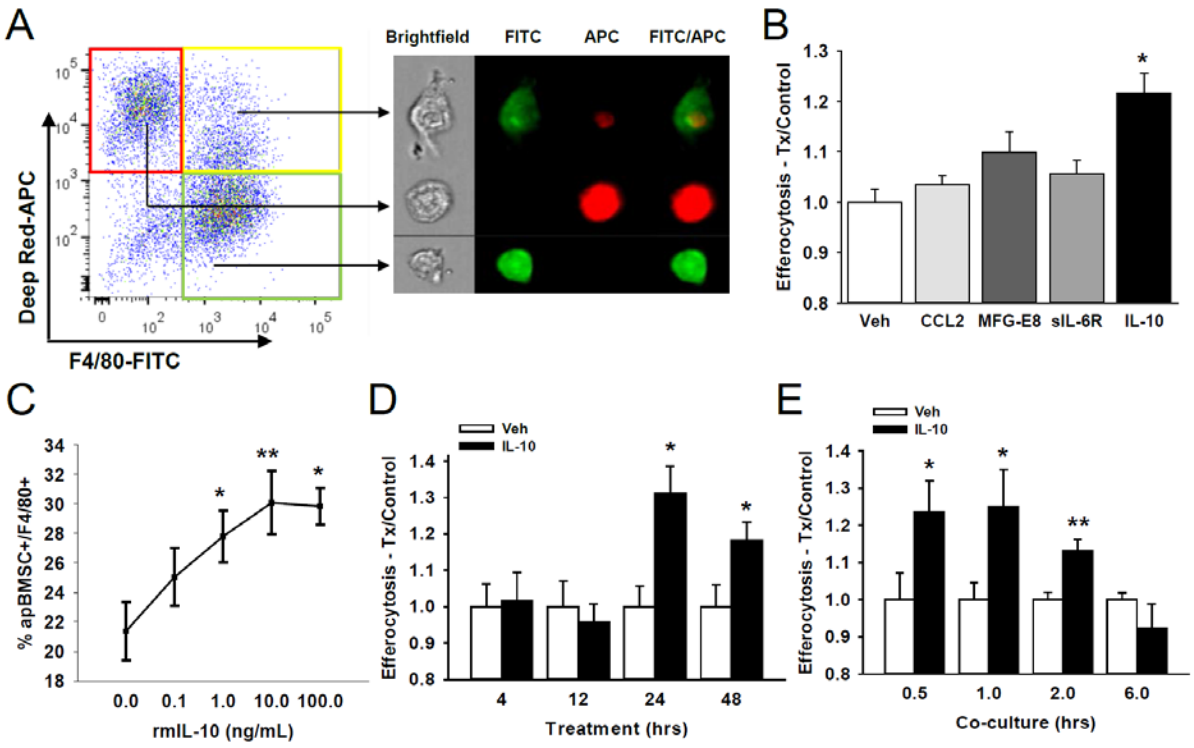


Figure 3.1. IL-10 treatment enhanced efferocytosis of apoptotic bone marrow stromal cells (apBMSCs) by bone marrow derived macrophages (BMMs). (A) BMMs were stained for F4/80-FITC and apBMSCs stained with Cell Tracker Deep Red, co-cultured for 1hr and analyzed via flow cytometry. Representative fluorescence-activated cell sorting (FACs) dot plots (left) indicate macrophages alone (green gate), apBMSCs alone (red gate), or macrophages with internalized apBMSCs (yellow gate). Representative photo from Image Stream which captures single cell images showing either single cells or engulfment. (B) BMMs treated for 24hrs with vehicle, rmIL-10 (10ng/mL), rmMCP-1 (10ng/mL), rmMFG-E8 (500ng/mL) or sIL-6R (500ng/mL) and co-cultured with apBMSCs for 1hr. Efferocytosis was measured as percentage of $F4/80^{+}$ cells which were $apBMSC^{+}$. IL-10 treatment increased efferocytosis compared to vehicle control. (C) BMMs were treated for 24hrs with rmIL-10 (0.1-100ng/mL) and co-cultured with apoptotic BMSCs for 1hr. IL-10 (1.0-100ng/mL) increased efferocytosis of apBMSCs. (D) BMMs were treated with IL-10 (10ng/mL) for 4-48hrs and co-cultured with apBMSCs for 1hr. Treatment of macrophages with rmIL-10 for 24-48hrs induced a larger increase in efferocytosis relative to shorter treatment times. (E) BMMs treated for 24hrs with IL-10 or vehicle control and co-cultured with apoptotic BMSCs for 0.5-12hrs. IL-10 increased engulfment of apBMSCs after 0.5-2hrs co-culture. n=6/gp, data is mean ± SEM. *p <0.05, ** p <0.01 versus vehicle treatment.

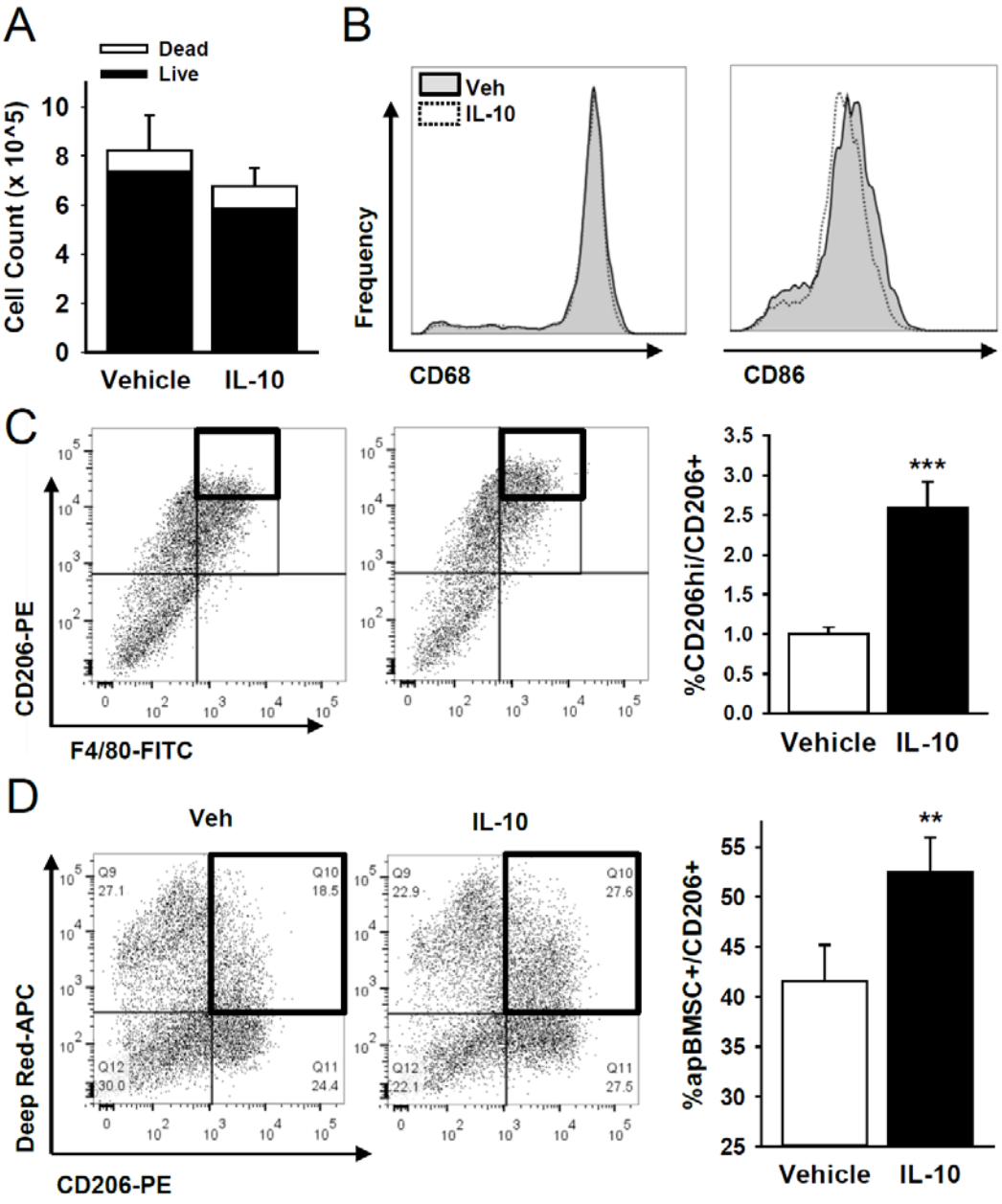
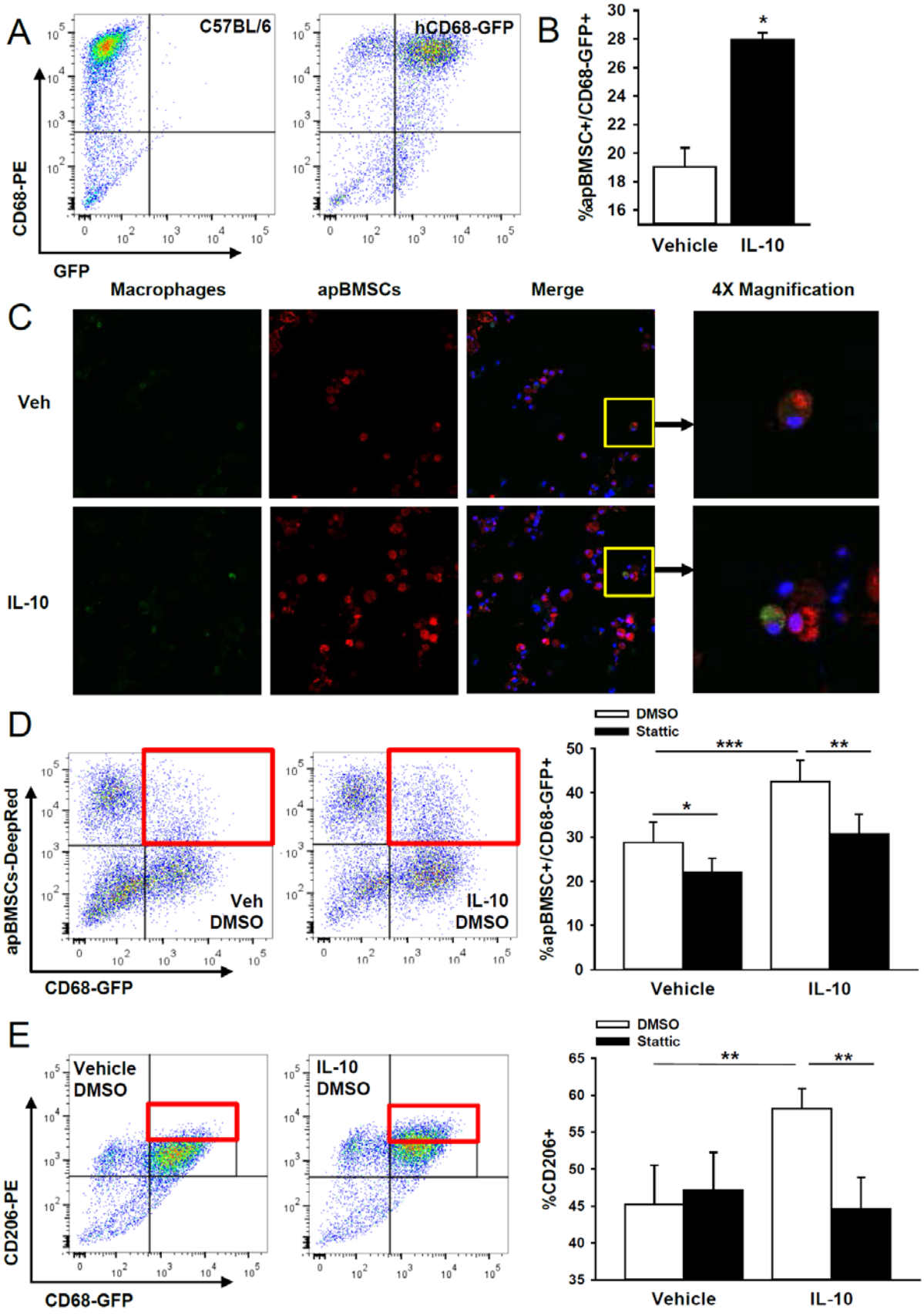


Figure 3.2. IL-10 polarizes BMMs to alternatively activated (M2) phenotype. (A) BMMs were treated with rmIL-10 (10ng/mL) for 24hrs and enumerated. IL-10 did not change cell number compared to vehicle controls or viability, (B) BMMs treated for 24hrs with rmIL-10 were analyzed via FACs for phagocytic cells (CD68) and M1 macrophage phenotype (CD86). No significant changes were observed in these cell populations with IL-10 treatment. (C) BMMs treated with IL-10 were analyzed via FACs analysis for alternatively activated M2 macrophages (CD206). IL-10 treatment significantly increased the $CD206^{hi}$ population per $CD206^{+}$. (D) BMMs treated with IL-10 showed enhanced efferocytosis of apBMSCs within the $CD206^{+}$ population after 1hr co-culture as evidences by increased $CD206^{+}apBMSCs^{+}$ double positive cells. n = 6/gp, data is mean ± SEM. **p<0.01, ***p <0.001 versus vehicle treatment.


A
C57BL/6
hCD68-GFP
CD68-PE
GFP
B
%apBMSC+/CD68-GFP+
Vehicle
IL-10
C
Macrophages
apBMSCs
Merge
4X Magnification
Veh
IL-10
D
apBMSCs-DeepRed
CD68-GFP
Veh DMSO
IL-10 DMSO
DMSO
Stattic
%apBMSC+/CD68-GFP+
Vehicle
IL-10
E
Vehicle DMSO
IL-10 DMSO
CD206-PE
CD68-GFP
DMSO
Stattic
%CD206+
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IL-10

Figure 3.3. Stat3 inhibition diminished IL-10 effect. (A) BM cells were harvested from 4-8wk old hCD68-GFP mice, enriched to macrophages with M-CSF then analyzed for GFP retention. FACs analysis showed that the majority of CD68-PE+ cells were also GFP+, indicating GFP was retained during *in vitro* culture. (B) hCD68-GFP BMMs were treated with IL-10 for 24 hours and efferocytosis of apBMSCs harvested from C57BL/6J analyzed via FACs analysis. IL-10 significantly increased the percentage of GFP^+ cells which were also positive for apBMSCs (Deep Red). (C) hCD68-GFP BMMs were treated with IL-10 for 24 hours and efferocytosis of apBMSCs analyzed via confocal microscopy. (D) hCD68-GFP BMMs were treated with Stat3 inhibitor (Stattic, 6.25μM) or DMSO for 2 hours followed by IL-10 or vehicle treatment for 24 hours. Efferocytosis of apBMSCs was analyzed via FACs analysis for double positive GFP/apBMSCs cells (representative dot plots on left). Stat3 inhibitor decreased efferocytosis of apBMSCs in vehicle and IL-10 treated BMMs. (E) Alternatively activated macrophage phenotype (CD206-PE) was increased in IL-10 treated BMMs and the effect was diminished with Stattic pre-treatment. n = 6/group, data is mean ± SEM. *p <0.05, ** p <0.01, ***p <0.001 versus vehicle treatment.

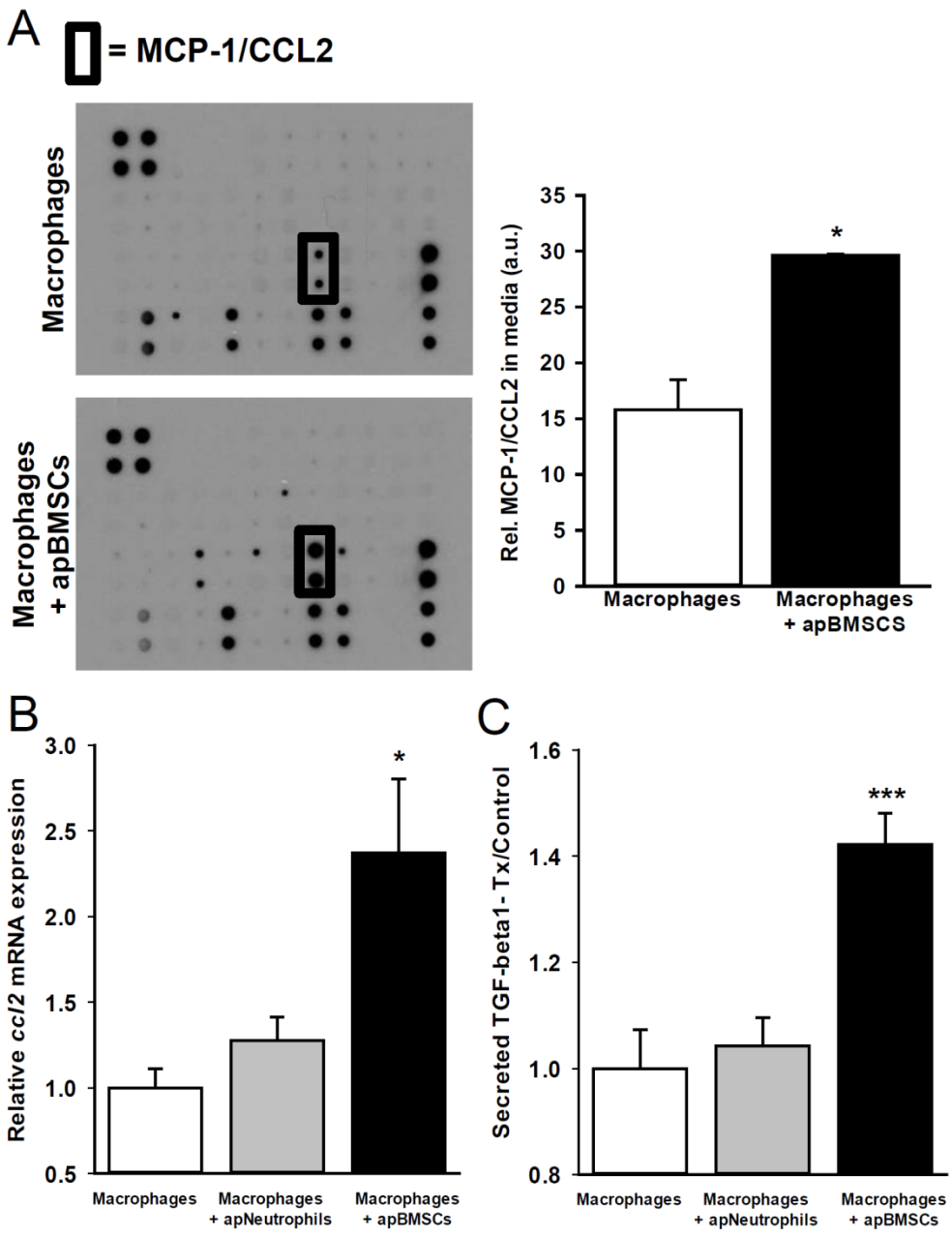

Figure 3.4. Macrophage efferocytosis induces CCL2 and TGF-β1 secretion. (A) Supernatants were harvested from BMMs, apBMSCs or co-culture of BMMs with apBMSCs for 18hrs and analyzed for secreted proteins using a murine inflammatory cytokine array. Co-culture of BMMs with apBMSCs showed significantly increased secreted CCL2 compared to BMMs or apBMSCs cultured alone (arbitrary units, a.u.). (B) RNA was isolated from BMMs alone or co-cultured with apBMSCs or apoptotic neutrophils and QRT-PCR performed for *ccl2* mRNA. *Ccl2* gene expression was increased in co-culture of BMMs with apBMSCs compared to BMMs alone. (C) ELISA for total TGF-β1 levels in the supernatants showed increased secreted TGF-β1 after 18hrs of co-culture of BMMs with apBMSCs compared to BMMs, n=6/gp data is mean ± SEM. * $p < 0.05$, ***$p < 0.001$ versus macrophages cultured alone.

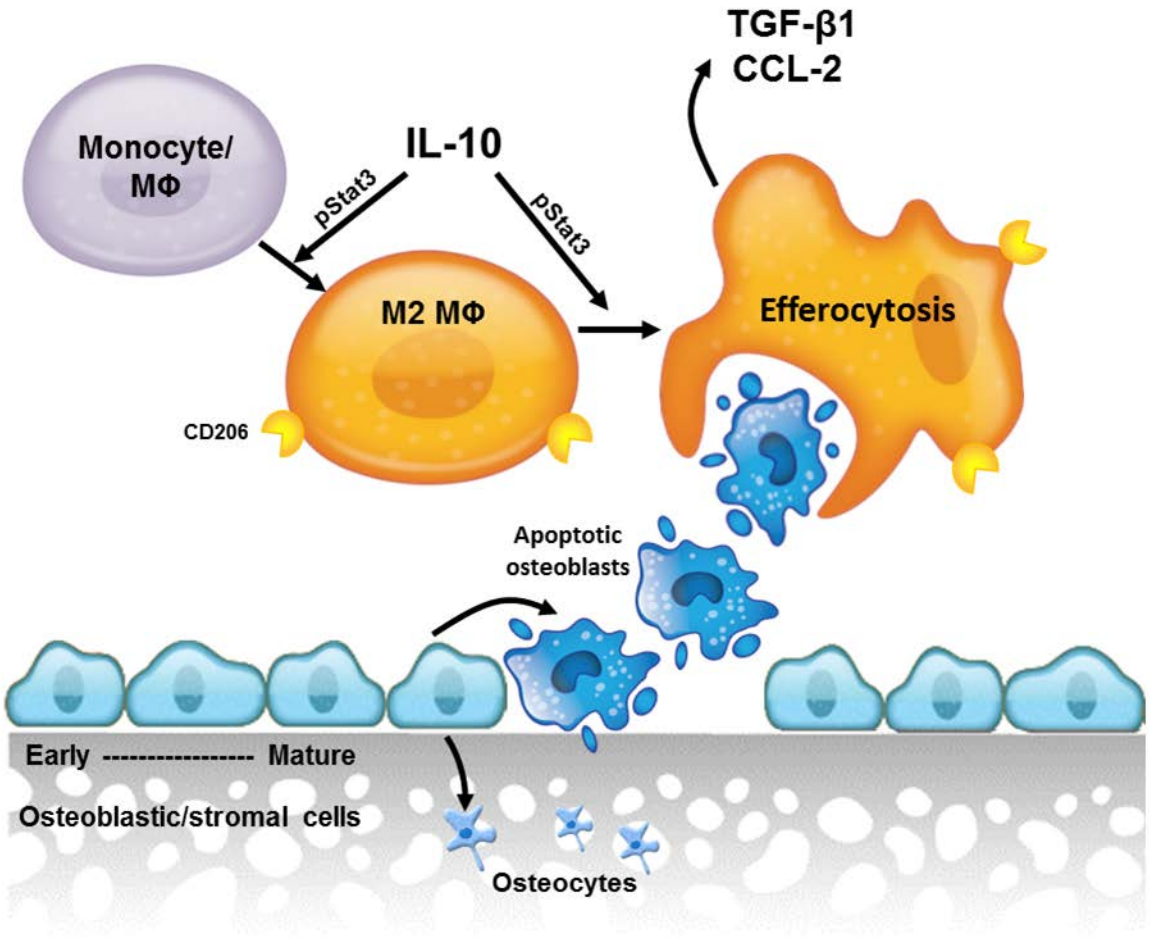

Figure 3.5. Model. IL-10 promotes M2 macrophage polarization and enhances clearance of apoptotic bone marrow stromal cells in a STAT3-phosphorylation dependent manner. Clearance of apoptotic cells results in release of anti-inflammatory and osteoinductive factors (TGF-β1 and CCL2) which aid in the recruitment of monocytes and osteoclast precursors.

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CHAPTER 4

MFG-E8 DEFICIENCY: A MODEL OF INFLAMM-AGING ASSOCIATED BONE LOSS RESCUED BY TERIPARATIDE

Abstract

Multiple processes become less efficient with age leading to chronic increases in pro-inflammatory cytokines, termed inflamm-aging [1]. This process has been associated with osteoporotic- and autoimmune-associated bone loss. Crucial components of anti-inflammatory pathways are associated with age-related bone loss. Milk fat globule-EGF 8 (MFG-E8) is a glycoprotein that is pro-resolving, regulates apoptotic cell clearance and has recently been linked to autoimmune disease and skeletal homeostasis. The role of MFG-E8 in the skeleton was determined with age in mice deficient in MFG-E8 (KO). In vivo, trabecular bone was similar in MFG-E8 KO and wildtype (WT) mice at 6 and 16wks, whereas 22wk MFG-E8 KO mice displayed significantly reduced trabecular bone. Osteoclast number per bone surface was increased in 22wk KO vs. WT and osteoclasts treated with recombinant murine MFG-E8 were decreased in number and size. Adult MFG-E8 KO spleen weight/body weight was increased compared to WT and FACs analysis showed significantly increased myeloid derived suppressor cells ($CD11b^{hi}GR\text{-}1^{+}$) and neutrophils ($CD11b^{hi}Ly6G^{+}$) in MFG-E8KO bone marrow, suggesting an increase in inflammation. Interestingly, iPTH-treated MFG-E8 KO mice showed a robust anabolic response in trabecular BV/TV, exceeding the response in iPTH-treated WT mice. The strong anabolic response in MFG-E8 deficient mice demonstrates that iPTH therapy may be

highly effective in models of age-related bone loss and/or inflammatory states. These data give insight into the role of MFG-E8 in aging, provide a new model of age-associated bone loss, and suggest anabolic PTH may be a valuable therapeutic approach for autoimmune-associated skeletal disease.

Introduction

Milk fat globule epidermal growth factor 8 (MFG-E8) is a secreted glycoprotein which was first identified in the mammary gland and then studied extensively in many other tissues [2-5]. One of the most prominent functions of MFG-E8 is to act as a bridge between apoptotic cells and phagocytic cells thus coordinating the engulfment of apoptotic cells, a process termed efferocytosis [6-9]. MFG-E8 has also been linked to other functions in the body including collagen clearance by lung alveolar macrophages [3], angiogenesis in cutaneous wound healing [10], phagocytosis in the retina [5], and polarization of tumor associated macrophages [11]. MFG-E8 is also an important regulator of the inflammatory response, and mice deficient in MFG-E8 have inflammatory phenotypes including intestinal colitis and systemic lupus erythematous (SLE)-like symptoms [7, 12, 13]. In humans, a genetic polymorphism of MFG-E8 correlated significantly to human SLE [14].

The role of MFG-E8 has recently emerged in bone where it was found to be a positive regulator of bone turnover [15] and a protective factor against rheumatoid arthritis [16]. MFG-E8 deficient mice have reduced bone mass as well as accelerated bone loss in a ligature-induced periodontitis model [17], yet the exact functional role of MFG-E8 in bone turnover is still unclear. MFG-E8 expression leads to an anti-inflammatory response. Chronic inflammatory states increase with age and support osteoclast differentiation and activity. The aging skeleton presents with reduced osteoblast activity and increased osteoclast activity leading to a net

reduction in bone [18, 19]. Additionally, with age, the body has reduced efficiency of a variety of processes and associated chronic elevation of pro-inflammatory cytokines. Age associated inflammation has been termed inflamm-aging [1]. The contributions of many inflammatory cytokines to age-associated osteoporosis have been studied and well characterized, however the role of resolving or anti-inflammatory cytokines are less well characterized. Due to the resolving nature of MFG-E8 it is hypothesized that it plays a role in age-associated inflammation and bone loss.

Teriparatide (human parathyroid hormone, hPTH 1-34) is an FDA approved injectable anabolic therapeutic used in cases of severe osteoporosis. Its mechanism has been widely studied and intermittent daily treatment (iPTH) results in an increase in overall bone turnover and a resultant increase in bone formation and bone mass. PTH effects on bone marrow cell populations have also been investigated and have been shown to alter marrow neutrophils and macrophages [20, 21]. The therapeutic potential of PTH in rescuing inflammation-induced bone loss is less understood. Additionally, therapeutic interventions to rescue MFG-E8 associated bone loss have not been investigated. The purpose of the present study was to assess the role of MFG-E8 in the aging skeleton and investigate an anabolic therapeutic intervention in MFG-E8 deficient mice.

Materials and Methods

Animals

All mice were maintained in accordance with institutional animal care and use guidelines, and experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Michigan. MFG-E8 deficient (KO) mice were originally created using a gene

trap vector and backcrossed into C57BL/6 background (WT controls) [2, 3]. Female mice were used for *in vitro* or *in vivo* experiments at ages 6, 16 or 22 weeks. To test the anabolic effect of intermittent parathyroid hormone (iPTH), 16 week old KO and WT mice were treated daily with recombinant human PTH (1-34) (Bachem, Torrance, CA) (50μg/kg, SC) or vehicle (0.9% saline, SC) for 6 weeks. MFG-E8KO mice were crossed with Mertk KO mice (Jackson Laboraties) to create double MFG-E8/Mertk KO mice (dKO). Skeletal phenotypes were assessed at 6, 16, and 22 weeks and were treated in the same manner as KO mice with iPTH.

Complete Blood Counts (CBC)

Blood was harvested at time of euthanasia via intracardiac puncture from mice at ages 6, 16, and 22wks, collected in BD Microtainer Tubes with K2E (K_2EDTA), and analyzed for CBC with differential.

Serum ELISAs

Mice underwent food and water restriction for 6 hours prior to serum collection. Blood was harvested as above, and placed in non-EDTA containing microcentrifuge tubes, allowed to coagulate for at least one hour at room temperature, spun down at 8000 rpm for 10 minutes, and liquid serum collected into new microcentrifuge tubes. Samples were stored at -20°C until use. Enzyme immunoassays were used to measure the serum concentrations of tartrate-resistant acid phosphatase form 5b (TRAcP 5b), propeptide of type I procollagen (P1NP) and C-telopeptide of type I collagen (CTX-I) according to manufacturer instructions (IDS) and measured on an EZ Read 400 microplate reader (Biochrom).

Fluorescence Activated Cell Sorting (FACs) Analysis

Bone marrow was isolated from the femur via flushing in FACs buffer (1X PBS with 2%FBS, 0.5mMEDTA), and 10^6 cells stained with anti-mouse F4/80 (APC, Abcam, A3-1), anti-mouse CD68 (FITC, Biolegend, FA-11), anti-mouse CD11b (APC, Biolegend, M1/70), anti-mouse Gr-1 (FITC, Biolegend, RB6-8C5), and/or anti-mouse Ly6G (FITC, Biolegend, 1A8). Isotype controls were used to confirm antibody specificity. FACs analysis was performed using a FACs Aria III (BD).

MicroCT (μCT)

Tibiae were harvested from 6, 16 and 22wk old mice and fixed in 10% NBF for 24-48hrs at 4°C, then stored in 70% ethanol. Tibiae were scanned by micro-computed tomography at a 12μm voxel size (Scanco μCT-100) as previously described [20] and following established guidelines [22]. Trabecular bone was measured starting 360μm distal to the top of the proximal growth plate and extending 600μm distally with a threshold of 180 mg/cm^3. Trabecular bone morphometric variables analyzed included bone volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular spacing (Tb.Sp) and trabecular bone mineral density (Tb.BMD). Cortical bone was measured starting 3mm proximal to the tibia-fibula junction and extended 360μm with a threshold of 280 mg/cm^3. Cortical bone morphometric variables analyzed included total volume (Tt.V), cortical volume (Ct.V), cortical volume fraction (Ct.V/Tt.V), cortical thickness (Ct.Th), and bone mineral density (BMD).

TUNEL Staining

Spleens and tibiae were fixed in 10% NBF for 24-48hrs at 4°C. Tibiae were decalcified in 14%

EDTA for 10-14 days. Spleens and tibiae were processed, embedded in paraffin, and sectioned at 5μm. Sections were stained for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) positive cells (*In Situ* Cell Death Detection Kit, TMR red, Roche). TUNEL positive cells were quantified in the white pulp of the spleen and in the bone marrow of 22wk old WT and KO mice.

Static Histomorphometry

Tibiae were fixed in 10% NBF for 24-48hrs at 4°C, decalcified in 14% EDTA for 10-14 days, embedded in paraffin, and sectioned at 5μm. A central slice of the proximal tibiae was stained with hematoxylin and eosin (H&E) or tartrate resistant acid phosphatase (TRAP, Sigma 387A) and bone morphometry or osteoclast quantification performed as described [23] and according to standards set by the ASBMR [24]. The ROI was manually defined, beginning 200μm distal to the proximal growth plate and extending 1200μm distally.

Dynamic Histomorphometry

Five and two days prior to sacrifice, mice were administered calcein (Sigma Aldrich, 30mg/kg, IP). Tibiae were harvested and fixed in 10% NBF for 24-48hrs and stored in 70% ethanol. Undecalcified tibiae were embedded in methylmethacrylate, sectioned (8μm), and dual-labeled surfaces quantified as previously described [25]. Bone formation rate (BFR/BS) and mineral apposition rate (MAR) were analyzed.

In vitro osteoblast mineralization assays

Calvarial osteoblasts were isolated from day 4-10 old KO and WT mice, expanded and induced

to differentiate and mineralize as previously described [26]. RNA was harvested and processed using RNeasy Mini Kit (Qiagen). Reverse transcription PCR was conducted, and the cDNA products were amplified and detected using TaqMan Universal PCR master mix (Applied Biosystems) and TaqMan probes, including mouse *bglap* (Mm03413826_m1), *runx2* (Mm00501584_m1) and mouse *actb* (Mm02619580_g1) as an endogenous control. Realtime PCR was analyzed on ABI PRISM 7700 (AppliedBiosystems).

Bone marrow stromal cells (BMSCs) were obtained from flushing bone marrows of 6 and 22wk old KO and WT mice with plain α-MEM, centrifuging and resuspending with α-MEM medium (20% FBS, Pen/Strep, glutamine) containing 10nM dexamethasone (Sigma). BMSCs were seeded at 2 x 10^5 cells/well in 24-well plates, grown to confluency, and treated with β-glycerophosphate and ascorbic acid to induce mineralization. Cells were fixed and stained using the von Kossa method to assess mineralized nodules at day 7, 14, and 21 [27].

In vitro osteoclast assays

Bone marrow from 6 week old KO or WT mice was extracted into 100mm dishes in complete α-MEM medium (10% FBS, Pen/Strep, glutamine). The following day, non-attached cells were re-plated onto petri dishes and treated with murine M-CSF (30ng/mL eBioscience) for 4-5 days. Cells were then split with 10μM EDTA in ice cold PBS and re-plated at 60,000/cm2 in 48 or 96 well plates with M-CSF (30ng/ml) and murine RANKL (50ng/mL) (Peprotech). Osteoclastic cells were identified via TRAP staining (Sigma 387A kit) or seeded onto Corning Osteoassay plates to measure resorptive functional activity. Osteoclast assays were performed as above using bone marrow from 22wk old WT mice. At the time of osteoclast differentiation induction using

RANKL, cell cultures were treated with rmMFG-E8 (500ng/mL, R&D Systems) or BSA control (500ng/mL, Millipore). Cultures were stained for TRAP and quantified.

Efferocytosis

Bone marrow macrophages were assessed for efferocytic capacity of apoptotic BMSCs or apoptotic thymocytes. Bone marrow from 6 or 22 week old WT or KO mice was harvested and cultured in macrophage differentiation media (α-MEM, 10% FBS, Pen/Strep, glutamine, 30ng/mL M-CSF) for 7 days and replated in 6 well plates at a density of 1.5 x 10^6/well. BMSCs were harvested from 6-8 week old KO and WT mice via bone marrow flush and cultured in α-MEM medium (20% FBS, Pen/Strep, glutamine) containing 10nM dexamethasone (Sigma). BMSCs were grown to confluency, dissociated from tissue culture plate using 0.25% trypsin-EDTA and resuspended in 1X PBS. BMSCs were stained with CellTrace™ CFSE Cell Proliferation Kit (2μM, Invitrogen). Apoptosis was induced by exposure to UV light for 30 min and cells recovered at 37°C for 2hrs. The thymus was dissected from 6-10 wk old KO or WT mice in ice cold 1X PBS, pressed though a 70μm cell strainer, red blood cells lysed with 1X ACK, and resuspended in complete α-MEM plus 0.1μM dexamethasone. Thymocytes were incubated at 37°C for 16hrs to induce apoptosis, then stained with pHrodo™ succinimidyl ester (SE) (20ng/mL, Invitrogen)[28]. Apoptotic BMSCs and thymocytes were counted via trypan blue exclusion and resulted in 80-95% apoptosis. Apoptotic cells (BMSCs or thymocytes) were cultured with macrophages at a 1:1 ratio in plain α-MEM for 2 hours, fixed in 1% PFA, stained for F4/80-APC (Abcam, A3-1). Efferocytosis was measured via flow cytometric analysis for double labeling APC-CFSE (engulfed apoptotic BMSCs) or APC-pHrodo-SE (engulfed thymocytes).

Statistical Analysis

Statistical analyses were performed by unpaired Student's t test to compare two groups or ANOVA to compare three or more groups with a significance of $p < 0.05$. Data are presented as mean ± standard error of mean (SEM).

Results

MFG-E8 deficiency leads to age-associated osteopenia

The skeletal phenotypes of female MFG-E8 deficient mice were assessed at 6, 16 and 22 weeks and compared to age matched WT controls. At 6 weeks of age, MFG-E8 and WT mice had similar trabecular BV/TV (**Figure 4.1A, B**) and Ct.V/Tt.V (**Figure 4.1C**). With age, MFG-E8 deficient mice had lower bone mass compared to WT, with significantly decreased trabecular BV/TV at 22 weeks (**Figure 4.1A, B**) and significantly reduced cortical bone at 16 and 22 weeks (**Figure 4.1C**). Serum ELISAs of bone formation (**Figure 4.1D**) and resorptive markers (**Figure 4.1E, F**) showed similar trends in KO and WT with age. Serum TRAcP 5b, a marker reflecting osteoclastic cells quantitatively in the body, was decreased in MFG-E8 KO mice at 22wks of age compared to WT (**Figure 4.1E**). In contrast, CTX-I, a marker of osteoclast functional activity was increased in MFG-E8 KO mice at 22wks compared to WT controls (**Figure 4.1F**).

Increased osteoclasts in 22wk KO mice

Trabecular bone analysis of the tibia confirmed KO mice had statistically decreased trabecular BV/TV (-32%, **Figure 4.2A**), unchanged Tb.Th (-5%, **Figure 4.2B**), decreased Tb.N (-15%, **Figure 4.2C**), unchanged trabecular spacing (+8%, **Figure 4.2D**), and decreased trabecular BMD (-33%, **Figure 4.2E**) compared to WT control mice. Cortical volume fraction

and cortical thickness were statistically decreased 4% and 9% respectively in KO mice compared to WT (**Figure 4.2F, G**). Dynamic histomorphometry was performed in mice at 22wks age with no significant changes observed in bone formation (BFR/BS) or mineral apposition rate (MAR) between KO and WT mice (**Figure 4.2 H, I**). Osteoclast number (N.Oc/BS) per bone surface was increased in 22wk old KO mice compared to WT (**Figure 4.2J**). These data suggest the reduced bone phenotype in 22wk old KO mice is in part due to an increase in osteoclasts.

Loss of MFG-E8 results in altered immunologic profile in spleen and bone marrow

In order to evaluate the immunologic impact of MFG-E8, spleens were harvested and weighed from 22wk old KO and WT mice. KO mice had significantly increased spleen weight per body weight compared to WT mice (**Figure 4.3A**). FACs analysis of bone marrow populations revealed KO mice had significantly increased neutrophils ($CD11b^{hi}Ly6G^{+}$, **Figure 4.3B**) and myeloid-derived suppressor cells (MDSCs, $CD11b^{hi}Gr\text{-}1^{+}$, **Figure 4.3C**) compared to WT. $F4/80^{+}$ (murine macrophages) and $CD68^{+}$ (macrophage and dendritic cells) populations were not changed in KO mice compared to WT (data not shown). Spleens and tibiae were fixed, embedded in paraffin, sectioned and stained for TUNEL positive cells reflecting cell death. TUNEL-positive cells were unchanged in the bone marrow of 16 and 22wk old KO and WT mice, suggesting apoptotic cells clearance in the marrow may be facilitated via other efferocytic pathways (**Figure 4.3D**). *In vitro* efferocytosis studies were performed to assess the effect of MFG-E8 deficiency on bone marrow macrophage engulfment of apoptotic cells. No significant alterations in engulfment of apoptotic BMSCs or thymocytes were seen *in vitro* (data not shown). Interestingly, TUNEL positive cells were increased in the white pulp of spleens from

22wk old KO mice compared to WT (**Figure 4.3E**) which might explain a site specific efferocytic function of MFG-E8.

Impact of MFG-E8 on cellular activity: osteoblastic and osteoclastic cells

To better understand the cellular contributions to the age-related skeletal phenotype seen in the MFG-E8 deficient mice, a series of *in vitro* assays were performed. Calvarial osteoblasts were isolated from 4-10 day old KO or WT mice, expanded and mineralization or gene expression analyzed. KO calvarial osteoblast preparations showed increased mineralized nodules compared to WT but no changes in *runx2* or *osteocalcin* gene expression were detected (**Figure 4.4A**). BMSCs were cultured from 6 and 22 week old WT and KO mice and induced to differentiate. BMSCs from 6 week old KO mice had increased mineralized nodules compared to WT mice after 14 days of culture (**Figure 4.4B, left**). Interestingly, BMSCs from 22 week old mice showed the opposite trend. KO BMSCs displayed decreased mineralized nodules compared with WT mice at 14 and 21 days of culture (**Figure 4.4B, right**). *In vitro* osteoclast assays from 6 week old mice revealed no significant alterations in osteoclast differentiation or resorptive capacity between WT and KO mice (**Figure 4.4C**). Osteoclasts from 22 week old mice WT were cultured with rmMFG-E8 or BSA control, stained for TRAP and quantified. Treatment with rmMFG-E8 significantly reduced osteoclast formation and size (**Figure 4.4D**).

Intermittent PTH treatment leads to robust anabolic response in KO mice

MFG-E8 KO and WT mice (16 week) were treated daily with iPTH or vehicle for 6 weeks to evaluate the therapeutic potential of a known anabolic bone agent (**Figure 4.5A**). All data are presented as treatment (PTH) over control (vehicle). PTH increased spleen weight in

both WT and KO mice (tx/control > 1.0, **Figure 4.5B**). PTH did not alter the $CD11b^{hi}Ly6G^{+}$ populations in WT and KO mice whereas PTH increased marrow $CD11b^{hi}Gr\text{-}1^{+}$ cells in WT but not KO mice (**Figure 4.5C**). Complete blood counts from mice WT and KO treated with vehicle or PTH showed PTH treatment decreased the percent neutrophils in the peripheral blood in both WT and KO treated mice (**Table 4.1**). Red blood cell MCV and MCH were significantly increased in KO mice compared to WT mice, and PTH further increased these parameters in KO mice (**Table 4.1**).

Adult mice treated with vehicle or PTH were assessed for skeletal phenotypes. Static histomorphometry of tibia showed an anabolic response in both WT and KO mice in the proximal tibia (**Figure 4.5D**). Trabecular bone analysis of the tibia via μCT showed both WT and KO mice responded to iPTH treatment (tx/control > 1.0, **Figure 4.5 E-H**). KO mice showed a stronger anabolic response to PTH in BV/TV (**Figure 4.5E**), Tb.N (**Figure 4.5G**) and Tb.BMD (**Figure 4.3H**) than PTH treated WT mice. KO and WT had similar cortical bone anabolic responses to PTH (**Figure 4.5I, J**).

Mertk is an efferocytic receptor on macrophages. MFG-E8/Mertk double KO (dKO) mice were generated to assess the effect of further decreasing a different efferocytic pathway. The resulting phenotype was similar in the dKO mice as the MFG-E8KO phenotype. Double KO mice had decreased bone with age (**Figure 4.7A, B**) and responded to iPTH treatment to a greater extent than WT controls (**Figure 4.7C**). These data suggest Mertk deficiency does not lead to a stronger phenotype than MFG-E8 deficiency alone.

KO mice display increased osteoclasts per surface, rescued by PTH treatment

The serum formation marker P1NP and serum resorptive markers TRAcP 5b and CTX-I

were all increased with iPTH treatment in both KO and WT mice (**Figure 4.6A-C**). The increase in serum P1NP was larger in iPTH treated KO than WT mice (**Figure 4.6A**). Bone formation (BFR/BS) and mineral apposition rate (MAR) were increased in both iPTH treated WT and KO mice, but KO mice showed a lower response in dynamic bone formation parameters with iPTH treatment versus WT (**Figure 4.6D,E**). The increase in osteoclast number (N.Oc/BS) per bone surface in KO mice compared to WT was reduced with iPTH treatment in KO mice (**Figure 4.6F**).

Discussion

MFG-E8 is a known anti-inflammatory mediator. It is well accepted that chronic inflammation increases during the aging process leading to the upregulation of pro-inflammatory mediators. Chronic increases in inflammatory cytokines are seen in post-menopausal osteoporosis, a disease whose pathology is related to increased osteoclast differentiation and activity [29]. The current study describes the contributions of MFG-E8 in the aging skeleton. MFG-E8 deficient mice developed a skeletal phenotype that became apparent with age. At 16 weeks of age, MFG-E8 KO mice showed a trend of decreased trabecular bone and significantly decreased cortical bone compared to WT and displayed significantly decreased trabecular and cortical bone at 22 weeks of age. These data suggest that MFG-E8 is a contributor to bone turnover in adult bone. Interestingly, a previous report of MFG-E8 contributions to bone showed decreased vertebral trabecular bone volume fraction in MFG-E8 deficient mice as early as 6 weeks of age [15], and may describe a location specific effect. The difference of onset of osteopenic phenotype between these two models may also represent differences in the development of the genetic knockouts. The genetic model presented here was developed by

inserting the pGT1-pfs gene trap vector in intron 7 of *Mfge8*, leading to protein degradation [2]. Sinningen et al. [15] used a KO model that was generated by replacing exons 2 to 6 of *Mfge8* with a neomycin resistance cassette [30]. This suggests that disrupting the proper transcription of the gene may result in a more dramatic phenotype present at an earlier age whereas the prevention of protein secretion results in a phenotype that becomes apparent with time.

Interestingly, while male mice did have a reduction in trabecular BV/TV at 22wks of age (data not shown), the phenotype was more dramatic in female mice. Sinningen et al. [15] also saw a reduced bone phenotype in female mice, suggesting a possible sex-specific phenotype which should be further explored; however information about a male phenotype was not discussed. Given that MFG-E8 is highly expressed in mammary gland tissue and is important in mammary gland development and involution [2], it may be associated with hormonal controls related to sex steroids that in turn impact a gender specific effect on the skeleton.

The reduced bone phenotype in MFG-E8 KO mice has been attributed to increased osteoclast numbers; via not clearly delineated mechanisms [17]. In concert with an osteoclastic phenotype, administration of recombinant MFG-E8 protein to inflammation-induced periodontal bone defects decreased bone loss [17]. An inflammatory phenotype was not previously detailed in the bones of MFG-E8 deficient mice. Given the relationship between inflammation and osteoclast activation, we aimed to identify if the reduced bone phenotype in MFG-E8 deficient mice was due in part to an enhanced inflammatory environment. Adult MFG-E8 KO mouse spleen weights were increased and bone marrow neutrophils and MDSCs were increased, which is consistent with increased inflammatory phenotype. These data are consistent with previous findings that MFG-E8 KO mice have increased spleen size at 40 weeks of age [7] and display signs of inflammation in other tissues [13]. In the present study, MFG-E8 KO mice showed

increased osteoclast number per bone surface and increased serum CTX-I levels consistent with an inflammatory induced osteoclastogenesis. Proinflammatory cytokines increase osteoclastic differentiation and activity via upregulation of RANKL [31]. Increased proinflammatory cytokine production has been associated with systemic and/or local bone loss in patients with inflammatory diseases [32, 33] including systemic lupus erythematous (SLE) [34], rheumatoid arthritis [35-37], inflammatory bowel disease [38, 39], and periodontal disease[40]. Recombinant MFG-E8 protein decreased osteoclast differentiation suggesting that MFG-E8 signaling directly affects pathways important to osteoclast differentiation and may be a potential targeted treatment for inflammatory bone loss. These findings are similar to recently published articles detailing the contributions of MFG-E8 to osteoclast differentiation and function [15-17], and extends these findings into an adult mouse model.

A distinct function of MFG-E8 is to act as a bridge between apoptotic cells and phagocytes to facilitate engulfment of dead cells [6]. Accumulations of apoptotic cells lead to increased pro-inflammatory cytokine production. Polymorphisms of MFG-E8 have been found in cases of SLE, which is characterized by decreased apoptotic cell clearance as well as decreased bone mass [41]. The efferocytic capacity of bone marrow macrophages was unchanged *in vitro* in MFG-E8 deficient mice. TUNEL positive cell populations trended downward in the bone marrow and TUNEL staining of spleens, revealed increased apoptotic bodies in the white pulp of the spleen. This phenotype, consistent with previous literature [7], suggests that *in vivo* there is an alteration in apoptotic cell clearance in MFG-E8 deficient mice. The increase in TUNEL positive cells in MFG-E8 deficient spleens but not so in the bone marrow suggests compensatory efferocytic mechanisms may be more operative and critical in the bone marrow environment. Alternatively, the data presented here may also suggest MFG-E8

is more dispensable in bone in regards to efferocytic clearance of apoptotic cells. However, the increase in marrow neutrophils and MDSCs may in fact reflect a reduced efferocytic environment in the marrow. Ineffective efferocytosis leads to increased inflammatory cytokines that support the increase in these cells [42]. Further understanding of the pathways which are most important in the marrow space will help delineate if and how the process of apoptotic cell clearance regulates bone turnover.

MFG-E8 deficiency resulted in increased osteoclasts due to an enhanced inflammatory environment. Currently, therapeutic interventions for patients with inflammatory bone loss include anti-resorptives such as bisphosphonates as well as anti-inflammatory targeted therapies. Intermittent PTH administration has been extensively studied for its anabolic effects in bone. It is FDA approved but is limited to use in cases of severe osteoporosis. Better understanding of phenotypes in which PTH may be a beneficial therapeutic could lead to more targeted use of the anabolic agent as well as the latest anabolic agent abaloparatide which interacts with the same receptor at PTH [43]. In adult MFG-E8 KO mice, PTH was an effective therapeutic and surprisingly resulted in a larger anabolic response in KO versus WT mice. Additionally, iPTH treatment decreased the number of osteoclasts per bone surface in the adult KO mice and brought osteoclast numbers to the level of WT vehicle mice. Treatment with iPTH has been shown to both decrease peripheral neutrophils [20] as well as polymorphonuclear leukocyte infiltration in healing oral tissue [44]. In our model, iPTH treatment similarly decreased peripheral neutrophil numbers. These data suggest iPTH therapy may alter the inflammatory phenotype and be beneficial in treatment bone loss due to inflammation. PTH increases specialized pro-resolving factors in the bone marrow including resolvin D1, D2 and lipoxins suggesting iPTH therapy aids the resolution of inflammation [45]. A previous study of the application of iPTH in a model of

rheumatoid arthritis showed PTH repaired local erosions [46] and a clinical trial of local PTH application to periodontal defects showed enhanced bone regeneration in PTH treated lesions [47]. Collectively, these data suggest PTH may be a particularly effective therapeutic in certain types of inflammatory bone disease, yet future studies are necessary to confirm its therapeutic benefit and further delineate mechanisms.

In conclusion, our data show that MFG-E8 deficiency leads to an altered immunologic profile in the bone marrow, is associated with bone loss with age, and is responsive to intermittent PTH therapy.

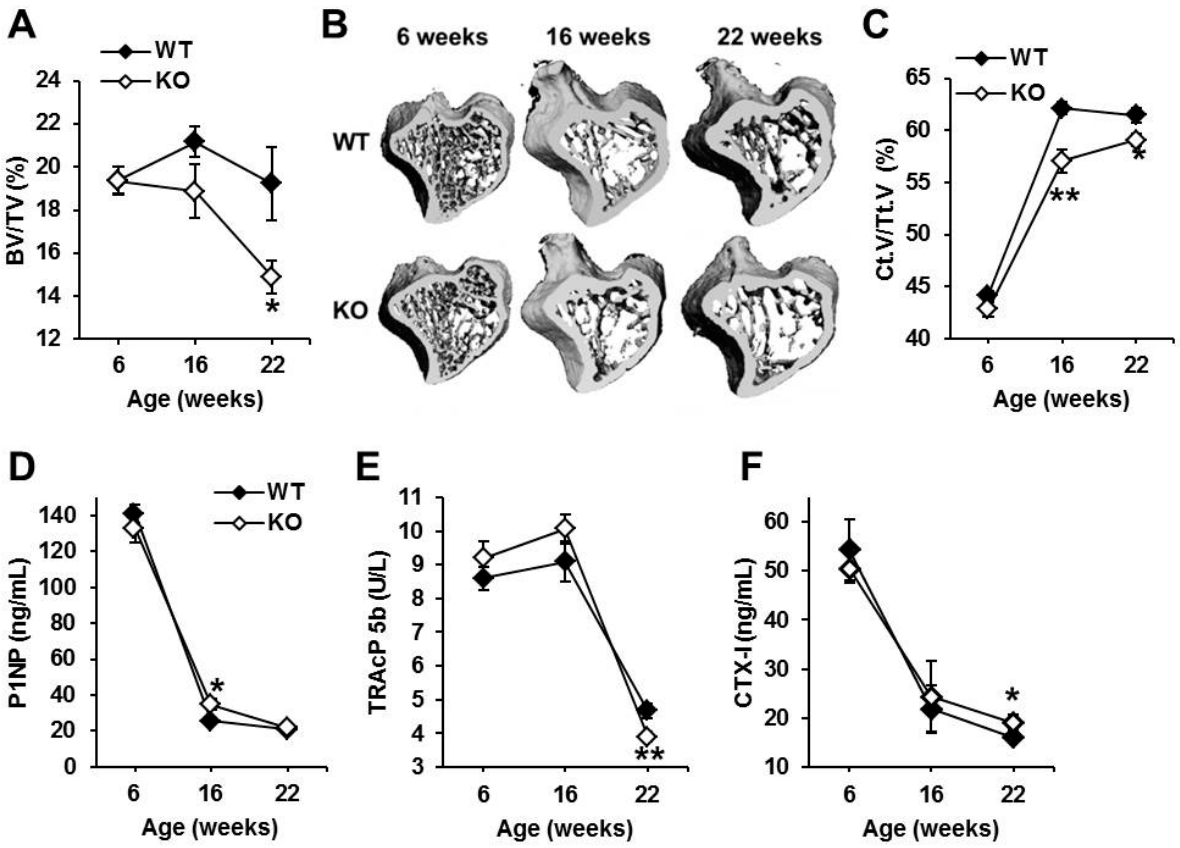

Figure 4.1. MFG-E8 KO mice have reduced bone mass with age. Tibiae harvested from 6, 16, and 22wk old MFG-E8 KO and WT female mice were analyzed for trabecular BV/TV (A, B) and cortical volume per total volume (Ct.V/Tt.V) (C) by μCT. (D) Serum formation marker P1NP and serum resorptive markers TRAcP 5b and CTX-I were measured via ELISA at the time of sacrifice. n=8-13/group, *$p<0.05$, **$p<0.01$, data are mean ± SEM.

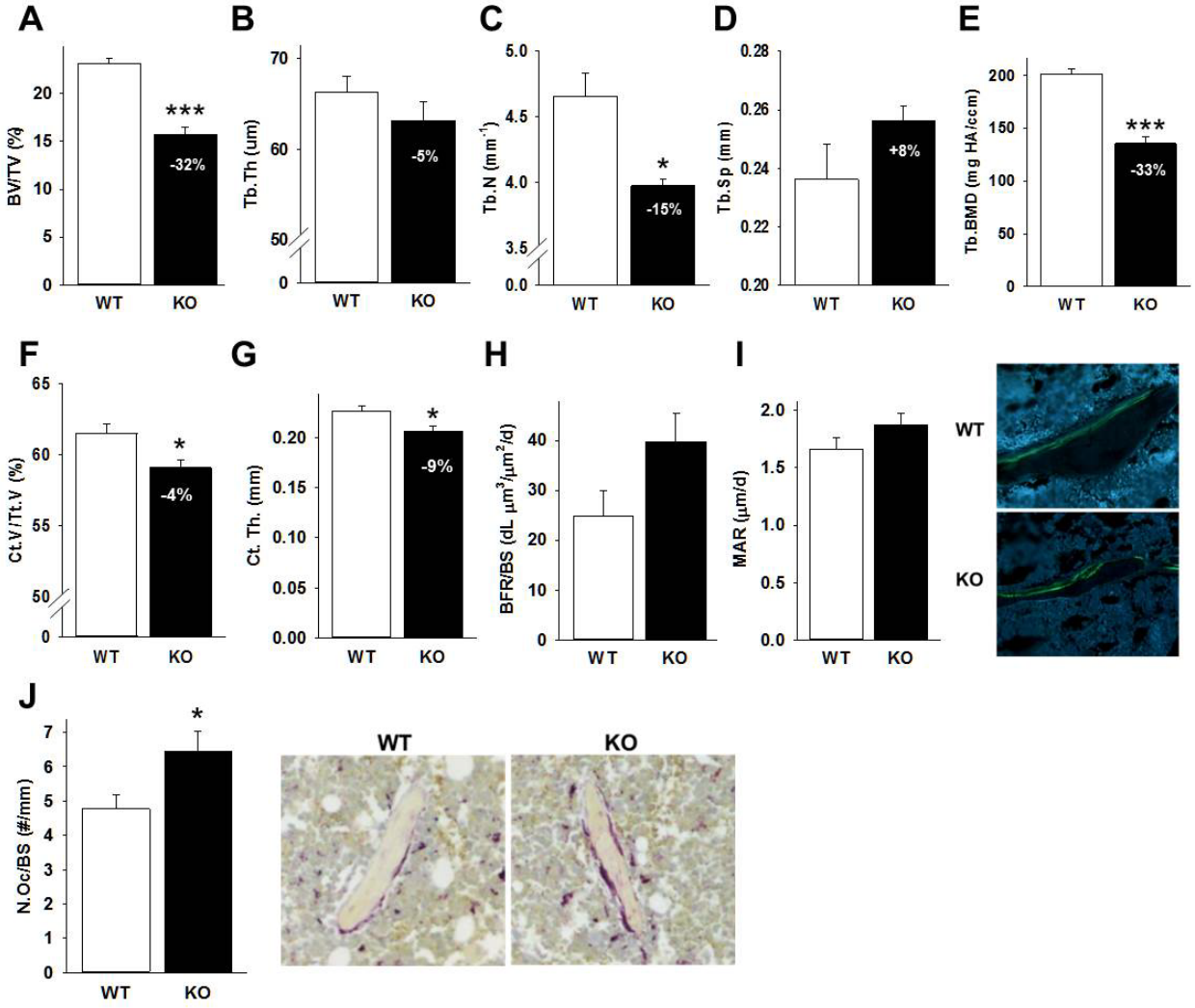

Figure 4.2. Adult (22wk) KO mice have reduced bone mass and increased osteoclast numbers. (A-E) Tibiae were harvested from female 22 week old WT and KO mice and trabecular parameters quantified via µCT. KO mice displayed significantly reduced trabecular BV/TV (A), Tb.N (C), and Tb.BMD (E). Trabecular thickness (B) and trabecular spacing (D) were not significantly different in WT and KO mice. (F, G) Cortical bone was measured in the midshaft of the tibia of 22 week old WT and KO mice. KO mice had reduced Ct.V/Tt.V (F) and Ct.Th (G) compared to WT control mice. (H, I) Mice were administered calcein (30mg/g, I.P.) 5 and 2 days prior to sacrifice. Dynamic histomorphometric analyses were performed in the cancellous bone of the proximal tibia. Sections were analyzed for bone formation rate (BFR/BS) and mineral apposition rate (MAR). No significant differences were seen between WT and KO mice. (J) Paraffin embedded tibiae were sectioned and stained for TRAP. TRAP+ multinucleated osteoclasts were quantified per bone surface (N.Oc/BS). Adult KO mice had increased N.Oc/BS compared to WT controls. Representative images on right. n=6-11/group, *p<0.05, **p<0.01, ***p<0.001, data are mean ± SEM.

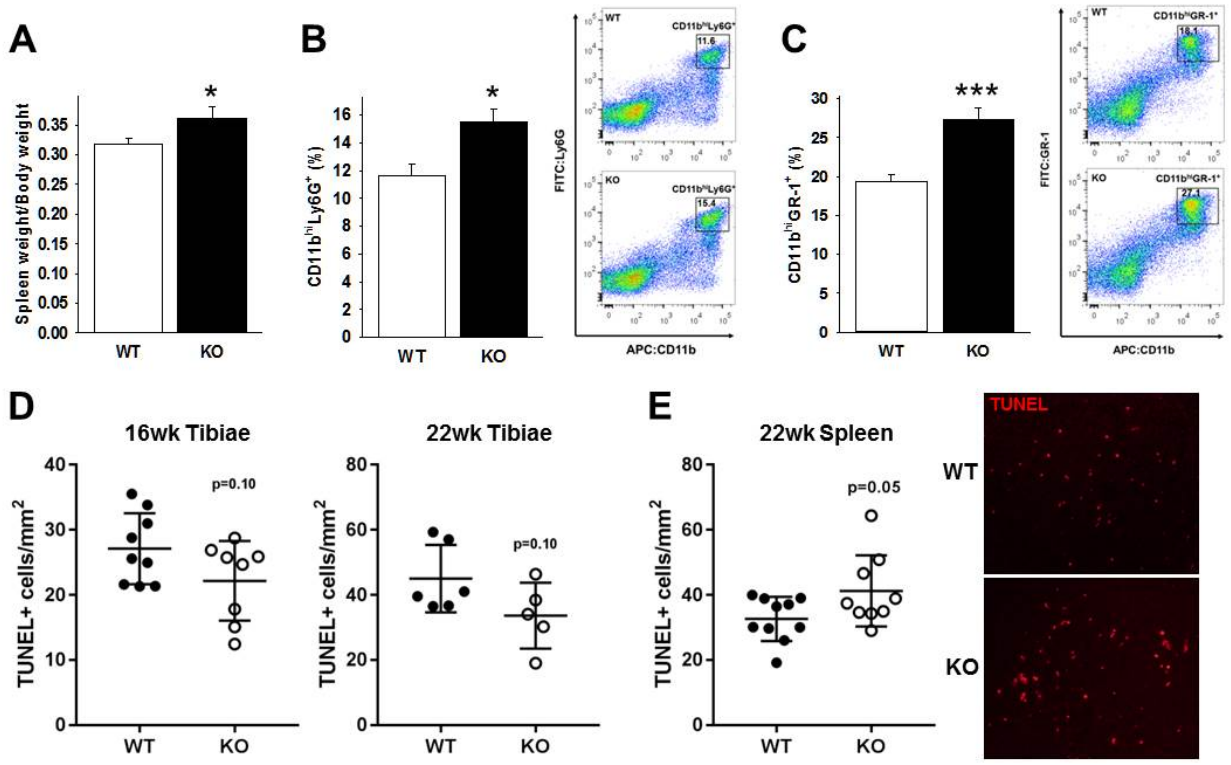

Figure 4.3. Adult KO mice have increased spleen size and greater numbers of marrow neutrophils and myeloid cells. (A) Spleens were harvested at time of sacrifice, weighed and compared to body weight. Spleen weight per body weight was increased in 22 week old KO versus WT mice. n=10-11/group, *p<0.05, ***p<0.001, data are ± SEM. (B, C) Marrow was flushed from femora of 22wk old WT and KO mice and stained for flow cytometric analysis of $CD11b^{hi}Ly6G^{+}$ (neutrophils) and $CD11b^{hi}Gr\text{-}1^{+}$ (immature myeloid cells) populations. KO mice displayed significantly increased $CD11b^{hi}Ly6G^{+}$ and $CD11b^{hi}Gr\text{-}1^{+}$ cells compared to WT. n=6-8/group, *p<0.05, ***p<0.001, data is mean ± SEM. (D , E) Tibiae and spleens were processed, embedded in paraffin, sectioned and stained for TUNEL positive cells reflecting cell death.

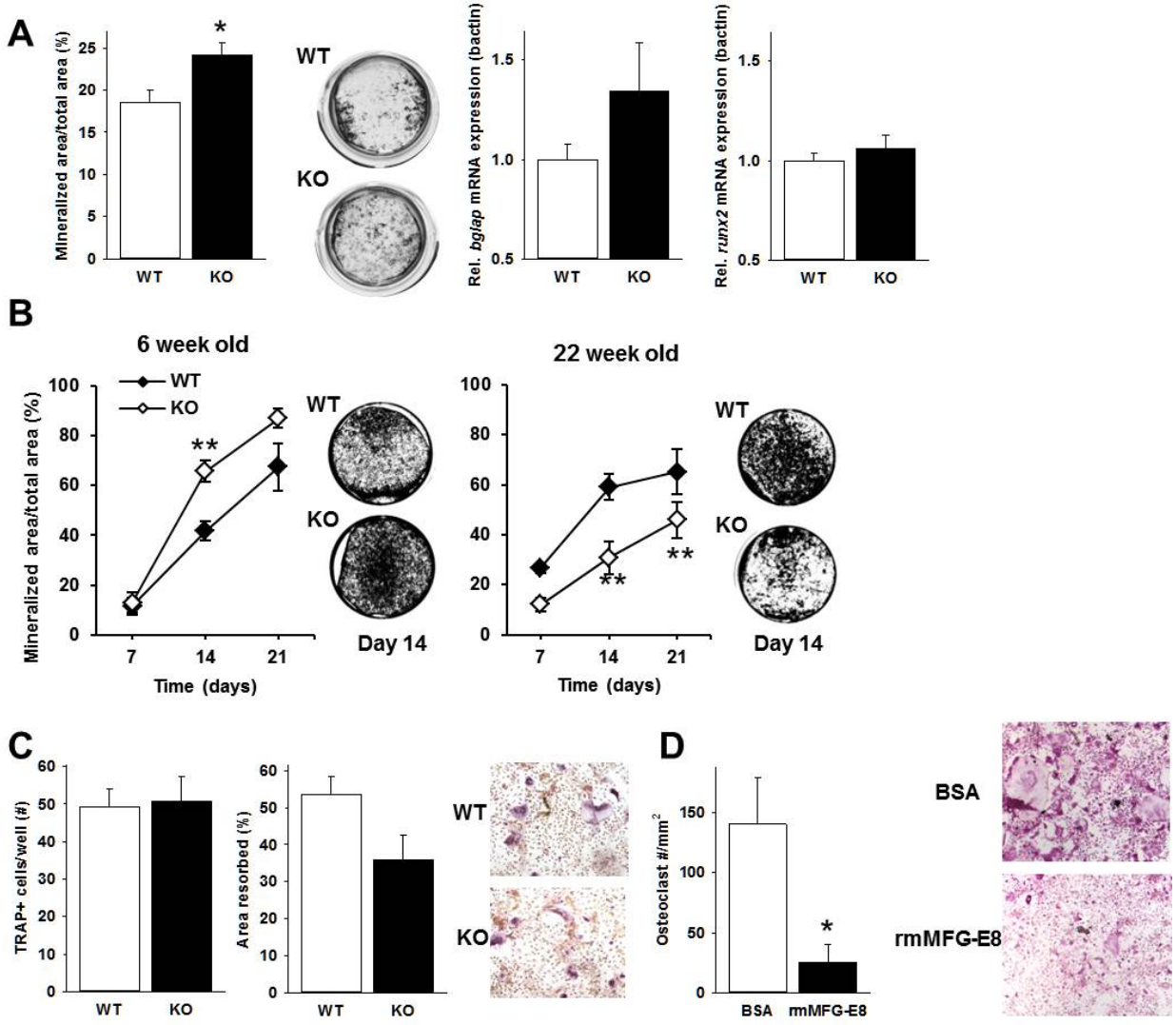

Figure 4.4. Aged KO BMSCs had decreased mineralization. (A) Calvarial osteoblasts were cultured from day 4-10 old KO and WT mice, expanded and induced to differentiate and mineralize. KO calvarial osteoblasts displayed increased mineralization. *Runx2* and *bglap* expression were measured at day 7 of differentiation and no changes were observed. (B) BMSCs were cultured from 6 and 22wk mice, induced to differentiate toward osteoblasts, and mineralized nodules measured via von Kossa staining. Six week KO BMSCs had similar mineralization to WT at d7 and d21 of culture with increased mineralization at d14. Twenty-two wk KO BMSCs showed decreased mineralization at d14 and d21 compared to WT. Representative von Kossa stained wells from 6 and 22wk WT and KO BMSCs (d14). n=4-5/group, **p<0.01, data is ± SEM. (C) Osteoclasts were derived from 6wk old KO and WT mice. Osteoclast differentiation and resorptive activity were unchanged between WT and KO. (D) Osteoclasts were derived from 22wk old mice and treated with BSA or rmMFG-E8 (500ng/mL). Osteoclasts were stained for TRAP. Treatment with rmMFG-E8 significantly reduced osteoclast number and size.n=3/group repeated 2-3 times, *p<0.05, data are mean ± SEM.

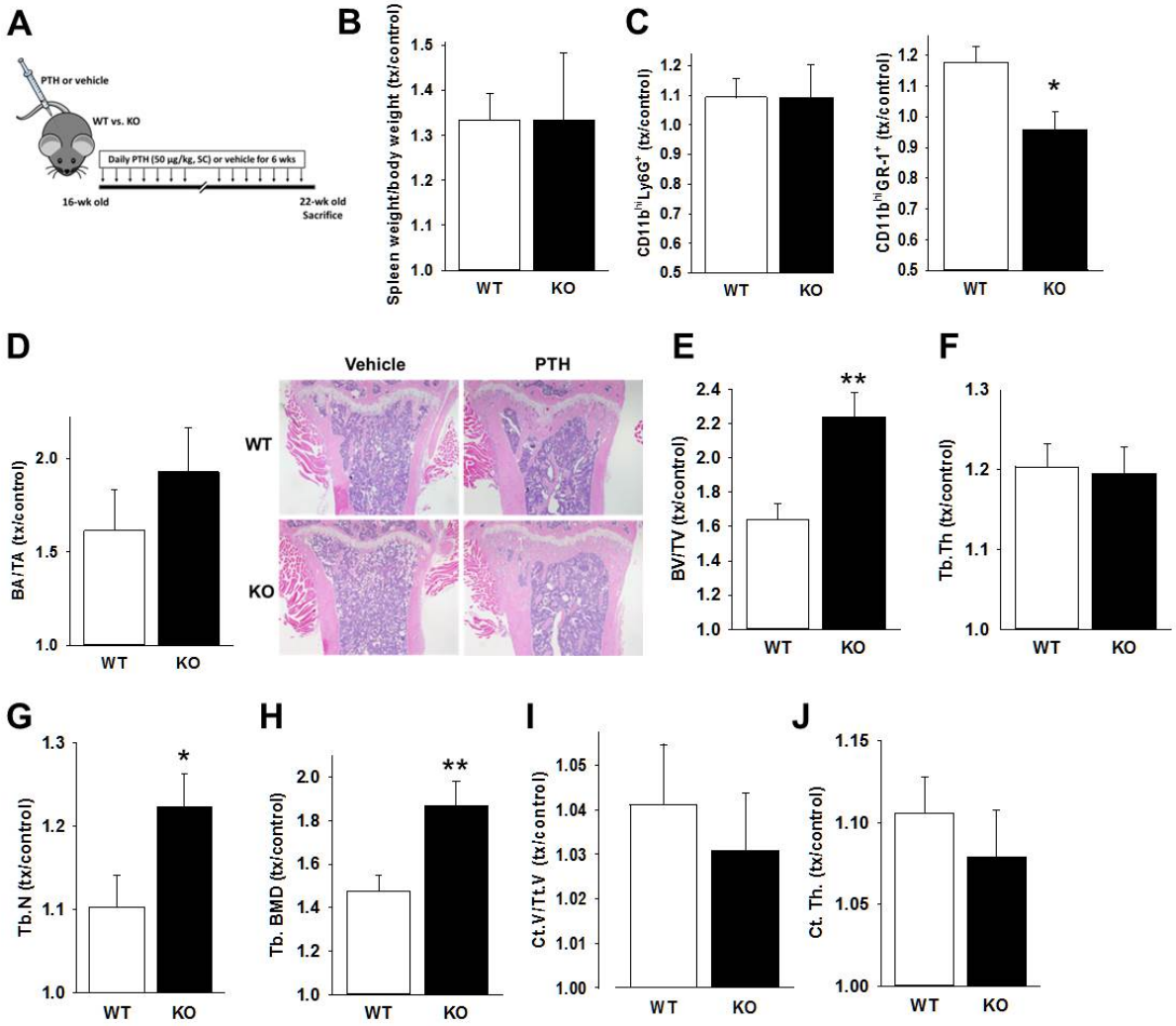

Figure 4.5. Anabolic response to iPTH is greater in adult KO mice than WT. (A) Experimental design. KO and WT mice (16wks) were administered daily injections of PTH (50µg/kg, SC) or vehicle (0.9% saline) for 6 weeks and sacrificed at age 22wks to assess spleen and marrow phenotypes. (B) Spleens were harvested from vehicle and PTH treated KO and WT mice and weighed. PTH treatment similarly increased spleen weight per body weight in both WT and KO mice (tx/control > 1.0). (C) Marrow flow cytometric analysis for $CD11b^{hi}Ly6G^{+}$ and $CD11b^{hi}Gr\text{-}1^{+}$ populations showed that PTH treatment of WT mice resulted in increased $CD11b^{hi}Gr\text{-}1^{+}$ populations (tx/control > 1.0) but no change was seen in PTH treated KO compared to vehicle treatment (tx/control ~1.0). (D) H&E stained paraffin sections of KO and WT tibiae treated with PTH or vehicle were quantified for bone area per total area. Both WT and KO mice had an anabolic response to PTH treatment (tx/control > 1.0). (E) Trabecular BV/TV analysis of proximal tibia via µCT. Both KO and WT displayed significantly increased BV/TV with PTH treatment. PTH treatment showed a greater anabolic effect in KO mice compared to WT PTH treated.

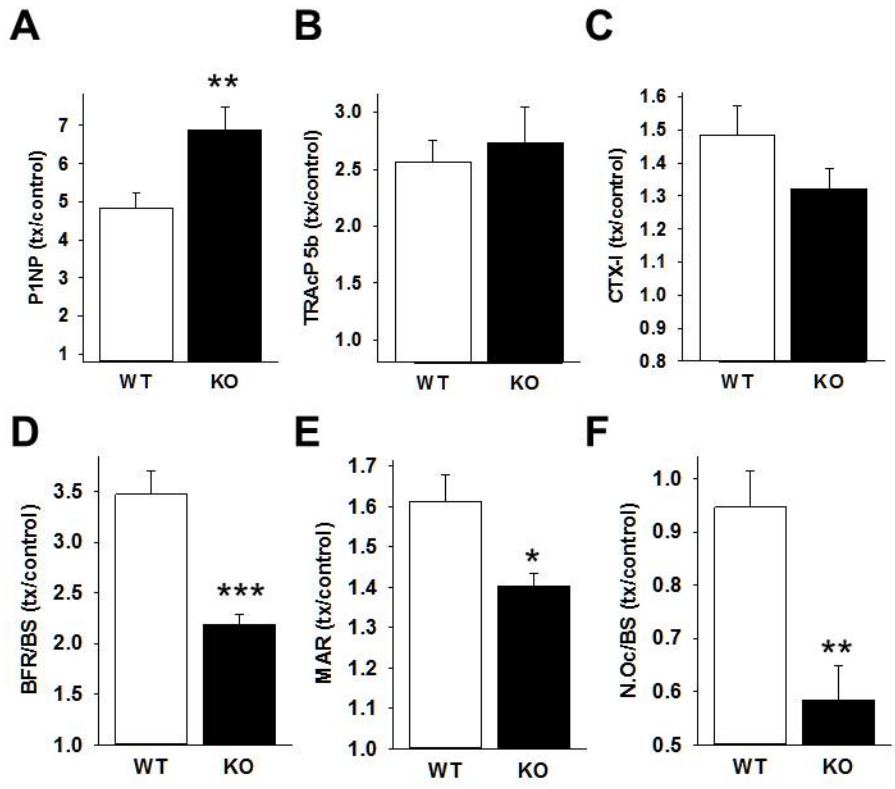

Figure 4.6. Bone formation and resorption analyses in PTH and vehicle treated KO and WT mice. (A, B, C) Serum was collected at time of sacrifice and P1NP, TRAcP 5b, and CTX-I were measured via ELISA. n=10-11/group, *p<0.05, **p<0.01, ***p<0.001, data is ± SEM. (D, E) Calcein (30mg/g, I.P.) was administered 5 and 2 days prior to sacrifice. Undecalcificed tibiae were collected, fixed, embedded, and sections were analyzed for bone formation rate (BFR/BS) and mineral apposition rate (MAR). iPTH treatment increased BFR/BS and MAR in both KO and WT (tx/control >1.0) but to a greater extent in the WT mice compared to KO. (F) TRAP stained paraffin embedded tibiae sections were quantified for TRAP positive multinucleated cells. KO mice treated with iPTH showed decreased osteoclast number/bone surface (N.Oc/BS) (tx/control < 1.0). n=8-11/group, *p<0.05, **p<0.01, ***p<0.001, data are mean ± SEM.

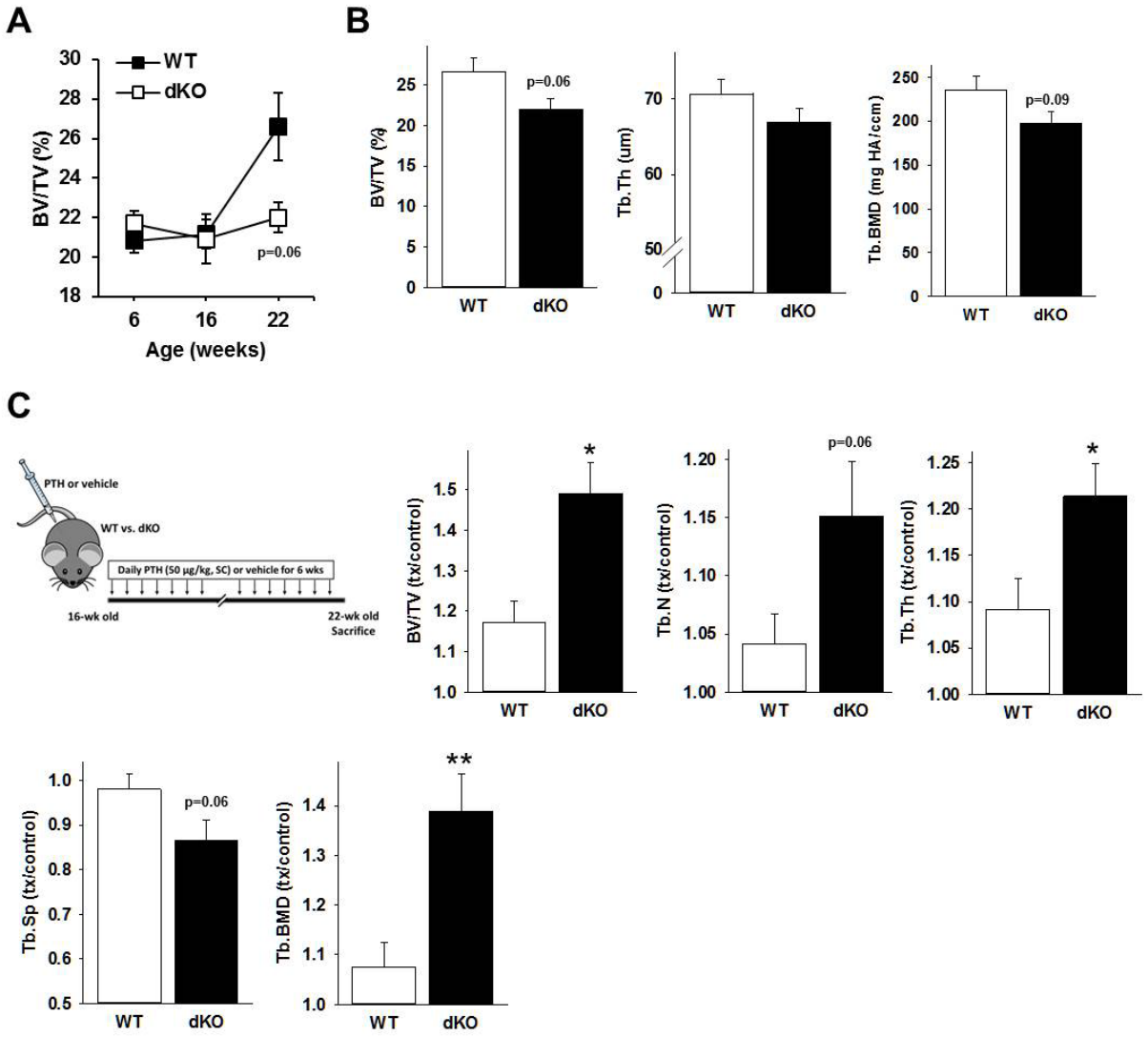

Figure 4.7. Double MFG-E8/Mertk KO mice display a similar phenotype to MFG-E8 KO mice. To assess any additive effects of Mertk KO in our MFG-E8 mice, a double MFG-E8/Mertk KO (dKO) mouse was generated. (A,B) WT and dKO mice had similar trabecular bone at 6 and 16 weeks, and a trend of decreased bone in dKO mice at 22 wks of age. (C) Sixteen wk old WT and dKO were treated with vehicle or iPTH daily for 6 weeks and skeletal phenotypes assessed. WT and dKO mice had anabolic responses to iPTH treatment, and the dKO anabolic response exceeded the response in WT mice. n=7-10/group, *p<0.05, **p<0.01, data are mean ± SEM

Table 4.1. Complete blood counts.

		WT Vehicle n=10	WT PTH n=11	KO Vehicle n=10	KO PTH n=10
WBC	**K/uL**	4.7 ± 0.5	5.9 ± 0.6	4.7 ± 0.3	6.1 ± 0.6
NE#	**K/uL**	0.85 ± 0.10	0.91 ± 0.13	0.98 ± 0.11	0.96 ± 0.12
LY#	**K/uL**	3.7 ± 0.4	4.8 ± 0.4	3.6 ± 0.3	**5.0 ± 0.5[c]**
MO#	**K/uL**	0.09 ± 0.01	0.11 ± 0.01	0.10 ± 0.02	0.10 ± 0.02
EO#	**K/uL**	0.03 ± 0.01	0.07 ± 0.02	0.05 ± 0.04	0.04 ± 0.01
BA#	**K/uL**	0.01 ± 0.004	0.03 ± 0.008	0.02 ± 0.01	0.01 ± 0.003
NE%	**%**	18.1 ± 1.1	**15.0 ± 0.9[a]**	20.4 ± 1.4	**15.5 ± 1.0[c]**
LY%	**%**	79.0 ± 1.3	81.7 ± 1.1	76.3 ± 2.1	**82.1 ± 1.0[c]**
MO%	**%**	1.9 ± 0.3	2.0 ± 0.2	1.9 ± 0.2	1.7 ± 0.04
EO%	**%**	0.65 ± 0.20	0.95 ± 0.29	0.95 ± 0.59	0.58 ± 0.18
BA%	**%**	0.27 ± 0.06	0.38 ± 0.12	0.37 ± 0.20	0.14 ± 0.04
RBC	**M/uL**	8.8 ± 0.1	8.5 ± 0.2	8.7 ± 0.1	**7.8 ± 0.3[c]**
HB	**g/dL**	13.1 ± 0.2	12.7 ± 0.2	13.3 ± 0.1	12.3 ± 0.5
HCT	**%**	44.8 ± 0.9	43.9 ± 0.9	46.9 ± 0.7	43.0 ± 1.8
MCV	**fL**	50.8 ± 0.7	51.5 ± 0.5	**53.9 ± 0.2[b]**	**55.2 ± 0.3[d]**
MCH	**Pg**	14.8 ± 0.2	15.0 ± 0.2	**15.3 ± 0.1**[a]	**15.8 ± 0.1[c]**
MCHC	**g/dL**	29.2 ± 0.3	29.1 ± 0.3	28.4 ± 0.3	28.5 ± 0.2
RDW	**%**	17.1 ± 0.7	18.2 ± 0.1	16.0 ± 0.1	**17.2 ± 0.1[e]**
PLT	**K/uL**	747 ± 16	746 ± 22	744 ± 27	752 ± 14
MPV	**fL**	4.1 ± 0.06	4.1 ± 0.06	4.0 ± 0.03	4.0 ± 0.03

WBC = white blood cell, NE = neutrophil, LY = lymphocyte, MO = monocyte, EO = eosinophil, BA = basophil, RBC = red blood cell, HB = hemoglobin, HCT = hematocrit, MCV = mean corpuscular volume, MCH = mean corpuscular hemoglobin, MCHC = mean corpuscular hemoglobin concentration, RDW = red cell distribution width, PLT = platelet, MPV = mean platelet volume

Data are expressed as mean ± SEM

[a]$p<0.05$ vs. WT Vehicle, [b]$p<0.01$ vs. WT Vehicle, [c]$p<0.05$ vs. KO Vehicle, [d]$p<0.01$ vs. KO Vehicle, [e]$p<0.001$ vs. KO Vehicle

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CHAPTER 5

INCREASED TOOTH EXTRACTION SOCKET BONE FILL WITH CLODRONATE-LOADED LIPOSOME TREATMENT

Abstract

Ineffective oral wound healing is detrimental to patients' oral health related quality of life. Delineating the cellular mechanisms involved in optimal healing will elicit better approaches to treating patients with compromised healing. Osteal macrophages have recently emerged as important positive regulators of bone turnover. The contributions of macrophages to long bone healing have been studied but their role in oral osseous wound healing following tooth extraction are less appreciated. Clodronate-loaded liposomes were used a tool to deplete phagocytic macrophages and assess oral osseous bone fill after extraction. In addition to macrophage depletion, off target osteoclast depletion occurred. Interestingly, depletion of macrophages and osteoclasts via clodronate treatment had different effects based on the location and type of bone turnover. In the tibiae, clodronate treatment drastically increased trabecular bone volume fraction after 7 and 14 days of treatment, which correlated with increased CD68+ cells and decreased F4/80+ cells in the marrow. In extraction sockets, clodronate treatment had no effect on bone fill at 7 days after surgery but increased extraction socket bone fill 14 days after surgery. This increase was to a much lesser extent than the large changes seen in homeostatic bone turnover in the long bone. These data suggest a temporal and spatial specificity in the roles of macrophages in normal turnover and healing.

Introduction

Compromised wound healing in the oral cavity leads to prolonged infection, pain and overall decreased quality of life. Proper healing of bony defects in the oral cavity from tooth extractions, periodontal procedures, congenital diseases or due to surgical reconstruction is crucial to restoring function. Compromised immune systems, radiation therapy or IV bisphosphonate cancer therapies can lead to ineffective bone healing in the jaw, causing chronic issues such as osteonecrosis [1]. Having a better understanding of the mechanisms of osseous wound healing will help to abrogate the issues seen in these inadequate healing states, and aid in the design of successful therapeutic strategies.

Extraction sockets are unique locations for healing as they require both bony and soft tissue healing and are exposed to the oral flora. Several cell types are crucial to the osseous healing of extraction sockets including traditional bone cells (osteoclasts, osteoblasts and osteocytes) and immune cells such as macrophages and neutrophils [2]. Soft tissue closure requires the coordination of fibroblasts and epithelial cells, along with immune cells aiding in tissue repair. Understanding the individual roles of each cell in the healing response will aid in creating targeted therapies to assist in the healing process.

Clodronate-loaded liposomes have been extensively studied for their effectiveness as a macrophage-ablation tool [3]. Clodronate bisphosphonate is encapsulated into liposomes which are readily engulfed by phagocytic cells. Once engulfed, the phagocytic cell machinery degrades the liposome and releases clodronate. Clodronate then is converted to a non-hydrolizable analogue of ATP resulting apoptosis of the cell which engulfed the liposomes. Mice treated with clodronate-loaded liposomes have depleted macrophages in several tissues in the body, including the bone marrow of the long bones [4, 5]. The knowledge of the impact of clodronate-loaded

liposomes or oral osseous wound healing is lacking. The aim of this study was to delineate the role of macrophages in oral wound healing of extraction sockets via clodronate treatment.

Materials/Methods

Animals

All mice were maintained in accordance with institutional animal care and use guidelines, and experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Michigan. Six week old male C57BL6/J mice were ordered from Jackson Laboratories. Mice were anesthetized with ketamine/xylazine (90mg/kg (K), 5mg/kg (X), IP). Once adequate anesthesia was achieved, the mandible was retracted and left and right maxillary M1 molars extracted. Mice were given a dose of buprenorphine (0.05-1.0 mg/kg, SC) at time of surgery and every 8-12 hours for 48 hours following surgery. PBS- or clodronate-loaded liposomes (clodronateliposomes.com) were administered locally in extraction sockets at time of surgery as well as daily intraperitoneal injections at a dose of 10μL/g from day 0-6 and 6μL/g from day 7-13. Mice were euthanized at day 7 or 14 following tooth extraction and tissues harvested for analysis.

Serum ELISAs

Blood was harvested at time of euthanasia via intracardiac puncture under anesthesia (ketamine/xylazine), allowed to coagulate for at least one hour at room temperature, spun down at 8000 rpm for 10 minutes, and liquid serum collected. Samples were stored at -80°C until use. Enzyme immunoassays were used to measure the serum concentrations of tartrate-resistant acid phosphatase form 5b (TRAcP 5b) and propeptide of type I procollagen (P1NP) according to

manufacturer instructions (IDS) and measured with an EZ Read 400 microplate reader with Galapagos software (Biochrom US, Holliston, MA).

Fluorescence Activated Cell Sorting (FACs) Analysis

Bone marrow was isolated from the tibia via flushing in FACs buffer (1X PBS with 2%FBS, 0.5mM EDTA), red blood cells lysed with 1x ACK, and 10^6 cells stained with anti-mouse F4/80 (APC, Abcam, A3-1), anti-mouse CD68 (FITC, Biolegend, FA-11), and anti-mouse CD206 (FITC, Biolegend, C068C2). Isotype controls were used to confirm antibody specificity. FACs analysis was performed using a BD FACs Aria II (BD Biosciences).

MicroCT (μCT)

Tibiae and maxillae were harvested at time of sacrifice (7 or 14 days) and fixed in 10% neutral buffered formalin for 24-48hrs at 4°C, then stored in 70% ethanol. Samples were scanned by micro-computed tomography at a 12μm voxel size (Scanco μCT-100) as previously described [6] and following established guidelines [7]. Tibial trabecular bone volume of interest was defined as starting 360μm distal to the top of the proximal growth plate and extended 600μm distally. A threshold of 180 mg/cm^3 was used for trabecular analysis. Extraction socket bone fill was measured in mesial, distal and palatal extraction sockets. Trabecular bone parameters analyzed included trabecular bone volume fraction (Tb. BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular spacing (Tb.Sp) and trabecular tissue mineral density (Tb.TMD). Tibial cortical bone volume of interest included a region of the midshaft of the tibia starting 3mm proximal to the tibia-fibula junction and extending 360μm distally. The cortical

bone threshold was 280 mg/cm^3. Cortical bone morphometric variables analyzed included cortical volume fraction (Ct.V/Tt.V) and cortical thickness (Ct.Th).

Histomorphometry

Maxillae and tibiae were fixed in 10% neutral buffered formalin for 24-48hrs at 4°C, decalcified in 14% EDTA for 10-20 days, embedded in paraffin, and sectioned at 5μm. A transverse section of the maxilla was cut to view bilateral extraction sockets and stained with hematoxylin and eosin (H&E), tartrate resistant acid phosphatase (TRAP, Sigma 387A), or F4/80 (antibody information). F4/80 immunohistochemical staining was performed following previously published protocols [8, 9]. Briefly, slides were de-paraffinized in xylene and rehydrated in ethanol series. Slides were then treated with H_2O_2 (3% in TBS) for 10 minutes at room temperature, washed in TBS, then digested in sodium citrate (10mM, pH 6.0) which was brought up to 71-75°C then cooled to room temperature overnight. Slides were washed, serum blocked with BioCare Background Sniper for 15 minutes, and incubated with anti-F4/80 (abcam 6640, clone CI:A3, diluted 1/350 in BioCare Da Vinci Green diluent) for 1.5 hours at room temperature. Slides were washed with TBS then incubated with goat anti-rat secondary antibody for 30 min at room temperature, followed by SA-HRP incubation and DAB chromogen. Slides were counterstained with hematoxylin and dehydrated before coverslip. Tibial sections were stained with hematoxylin and eosin (H&E) or tartrate resistant acid phosphatase (TRAP, Sigma 387A). Osteoclast quantification in tibiae and extraction sockets was performed as described [4] and according to ASBMR histomorphometric analysis standards [10].

Statistical Analysis

Statistical analyses were performed by unpaired Student's t test to compare PBS-liposome versus clodronate-liposome treatments at each timepoint with a significance of $p < 0.05$. Data are presented as mean ± standard deviation (SD).

Results

Clodronate loaded liposomes deplete bone marrow F4/80+ macrophages

Maxillary M1 molars were extracted bilaterally and mice were treated with a one-time intra-defect application of clodronate (CLOD)- or PBS-loaded liposomes in addition to daily intraperitoneal injections of clodronate- or control PBS-loaded liposomes (**Figure 5.1**). Treatment with clodronate loaded liposomes resulted in body weight reductions compared to PBS-loaded liposomes after 7 days of treatment, whereas no changes were seen at 14 days (**Figure 5.2A)**. Spleen weights were reduced in clodronate treated mice at both 7 and 14 days of treatment (**Figure 5.2B**). Flow cytometric analysis of long bone marrow showed clodronate treatment decreased F4/80^{+} macrophages and M2-like (F4/80-CD206^{+}) macrophages (**Figure 5.2C-D**). In contrast, CD68^{hi} cell populations were increased with clodronate treatment compared to control PBS (**Figure 5.2E**).

Bone fill following tooth extraction was increased in clodronate loaded liposome treated mice

Maxillary M1 molars were extracted and extraction sockets were analyzed for bone fill at 7 and 14 days post-surgery using micro-CT analysis. Clodronate treatment had no effect on trabecular bone parameters after 7 days of treatment (**Figure 5.3A-F**), with the exception of trabecular tissue mineral density (TMD), which was significantly decreased with clodronate

treatment. After 14 days of daily treatment with clodronate- or PBS-loaded liposomes, clodronate treated mice had increased extraction socket BV/TV compared to PBS treated mice (**Figure 5.3G,M**). Given that trabecular number was decreased with clodronate treatment (**Figure 5.3H**), the increase in trabecular bone volume fraction with clodronate treatment was due to an increase in trabecular thickness (**Figure 5.3I**). The increase in bone volume suggests clodronate depleted cells which are negative regulators of bone fill following tooth extraction. Alternatively, upregulated cell types may be positive regulators. The significant increase in CD68 positive cells in the long bone suggests these may be positive regulators of bone turnover and oral osseous repair.

Clodronate treated mice have increased tibial trabecular bone associated with decreased resorption

In addition to assessing the effects of clodronate treatment on maxillary extraction socket healing, systemic skeletal effects were determined. Clodronate treatment resulted in dramatic increases in trabecular bone in the proximal tibia as early as 7 days after treatment, and further increased trabecular bone after 14 days (**Figure 5.4A-F,J**). Trabecular bone tissue mineral density was decreased with clodronate treatment (**Figure 5.4E**), which suggests that although there was increased bone, the average density of the bone in the clodronate treated mice was lower than that in PBS treated. Cortical bone was unchanged in clodronate treated mice at both time points (**Figure 5.4G**). The serum formation marker P1NP and to an even greater extent, the serum resorptive marker TRAcP 5b were significantly reduced in clodronate treated mice at 7 and 14 days (**Figure 5.4H-I**). Osteoclasts are known phagocytes and have the ability to engulf foreign material (i.e. liposomes). Given the reduction in serum TRAcP 5b, the increased bone

phenotype may be largely due to alterations in osteoclast number and/or activity. To further assess the effect of clodronate on osteoclasts, we measured TRAP+ cells in tibial histology sections. Clodronate treatment decreased TRAP+ osteoclasts in tibiae sections (**Figure 5.4K**). The reduction in formation marker P1NP may be due to a coupling response in which decreased osteoclast signaling feeds back to decrease osteoblast formation and activity. Alternatively and potentially in addition to the coupling response, the reduction in bone formation could be attributed to the reduction in macrophages. TRAP+ osteoclasts and F4/80+ macrophages were assessed in extraction sockets of mice treated with PBS- or clodronate-loaded liposomes.

Treatment with clodronate-loaded liposomes decreased F4/80+ and TRAP+ cells in the bone

Mice treated with clodronate- or PBS-loaded liposomes were assessed for TRAP+ (osteoclasts) and F4/80+ (macrophages) cells in the maxillary extraction sockets at 7 and 14 days after surgery. TRAP+ (purple) multinucleated osteoclasts increased in extraction sockets at 14 days compared to 7 days in both PBS and clodronate treated mice (**Figure 5.5**). There was a decrease in TRAP+ osteoclasts at both 7 and 14 days after extraction in the clodronate treated mice compared to PBS. F4/80 is a marker for murine macrophages. F4/80+ (brown) cells were present in the marrow at both 7 and 14 days after extraction and were decreased with clodronate treatment relative to PBS (**Figure 5.6**).

Clodronate treatment did not affect non-wounded oral bone volume

Given the changes seen in steady state long tibiae bone volume and extraction socket bone fill, non-wounded bone in the maxilla was analyzed to determine the effects of clodronate on bone in other oral sites. Trabecular bone volume was measured between the maxillary M2 and

M3 molars (**Figure 5.7, right**). No significant differences were seen in non-wounded oral bone volume after 14 days of treatment with clodronate-loaded liposomes (**Figure 5.7, left**), suggesting a site-specific effect. **Table 5.1** summarizes the effects of clodronate on bone volume, TRAP+ osteoclasts, F4/80+ macrophages, and CD68+ macrophages in the tibia and maxilla.

Discussion

Macrophages are positive regulators of bone turnover, yet the mechanisms by which they exert these effects are currently unknown. The macrophage depletion model clodronate-loaded liposomes, results in F4/80-positive macrophage cell depletion in several tissues including the lung, liver, spleen and bone marrow [4, 5, 11, 12]. Interestingly, a subset of bone marrow macrophages and dendritic cells positive for the marker CD68 are increased in clodronate-treated mice, consistent with previous studies [4]. Given the large influx of apoptotic cells from the induction of phagocytic cell death by clodronate, it is hypothesized that CD68-positive cell populations are increased to aid in the clearance of apoptotic cell bodies via some type of feedback mechanism.

It was previously shown that macrophages secrete osteogenic factors during the process of apoptotic cell clearance (termed efferocytosis) [13]. In this study as well as others, mice treated with clodronate-loaded liposomes had increased bone volume in the appendicular skeleton [4, 5], suggesting the increase of CD68+ cells may contribute positivity to bone turnover. Widespread depletion of monocytes/macrophages including CD68+ and F4/80+ cells using the MAFIA mouse model results in overall decreased bone volume [4, 8, 12, 14, 15]. These contrasting phenotypes in which CD68+ cells are high (in clodronate) and low (in MAFIA) suggest that CD68+ macrophage populations are positive regulators of bone

homeostasis compared to the F4/80+ population. It is important to note, however, that osteoclasts were also decreased with clodronate-loaded liposome treatment. Given that osteoclasts are mediators of bone resorption, decreasing their numbers undoubtedly decreases resorption and may lead to increased bone volume. Targeted depletion of macrophage population subsets to rule out osteoclast effects can aid in delineating the contributions of each cell type to homeostatic bone turnover as well as healing.

Mice treated with clodronate-loaded liposomes had significantly increased trabecular bone in the tibiae. The trabecular bone volume fraction was increased 81% and 152% compared to PBS treatment at days 7 and 14, respectively. Alternatively, while oral extraction socket bone fill was significantly increased at day 14 following surgery, the increase in bone was only 7%. This suggests that macrophage and osteoclast depletion via clodronate-loaded liposomes has site specific effects as well as effects on healing versus homeostatic turnover. Clodronate-liposome treatment has been investigated in the context of long bone injury healing. Treatment with clodronate-loaded liposomes significantly decreased new bone formation in a tibial injury model [12, 16] and in femoral fracture healing [17, 18], further supporting site specific responses to clodronate treatment. To better understand how the changes seen in extraction socket bone fill are mediated, it is helpful to understand the sequence of events in extraction socket healing.

After a tooth is extracted a well-orchestrated series of inflammatory and healing events occurs. In mice, immediately after the tooth is removed, a clot forms [2]. Early in healing of a mouse extraction socket (d0-5), inflammatory cells and MSCs migrate and proliferate. Around day 3 after extraction, fibroblasts proliferate and osteoblasts differentiate to aid in collagen synthesis and bone formation, respectively. The levels of inflammatory cells and fibroblasts peak at around day 7 and osteoblasts peak at day 14 [2]. Blood vessels begin to form around day 5 and

continue to increase until day 21. Osteoclasts differentiate and begin to remodel the new bone around day 14. This sequence of events leads to a rapid increase in bone tissue from day 5 to 14 at which time bone fill levels off as osteoclast remodeling takes over. Our findings are consistent with the published literature where control mice had a clear increase in TRAP+ osteoclasts in extraction sockets at day 14 compared to day 7. While clodronate-loaded liposomes decreased osteoclasts in extraction sockets at both time points, the effect of depleting osteoclasts may not be appreciated until later time points. The difference in extraction socket bone fill may instead be due to an increase in CD68+ cells as was seen in the bone marrow. Future studies should include temporal depletion of macrophages and osteoclasts via clodronate-loaded liposomes, including before and/or after tooth extraction surgery.

The location specific changes seen with clodronate are outlined in **Table 5.1**, and highlight the difference between the response in the tibia and maxilla. These differences observed in the long bone site versus the oral cavity may be due to many factors including differences in the cell populations in the marrow in these sites, origin of these cells, mechanical stimulation and exposure to different environmental cues. Bone marrow stromal cells (BMSCs) from the mandible have been shown to differentiate and mineralize more than BMSCs from long bone sites [19, 20]. Additionally, osteoclasts from the mandible and tibia have similar resorptive activity, but osteoclasts from the mandible differentiate faster than those from the long bone [21-23]. These findings support the differences seen in the oral wound healing sites compared to long bone sites. Furthermore, although these cells look similar histologically [24], they arise from different origins during embryonic development. The bones of the mandible and maxilla arise from neural crest cells of the neuroectoderm layer, and long bones arise from the mesoderm [25]. Also, the mandible and maxilla undergo intramembranous ossification compared to

endochondral ossification of the long bones [26]. Lastly, the bones in the jaws are exposed to stronger forces during mastication than those generated during walking [27, 28], and mechanical stimulation is an important regulator of bone turnover.

Interestingly, we observed a decrease in tissue mineral density (TMD) in the tibiae and extraction socket with clodronate-loaded liposome treatment. One factor that could drive this change in TMD is a difference in the rate of mineralization. While clodronate liposomes may not directly interact with the mineralization process in a significant way, they did inhibit macrophages which have a known role in regulating bone mineralization [8, 12, 15, 29]. Specifically, macrophages have been shown to secrete osteogenic factors such as TGF-β1 [13], oncostatin M [30-32] and BMP-2 [33], which could impact mineralization. In addition to direct impacts on the bone mineralization process altering TMD, in a rapidly healing model, TMD is also likely to be driven in large part by the average age of the tissue present, or how much time it has been mineralized. A bone with a higher average tissue age could be expected to have a higher TMD. In our clodronate-treated healing socket, a potential anti-osteoclast effect could yield a greater tissue age due to decreased resorption of the aged bone. In extraction sockets, TMD was decreased at day 7 with clodronate treatment, suggesting less mineralization. In our model, the effect of TMD is likely not singular, but rather the complex result of multiple distinct, and even opposing, biological actions that combine to yield observed TMD phenotype.

It should not be overlooked that clodronate is a bisphosphonate. Bisphosphonates have been studied in the context of extraction socket healing due to their potential ability to lead to osteonecrotic lesions in the oral cavity. Because bisphosphonates are anti-resorptives, they prevent osteoclasts from clearing bone that has been haphazardly formed. Treatment with bisphosphonates leading to necrotic bone has been characterized by empty osteocyte lacunae [34,

35]. Treatment with clodronate-loaded liposomes did not alter the presence of osteocytes in lacunae (data not shown). Additionally, bisphosphonate treatment increases extraction socket bone fill [35, 36], but typically to a greater extent than seen in the present study. Although the increase in CD68+ macrophages and decrease in osteoclasts explains an overall increase in bone, the percent increase with clodronate treatment may be less than that seen with other bisphosphonates due to the altering of other macrophage populations, namely the F4/80+ cells. In a healing model, decreased F4/80+ cells may compromise the ability of the bisphosphonate to increase bone. This may support the hypothesis that specific macrophage subsets have distinct and necessary roles in the context of healing. This is supported by data in long bone healing in which F4/80+ cell depletion impaired healing [12]. Perhaps, the depletion of the F4/80+ cells prevents the clodronate-loaded liposome treatment from reaching the same level of bone fill result seen with non-liposome bisphosphonates.

Clodronate-loaded liposome treatment resulted in increased bone in the tibiae as well as increased bone fill in the extraction sockets. The extent to which clodronate exerted its effect on bone was variable according to the location and whether the bone was under normal homeostatic turnover or if it is was new bone in response to a wound. Macrophage and osteoclast populations were altered with clodronate and suggest a crucial balance of these cells to maintain proper oral wound healing. In regards to clinical treatment of oral osseous wounds, it is important to understand what cell populations may be potential targets to aid in osseous healing without compromising the quality of the bone. Further insight into the temporal and spatial specificities of osteal macrophages and osteoclasts will ultimately lead to better methods to target cells.

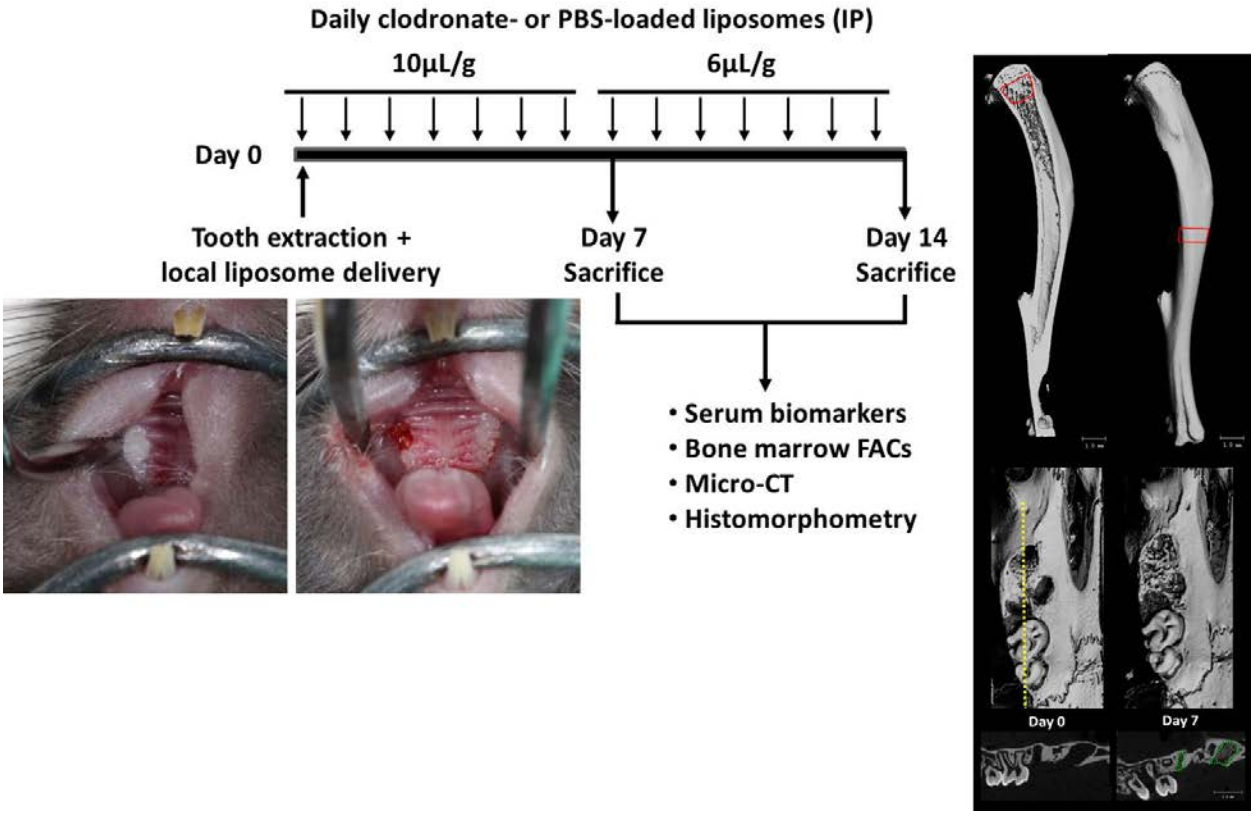

Figure 5.1. Experimental model. Six week old male C57BL/J mice were anesthetized with ketamine and xylaxine, mandible retracted and maxillary M1 molars extracted bilaterally. Mice received local delivery of clodronate- or PBS-loaded liposomes in extraction sockets at time of surgery. Intraperitoneal injections of clodronate- or PBS-loaded liposomes were given daily at a dose of 10µL/g from days 0-6 and 6µL/g days 7-13. Mice were sacrificed at day 7 or 14 after surgery and tissues harvested for serum biomarkers, bone marrow flow cytometric analysis (FACs), micro-CT, and histomorphometric analysis. n=8-10/group.

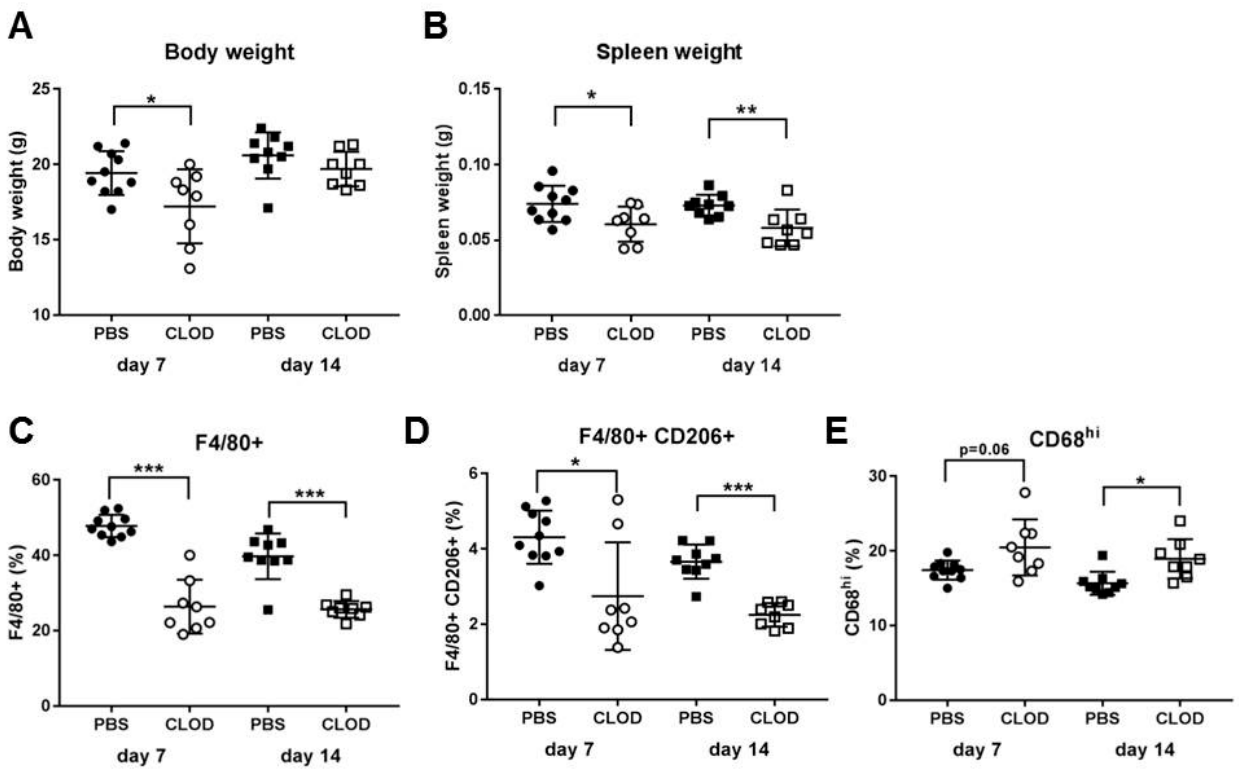

Figure 5.2. Clodronate treated mice have decreased spleen weight and altered bone marrow macrophage populations. (A) Body weight was measured at the time of sacrifice. Clodronate treated mice displayed decreased body weight at 7 days post-extraction surgery and mice sacrificed at 14 days after surgery showed no differences in body weight. (B) Spleens were harvested at time of sacrifice, weighed and compared to body weight. Spleen weight was decreased in clodronate treated mice at both time points. (C-E) Bone marrow was flushed from tibiae at time of sacrifice and stained for flow cytometric analysis of F4/80+ (macrophages), CD206+ (M2-like macrophages) and CD68hi (monocytes, dendritic cells). Clodronate-loaded liposome treated mice displayed decreased F4/80+ and F4/80+/CD206+ and increased CD68hi marrow populations compared to PBS-treated mice. n=8-10/group, *$p<0.05$, **$p<0.01$, ***$p<0.001$, data are mean ± SD.

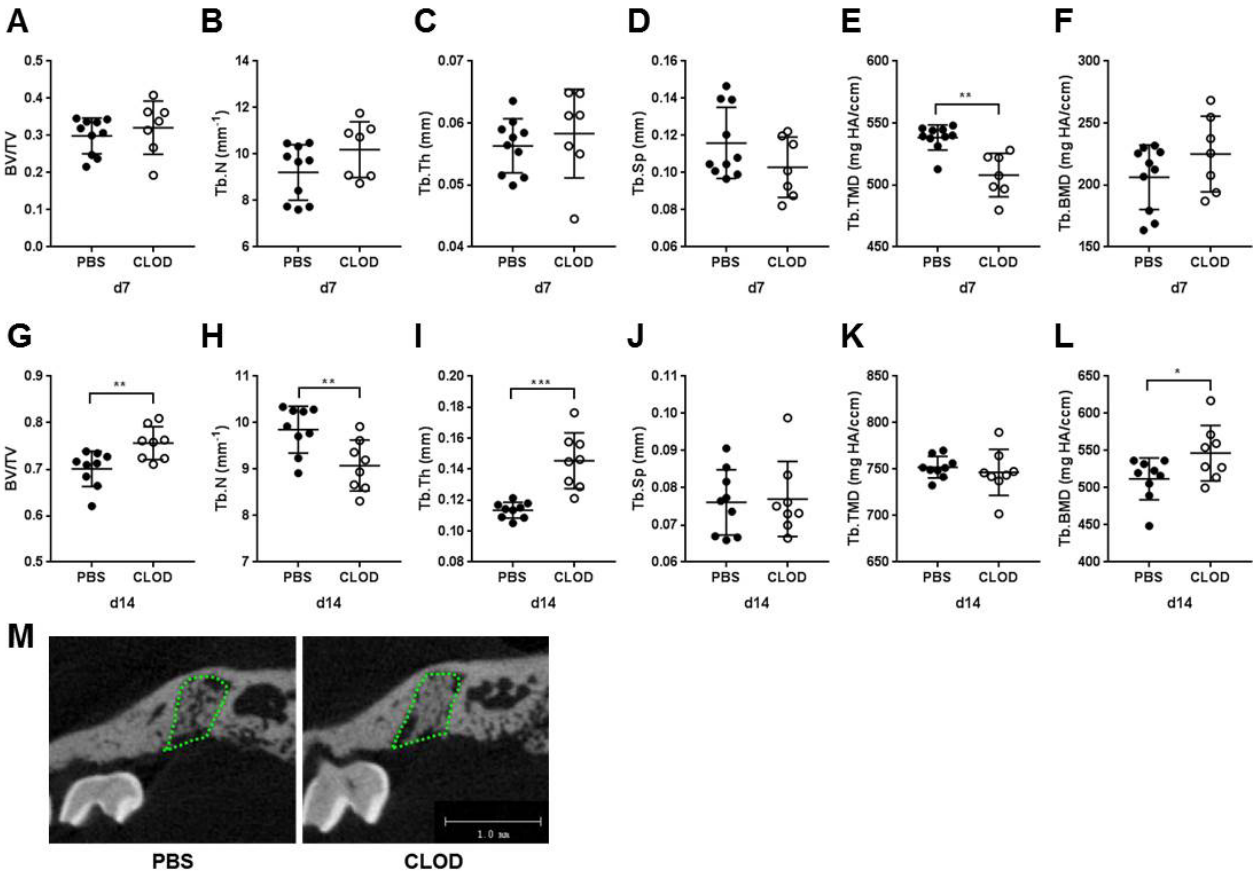

Figure 5.3. Clodronate treatment increases extraction socket bone fill at 14 days post tooth extraction. Maxillae were harvested at time of sacrifice and analyzed via micro-CT (μCT). (A-F) Extraction socket bone fill was unchanged after 7 days of treatment with clodronate. (G-L) Clodronate liposome treatment significantly increased extraction socket bone fill after 14 days of treatment compared to PBS treated mice due to increased trabecular thickness. (M) Representative images of bone fill in extraction socket after 14 days of healing. n=8-10/group, **p<0.01, data are mean ± SD.

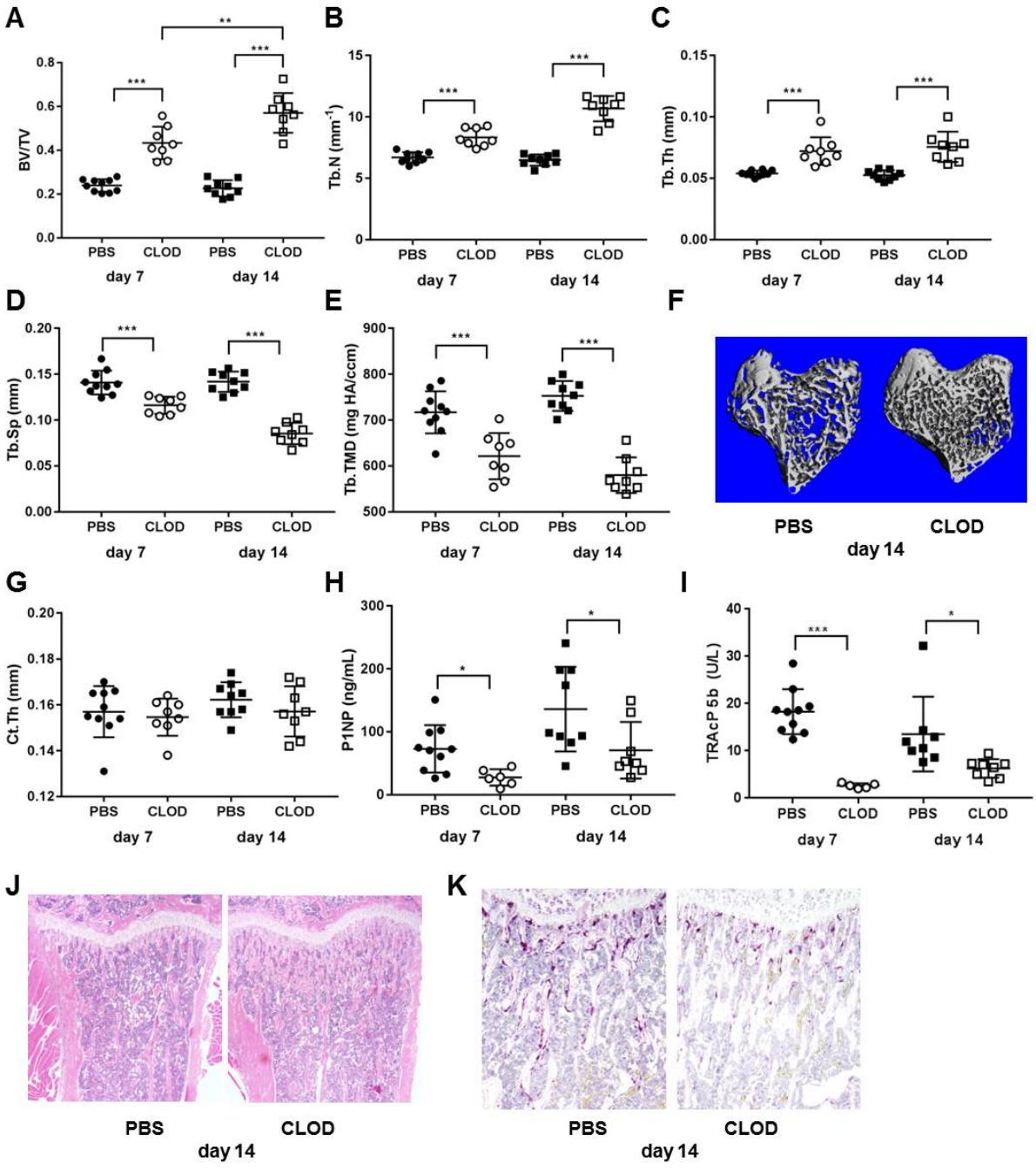

Figure 5.4. Clodronate treatment increases tibial trabecular bone. (A-F) Tibiae were harvested at time of sacrifice and trabecular parameters quantified via μCT. Clodronate-loaded liposome treated mice had significantly increased trabecular trabecular BV/TV (A), trabecular number (Tb.N, B), trabecular thickness (Tb.Th, C) and decreased trabecular spacing (Tb.Sp, D) and trabecular tissue mineral density (Tb.TMD, E) at day 7 and 14 compared to PBS treated mice. Clodronate treatment increased trabecular bone more at 14 days than at 7 days of treatment. (F) Representative images of trabecular bone in proximal tibiae after 14 days of treatment with PBS or clodronate. (G) Cortical bone was measured in the midshaft of the tibia and was unchanged with clodronate treatment. (H, I) Serum formation (P1NP) and resorptive

(TRAcP 5b) markers were measured. Clodronate treatment significantly reduced both formation and resorption compared to PBS-treated mice. (J) Representative image of H&E section showing increased bone in clodronate treated mice. (K) Tibiae sections were stained for TRAP. Clodronate treatment decreased TRAP+ cells in tibiae. n=8-10/group, *$p<0.05$, **$p<0.01$, ***$p<0.001$, data are mean ± SD

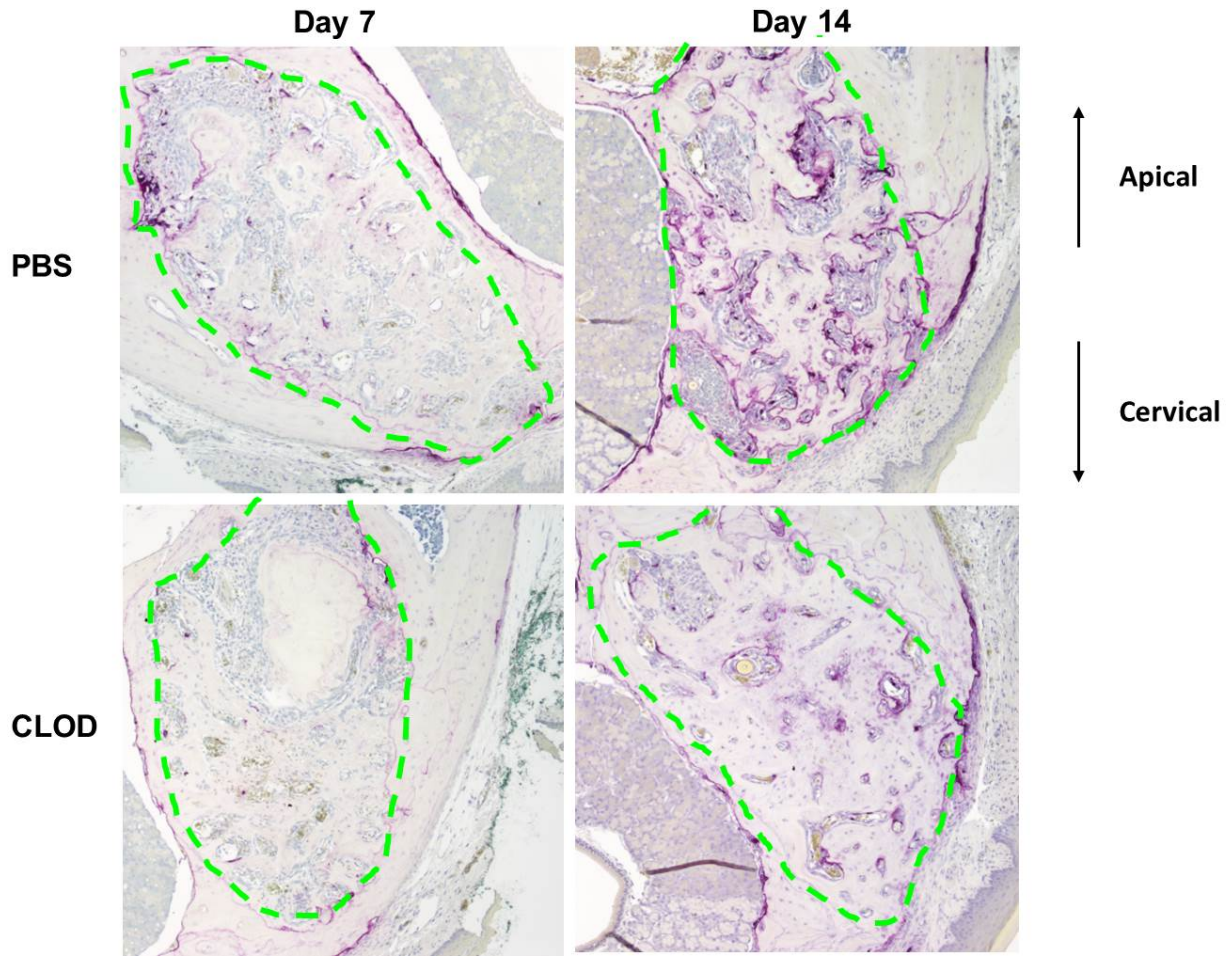

Figure 5.5. Clodronate treatment decreases TRAP+ osteoclasts in extraction sockets. TRAP+ multinucleated osteoclasts are increased in extraction sockets at day 14 compared to day 7 after surgery. Treatment with clodronate-loaded liposomes decreases TRAP+ multinucleated cells at 7 and 14 days post-extraction compared to PBS-loaded liposome controls.

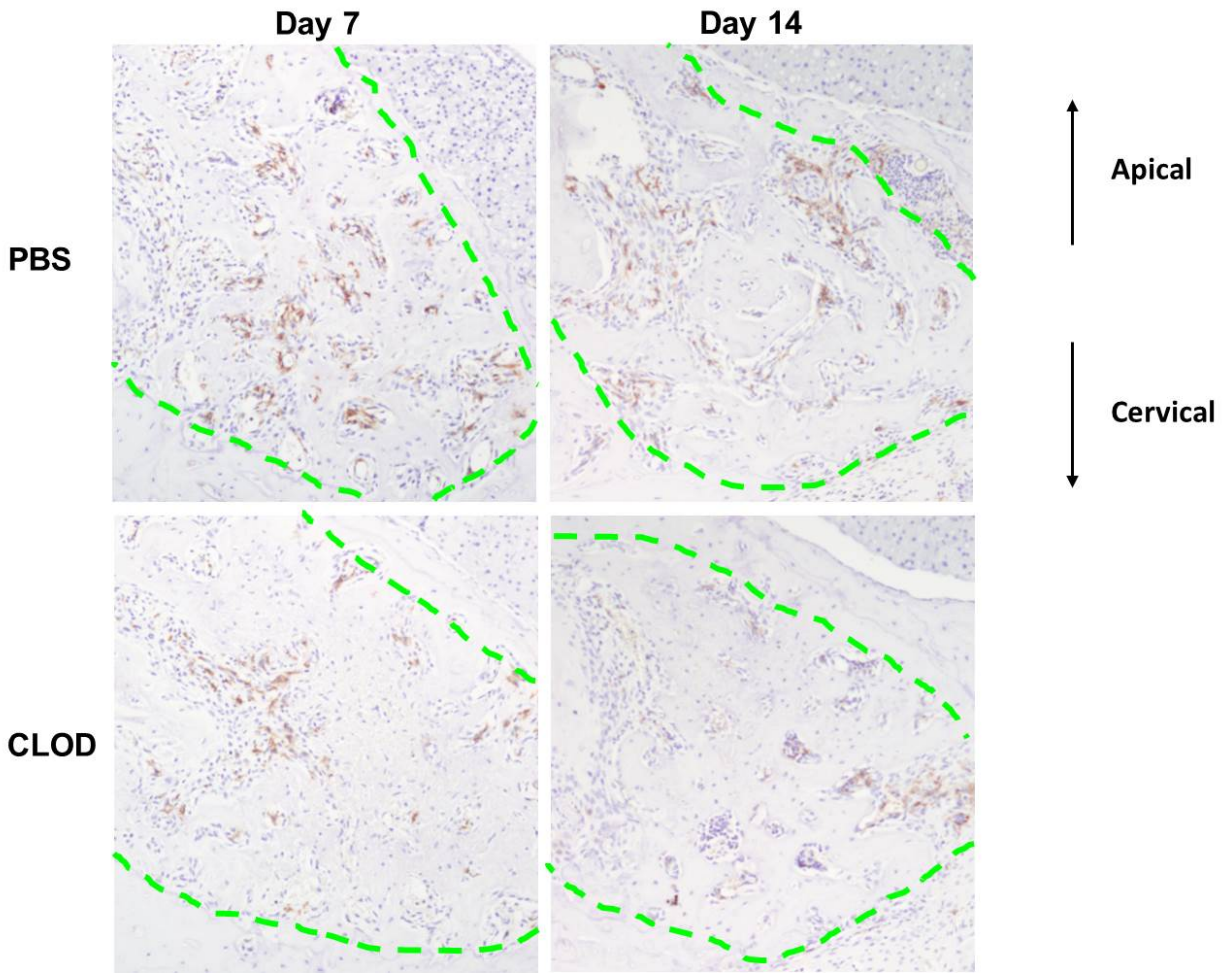

Figure 5.6. F4/80-positive cells are reduced in extraction sockets with clodronate treatment. F4/80+ cells are located in the marrow spaces at 7 and 14 days post-extraction and clodronate decreased F4/80+ macrophages compared to PBS at both time points.

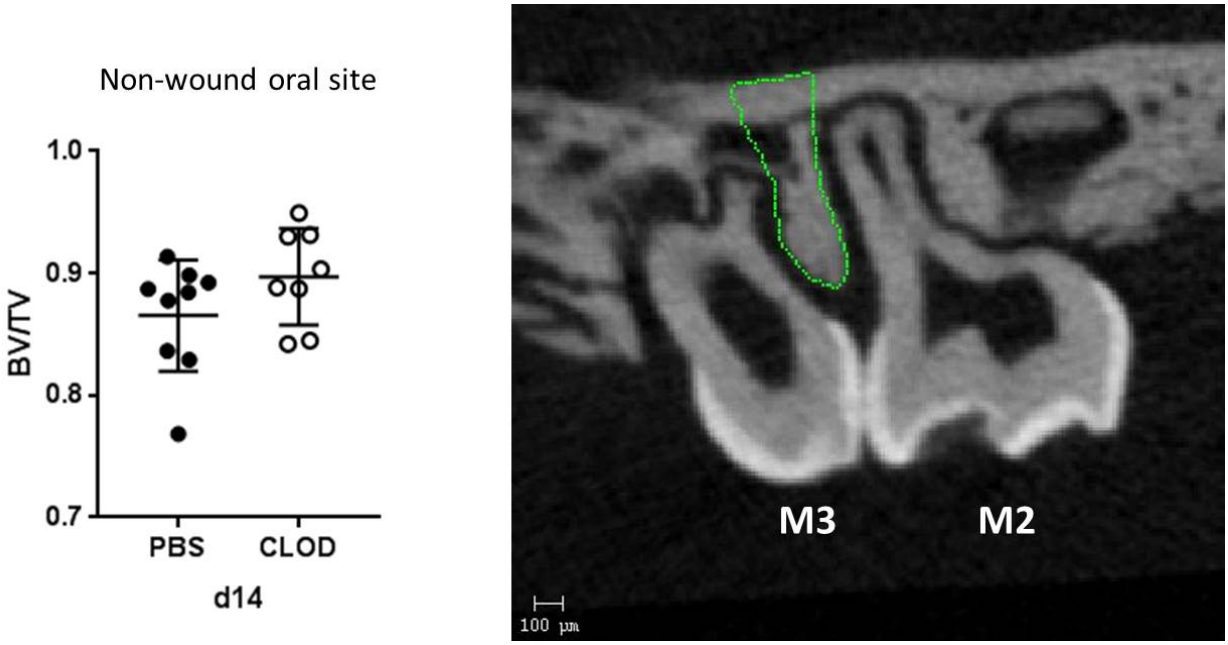

Figure 5.7. Clodronate treatment had no effect on non-wounded oral bone. Interseptal bone volume fraction was measured between M2 and M3 molars in the maxilla with no statistically significant changes with clodronate treatment.

Table 5.1. Clodronate effects on tibia versus maxilla.

Location	Bone Volume		TRAP+ Osteoclasts	F4/80+ Macrophages	CD68+ Macrophages
	Non-wound	Wound			
Tibia	↑↑↑	*↓↓	↓	↓	↑
Maxilla	No change	↑	↓	↓	Not measured

* References [12, 16-18]

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CHAPTER 6

DISCUSSION AND CONCLUSION

Discussion

Understanding the specific roles of individual cells in tissue homeostasis is the basis of decades of basic science and translational research. The ultimate goal of identifying the mechanistic role of cells under normal conditions is to then be able to identify how these roles change in disease. New tools can then be developed to manipulate these mechanisms to promote a desired outcome. Each tissue is different and houses its own unique cellular profile which affects how that tissue functions, what diseases it is susceptible to and how it responds to injury. In the context of the skeletal system, there are 2 major organs, the bone and the bone marrow. The bone and bone marrow consists of many cells types that must communicate effectively with one another to maintain homeostasis. When one cell type in the bone does not function properly, the balance of resorption and formation is affected, leading to potentially undesirable outcomes.

One particularly influential cell in bone is the macrophage. Macrophages are myeloid lineage cells and function as phagocytes. They are immune cells that can exert pro- and anti-inflammatory functions. When early and late macrophages are depleted using macrophage ablation models, bone forming osteoblasts no longer secrete matrix for mineralization, but the mechanisms by which macrophages exert these positive effects are not clear. The work presented in this dissertation adds to the current literature on bone marrow macrophage function via *in vitro* and *in vivo* experimental approaches.

A comprehensive review of the current literature on the bone marrow macrophage indicates that the pro-resolving nature of macrophages may explain some of their positive effects on bone. To better understand the resolving nature of bone marrow macrophages, a series of *in vitro* experiments were performed to identify regulators of macrophage activity, specifically their ability to phagocytose dead and dying cells. In non-bone tissues, anti-inflammatory cytokines increase macrophage efferocytosis [1, 2]. It was hypothesized that these anti-inflammatory proteins exert similar effects on bone marrow macrophages. Interleukin-10 (IL-10) was a candidate cytokine to assess efferocytosis of apoptotic bone cells. IL-10 regulates osteoclastogenesis and mice deficient in IL-10 have reduced bone mass [3, 4], yet it was unclear how IL-10-associated efferocytosis by bone marrow macrophages may contribute to these effects. Consistent with our hypothesis, IL-10 treatment increased macrophage uptake of apoptotic bone marrow stromal cells (apBMSCs). IL-10 enhanced efferocytosis by shifting macrophage polarization toward the pro-resolving M2 phenotype and increasing the M2 macrophage ability to uptake apoptotic cells. Next, further information was gathered on the signaling pathways affected by the treatment of macrophages with IL-10. Macrophages were treated with an inhibitor of STAT3 phosphorylation (Stattic) prior to IL-10 treatment. This significantly reduced efferocytosis of apBMSCs, confirming IL-10 increases efferocytosis and M2 polarization in a pSTAT3-dependent manner.

Efferocytosis of apBMSCs versus neutrophils led to a unique profile of cytokines released by the bone marrow macrophages. The cell specific cytokine response may indicate what effects efferocytosis has on bone modeling and remodeling. Following efferocytosis of apBMSCs, macrophages displayed increased secretion of monocyte chemoattractant protein-1/CC chemokine ligand 2 (MCP-1/CCL2) and transforming growth factor beta-1 (TGF-β1).

CCL2 promotes osteoclastogenesis in part due to the recruitment of monocyte/osteoclast precursors [5]. The release of CCL2 by macrophages engulfing apoptotic bone cells may increase bone turnover by recruitment of new osteoclasts and bone macrophages. TGF-β1 is a known mesenchymal stem cells recruitment factor [6, 7]. Macrophage secretion of TGF-β1 in response to apBMSCs cells may contribute to repopulating the bone forming units.

Together, the data presented in Chapter 3 show one modulator of macrophage efferocytosis of apBMSCs and detail two important cytokines which are upregulated in response. While this information tells us more about mediators of efferocytosis in bone, *in vivo* work should be performed to confirm the biologic relevance of these findings.

The goal of Chapter 4 was to assess the contributions of MFG-E8, a known anti-inflammatory mediator, to bone turnover. One well established function of MFG-E8 is to bridge between dead and dying cells and macrophages to coordinate engulfment of the apoptotic cell. Interestingly, bone marrow macrophages deficient in MFG-E8 did not display altered engulfment phenotype *in vitro*. Additionally, TUNEL positive cells were increased in the spleen and slightly but not significantly decreased in the marrow of MFG-E8 KO mice compared to controls. This suggested that MFG-E8 may not be the predominant regulator of efferocytosis in the bone marrow microenvironment. Given the high turnover rate of cells in the marrow, it would not be surprising to see other efferocytic mechanisms upregulated to compensate for the lack of MFG-E8. Additionally, an MFG-E8 homologue, developmental endothelial locus-1 (DEL-1), has been shown to function similarly to MFG-E8 and regulates efferocytosis to a larger extent than MFG-E8 in certain tissues [8, 9]. Measuring the relative MFG-E8 and DEL-1 expression in the bone and bone marrow may provide insight on key efferocytic pathways in bone. Future studies can provide further insight into the important regulators of efferocytosis in this highly plastic tissue.

While an efferocytic phenotype was not observed in the bone of the MFG-E8 deficient mice, other inflammatory phenotypic changes were observed. In addition to its role in apoptotic cell clearance, MFG-E8 is an important anti-inflammatory mediator. MFG-E8 has been shown to down-regulate nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and osteopontin signaling, which are both key regulators of osteoclastic cell differentiation, activation and adhesion [10, 11]. Adult MFG-E8 KO mice had increased bone marrow neutrophils and myeloid derived suppressor cells as well as increased spleen weights. Inflammatory environments have been shown to upregulate osteoclast differentiation and activity. Consistent with an increase in osteoclastic bone resorption, MFG-E8 deficient mice developed a skeletal phenotype of reduced bone. This phenotype became more apparent with age. No phenotype was seen at 6wks of age, but 16wk old mice had decreased cortical bone and a trend of decreased trabecular bone. At 22wks, the trabecular phenotype was significant. Other groups have also reported on a reduced bone phenotype with MFG-E8 deficiency and found this to be consistent with an increase in osteoclast number [12]. In our model, serum from 22wk old MFG-E8 KO had increased CTX-I levels which is a marker for osteoclast resorptive activity. *In vivo,* osteoclast number per bone surface was also increased in 22 wk old KO mice compared to WT.

A therapeutic intervention was tested in adult MFG-E8 mice. Intermittent PTH (iPTH) is an anabolic bone agent with use limited to severe cases of osteoporosis. Adult MFG-E8 KO mice responded robustly to iPTH treatment. Treatment with iPTH decreased osteoclast number per bone surface in the adult KO mice and decreased peripheral neutrophil numbers. It is hypothesized that iPTH therapy may alter the inflammatory phenotype by mechanisms which remain unclear. The insight gained from further studies characterizing the effect of iPTH on

inflammation should prove valuable in identifying new patient groups that may benefit from this anabolic bone agent as well as the newest anabolic agent, abaloparatide, which utilizes the same receptor.

Steady state bone turnover and bone healing require the presence of macrophages to aid in the formation of newly mineralized tissue [13, 14]. Oral osseous wound healing is complicated by the unique environment of the oral cavity as well as the need for soft tissue closure. Chapter 5 delves into the contributions of macrophages and osteoclasts to oral osseous wound healing following tooth extraction. Clodronate-loaded liposomes were used to preferentially target phagocytic cells and induce apoptosis. Treatment with clodronate-liposomes decreased F4/80+ cells in the marrow and in extraction sockets. However, likely due to compensatory mechanism, CD68 positive cells were increased in the marrow. CD68 positive cells are mature phagocytic macrophages and dendritic cells. This population may be increased to effectively clear the insult of apoptotic cell bodies from the clodronate treatment. Additionally, osteoclasts were decreased in histologic sections of extraction sockets in mice treated with clodronate and was confirmed by decreased serum TRAcP 5b, a marker for osteoclastic bone resorption.

Mice treated with clodronate displayed location specific alterations in bone volume. In the long bones, clodronate dramatically increased trabecular bone volume fraction by 152% after 14 days of treatment. Alternatively, in extraction sockets, clodronate increased bone fill to a much lesser extent; there was an increase in bone fill of 7% compared to PBS treated mice. Interestingly, others studies have reported that clodronate-loaded liposome treatment decreased new bone formation in a long bone healing [13, 15-17]. **Table 6.1** outlines the site-specific effects of clodronate treatment as well as MFG-E8 deficiency. As described above, MFG-E8 deficiency leads to osteopenia in adult mice. Oral osseous wound healing was measured in mice

with MFG-E8 deficiency and no alterations to bone fill in extraction sockets or drill hole defects were seen (data not shown, summarized in **Table 6.1**). Because there is an apparent site-specific effect of clodronate and MFG-E8 deficiency, it is important to understand the sequence of events in tooth extraction socket healing. Briefly, in a mouse extraction socket inflammatory cells infiltrate early (d0-3). Osteoblasts form newly mineralized bone around day 5 which is then remodeled by osteoclasts after day 7 [18]. In our model, there is a clear increase in TRAP+ osteoclasts in extraction socket healing at day 14 compared to day 7, and clodronate decreased TRAP+ cells at both time points. Because osteoclast remodeling occurs at or after the latest time point measured, the resulting phenotype may not reflect an osteoclast specific outcome. These data support macrophages and osteoclasts function at different times to aid in the healing process. Future studies should be performed to deplete macrophages and osteoclasts at different stages of healing, including before and/or after tooth extraction surgery.

In addition to depletion of these cells at different stages, the profiles of these cells need to be characterized. Previous studies have shown that bone marrow stromal cells (BMSCs) and osteoclasts from oral versus long bone sites have different differentiation and activity [19-23]**.** Also, the oral bones and long bones derive from different embryological origins. The mandible and maxilla arise from the neuroectoderm layer and undergo intramembranous ossification while the long bones arise from the mesoderm and undergo endochondral ossification [24, 25]. Bone marrow macrophages from different skeletal sites should also be investigated for their ability to differentiate, phagocytose and efferocytose. Given the oral cavity is unique in its exposure to bacteria and pathogens, the macrophages in these sites may be primed to be more active phagocytes. Additionally, mechanical forces from mastication are almost double the forces seen during walking, and mechanical stimulation positively supports bone formation [26, 27]. The

different forces in oral versus long bones may contribute to the relative activity of cells from these different skeletal sites. Clearly, more information about how the location of these cells affects their behavior is needed.

Bisphosphonates are anti-resorptive therapeutics that target osteoclasts to prevent resorption and further bone loss in patients with osteoporosis. Clodronate is a bisphosphonate drug. Treatment of mice with bisphosphonates prior to tooth extraction leads to increased extraction socket bone fill [28, 29]. These effects seen with non-liposome bisphosphonates tend to lead to a greater increase in extraction socket fill than seen in the clodronate-loaded liposome model. Because clodronate-loaded liposomes target macrophages in addition to osteoclasts, this may explain the difference between liposome-loaded and non-loaded bisphosphonate extraction socket bone fill outcomes. F4/80+ cells are positive regulators of bone turnover [14]. With clodronate-loaded liposome treatment, F4/80+ cells are decreased in extraction sockets. The clodronate-loaded liposome depletion of F4/80+ cells may therefore negatively impact bone formation and explain why clodronate-loaded liposome treatment resulted in differential healing than previously published non-liposome bisphosphonate treatments.

A better understanding of the cell populations crucial to proper healing will lead to the development of potential targeted therapies to enhance healing.

Conclusions

The information gained from these studies has the potential to positively affect patient care. Understanding the link between efferocytosis, inflammation and bone volume can lead to new screening methods to detect individuals susceptible to inflammation-induced osteoporosis. For example, the data presented in Chapter 4 suggests that screening for polymorphisms in the

gene encoding MFG-E8 or measuring serum MFG-E8 levels may identify patients at risk of developing reduced bone volume. Additionally, other studies have investigated the effect of recombinant MFG-E8 protein to decrease the severity of inflammation-induced bone loss with promising results [12]. Future studies are needed to investigate MFG-E8 as a drugable target. Furthermore, an FDA approved anabolic bone agent, iPTH, was highly effective at increasing bone in MFG-E8 deficient mice. This suggests iPTH may be useful in treating patients with inflammatory bone loss. Clearly, more studies need to be conducted to assess the effects of iPTH in other models of inflammation-induced osteoporosis.

Compromised oral wound healing leads to decreased oral health related quality of life. It is important to understand what cell populations may be potential targets to aid in osseous healing without compromising the quality of the bone. Further insight into the temporal and spatial specificities of osteal macrophages will ultimately lead to better methods to target cells important in the healing process of oral wounds. More information in the quality of the bone that is produced by treatment with clodronate-loaded liposomes as well as how timing of depletion of macrophages affects healing, may lead to effective treatments for patients susceptible to compromised healing.

Table 6.1. Clodronate and MFG-E8 deficiency: oral versus long bone changes.

Location	Clodronate		MFG-E8 KO	
	Non-wound	Wound	Non-wound	Wound
Tibia	↑↑↑	*↓↓	↓	?
Maxilla	No change	↑	No change	No change

* References [13, 15-17]

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