SIGNIFICANCE AND REGULATION OF CD68 EXPRESSION IN THE OSTEOCLAST

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MOLECULAR AND CELLULAR PATHOLOGY

ABSTRACT

The mucin-like Lysosome Associated Membrane Protein (LAMP) family member CD68 is a primarily myeloid lineage restricted transmembrane protein that is expressed in macrophages and osteoclasts. While the existence and expression pattern of human CD68 and mouse CD68 (sometimes called macrosialin) are well-known, and these molecules are routinely used as histological markers of tissue macrophages, the functional significance of CD68 expression remains an unanswered question. Our overall goal is to determine the significance and characterize the function of CD68 in osteoclasts and explore the effects of Receptor Activator of Nuclear Factor κB (RANK) signaling on CD68 post-translational modification. To achieve this goal, I used homologous recombination to generate a mouse line that lacks expression of CD68. Mice that lack CD68 expression have increased trabecular bone and dysfunctional, morphologically aberrant osteoclasts demonstrating the importance of CD68 expression for proper osteoclast function. Next, altered glycosylation of CD68 that occurs with RANK ligand (RANKL)-stimulated osteoclastogenesis was investigated. I found that RANKL induces an increase in terminal sialylation of CD68, and this change is dependent upon the signaling of RANK Tumor Necrosis Factor Associated Factor (TRAF) binding motifs $^{539}PVQET^{564}$ and $^{604}PVQEQQ^{609}$ and the non-TRAF motif $^{535}IVVY^{538}$ perhaps through the action of the
sialating enzyme ST3Gal1. Finally, I developed an assay to identify inhibitors of the signaling from $^{539}\text{PVQEE}^{564}$ and $^{604}\text{PVQE}^{609}$. In addition to contributing to the RANKL-induced modification of CD68, these signaling motifs are also necessary for the normal formation of osteoclasts, and inhibitors of these motifs may serve as valuable new anti-resorptive therapeutics. Importantly, the work of this dissertation establishes CD68 as an important molecule for osteoclast function and marks it as a potential therapeutic target for treating disorders of bone loss. In addition, this work should open new avenues of research into functions of CD68 and the significance of its RANKL-induced alternative glycosylation.
DEDICATION

I dedicate this dissertation to my mother, Kay Young Ashley, who has ever been my strongest advocate and to whom I owe all my past and future successes.

“What stands closer to a man all his days than his bones?”

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1 - INTRODUCTION

Bone Biology

Bone structure and composition

The skeleton is an essential organ system with significant impact on both physiological and pathological states. While generally considered unchanging inorganic masses that support body structure and provide physical protection for delicate soft tissues such as those of the heart, lungs, and brain, the bones are, in fact, highly dynamic organs whose functions extend beyond biomechanics and into complex processes such as hematopoiesis, immune function, and energy metabolism[1-3]. The composition of bone is intimately tied to these divergent and seemingly unrelated processes. The strength of the bone is owed to the crystalline matrix of inorganic hydroxyapatite (Ca$_5$(PO$_4$)$_3$(OH)) that constitutes roughly 70% of bone and type I collagen, bone’s most abundant protein. The remainder of the bone is composed of water, non-collagenous proteins, and cells[4].

Bone strength is not wholly dependent on material composition, however; the architecture of bone is also a vital contributor to bone quality. Indeed, pathologies with both associated increases and decreases in the amount of mineral in bone often result in increased fractures. This is due to aberration in macro- and micro-architectural features of the bone. On the macro level, bone can be subdivided into two classes: (1) cortical bone, which is the densely packed matrix that defines a bone’s overall shape and (2) trabecular bone, which is a less dense network of interlocking rods running through the marrow cavity that are found at high concentrations at the ends of bones and at lower amounts
towards the center[5,6]. At microscale, the matrix of bone can be ordered in one of two ways: (1) lamellar bone in which the matrix is highly organized into parallel layers and (2) woven bone which is less organized with successive matrix layers intersecting at un-uniform and random angles[7-9].

It is easy to imagine how reductions in bone matrix would cause structural problems at the macro level that result in increased bone fragility and risk of fracture. Reduced matrix translates to less material maintaining the cortical and/or trabecular bone. The severity of bone loss defines the disorder as osteopenia (less severe; defined statistically as bone mineral density 1.0 - 2.5 standard deviations below average peak bone mineral density) or osteoporosis (more severe; greater than 2.5 standard deviations below average peak bone mineral density)[10,11]. In the context of reduced bone mineral density, bones are more prone to fracture. The loss of bone strength in pathologies with increased bone is more subtle and requires consideration of the microarchitecture. In many cases, although bone is increased, there is a higher proportion of woven bone present[12]. While the stacked layers of lamellar bone amplify the force required to break the bone, woven bone does not have such synergy[13]. The result is increased, but poorly organized, bone that is more brittle than less dense but better organized bone. The cells that establish and maintain the bone matrix architecture will be discussed later in this section.

The cell content of bone is the main driver for its interaction with other systems. All hematological cells have their origin within the bone marrow[1]. These cells include not only erythrocytes and megakaryocytes, but also the cells of immunity. Indeed, the vertebrate immune system would not exist in its presently known form in the absence of
bone, as dendritic cells, macrophages, B cells, and T cells all originate from the same hematopoietic precursors that reside in the bone marrow[2]. Similarly, mesenchymal stem cells in the bone marrow can give rise to adipocytes which contribute to the regulation of energy metabolism[14,15]. There is also growing evidence of an ongoing chemical conversation between energy-regulating tissues and bone cells with metabolic factors such as insulin and insulin-like growth factor influencing bone architecture and density[16,17]. Bone-derived factors such as osteocalcin, in turn, have been shown to stimulate changes in systemic insulin sensitivity and fat metabolism[18-20].

Bone is important in maintaining body structure as well as in immunity and energy metabolism, but the skeleton is not an unchanging vessel for cells and regulatory factors. The skeleton must both grow and repair itself. The process of bone modeling occurs during development and growth, while remodeling persists throughout life. So dynamic is the bone remodeling process that, on average, the entire skeleton is replaced every ten years[21]. As with other processes, a bone’s shape, density, structure, and strength are dependent upon the cells within it. Thus, a discussion of the cells of bone remodeling is warranted to shed light on this process.

Osteoblasts and osteocytes

Within the bone marrow reside mesenchymal stem cells. As previously mentioned, these progenitors can give rise to adipocytes. These cells can also differentiate into myocytes and chondrocytes, but, importantly, these cells can become osteoblasts, which are responsible for generating new bone. The decision for a mesenchymal stem cell to become an adipocyte or osteoblast ultimately hinges upon the activation of one of
two master regulators: peroxisome proliferator-activated receptor gamma (PPARγ) drives adipogenesis where runt-related transcription factor 2 (Runx2) induces osteogenesis[22-25]. The factors and mechanical forces that stimulate PPARγ and/or Runx2 are diverse and numerous, and detailed description is beyond the scope of this discussion. The differentiation decision between adipocyte (fat) and osteoblast (bone) is not made in the bone alone; signals from adipose tissue, pancreas, digestive tract, brain, and even the external environment can influence the fate of a differentiating mesenchymal stem cell[26-30].

Once differentiated, an osteoblast is cuboidal in shape and adheres to the bone matrix[31-33]. Osteoblasts form bone in two steps. First, the cells secrete type I collagen fibrils that associate and form a fibrous matrix. Mixed with this collagenous matrix is a collection of growth factors such as bone morphogenetic proteins (BMPs) and transforming growth factor β (TGFβ) and matrix-associated proteins such as osteopontin, bone sialoprotein, and dentin matrix acid phosphoprotein[34-37]. This amalgamation of matrix and non-matrix proteins is termed the osteoid and represents the non-mineral component of the bone matrix. Next, the osteoblasts add a “cement” of calcium and phosphate in the form of hydroxyapatite to the osteoid, effectively mineralizing it[38].

A mineralizing osteoblast faces three potential fates. With its work finished, an osteoblast may simply die via the programmed suicide of apoptosis. Osteoblasts may also flatten, lose their bone-forming abilities, and persist as bone-lining cells. The third and most dramatic end involves a mineralizing osteoblast becoming buried in the growing matrix. Once buried within the bone, the osteoblast undergoes another differentiation process that changes it into a terminally differentiated osteocyte[39]. The osteocytes, which are the most numerous type of cell in the bone, remain entombed inside lacunae
within the bone matrix. These lacunae are connected by a network of tunnels, called canaliculi, into which the osteocytes extend tendril-like membrane projections[40]. In this way, osteocytes are able to communicate with one another and establish a cellular network throughout the bone from within the mineralized matrix itself. Osteocytes are the most poorly understood cells of bone remodeling, but studies have suggested that these cells are involved in sensing and directing responses to mechanical stress[41-43].

**Osteoclasts**

During bone organogenesis and growth, the growing bone matrix must be sculpted into its proper form, and during remodeling, which maintains the integrity of bone, damaged matrix must be removed. The cells responsible for these processes are not mesenchymal in origin, but hematopoietic. Among the great diversity of lineages arising from hematopoietic stem cells of the bone marrow, there is a subset of mononuclear cells that are capable of differentiating into bone-adherent macrophages upon stimulation with monocyte/macrophage-colony stimulating factor (M-CSF)[44,45]. These so-called bone-marrow macrophages (BMMs) persist within the bone and are capable of proliferation as long as M-CSF is available. The presence of a second cytokine, Receptor Activator of Nuclear Factor κB ligand (RANKL, also known as OPGL, ODF, TRANCE, TNFSF11) slows proliferation of BMMs and induces another differentiation[46]. During this process, there is increased expression of multiple genes involved in the breakdown of bone matrix including matrix metalloproteinase 9 (MMP9), cathepsin K (CtsK), carbonic anhydrase II (Car2), and tartrate-resistant acid phosphatase (TRAP)[47-52]. These cells, sometimes called mononuclear osteoclasts, are able, albeit weakly, to resorb bone, but
they are immature and inefficient[53]. In order to achieve maximum efficiency, these mononuclear cells must fuse into a mature, multinuclear syncytium – the osteoclast.

The osteoclast, with diameters often greater than 50 microns and dozens of nuclei, is commonly and appropriately described as a giant cell. These unique cells attach to the bone surface via rings of adhesion molecules that link with similarly shaped rings of actin and tubulin cytoskeletal fibers. The tight adhesion of the osteoclast to the bone matrix below these actin rings effectively forms a sealing zone that isolates the bone area within the ring from the rest of the environment[54]. Within this ring, on the membrane of the osteoclast, is a ruffled border formed by the fusion of specifically targeted vesicles that deliver the aforementioned MMP9 and CtsK to the encircled bone surface[55]. These vesicular fusions produce a folded membrane with tremendous surface area that is dotted with vacuolar ATPases that pump protons into the isolated space. The acidic pH and secreted proteolytic enzymes establish, essentially, a large extracellular lysosome[56]. In this space, called Howship’s lacuna, enzymes degrade the proteinaceous components of the bone while the secreted acid aids in the solubilization of the hydroxyapatite/mineral component.

As components are released from the bone matrix, they are endocytosed at the ruffled border and, in a highly-regulated process of vesicular trafficking, transcytosed through the osteoclast and released into the environment upon vesicular fusion with the apical membrane at a region called the functional secretory domain[57,58]. The length of time an osteoclast resorbs and, thus, the amount of bone broken down is subject to regulation, but, for the osteoclast, the resorption process always concludes in the same fashion: death via apoptosis[59].
**Bone remodeling**

As is true for all other tissues and processes, the cells of bone remodeling do not operate in isolation. Indeed, for bone remodeling to be properly regulated and for the homeostatic balance of bone formation and resorption to be maintained, the activities of the osteoblasts and osteoclasts must be closely paired. The phenomenon of balanced bone resorption and formation is referred to as coupling, and its basis lies in communication between osteoclasts, osteoblasts, and osteocytes.

As described above, the critical factor for osteoclast precursor differentiation into mature osteoclasts is RANKL, and osteoblasts are a major source of RANKL in the bone[60]. In this way, an increase in the number of bone-forming osteoblasts can have a proportionate increase in the local concentration of RANKL that will stimulate osteoclastogenesis in the region of the bone where formation is occurring. Osteoclastogenic signals from outside the bone are often also dependent upon the osteoblasts. One such systemic factor is parathyroid hormone, which is normally secreted by the parathyroid glands into the blood stream in response to decreases in blood calcium concentration. Clinically, it has been found that intermittent parathyroid hormone infusion can stimulate osteoblast activity by activating its parathyroid hormone receptor. Continuous receptor activation, however, results in osteoclast-driven calcium liberation from the bone by increasing osteoblast expression of RANKL[61-63].

As an increase in osteoblast activity can stimulate bone resorption, so too can osteoclast activity stimulate bone formation. During bone formation, osteoblasts secrete multiple factors that become sequestered in the growing mineralized matrix. These fac-
tors, importantly TGF-β, are released from the bone as it is resorbed[35]. The local increase in these factors attracts and drives the recruitment of osteoblast precursors that differentiate into mature osteoblasts. In addition to these bone-derived coupling factors, the osteoclasts themselves secrete osteoblast-stimulatory molecules, though the identities of these signals remain undetermined[64].

As described, local bone remodeling seems likely to be unending with osteoblasts and osteoclasts continuously promoting each others’ activities. While global bone remodeling is a continuous process, local resorption and formation does not proceed ad infinitum. The key cellular regulator of these processes is the osteocyte. From within their lacunae dispersed throughout the bone, osteocytes are capable of detecting mechanical forces and the microfractures that can result from them. When local damage is severe enough to require repair, nearby osteocytes secrete factors to recruit osteoclast precursors to the damaged area. There is evidence that osteocytes release these factors while undergoing apoptosis, and it has been shown that conditioned medium from apoptotic osteocytes can stimulate osteoclast precursor migration and differentiation in vitro[65,66]. Conversely, osteocytes are major source of osteoprotegerin (OPG), which is a soluble decoy receptor for RANKL that can inhibit osteoclastogenesis[67]. Between these two mechanisms, osteocytes regulate the activity of the osteoclasts. With respect to osteoblasts and bone formation, osteocytes can secrete the potent Wnt signaling inhibitor, sclerostin. The importance of Wnt signaling in osteoblast function and the strength of sclerostin as an inhibitor of bone formation are apparent in humans and mice lacking sclerostin that suffer severe increases in bone mass[68-70]. By these mechanisms and, likely, others, osteocytes effectively prevent an unending cycle of local bone remodeling.
Taken together, the bone remodeling process can be thought of as five phases: activation, resorption, reversal, formation, and termination[71]. During activation, local or systemic signals trigger the recruitment and differentiation of osteoclast precursors. During resorption, mature osteoclasts break down the mineral and proteinaceous matrix of bone releasing both osteoclast- and matrix-derived factors. The reversal phase is marked by a reduction in osteoclast activity and recruitment of osteoblast precursors by factors released during resorption. Osteoblasts then form and mineralize new bone, with some of the osteoblasts differentiating into osteocytes during the formation phase. At termination, bone formation concludes, and the remodeled bone enters into a quiescent state.

Diseases of Bone

*Osteoporosis*

In normal physiological states, this process of remodeling is necessary for the health and quality of bone. In pathological states, however, the homeostatic balance of resorption and formation can be disturbed resulting in either abnormal increases or decreases in bone. Two common disorders resulting from general increases in bone resorption are osteopenia and osteoporosis. As described above, both osteopenia and osteoporosis are defined clinically by statistical criteria. When a patient’s bone mass is measured, it is compared to a normal distribution of peak bone masses measured in young adults. A bone mass between 1 and 2.5 standard deviations below the mean defines osteopenia, while the more severe osteoporosis is marked by bone masses more than 2.5 standard deviations below the mean[72]. The cause of enhanced resorption can be broken down
into 5 categories: (1) unloading, (2) vitamin D/calcium deficiency, (3) corticosteroid use, (4) immune dysfunction, and (5) hormone dysfunction. Among these, the decrease in sex hormones associated with aging is the most common cause of osteoporosis. In particular, the reduction in estrogen that accompanies menopause is a major contributor; thus, osteoporosis is most common in women over fifty [73]. Reductions in testosterone (which can be converted to estrogen in the bone) during andropause raise the risk of osteoporosis in men over seventy [74,75]. The hormone estrogen is a systemic inhibitor of osteoclast activity, and the decrease in estrogen levels during menopause reduces systemic regulation of bone resorption [76,77]. This, combined with other factors such as inflammation, can result in bone loss.

Cancer metastasis to bone

The presence of metastatic tumor cells within the bone is also often associated with aberrations in bone density and/or architecture. Metastases that tend to stimulate osteoclast produce lytic lesions characterized by large, focal bone loss; conversely, those metastases that promote osteoblast activity produce lesions that have increased bone density that is often woven and of poor material quality [78,79]. Nevertheless, even in cases of osteoblastic metastases, there is evidence that osteoclast activity is necessary for the growth of such bony lesions [80].

Osteopetrosis

Disorders of pathological increases in bone density are often due to genetic defects that result in decreased osteoclast function. An exception to this is a familial osteo-
petrosis that originates from mutations in the \textit{SOST} gene which encodes a Wnt signaling inhibitor called sclerostin\cite{69}. In this disease, bone is increased due to over-activity of the osteoblasts. Most cases of familial osteopetrosis are due to loss of functional carbonic anhydrase, chloride channel 7, and the vacuolar ATPase all of which are involved with the production or secretion of acid during bone resorption\cite{81-83}. Whether the disorder is acquired or familial, characterized by increases or decrease in bone mass, the osteoclast is a critical cell in the pathophysiology of the bone, and investigation and understanding of these unique cells is critical for future management of these diverse diseases of bone.

\textit{Current osteoporosis therapies}

Treatments for osteoporosis fall into 2 broad categories: (1) anti-resorptives and (2) anabolics. Anti-resorptives target the osteoclasts and include estrogen hormone replacement therapy, bisphosphonates, and RANKL inhibitors. Hormone replacement was one of the earliest effective treatments of osteoporosis, but it has largely fallen out of use due to an increased risk of cancer that is associated with estrogen replacement\cite{84,85}. Bisphosphonates, which are molecules defined by a pyrophosphate group linked to a variable organic side chain, function by incorporating into the inorganic bone matrix and directly inhibiting osteoclasts. Due to their relatively low cost and multiple delivery options, bisphosphonates are the most common treatment for osteoporosis. Bisphosphonates are not without problems, however, as there are concerns regarding risk of osteonecrosis of the jaw, atypical fractures, and esophageal cancer\cite{86-90}. The newest anti-resorptive therapy approved for use is a humanized monoclonal antibody called denosumab (trade name: Prolia). This antibody binds and inhibits the osteoclast differentiation factor,
RANKL, thereby reducing the number of functional osteoclasts[91]. While denosumab is effective in osteoclast inhibition, the diversity of cells utilizing the RANK signaling system (in particular, T, B, and dendritic cells) raises the possibility of undesirable off-target effects[92,93]. Thus, while there are a variety of anti-resorptive therapies available, no therapy lacks drawbacks, and this should drive work towards new, more specific anti-resorptive therapies.

Anabolic agents counter the bone loss of osteoporosis by stimulating new bone formation. The most commonly used anabolic therapy is intermittent treatment with a recombinant fragment of parathyroid hormone called teriparatide (trade name: Forteo)[94]. Physiologically, parathyroid hormone stimulates calcium release from the bone. Therapeutically, and for reasons not entirely understood, intermittent rather than continuous teriparatide treatment results in short term increases in osteoblastic bone formation[95]. This treatment is useful in restoring some of the bone that is lost in osteoporosis. The primary drawback of teriparatide is its limited window of usefulness – the anabolic effect diminishes over time[96]. Thus, there is great pressure for new anabolics.

One such new anabolic is an antibody against sclerostin, which is an endogenous inhibitor of Wnt signaling in bone[97]. Familial loss of sclerostin function results in osteopetrosis due to enhanced osteoblast activity[68,98]. In pre-clinical models, an antibody that inhibits sclerostin has been shown to promote new bone formation and reverse bone loss in models of post-menopausal osteoporosis[99,100]. It is likely that future clinical studies of sclerostin inhibition will determine whether this approach represents a long sought success in the search for a new bone anabolic.
Receptor Activator of Nuclear Factor κB

Historical overview

Prior to 1998, the only consistently reliable method for differentiating osteoclasts from mononuclear precursors was to co-culture the cells with osteoblasts or the osteoblast-like cell line ST2. In 1997, Anderson et al. identified a novel TNF receptor family member, which they termed Receptor Activator of Nuclear Factor κB (RANK), during a screen of a myeloid dendritic cell cDNA library[101]. In the same study, the investigators also identified a ligand for RANK (RANKL) expressed by a T-cell lymphoma line using a soluble RANK-Fc screen followed by cDNA cloning. Functionally, this study revealed a role for RANKL and RANK in dendritic cell activation. RANKL was simultaneously identified as an activator of c-Jun-N-terminal kinase (JNK) in T-cells by Wong et al., who termed it TNF-related activation-induced cytokine (TRANCE)[102]. In the same year, Simonet et al. and Tsuda et al. both identified factors that could inhibit osteoclast formation calling them osteoprotegerin (OPG) and osteoclastogenesis inhibitory factor (OCIF)[103,104]. Sequence analysis later revealed that OPG and OCIF were the same protein[105].

A year later, Lacey et al. and Yasuda et al. simultaneously identified soluble factors that stimulate osteoclast formation calling them OPGL and osteoclast differentiation factor (ODF), respectively[46,106]. Upon deeper analysis, it was determined that not only were OPGL and ODF the same molecule, but this molecule had already been identified as TRANCE and RANKL. Thus, in the span of two years, the primary factor of osteoclastogenesis, its receptor, and its soluble inhibitory decoy receptor were identified. These simultaneous discoveries resulted in multiple names for the same molecule. By
convention of assigning nomenclature on order of discovery, the American Society for Bone and Mineral Research President’s Committee on Nomenclature defined the names of these molecules as RANK, OPG and RANKL[107]. NCBI nomenclature defines the genes encoding these factors as TNFRSF11a, TNFRSF11b, and TNFSF11, respectively.

RANKL and RANK knockout mice generated in 1999 and 2000, respectively, reiterated the importance of both receptor and ligand in osteoclast differentiation [108,109]. In both instances, knockout mice demonstrated a severe osteopetrosis marked by the total absence of osteoclasts in their bones, a significant reduction in the size of the marrow cavity, and an enlarged spleen due to its assumption of hematopoietic responsibilities following the failure of adequate marrow-based hematopoiesis. Interestingly, while homozygotes are fertile, pups from knockout animals often perish shortly after birth due to inadequate nutrition. This is unrelated to osteoclast function as, in the absence of RANK signaling in mammary epithelial cells, mammary gland maturation cannot occur. It is, however, important evidence that RANK signaling has roles in systems other than bone. In the following section, the known signaling initiated by the interaction between RANKL and RANK as it pertains to osteoclasts will be discussed.

_Tumor Necrosis Factor Receptor Associated Factor (TRAF) motif signaling_

Similar to other members of the Tumor Necrosis Factor Receptor (TNFR) superfamily, RANK initiates signaling through adaptor proteins known as Tumor Necrosis Factor Receptor Associated Factors (TRAFs)[110]. TRAFs serve as both merging and branching points for receptor-mediated intracellular signaling, and different members of the TRAF family mediate different signaling cascades. In the case of RANK, TRAFs 2,
5, and 6 are of particular functional significance. TRAF6 has been shown to be the most important single TRAF in the process of osteoclastogenesis as TRAF6 knockout mice are the only TRAF deficient animals that recapitulate the bone phenotype seen in RANK knockout animals[111]. TRAF6 binds to consensus sequences on RANK and, through its adaptor function and intrinsic ubiquitin ligase activity, initiates signaling through JNK, p38, nuclear factor κB (NF-κB), extracellular signal-related kinase (ERK), c-Fos, and nuclear factor of activated T-cells c1 (NFATc1).

Individual genetic ablation of TRAF2 and TRAF5 has been less informative as single deletion of either TRAF does not result in a large defect in osteoclastogenesis in vitro. This is likely due to compensation as TRAF2 and TRAF5 recognize the same consensus amino acid sequence. Nevertheless, the RANK signaling initiated by TRAF2/5 is important in osteoclastogenesis, and this importance is seen when RANK’s TRAF2/5 binding motifs are mutated. Mutation of two motifs (PVQEET\(^{560-565}\) and PVQEQQ\(^{604-609}\)), in particular, results in a severe defect in osteoclastogenesis both in vitro and in vivo[112]. Work with single mutant receptors revealed that, of the six-recognized RANK-initiated signal cascades, PVQEET\(^{560-565}\) and PVQEQQ\(^{604-609}\) are able to activate only NF-κB. This common pathway coupled with the importance of these motifs in osteoclastogenesis has proven useful in developing a screening assay for new anti-osteoclast drugs, and discussion of this assay will be presented later in this dissertation[113].

**Signaling from non-TRAF motif IVVY**

It is generally recognized that the six TRAF-mediated signaling pathways JNK, ERK, p38, NF-κB, c-fos, and NFATc1 are essential to the normal development of osteoc-
lasts. There is evidence, however, that activation of these pathways and others by factors other than TRAF are also critical. The most compelling example of this comes from experimental manipulation of the RANK motif IVVY. Point mutation of this motif (IVVY → IVAF) or application of an exogenous cell permeable peptide that mimics this sequence is sufficient to inhibit osteoclastogenesis[114-116]. What differentiates IVVY inhibition from inhibition of RANK’s known TRAF-binding motifs is the maintenance of all previously-mentioned signaling pathways except NFATc1. The inhibition of an accepted TRAF6-initiated pathway via inhibition of a non-TRAF motif is compelling, and evidence points to IVVY-mediated activation of Gab2 and phospholipase Cγ2 as the mechanism of NFATc1 activation[117]. IVVY may also be responsible for other osteoclast-critical signaling, as one study has demonstrated that interaction of a guanine nucleotide exchange factor called Vav3 with IVVY is critical for normal actin ring formation, and work that will be presented in this dissertation suggests other NFATc1-independent roles for IVVY, as well[116].

CD68

*Historical overview*

In 1987, Smith and Koch developed an antibody against a macrophage glycoprotein by injecting a rat with concanavalin A-purified macrophage proteins and fusing the resultant antibody-secreting cell clones with immortalized plasma cells[118]. The antibody from clone FA/11 was found to recognize a predominantly intracellular glycoprotein of molecular mass between 87 and 115kDa in macrophage-like cell lines, and the authors tentatively named this protein macrophage glycoprotein antigen (MAG). Shortly
after, MAG was renamed macrosialin by Rabinowitz and Gordon following their observations that it is differentially glycosylated under varying immunological states in a manner similar to a leukocyte glycoprotein called leukosialin[119]. Similarly, in 1989, six antibodies (Y2/131, EBM11, Ki-M6, Ki-M7, KP1, and Y1/82A) generated using lung and lymph node macrophages as immunogens that recognize a human macrophage-specific protein were classified as cluster of differentiation 68 (CD68) by the Fourth Workshop on Leukocyte Differentiation Antigens, and the protein they recognize was designated CD68 antigen[120]. By literary convention, however, CD68 generally refers to the protein and not the antibody group.

Following cDNA cloning of macrosialin and CD68, it was revealed via sequence analysis that macrosialin and CD68 are the mouse and human forms of the same protein[121,122]. In early publications, macrosialin referred to the mouse molecule while CD68 was reserved for studies using the human form. Recently, however, CD68 has come to refer to both the human and mouse forms, and the term CD68 will be used almost exclusively throughout this dissertation. Whatever terminology is used, CD68, from nearly the time of its designation, has proven highly useful in tissue studies. Its predominant restriction to cells of myeloid origin has made it the histological marker of choice for macrophages in tissue sections[123]. Indeed, identifying functional studies of CD68 in the literature is a challenge because of the over five thousand publications that reference CD68, the greatest majority are not studies of CD68, but rather use CD68 as a tool for identifying macrophages. Nevertheless, structural and functional studies of CD68 have been published and will be discussed in the following sections.
Structure and expression of the CD68 gene and protein

CD68 is classified as a member of the lysosome associated membrane protein (LAMP) family due to its primarily lysosomal localization and its partial sequence homology to other LAMP family members[121,122]. This type I transmembrane protein greatly resembles LAMPs in that it consists of a short, ten amino acid carboxy-terminal tail, transmembrane domain, and N-glycosylated membrane proximal domain marked with intramolecular disulfide bonds followed by a proline-rich region. Beyond the proline-rich region, however, CD68 displays a “bottle brush” structure not seen in any other LAMP. This amino-terminal region is rich in serine and threonine residues that are heavily mucin-type O-glycosylated[124]. Because of this, CD68 can best be considered a LAMP with mucin characteristics.

The gene encoding CD68 consists of 6 exons, and study of its promoter has provided insights into its regulation and macrophage specificity[121,122]. Through sequence analysis and transcription factor binding experiments, it has been determined that CD68 expression is activated by members of the E-twenty-six (ETS) transcription factor family, particularly Elf-1, Fli-1, and PU.1[125]. While ETS members can be found in multiple lineages, Elf-1, Fli-1, and PU.1 are found more commonly in hematopoietic, lymphoid, and myeloid lineages. Nevertheless, CD68 expression has only been consistently detected in myeloid lineage cells such as macrophages and osteoclasts. This specificity is explained, in part, by the presence of interferon regulatory factor (IRF) binding sites in the CD68 promoter. Binding of PU.1/IRF-4 complexes to the CD68 promoter represses CD68 expression, and this factor is found highly expressed in lymphoid, but not myeloid, lineages. In addition, PU.1 expression is elevated in myeloid lineages as compared to
lymphoid lineage cells and is critical to macrophage differentiation. In this way, elevated
PU.1 along with Elf-1 and Fli-1 promote CD68 expression in myeloid-lineage cells as they
differentiate into macrophages while expression of IRFs prevent similar up-
regulation of CD68 in other cell types.

Other than lineage-restricted expression, the levels of CD68 in a tissue is also
regulated by environmental factors. The most studied stimulus of CD68 expression is
dietary fat. It has been demonstrated that animals on a high-fat diet demonstrate increased
hepatic CD68 expression[126]. This has also been shown on a cellular level, with macro-
phage expression of CD68 increasing upon stimulation with oxidized low density lipo-
protein (oxLDL)[127]. This up-regulation can be blunted via the application of LDL-
lowering drugs. This apparent regulation by fat, along with other characteristics that will
be described later, has led to speculation of a role for CD68 in lipid metabolism. Fur-
thermore, not only is CD68 level of expression subject to regulation, but its post-
translational modification can also be altered in different circumstances.

_Glycosylation of CD68_

It was stated earlier that CD68 is a glycosylated protein, but, to fully appreciate
the degree to which CD68 is modified by sugar, one must consider the predicted molecu-
lar mass of the primary amino acid sequence of 36 kDa in comparison to its apparent
molecular mass of over 90 kDa. With, in some cases, over two-thirds of its mass attribu-
table to sugar, CD68 is a heavily glycosylated molecule. Furthermore, when detected via
Western immunoblotting, CD68 appears as a smear rather than a single band, indicating
that multiple glycoforms may exist in a population, though many of these forms may be intermediates in the process of modification[122].

While CD68 is both N-glycosylated at nitrogen-containing asparagine residues and O-glycosylated at serine and threonine residues, its O-glycosylation is the best understood. Sometimes called mucin-type glycosylation, the O-glycosylation of CD68 occurs in the Golgi complex and begins with the addition of an N-acetylgalactosamine (GalNAc) residue to the hydroxyl group of a serine or threonine[124]. Next, galactose is added joining C1 of galactose to C3 of the already-attached GalNAc. This is the most basic α-glycan moiety and is referred to as core 1 (Figure 1, Core 1). Additional sugars can be added to the galactose to generate extended core-1 glycosylations or an N-acetylglucosamine (GlcNAc) can be added at its C1 to GalNAc’s C6 to form the basic core 2 structure (Figure 1, Core 2). Similar to core 1, core 2 glycans can be extended by adding sugars to the terminal GlcNAc. Other core structures exist, but only core 1 and core 2 based chains are typically seen on membrane-bound proteins.

Figure 1. Structure of core 1 and core 2 glycans
The glycobiology of CD68 has been most thoroughly studied by a group headed by Siamon Gordon. Through their studies, this group found that CD68 glycosylation is altered by inflammatory stimuli[119]. Indeed, the parallel between the alternative glycosylation of CD68 and another glycoprotein, leukosialin, according to inflammatory states and cell activation led to the investigators proposing the name macrosialin for CD68. By comparing resting peritoneal macrophages to actively phagocytosing macrophages, they revealed that CD68 expressed by resting macrophages has primarily core 1 glycosylation where phagocytosis stimulates a conversion to core 2[128]. They further reported that macrophages of the bone marrow (i.e. osteoclast precursors) express the core 2 “phagocytic glycoform” of CD68. To date, however, the significance of these alternate glycoforms has not been determined.

Established properties and proposed functions of CD68

When cells are stained for CD68 expression either in tissue sections or cell culture, the majority of reactivity can be found intracellularly. Closer examination reveals that CD68 localizes primarily to intracellular vesicles such as lysosomes and endosomes[129]. Nevertheless, a small percentage of total CD68 expression can be found on the cell surface. The surface fraction of CD68 is rapidly internalized and recycled, and this cycling coupled with observed upregulation of CD68 by oxLDL has led to speculation that CD68 is a scavenger receptor involved in the uptake up oxLDL[130]. Further supporting this hypothesis, affinity purification and ligand blotting studies have revealed that CD68 can bind oxLDL[131,132]. This property has been exploited in a pre-clinical
model of atherosclerosis where a soluble fusion protein of CD68 and the Fc antibody chain was used to slow the accumulation of lipid in atherosclerotic lesions and foam cell formation[133,134].

This potential role for CD68 in lipid uptake is a complex one, however, as there are conflicting data from in vitro studies. One of the earliest studies reported that antibody blockage of CD68 resulted in reduced oxLDL binding and uptake by THP-1 macrophage-like cells[132]. Later however, these findings could not be replicated in either RAW264.7 macrophage-like cells or primary mouse macrophages that had been treated with small interfering RNA against CD68[126]. The question of whether CD68 is involved in oxLDL uptake in macrophages seems to have been answered by investigation of the oxLDL scavenging abilities of macrophages derived from CD68 knockout mice[135]. These macrophages are not deficient in their ability to take up oxLDL, which would suggest the lack of a role for CD68 in oxLDL uptake or redundancy in the mechanisms of oxLDL uptake in macrophages. This does not, however, preclude potential intracellular functions related to its oxLDL binding properties.

With much of the focus on the oxLDL affinity of CD68 and its expression in macrophages, there has been little attention to its expression by osteoclasts. Furthermore, to date, there has been no published example of cellular dysfunction due to CD68 inhibition in any cell type. Chapters 2 and 3 of this dissertation describes the osteoclast dysfunction that results from genetic deletion of CD68 followed by a brief exploration of RANK’s role in CD68 glycosylation[136]. Chapter 4 describes a cell-based method developed to identify inhibitors of a RANK signaling pathway that contributes to regulation of CD68[113].
Summary and Dissertation Objective

Osteoclasts are a critical component of bone health, but when these cells function in an unregulated fashion, disease can result. Over the past two decades research into the mediators of bone resorption and osteoclast differentiation have greatly enhanced understanding of this unique cell and created a toolkit for managing bone loss due to excessive resorption. Though much has been learned regarding the signaling of RANK and the molecular machinery of the osteoclast, there is still more to be determined. During the course of the work contributing to this dissertation, 3 goals were pursued: (1) explore the significance of CD68 expression in the osteoclast, (2) investigate the effect of RANK signaling on CD68, and (3) develop an assay that can exploit RANK’s motif-specific signaling for drug identification.
2 - GENETIC ABLOCATION OF CD68 RESULTS IN MICE WITH INCREASED BONE AND DYSFUNCTIONAL OSTEOCLASTS

by

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Abstract

CD68 is a member of the lysosome associated membrane protein (LAMP) family that is restricted in its expression to cells of the monocyte/macrophage lineage. This lineage restriction includes osteoclasts, and, while previous studies of CD68 in macrophages and dendritic cells have proposed roles in lipid metabolism, phagocytosis, and antigen presentation, the expression and function of CD68 in osteoclasts have not been explored. In this study, we investigated the expression and localization of CD68 in macrophages and osteoclasts in response to the monocyte/macrophage-colony stimulating factor (M-CSF) and the receptor activator of NF-κB ligand (RANKL). We found that M-CSF stimulates CD68 expression and RANKL alters the apparent molecular mass of CD68 as measured by Western immunoblotting. In addition, we explored the significance of CD68 expression in osteoclasts by generating mice that lack expression of CD68. These mice have increased trabecular bone, and in vitro assessment of CD68−/− osteoclasts revealed that, in the absence of CD68, osteoclasts demonstrate an accumulation of intracellular vesicle-like structures, and do not efficiently resorb bone. These findings demonstrate a role for CD68 in the function of osteoclasts, and future studies will determine the mechanistic nature of the defects seen in CD68−/− osteoclasts.

Introduction

CD68, also known as macrosialin, is a heavily glycosylated LAMP family member that is commonly used as a histological marker of macrophage lineage cells. Indeed,
CD68 expression can be found in the resident macrophages of multiple tissues such as microglia in the brain, Kupffer cells in the liver, and bone marrow macrophages (BMMs) [1-3]. Furthermore, immunohistochemical staining of bone tissues has demonstrated expression of CD68 in osteoclasts, which are multinucleated, bone-resorbing giant cells of monocyte/macrophage origin [3,4]. Infiltration of CD68-positive cells is also used clinically as a marker of inflammation and tumor progression [5-8]. The apparent monocyte/macrophage lineage specificity of CD68 expression has not only led to its routine use in histological identification of macrophages, but to the proposed use of the CD68 promoter to specifically direct transgene expression in both in vitro and in vivo model systems and gene therapy [9-11]. Though both CD68 protein and gene have been and continue to be used as tools in both research and clinical settings, studies of the physiological function(s) of CD68 remain inconclusive.

Despite the lack of a specific, defined role for CD68 in cells, studies of the characteristics and regulation of the CD68 gene and protein have inspired many of the early studies into its function. Ramprasad et al. demonstrated via ligand blotting and affinity purification experiments that CD68 is capable of binding oxidized low density lipoprotein (oxLDL) and can be detected on the surface of macrophages [12-14]. This oxLDL binding property was found by multiple groups, and, recently, the oxLDL binding properties of CD68 were exploited in a pre-clinical model wherein atherosclerotic lesion progression was retarded by application of a soluble Fc receptor-CD68 fusion protein [15,16]. The binding of oxLDL by CD68 appears to be independent of associated sugar moieties as even deglycosylated CD68 retains much of its oxLDL binding affinity. CD68 not only binds oxLDL, but its expression also appears to be upregulated by oxLDL [17].
de Beer et al. found that levels of CD68 in the livers of mice fed a high fat diet were increased, and a direct effect of lipid on macrophage expression of CD68 was confirmed by Llaverias et al. [18,19]. These studies of the oxLDL binding affinity of CD68 and its expression on the cell surface suggested a role for CD68 in the uptake of oxLDL, and, indeed, antibody blockage of CD68 on PMA-stimulated THP-1 monocytes resulted in reduced binding and uptake of oxLDL [13]. However, dsRNA-mediated silencing of CD68 expression in both primary peritoneal macrophages and macrophage-like RAW264.7 cells failed to reduce oxLDL binding by either cell type [18]. In addition, it was recently determined via examination of peritoneal macrophages from mice lacking expression of CD68 that neither oxLDL uptake nor microbe phagocytosis is dependent on CD68 expression [20]. Thus, despite its accepted oxLDL binding properties, the specific role of CD68 remains controversial, and there has been no further demonstration of the physiological significance of CD68 expression in any cell type.

Given that most studies of CD68 have focused on macrophages and macrophage-like cell lines, we set out to examine CD68 expression in osteoclasts, its regulation by the osteoclastogenic cytokines M-CSF and RANKL, and the consequences of its genetic ablation. Here we report that loss of CD68 results in morphological and functional defects in osteoclasts in vitro that result in increased bone in vivo.

Results

CD68 expression during osteoclastogenesis

We first assessed the expression of CD68 by BMMs and osteoclasts. To this end, we collected whole cell lysates from primary mouse BMMs cultured for varying periods
of time in M-CSF alone, which maintains the macrophage characteristics of the cells, and BMMs cultured with M-CSF and RANKL, which induces differentiation of BMMs to osteoclasts. We found that freshly isolated non-adherent bone marrow mononuclear cells do not express detectible levels of CD68, and addition of M-CSF stimulated expression of CD68 in a time-dependent manner (Fig. 1A). Interestingly, while addition of RANKL did not result in significantly altered levels of CD68 compared to M-CSF alone, RANKL treatment reduced the apparent molecular mass of CD68 as measured by its migration rate during polyacrilamide gel electrophoresis followed by Western blotting. Similar to primary BMMs, RAW264.7 cells, which are self-sufficient in their M-CSF receptor signaling, constitutively express CD68, and addition of RANKL resulted in a comparable shift in CD68 migration rate with no significant change in expression (Fig. 1B). CD68 can be found on the cell surface of macrophages, and this RANKL-induced form of CD68 may be subject to altered surface localization [21,22]. To determine whether the RANKL-induced form of CD68 can still be detected on the surface of BMMs, we analyzed primary BMMs treated for 72 hours with either M-CSF alone or M-CSF and RANKL via flow cytometry. We found that BMMs cultured with M-CSF alone express detectible levels of CD68 on their surface, and RANKL treatment does not appear to alter this surface expression (Fig. 1C – “Surface”). CD68 expression was also detected intracellularly by permeablizing cells prior to staining (Fig. 1C – “Total”).

Cellular localization of CD68 in osteoclasts

Our own immunoblotting and published tissue immunohistochemical studies have revealed expression of CD68 by osteoclasts. We next sought to determine the intracellu-
lar distribution of CD68 in mature, bone-adherent osteoclasts by performing immunofluorescent staining of osteoclasts differentiated on bovine cortical bone slices. Following staining with Alexa-488-conjugated phalloidin for actin (green), Hoechst for nuclei (blue), and either anti-CD68 antibody or non-immune Rat IgG2a (red), cells were visualized using confocal microscopy (Fig. 2). Staining revealed multiple nuclei and actin rings which are morphological features of mature osteoclasts and intense localization of CD68 around the periphery of the osteoclasts (Fig. 2A). CD68 could also be detected, though less intensely, towards the central regions of the cell. Visualization of osteoclasts along the Z-axis revealed a vertical concentration of CD68 at the osteoclast periphery with a more apical localization towards the center of the cell (Fig. 2B). Three-dimensional reconstruction of imaged osteoclasts confirmed this dome-like distribution with CD68 detected near both the bone-apposed, basolateral, and apical surfaces along the cell periphery, but only near the apical surface elsewhere (Fig. 2C).

Generation of CD68\(^{-/-}\) mice lacking expression of CD68

Detection of CD68 on the surface of BMMs and its distribution in osteoclasts prompted us to initiate deeper functional studies of this protein. To further explore the role of CD68 in bone specifically and elsewhere in general, we generated a line of mice that lack expression of CD68. Fig. 3A-C detail the strategy of homologous recombination within C57Bl/6J mouse embryonic stem (ES) cells to generate the CD68 knockout allele. This recombination event replaces exon 1, which contains the CD68 start codon, and part of exon 2 with a neomycin phosphotransferase expression cassette (Fig. 3A-B). Male chimeras generated by injecting the correctly targeted ES cells into albino C57Bl/6J
 blastocysts were mated with albino C57Bl/6J females, and black-coated offspring were genotyped using primers specific to the wild type (Fig. 3B) and knockout alleles (Fig. 3C) to identify CD68^{+/−} heterozygotes. Male and female heterozygotes were then mated to produce CD68^{+/-}, CD68^{+/−}, and CD68^{−/−} offspring as determined by PCR (Fig. 3D). CD68^{−/−} mice are viable and are born near to expected Mendelian frequencies (Table 1).

Similar to mice generated by Li et al., we observed no obvious behavioral or gross phenotypic abnormalities in the CD68^{−/−} animals [20]. We confirmed loss of CD68 expression in CD68^{−/−} mice by performing immunoblots on BMM protein extracts from mice of each genotype (Fig. 3E).

**Bone phenotype of CD68^{+/+}, ^{+/-}, and ^{-/-} mice**

To determine the consequences of genetic ablation of CD68 on the bone microarchitecture and tissue mineral density (TMD), we assessed the distal femurs of 6-month-old female mice using micro-computed tomography (μCT) and histology. TMD is the measure of mineral content of bone tissues only, as compared to bone mineral density (BMD), which includes surrounding non-bone tissues. This makes TMD a more accurate measurement of the mineral density of specific microarchitectural structures such as trabecular and cortical bone[23]. Quantitative μCT data revealed that, compared to CD68-expressing mice, mice lacking CD68 have increases in bone volume (BV/TV) and trabecular number (Tb. N) with a concurrent decrease in trabecular spacing (Tb. Sp.)(Fig. 4A-B). Interestingly, there was a significant reduction in trabecular TMD in CD68^{−/−} mice compared to CD68^{+/+} mice with CD68^{+/-} trending towards lower trabecular TMD (Fig. 4B); this is intriguing because, while there is more trabecular bone in CD68 deficient
animals, the level of trabecular mineral density appears to be abnormal. This decrease in TMD was not seen in cortical bone, however, nor was cortical thickness altered (Ct. Th.) (Fig. 4C-D). Histological examination of decalcified, TRAP-stained femur sections similarly showed increased trabeculae in CD68\(^{-/-}\) mice and abundant bone-adherent osteoclasts in all three genotypes (Fig. 5A-B) with no significant difference in osteoclast numbers (N. OC/BS) (Fig. 5C). These findings of increased trabecular bone without a corresponding decrease in osteoclasts suggest that loss of CD68 negatively impacts osteoclast function without reducing osteoclast numbers \textit{in vivo}.

As with the paraffin-embedded, TRAP-stained sections, plastic-embedded trichrome-stained sections demonstrated increased trabecular bone (Fig. 5D-E), and static histomorphometry values showed concordance with previous μCT-measured parameters (Fig. 5F – BV/TV and Tb. N). The decrease in trabecular TMD suggested a potential defect in osteoblast function, but quantification of osteoblast numbers showed no significant differences between the three genotypes (Fig. 5F – N. OB/BS). Furthermore, both CD68\(^{+/+}\) and CD68\(^{-/-}\) mice showed significant increases in mineral apposition rate (MAR) as measured by dynamic histomorphometry of double calcein-labeled sections (Fig. 5G). We found that wild type osteoblasts do not express CD68, which suggests that the observed changes in mineralization are likely due to indirect effects of CD68 ablation on osteoblast function (Fig. 6).

\textit{In vitro assessment of CD68\(^{+/+}\), \(^{+/}\), and \(^{-/-}\) osteoclast activity}

Given the increase in trabecular bone seen \textit{in vivo} following CD68 knockout, we next sought to determine the bone resorption efficiency of osteoclasts differentiated \textit{in vitro}.
BMMs were seeded onto bovine cortical bone slices with M-CSF and RANKL and allowed to differentiate into mature osteoclasts over 4 days. After an additional 3 days of resorption, cells were removed from the bone slices, and pits were imaged using confocal microscopy (Fig. 7A). We found that CD68⁻/⁻ osteoclasts resorbed a significantly smaller area than CD68-expressing osteoclasts by quantification of images generated from multiple bone slices (Fig. 7B). This confirmed our suspicion that CD68⁻/⁻ osteoclasts are less efficient at bone resorption than their CD68-expressing counterparts.

**In vitro morphological characterization of CD68⁺/⁺, +/−, and −/− osteoclasts**

In addition to preparing osteoclasts on bone slices to measure their resorption efficiency, we differentiated osteoclasts from precursors of each genotype in tissue culture plates to assess their *in vitro* morphology. Following differentiation, CD68⁻/⁻ osteoclasts appear as well spread as CD68-expressing osteoclasts, but osteoclasts lacking CD68 demonstrate an intracellular accumulation of vesicle-like structures that occur only rarely in CD68-expressing osteoclasts (Fig. 8A). Following TRAP staining, we found that many of the previously well-spread CD68⁺/− osteoclasts had reduced much of their membranes to spindle-like projections resulting in abnormally shaped cells (Fig. 8B). Furthermore, we found that CD68⁻/⁻ osteoclasts were more susceptible to EDTA-induced lifting than CD68-expressing cells. As measured by soluble TRAP activity assay, while 53 ± 4% of wild type osteoclasts remain attached to culture plates after a 10 minute treatment with 0.02% EDTA, only 8 ± 4% of CD68⁻/⁻ osteoclasts remained attached after a similar treatment (p < 0.001). These morphological and lifting data indicate that CD68 is necessary for the physical stability of osteoclasts, and, in its absence, osteoclasts acquire dys-
functions that are manifest in the form of aberrant morphology and reduced adhesion to culture substrates.

Discussion

Although CD68 is routinely used as a histological marker of macrophage lineage cells, its specific function(s) in these cells remain undefined. Multiple studies have demonstrated the oxLDL binding affinity of CD68, but deletion of CD68 has no significant inhibitory effect on the uptake of oxLDL by macrophages [13,16,18]. There was evidence in favor of a role in oxLDL uptake including surface expression of CD68 as well as its rapid recycling between the intracellular/endosomal compartment and cell surface [14]. Furthermore, initial antibody-blockade studies on PMA-differentiated THP-1 macrophages showed that inhibition of CD68 reduced binding and uptake of oxLDL [13]. Nevertheless, RNAi studies in peritoneal macrophages and macrophage-like RAW264.7 cells, however, suggested that CD68 inhibition does not reduce oxLDL uptake, and forced expression of CD68 in COS-7 kidney cells did not increase the ability of these cells to take up oxLDL [18]. Further evidence against a role for CD68 in oxLDL uptake can be seen in CD68−/− peritoneal macrophages which take up oxLDL as efficiently as CD68-expressing cells [20]. Thus, it appears that CD68 does not play an indispensible role in oxLDL uptake in macrophages. With the apparent resolution of this controversy, the question of the role of CD68 in cells, nevertheless, remains unanswered. To date, there has been no demonstration of cellular dysfunction due to CD68 inhibition, nor has there been prior evaluation of the significance of CD68 expression by cells other than macrophages and myeloid dendritic cells.
In this study, we examined the expression and localization of CD68 in bone marrow macrophages and osteoclasts and demonstrated that CD68 expression is critical to the normal morphology and function of the osteoclasts. This represents the first example of cellular dysfunction due to CD68 inhibition. Consistent with its status as a marker of macrophage lineage cells, we found expression of CD68 in both BMMs and osteoclasts, but not osteoblasts. Though CD68 expression levels in BMMs and osteoclasts were comparable, the migration rate CD68 from cells treated with RANKL was accelerated, suggesting an alteration in its glycosylation. The nature and significance of this altered glycosylation has not been defined, though its consistent appearance in both BMMs and macrophage-like RAW264.7 cells is compelling. It has been shown that CD8’s glycosylation is subject to alteration in response to inflammatory stimuli [21]. Phagocytosis, in particular, induces a change from a predominantly core 1 pattern of o-glycosylation to a core 2 state in peritoneal macrophages [22]. This “phagocytic glycoform” of CD68 has also been detected in BMMs. This alteration does not appear to have an effect on surface expression, however, as comparable amounts of CD68 can be detected on the surface of macrophages treated with either M-CSF alone or M-CSF and RANKL. This altered glycoform may, however, be related to the function of CD68 in osteoclasts, and future studies should explore this. Further examining localization, we found that CD68 has a dome-like distribution in osteoclasts cultured on bone slices. This pattern arises from a concentration of CD68 along the Z-axis of the osteoclast periphery with a more exclusively apical distribution elsewhere.

In order to examine the significance of CD68 expression in osteoclasts specifically and the consequences of its ablation in whole animals in general, we used targeted
genomic recombination to generate mice that lack expression of CD68. We found that CD68<sup>−/−</sup> pups appear near expected Mendelian frequencies and have no obvious physical or behavior abnormalities. Early studies of 6-week old female mice revealed no significant change in bone parameters measured by μCT. With aging, however, differences between CD68 knockout and CD68-expressing animals increased. Analysis of the distal femurs of six-month-old female mice revealed that knockout of CD68 resulted in increased trabecular bone that, nevertheless, has a reduced TMD. The mineral apposition rate of the knockout mice was increased, and this may relate to the observed decrease in trabecular TMD. Rapid bone formation could lead to insufficient mineralization, and there are examples of this in the literature[24]. We also found that CD68<sup>−/−</sup> osteoclasts differentiated in vitro demonstrated aberrant morphology including accumulation of abnormal intracellular vesicles and increased sensitivity to detachment forces. In addition, osteoclasts that lack CD68 expression showed reduced bone resorption in vitro. These in vitro abnormalities along with histological TRAP staining of femur sections suggest that the increases in trabecular bone in vivo are due to decreased osteoclast activity, not number. A decrease in bone resorption with an increase in bone formation is unusual, as these processes are often paired. There are, however, instances where non-resorbing osteoclast can stimulate osteoblast activity[25,26]. If this is the case for CD68 knockout mice, CD68 may prove to be a valuable target for an antiresorptive therapy that uncouples bone formation from bone resorption. The decreased trabecular TMD that results from the increase in MAR is a concern, and the biomechanical properties of bones from CD68 knockout animals should be assessed to determine any consequences of this reduction in TMD.
The *in vitro* phenotype of CD68⁻/⁻ osteoclasts is intriguing in that it recapitulates many of the abnormalities observed when the vesicular trafficking of osteoclasts is perturbed. Vesicular trafficking in osteoclasts is regulated by multiple factors including members of the Rab family of small GTPases [27]. Inhibition of individual Rab family members or their associated effectors results in varying degrees of defective vesicular trafficking and osteoclast dysfunction [28-31]. Lipid metabolism also contributes to normal vesicular trafficking in osteoclasts. Luegmayr et al. demonstrated that pharmacologic depletion of cholesterol from cultured osteoclasts resulted in cells with large vacuole-like accumulations and an increased rate of apoptosis, and osteoclasts with deficient LDL uptake demonstrated similar defects that were rescued by cholesterol enrichment [32]. Beyond inhibition of cholesterol uptake, sequestration of cholesterol within osteoclast late endosomes is sufficient to disrupt vesicular trafficking and ruffled border formation by preventing cholesterol enrichment in the ruffled border [33]. While Rab function and lipid metabolism likely have discrete roles in osteoclast vesicular trafficking, there is certainly overlap between these two aspects. Rab functionality is often dependent upon prenylation, which is a form of lipid modification. Indeed, pharmacological inhibition of Rab geranylgeranylation results in vesicular accumulation and decreased osteoclast activity, and inhibition of farnesyl pyrophosphate synthase, an enzyme of lipid metabolism, is a proposed mechanism of osteoclast-inhibiting nitrogen-containing bisphosphonates which have been shown to disrupt vesicular trafficking [34-36].

The morphological defects we have observed in CD68⁻/⁻ osteoclasts, in light of the significance of lipid in vesicular trafficking and the known LDL binding properties of CD68, makes a role for CD68 in lipid processing an attractive possibility. While it has
been demonstrated that CD68 is not a key contributor to LDL uptake, the uptake of lipid alone is not sufficient for the normal function of osteoclasts. Rather than LDL uptake, CD68 may have a role in delivery of lipids to their proper compartments or their addition to prenylated proteins. Determination of lipid distribution (i.e. cholesterol enrichment in the ruffled border) and GTPase prenylation in CD68\(^{-/-}\) osteoclasts will be an important step in investigating these possibilities. Another important vesicular trafficking event is the movement of bone-derived materials from the ruffled border to the apical surface via the process of transcytosis. As CD68 is arranged in a dome-like pattern proximal to the basolateral and apical membranes, it may play a role in the targeting of transcytosing vesicles to the apical membrane where their contents are released. Typing of the abnormal vesicles seen in CD68\(^{-/-}\) osteoclasts to determine their origin (e.g. late endosomes, transcytotic vesicles) is another way of exploring a role for CD68 in osteoclast trafficking. Coupled with studies of lipid distribution, these studies should further illuminate CD68 function in osteoclasts and the mechanism of osteoclast dysfunction in its absence.

In our study, we have demonstrated the importance of CD68 expression in osteoclasts, and, in the process, produced a tool in the form of the CD68 knockout mouse for examining not only the contribution of CD68 to normal skeletal physiology, but also for answering questions pertaining to CD68 function(s) in other tissues. Exploitation of known properties of CD68 have already been shown to have therapeutic value in preclinical studies of atherosclerosis, and future studies using CD68\(^{-/-}\) mice and their cells will hopefully shed light on new therapeutic opportunities.

Materials and Methods
Ethics Statement

Mice were maintained and the experiments involving mice were performed in accordance with the regulations of the University of Alabama at Birmingham Institutional Animal Care and Use Committee (animal protocol approval number: 100908911).

Chemical and Reagents

Chemicals were purchased from Sigma (St. Louis, MO) unless indicated otherwise. DMEM (Cat No 10-013-CV), L-glutamine (25-005-CI) were purchased from Mediatech. Fetal bovine serum was purchased from Invitrogen (26140-079). Recombinant GST-RANKL was purified as previously described [37]. Mouse M-CSF was prepared from an M-CSF-producing cell line, CMG14-12, which was constructed and kindly provided by Dr. Sunao Takeshita [38]. Anti-CD68 antibody (rat monoclonal; FA-11), RPE-conjugated anti-CD68 antibody, and RPE-conjugated Rat non-immune IgG2α were purchased from AbD Serotec (Raleigh, NC), anti-β-actin antibody (mouse monoclonal; AC-15) was purchased from Sigma, anti-Fc receptor antibody (clone 2.4G2; sc-18867) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), β-tubulin (rabbit polyclonal; ab6046) was purchased from Abcam (Cambridge, MA), and Alexa-647 conjugated anti-rat-IgG (goat polyclonal; 4418) was purchased from Cell Signaling Technology (Boston, MA).

Western immunoblotting

Cultured cells were lysed using cold cell lysis buffer with protease inhibitor cocktail (Cell Signaling Technology; 9803, 5871). Lysates were spun down to pellet inso-
luble precipitates and supernatant was transferred to clean tubes. Protein concentrations were measured by the Bradford method. 4X sample buffer (250mM Tris; 140mM sodium dodecyl sulfate [SDS]; 30mM bromophenol blue; 2% β-mercaptoethanol; 40% glycerol) was added to protein solutions to a final concentration of 1X, and samples were heated at 95°C for 10min to denature proteins. Samples were stored at -80°C prior to use.

Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 8.5% acrylamide: bis-acrylamide 30:1) in running buffer (25mM Tris; 25mM glycine; 3mM SDS) and transferred to nitrocellulose membranes in transfer buffer (50mM Tris; 40mM glycine; 1mM SDS; 20% methanol) using a semi-dry transfer apparatus for 30min at 23V. Membranes were blocked with 5% milk in Tris-buffered saline with .1% Tween-20 (TBS-T) for 1 hour at room temperature. Membranes were incubated with primary antibody (CD68, 5μg/mL; β-actin, 1μg/mL; α-Tubulin, 1μg/mL) at 4°C overnight. Membranes were washed 3x for 10 minutes with TBS-T, incubated with horse radish peroxidase-conjugated secondary antibody (.1μg/mL) for 1 hour at room temperature, and washed 3x for 10 minutes with TBS-T. Antibody-labeled proteins were detected with enhanced chemiluminescence and X-ray film. Densitometric analysis of films was performed using ImageJ software.

**Flow cytometric analysis**

All incubations and centrifugations were carried out at 4°C. 1×10^6 cells were fixed in solution with 4% paraformaldehyde/PBS for 20 minutes. Fixed cells were washed 3× with PBS for surface staining or PBS + 0.5% saponin for total staining, and Fc receptors were blocked with anti-Fc receptor antibody for 30 minutes. Cells were then
stained with either RPE-conjugated anti-CD68 antibody or non-immune IgG2a for 45 minutes. Non-permeabilized cells were stained with an RPE-conjugated anti-Actin antibody as a negative control for surface staining. After 3 more washes with PBS (+/− 0.5% saponin), cells were analyzed using a Becton Dickinson SLR II analytical flow cytometer. Flow histograms were generated using WinMDI.

*In vitro osteoclastogenesis*

BMMs were isolated from mouse long bones and were maintained in α-minimal essential medium (α-MEM) containing 10% heat-inactivated FBS with M-CSF (220 ng/mL) as previously described [39]. To generate osteoclasts from BMMs, 5×10⁴ adherent cells per well were plated in 24-well tissue culture plates (7.5×10³ cells for 96-well plates) and cultured in presence of 44ng/mL M-CSF and 100ng/mL of purified GST-RANKL for 3-5 days. The osteoclastogenesis cultures were stained for TRAP activity with Leukocyte Acid Phosphatase Kit (Sigma; 387-A).

*Culture of mouse calvarial osteoblasts*

Calvariae from 3-5 day old mouse pups were dissected and cultured in medium composed of α-MEM containing 10% fetal bovine serum, 1 mM sodium pyruvate, 100 IU/mL of penicillin, and 100 μg/mL streptomycin. Calvariae were digested in PBS with 0.1% collagenase (Wako Chemicals, #038-10531)/0.2% dispase II (Roche Biochemicals, #165859) and cells were isolated using straining and centrifugation. Osteoblast precursors were cultured and differentiated in of α-MEM containing 10% fetal bovine serum,
1 mM sodium pyruvate, 100 IU/mL of penicillin, 100 µg/mL streptomycin, 50 µg/mL ascorbic acid, and 10 mM β-glycerophosphate.

**Immunofluorescent staining of osteoclasts**

BMMs (5×10⁴ cells per well) were seeded directly into the wells of 24-well tissue culture plates or onto bone slices within 24-well culture plates and cultured under either macrophage-maintaining or osteoclastogenic conditions (with M-CSF only or M-CSF + RANKL). Cells were fixed with 4% paraformaldehyde solution in PBS for 20 minutes at room temperature. Cells were permeabilized and blocked with a 0.2% BSA/0.1% saponin/PBS solution (PBBS). Cells were incubated with anti-CD68 or non-immune control IgG₂a at a concentration of 10μg/mL in PBBS for 2 hours. Cells were then washed three times with PBBS and incubated with 1:400 Hoescht, 1:200 Alexa-488 conjugated phalloidin, and 5μg/mL Alexa-647 conjugated secondary antibody for 45 min. Cells were again washed three times with PBBS before mounting with 80% glycerol in PBS. Stained cells were imaged with a Zeiss LSM 710 confocal microscope.

**CD68 knockout mouse generation**

CD68 targeting vector was constructed using standard molecular cloning methods on a pBluescript-SK backbone. The targeting vector was electroporated into C57B6/J mouse embryonic stem cells, and transfected cells were positively selected with G418 and negatively selected with ganciclovir. Surviving clones were screened via PCR and Southern blotting. Positive clones were injected into albino C57B6/J blastocysts to gen-
erate CD68<sup>+/−</sup>/CD68<sup>+/+</sup> chimeras. Electroporation and embryonic stem cell injection was carried out at the UAB Transgenic Mouse Facility. Male chimeras were bred to albino C57B6/J females, and black-coated offspring were genotyped using PCR. CD68<sup>+/−</sup> siblings were mated to produce CD68<sup>+/+</sup>, CD68<sup>+/−</sup>, and CD68<sup>−/−</sup> mice.

*Micro-computed Tomography (μCT)*

Ethanol-fixed femurs were embedded in polymethylmethacrylate were scanned and using a ScanCo Medical μCT40 in the UAB Small Animal Phenotyping Core. The following parameters were measured using this system: BV/TV, Tb. N, Tb. Sp., trabecular TMD, cortical TMD, and cortical thickness. 5 samples per genotype were examined.

*Histological analysis*

Prior to sacrifice, mice were injected subcutaneously with 100μL 20mM calcein in PBS once and again 7 days later to label mineralization fronts for dynamic histomorphometric analysis. Formalin-fixed femurs were decalcified and embedded in paraffin blocks for sectioning. Slide-mounted sections were stained for Tartrate Resistant Acid Phosphatase activity and counterstained with hemotoxylin. Osteoclast number per mm bone surface was quantified using BioQuant software. 70%-ethanol fixed femurs were embedded in plastic and were sectioned and stained with Goldner’s trichrome. Static and dynamic histomorphometric analysis was performed using BioQuant software. Tissue processing and staining was carried out by the UAB Center for Metabolic Bone Disease Histomorphometry Core.
**In vitro bone resorption assay**

*In vitro* pit-forming assays were performed similarly as previously described [40]. $5 \times 10^4$ adherent BMMs were seeded onto bone slices within the wells of a 24-well culture plate under osteoclastogenic conditions. Osteoclasts were allowed to differentiate and mature over 4 days followed by an additional 3 days of resorption. Osteoclastogenic medium was refreshed every 2 days. At the conclusion of the resorption culture, cells were removed from the bone slices using 0.25M ammonium hydroxide and mechanical agitation. Slices were washed with water and glued to microscope slides for imaging using a Zeiss LSM 710 confocal microscope. Resorption area as a percent of total area was quantified from resulting images using Adobe Photoshop.

**Soluble TRAP microtiter assay**

Quantification of relative osteoclast number *in vitro* was carried out using the microtiter method of Minkin et al [41,42]. Osteoclasts were lysed in 100μL soluble TRAP assay buffer (50mM Sodium Acetate pH 5.0, .5mg/mL 4-Nitrophenyl phosphate disodium salt [Sigma; S0942], .5% Triton X-100 [Sigma; 93443], and 10mM L-Tartaric acid [Sigma; T109]) and incubated at 37°C for 5 min. Reaction was stopped and completed by addition of 50μL .1M NaOH. Absorbance was read at 405nm with a reference wavelength of 625nm.

**Statistical Analysis**

All graphs were generated using Microsoft Excel. P-values were calculated using a two-tailed Student’s t-Test. A p-value of less than 0.05 was considered significant.
Acknowledgements

This work was supported by a grant from the National Institute of Arthritis and Musculoskeletal and Skin Diseases (AR47830; to Xu Feng), and the University of Alabama at Birmingham Center for Metabolic Bone Disease T32 training grant (AR047512-09 to Jason W Ashley). We would like to thank the University of Alabama at Birmingham’s High Resolution Imaging Facility, ES/Transgenic Animal Facility, Animal Resources Program, Small Animal Phenotyping Core, and Bone Histomorphometry Core for their assistance in generating, maintaining, and phenotyping our CD68 knockout mice. We also thank the UAB Center for Metabolic Bone Disease for their ongoing support.
References


Figure 1. CD68 is expressed by macrophages and osteoclasts. (A) CD68 and β-actin expression in mouse bone marrow suspension cells treated with 44ng/mL M-CSF +/- 100ng/mL RANKL for indicated days (d). M-CSF increases expression of CD68 in a time dependent manner, and RANKL ligand induces an accelerated gel migration rate. Image is representative of 3 independent experiments. Quantification of relative band density is aggregate of 3 independent experiments; data shown is mean + standard deviation. (B) CD68 and β-actin expression in RAW264.7 cells with or without 100ng/mL RANKL treatment for indicated times. RAW264.7 cells have constitutive M-CSF-stimulated signaling and continuous expression of CD68. RANKL induces similar changes in gel migration as those seen in primary macrophages. Image is representative of 3 independent experiments. Quantification of relative band density is aggregate of 3 independent experiments; data shown is mean + standard deviation. (C) Flow cytometry histograms of BMMs with 3-day treatments of 44ng/mL M-CSF +/- 100ng/mL RANKL with (Total) and without (Surface) permeabilization with .5% saponin. CD68 can be detected on the surface of BMMs, and neither surface nor total detectible levels of CD68 are altered by addition of RANKL. Image is representative of 2 independent experiments.
Figure 2. CD68 can be found in a dome-like pattern in osteoclasts cultured on bovine cortical bone slices. (A) BMMs were seeded onto bone slices with 44ng/mL M-CSF and 100ng/mL RANKL and differentiated into osteoclasts over 4 days. Cells were fixed with 4% paraformaldehyde/PBS and stained with Alexa-488-conjugated phalloidin (actin, green), Hoescht (nuclei, blue), and either anti-CD68 (CD68, red) or rat non-immune IgG2a (IgG2a Merge) antibody followed by Alexa-647-conjugated anti-Rat IgG. Scale bars for CD68 staining are 40μm; scale bar for IgG2a staining is 20μm. Images are representative of 3 independent experiments. (B) Enlarged merge image from A. * and + indicate corresponding XZ and YZ cross sectional images, respectively. Scale bar is 40μm. Image is representative of 3 independent experiments. (C) 3-D reconstruction of osteoclast cross sectioned in B with actin in green, nuclei in blue, and CD68 in red.
Figure 3. BMMs from CD68<sup>−/−</sup> mice lack expression of CD68. (A) Vector diagram with a neomycin phosphotransferase expression cassette (PGK-NEO) flanked by sequences with homology to targeted genomic sequence. A thymidine kinase expression cassette (PGK-TK) lies outside the homology region of vector. (B) Structure of targeted wild type allele. A southern blot probe can hybridize to a genomic sequence outside of the homology region. P1 and P2 are primers that specifically amplify the sequence of CD68 targeted for replacement. (C) Recombined allele with exons 1 and 2 of CD68 gene replaced with PGK-NEO. Properly targeted recombined alleles do not contain the thymidine kinase expression cassette. P3 and P4 are primers that specifically amplify a region of PGK-NEO. (D) Tail tip extracts from each of three resultant genotypes were subjected to genotyping PCR using P1, P2, P3, and P4. Each genotype produced a unique pattern of PCR products. (E) Lysates from BMMs from each genotype cultured with 220 ng/mL M-CSF were immunoblotted with antibodies against CD68 and α-Tubulin. While expression of CD68 was seen in +/+ and +/- BMMs, no CD68 could be detected in lysates from -/- BMMs.
**Figure 4.** CD68<sup>−/−</sup> mice have increased trabecular bone and decreased trabecular tissue mineral density. (A) Representative trabecular μCT images from 6-month-old female mice of each genotype (5 mice per group). (B) μCT analysis of distal femoral trabecular bone revealed that CD68<sup>−/−</sup> mice have increased bone volume (BV/TV), increased trabecular number (Tb. N), decreased trabecular spacing (Tb. Sp.) and decreased trabecular tissue mineral density (Trabecular TMD). There was no significant difference in trabecular thickness (Tb. Th.). (C) Representative cortical μCT images from each genotype. (D) Quantification of measured parameters. There was no significant difference between genotypes in either cortical thickness (Ct. Th.) or cortical tissue mineral density (Cortical TMD). *, p<0.05; **, p<0.01. Data presented are means ± standard deviation.
Figure 5. Histological and histomorphometric analysis of 6-month old female CD68\(^{+/+}\), \(+/-\), and \(-/-\) mice. (A) Representative formalin-fixed, paraffin-embedded histological sections from each genotyped stained for TRAP activity and counterstained with hematoxylin at 40X magnification (5 mice per group). (B) 400X magnification of area defined in (A). (C) Quantification of number of osteoclasts per bone surface (N. OC/BS). There was no significant difference in N. OC/BS between genotypes. (D) Representative 70% ethanoll-fixed, plastic-embedded histological sections from each genotype stained with Goldner’s trichrome at 40X magnification (5 mice per group). (E) 400X magnification of area defined in (D). (F) Histomorphometric analysis of trichrome-stained sections. There was no significant difference between genotypes in numbers of osteoblasts (N. OB/BS) per bone surface. There was a significant increase in bone volume per total volume (BV/TV) in CD68\(^{-/-}\) mice compared to CD68\(^{+/+}\) animals and trabecular number (N. Tb.) in CD68\(^{-/-}\) mice compared to CD68\(^{+/+}\) and CD68\(^{+/-}\) animals. (G) Quantification of mineral apposition rate (MAR) via analysis of calcein double labeling. The MAR was significantly higher in CD68\(^{-/-}\) mice compared to both CD68\(^{+/+}\) and CD68\(^{+/-}\) animals, and MAR was significantly higher in CD68\(^{+/-}\) mice compared to CD68\(^{+/+}\) animals. *, p<0.05; **, p<0.01. Data presented are means + standard deviation.
Figure 6. CD68 is not expressed by osteoblasts. CD68 and β-actin expression in mouse BMMs(Mφ) cultured with 220ng/mL M-CSF and cultured mouse calvarial osteoblasts (OB) was determined by Western immunoblotting. While expression of CD68 is high in BMMs, expression of CD68 could not be detected in osteoblasts.
Figure 7. CD68\textsuperscript{−/−} osteoclasts do not efficiently resorb bone. BMMs from CD68\textsuperscript{++}, \textsuperscript{+/-}, and \textsuperscript{-/-} mice were seeded onto bovine cortical bone slices and differentiated into osteoclasts over 4 days. Differentiated osteoclasts were allowed to resorb the slices for an additional 3 days. (A) Representative images of resorbed bone slices generated using laser scanning confocal microscopy. A pit from each image is marked with an arrow. Scale bars are 70 μm. (B) Quantification of resorbed area. Data represented as means + standard deviation. *, p<0.001. 3 visual fields each from 3 separately resorbed bone slices were assessed. Data presented are means ± standard deviation. Images and data are representative of 2 independent experiments with 3 bone slices per genotype.
Figure 8. CD68<sup>−/−</sup> osteoclasts have abnormal morphology. (A) Prior to TRAP staining, osteoclasts of all three genotypes were of relatively similar size. CD68<sup>−/−</sup> osteoclasts demonstrated intracellular vacuole-like structures that were not present to such an extent in CD68-expressing cells. Scale bars are 100μm. Images are representative of 3 independent experiments. (B) During the fixation process (fixative: 25mL citrate solution [18mM citric acid, 9mM sodium citrate, 12mM sodium chloride, pH 3.6], 68mL acetone, 8mL 37% formaldehyde), many CD68<sup>−/−</sup> osteoclasts were reduced in size and partially detached from the culture substrate resulting in a smaller size following TRAP staining. Scale bars are 200μm. Images are representative of 3 independent experiments.
Table 1. Mouse Birth Ratios (Number animals/total)

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<th>Sex</th>
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<th>Sex/Total</th>
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<td>53/97; 0.55</td>
<td>23/97; 0.24</td>
<td>97/185; 0.52</td>
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<td>Female</td>
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<td>44/88; 0.5</td>
<td>28/88; 0.32</td>
<td>88/185; 0.48</td>
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Table 1. Mouse birth ratios. CD68<sup>++</sup> male and female mice were paired to produce CD68<sup>++</sup>, +/-, and -/- pups. Mice were genotyped at 2 weeks of age.
3 - RECEPTOR ACTIVATOR OF NUCLEAR FACTOR κB LIGAND STIMULATES ALTERNATIVE GLYCOSYLATION OF CD68

by

JASON W ASHLEY, HUIXIAN HONG, XU FENG

In preparation for Glycobiology

Format adapted for dissertation

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Abstract

CD68 is a heavily glycosylated, mucin-like member of the lysosome-associated membrane protein family that is primarily expressed by macrophages and osteoclasts. We previously determined that CD68 expression is critical to the normal function of osteoclasts, and osteoclasts express an altered form of CD68 compared to that of bone marrow macrophages. In this study, we sought to determine the nature of this altered form and its regulation. Lectin blotting revealed that the osteoclast form of CD68 showed reduced binding by *Galanthus Nivalis* agglutinin (GNA), which recognizes mannose moieties, and peanut agglutinin (PNA), which recognizes terminal core 1 galactose moieties. Blotting with *Maackia Amurensis* hemagglutinin (MAH), which recognizes terminal sialic acid residues, revealed a large increase in CD68 sialylation with addition of RANKL and cleavage of these residues with neuraminidase enhanced PNA binding and attenuated the RANKL-induced alteration of CD68 gel migration. Further, using BMMs that express mutant forms of the RANKL receptor (RANK) that have inactivating mutations in PVQEET<sup>560-565</sup> and PVQEQG<sup>604-609</sup> or IVVY<sup>535-538</sup> signaling motifs, we found that the altered migration of CD68 and, likely, its altered glycosylation are dependent upon signaling from these motifs. Finally, we found via quantitative real-time PCR that RANKL treatment upregulates the expression of the sialating enzyme ST3Gal1, and mutation of PVQEET<sup>560-565</sup> and PVQEQG<sup>604-609</sup> or IVVY<sup>535-538</sup> signaling prevents this upregulation. These data indicate that RANKL stimulates an increase in CD68 sialylation, and they provide correlative evidence that ST3Gal1 may be responsible for this change.
Introduction

CD68 is a myeloid lineage-restricted, heavily glycosylated type I transmembrane member of the lysosome associated membrane protein (LAMP) family[1-4]. The degree of its glycosylation is evidenced by its apparent molecular mass determined by Western immunoblotting. While the mass of CD68 predicted by its amino acid sequence is only 36kDa, blotted CD68 often has masses of 85 to 115kDa. With, in some cases, over two-thirds of the mass of CD68 attributable to sugars, the glycosylation of CD68 is likely of tremendous importance to its function. Partial sequence homology has classified CD68 as a LAMP, but the nature of its glycosylation differs from other LAMPs. The glycosylation of CD68 does indeed resemble most LAMPs in the membrane proximal region of its extracellular domain, which is highly homologous to LAMP family members, with primarily N-linked glycans. Beyond the proline-rich region of CD68, N-terminal to the membrane proximal region, is a mucin-like stalk that is marked by heavy O-linked N-acetylgalactosamine (GalNAc)-based glycan chains[5]. This stalk is highly homologous to mucin family proteins, and, because of this, CD68 could as easily be classified as a mucin with LAMP-like characteristics as LAMP with mucin-like characteristics.

All mucin-type O-glycosylations consist of a GalNAc residue α-linked covalently to the hydroxyl-oxygen of either a serine or threonine residue of the glycosylated protein. This moiety, often termed Tn antigen, forms the basis of eight known mucin glycan chains; the identity of the attached sugars and their configuration on the chain extended from the Tn antigen defines the chain as core 1-8[6]. While these divergent glycan structures have been identified physiologically, the most commonly occurring structures on
membrane-bound mucins and mucin-like proteins are core 1 and core 2. Core 1, also called Thomsen-Friedenreich or T antigen, is the simplest core structure and consists of a galactose glycan β3 linked to Tn antigen. Core 1 structures can be extended via the addition of glycans to the galactose and capping of the terminal core 1 galactose with sialic acid is common. The other common mucin glycan structure is core 2, and it is generated by addition of an N-acetylglucosamine (GlcNAc) via a β6 linkage to the GalNAc of a core 1 glycan. Core 2 glycan chains are extended via addition of sugars to this GlcNAc moiety.

Previous studies have demonstrated that CD68 glycosylation is subject to change under varying circumstances. The best understood among these events is macrophage phagocytosis[5,7]. Gordon et al., determined through studies using peanut agglutinin (PNA), *maackia amurensis* agglutinin (MAA), and *galanthus nivalis* agglutinin (GNA) and galactose and GlcNac incorporation, the O-glycans of CD68 expressed by naïve peritoneal macrophages are predominantly terminally sialated core 1, and phagocytosis stimulates a conversion to core 2. The significance of this switch and the enzymes responsible have not been determined. Interestingly, however, this same group surveyed resident macrophages from various tissues and found the same “phagocytic glycoform” of CD68 in bone marrow macrophages (BMMs).

In this study, we explored the glycosylation of CD68 in BMMs and osteoclasts. We also investigated the role of receptor activator of nuclear factor κB (RANK) signaling in the conversion of CD68 from its BMM glycoform to an osteoclast glycoform. The interaction between RANK and its ligand, RANKL, is critical to the differentiation of osteoclasts from mononuclear precursors, and we have found that signaling from RANK
intracellular signaling motifs PVQET\textsuperscript{560-565}, PVQEQ\textsuperscript{604-609}, and IVV\textsuperscript{535-538} are important players in both osteoclastogenesis and the alternative glycosylation of CD68. This study further highlights the malleability of CD68 glycosylation.

Materials and Methods

Chemicals and reagents

DMEM (Cat No 10-013-CV), L-glutamine (25-005-CI) were purchased from Mediatech. Fetal bovine serum was purchased from Invitrogen (Carlsbad, CA; 26140-079). Recombinant GST-RANKL was purified as previously described[8]. Mouse M-CSF was prepared from an M-CSF-producing cell line, CMG14-12, which was constructed and kindly provided by Dr. Sunao Takeshita[9]. Anti-CD68 antibody (rat monoclonal; FA-11 AbD Serotec (Raleigh, NC), anti-β-actin antibody (mouse monoclonal; AC-15) was purchased from Sigma, and anti-NFATc1 antibody (mouse monoclonal; 7A6) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Biotinylated PNA (L6135) was purchased from Sigma (St. Louis, MO), biotinylated MAH (B-1265) was purchased from Vector Laboratories (Burlingame, CA), NeutrAvidin (A-2664) was purchased from Invitrogen, and α2-3 Neuraminidase (P0728) was purchased from New England Biolabs (Ipswich, MA). Cyclosporin A (C3662) was purchased from Sigma.

Western immunoblotting

Cultured cells were lysed using cold cell lysis buffer with protease inhibitor cocktail (Cell Signaling Technology; 9803, 5871). Lysates were spun down to pellet insoluble precipitates and supernatant was transferred to clean tubes. Protein concentrations
were measured by the Bradford method. 4X sample buffer (250mM Tris; 140mM sodium dodecyl sulfate [SDS]; 30mM bromophenol blue; 2% β-mercaptoethanol; 40% glycerol) was added to protein solutions to a final concentration of 1X, and samples were heated at 95°C for 10min to denature proteins. Samples were stored at -80°C prior to use.

Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 8.5% acrylamide: bis-acrylamide 30:1) in running buffer (25mM Tris; 25mM glycine; 3mM SDS) and transferred to nitrocellulose membranes in transfer buffer (50mM Tris; 40mM glycine; 1mM SDS; 20% methanol) using a semi-dry transfer apparatus for 30min at 23V. Membranes were blocked with 5% milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T) for 1 hour at room temperature. Membranes were incubated with primary antibody (CD68, 5μg/mL; β-actin, 1μg/mL) at 4°C overnight. Membranes were washed 3× for 10 minutes with TBS-T, incubated with horse radish peroxidase-conjugated secondary antibody (.1μg/mL) for 1 hour at room temperature, and washed 3x for 10 minutes with TBS-T. Antibody-labeled proteins were detected with enhanced chemiluminescence and X-ray film.

**Immunoprecipitation and lectin blotting**

CD68 was immunoprecipitated from 1mg of extracted protein using 10μg anti-CD68 antibody (FA-11) and the Dynabead protein G magnetic immunoprecipitation kit from Invitrogen (100-07D) according to the manufacturer’s instructions. Precipitated proteins were run on an 8.5% SDS-PAGE gel and transferred as described above. Glycans on transferred proteins were detected using the DIG glycan differentiation kit from
Roche Applied Science (Indianapolis, IN; 11210238001) according to the manufacturer’s instructions.

**Neuraminidase digestion and chemiluminescent lectin blotting**

Total protein or immunoprecipitated CD68 was digested with α2-3 Neuraminidase according to the manufacturer’s instructions at 37°C overnight. Digested proteins were electrophoresed and transferred as described above. When lectins were used membranes with transferred proteins were blocked with 1% cold water fish gelatin in TBS-T overnight at 4°C. Glycans were bound with biotinylated lectins which were detected using horse radish peroxidase conjugated NeutrAvidin via enhanced chemiluminescence.

**In vitro osteoclastogenesis**

BMMs were isolated from mouse long bones and were maintained in α-minimal essential medium (α-MEM) containing 10% heat-inactivated FBS with M-CSF (220 ng/mL) as previously described[10]. To generate osteoclasts from BMMs, 5×10⁴ adherent cells per well were plated in 24-well tissue culture plates and cultured in presence of 44ng/mL M-CSF and 100ng/mL of purified GST-RANKL for 3-5 days. The osteoclastogenesis cultures were stained for TRAP activity with Leukocyte Acid Phosphatase Kit (Sigma; 387-A).

**Real-time quantitative PCR**

RNA was extracted from cultured cells using the RNeasy® RNA extraction kit (74104) from Qiagen (Valencial, CA). The reverse transcription and quantitative PCR
were carried out using the TaqMan® RNA-to-CT 1-step kit (4392938) from Applied Biosystems (Carlsbad, CA). Expression of ST3Gal1 was measured with a TaqMan® gene expression assay (4331182; Mm00501493_m1) normalized to β-actin (4331182; Mm00607939_s1). The reaction and analysis were carried out using the Applied Biosystems 7500 real-time PCR system.

Data presentation and statistical analysis

Charts were generated and calculations were performed using Microsoft Excel 2007. Significance was determined using Student’s T-test, and p-values of less than 0.05 were considered significant.

Results

RANKL treatment accelerates CD68 PAGE gel migration rate and alters its binding by PNA, GNA, and MAA

We previously found that treatment of freshly isolated bone marrow cells with M-CSF increased expression of CD68 and addition of RANKL resulted in an alteration in the apparent molecular mass of CD68[11]. To confirm these findings and to examine this alteration more closely, we cultured differentiated BMMs with either M-CSF alone or M-CSF and RANKL for 12, 24, 36, or 48 hours and detected CD68 via Western immunoblotting. As seen before, treatment with M-CSF alone resulted in an increase in CD68 expression. Culture with M-CSF and RANKL resulted in a gradual reduction in CD68 apparent molecular mass that was maximally apparent 48 hours post treatment initiation (Fig. 1A).
Given that much of the mass of CD68 is attributable to sugars, we sought to determine whether the alteration in CD68 migration rate was due to changes in glycosylation. To this end, we immunoprecipitated CD68 from BMMs culture in either M-CSF alone or M-CSF and RANKL for 48 hours, and subjected the precipitated proteins to blotting with or a panel of lectins. Table 1 details the lectins used along with their recognized sugar moieties. RANKL treatment caused a reduction in the binding of GNA and PNA and an increase in the binding of MAA (Figure 1B). This confirms that RANKL induces changes in the glycosylation of CD68 and those changes are likely the source of its altered electrophoretic mobility.

Table 1

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Lectin name</th>
<th>Recognized glycan</th>
</tr>
</thead>
<tbody>
<tr>
<td>GNA</td>
<td><em>Galanthus nivalis</em> agglutinin</td>
<td>Terminal mannose</td>
</tr>
<tr>
<td>SNA</td>
<td><em>Sambucus nigra</em> agglutinin</td>
<td>α6-linked terminal sialic acid of O- and N-linked glycans</td>
</tr>
<tr>
<td>MAA</td>
<td><em>Maackia amurensis</em> agglutinin</td>
<td>α3-linked terminal sialic acid of O- and N-linked glycans</td>
</tr>
<tr>
<td>PNA</td>
<td>Peanut agglutinin</td>
<td>Galactoseβ1-3GalNAc</td>
</tr>
<tr>
<td>DSA</td>
<td><em>Datura stramonium</em> agglutinin</td>
<td>Galactoseβ1-4GlcNAc</td>
</tr>
</tbody>
</table>

*The osteoclast glycoform of CD68 is marked by increased sialylation*

The most striking alteration of CD68 lectin binding following RANKL treatment was the complete elimination of binding by PNA. This could be due to either a reduction in the recognized core 1/core 2 galactose or the masking of this moiety by another sugar. As the apparent molecular mass of CD68 is reduced with RANKL treatment, a reduction in sugar is the initial obvious candidate. However, it has been shown that terminal sialyla-
tion of mucins and mucin-like proteins like CD68 can result in accelerated PAGE gel migration rates and, thus, decreased apparent molecular mass[5]. To test this possibility of increased sialylation, we used a more sensitive chemiluminescence-based detection technique to assess the binding of CD68 immunoprecipitated from macrophages and osteoclasts by PNA and *Maackia Amurensis* Hemagglutinin (MAH) with and without digestion with α2-3 neuraminidase, which preferentially cleaves α2-3 linked terminal sialic acids from glycoproteins (Figure 2A). The MAH used in this experiment was different from the MAA used in the previous experiment in that MAA is a mixture of *Maackia Amurensis* leukoagglutinin and hemagglutinin that recognizes α3-linked sialic acids of both O- and N-linked glycans, and MAH more exclusively recognizes α3-linked sialic acids of O-linked glycans[12-14].

Compared to α2-3 neuraminidase-digested CD68, there was less binding of macrophage-derived CD68 and no binding of osteoclast-derived CD68 by PNA. α2-3 neuraminidase digestion enhanced PNA binding of both macrophage and osteoclast-derived CD68, but, as with undigested CD68, PNA binding was higher on the macrophage glycoform (Figure 2A). Strikingly, where there was only a slight increase in MAA binding by the osteoclast glycoform of CD68 seen in the colorimetric detection (Figure 1B, MAA), chemiluminescent detection showed a high degree of MAH binding by the osteoclast glycoform of CD68 as compared to CD68 from macrophages which did not show detectible MAH binding. This suggests that MAA binding of macrophage-derived CD68 seen previously was due primarily to α3-linked sialic acid residues associated with N-linked glycan chains. MAH binding was reduced by α2-3 neuraminidase digestion supporting that CD68 O-linked terminal sialylation increases with RANKL treatment.
Furthermore, osteoclast-derived CD68 that remained detectible following α2-3 neuraminidase digestion (likely due to incomplete digestion) was shifted upwards relative to the undigested osteoclast-derived CD68. When whole cell lysates were subjected to α2-3 neuraminidase digestion, we found that the previous acceleration of CD68 migration rate induced by RANKL was reduced by roughly half (Figure 2B). The migration rate was also reduced in macrophage-derived CD68 by α2-3 neuraminidase digestion. These data demonstrate that RANKL stimulates an increase in CD68 sialylation, and, although not the only modification, this sialylation contributes to the altered migration rate seen in osteoclast-derived CD68.

_The altered glycosylation of CD68 is dependent upon signaling from RANK intracellular signaling motifs_

RANK has at least six putative Tumor Necrosis Factor Receptor Associated Factor (TRAF) motifs (PTMs) and at least one non-TRAF motif. Our group has shown that osteoclastogenesis is dependent upon signaling from two Tumor Necrosis Factor Receptor Associated Factor (TRAF) motifs (PVQET\(^{560-565}\) and PVQEQG\(^{604-609}\); PTM5 and PTM6) and a non-TRAF-binding motif (IVVY\(^{535-538}\)). To determine whether signaling from these motifs contributes to CD68 modification, we compared BMMs from wild type mice to those from mutant mice with either mutations in the two TRAF-binding motifs (PVQET\(^{560-565}\rightarrow LLNDDS\(^{560-565}\) and PVQEQG\(^{604-609}\rightarrow LLNDNA\(^{609-604}\); P56) or mutations in the non-TRAF-binding motif (IVVY\(^{535-538}\rightarrow IVAF\(^{535-538}\); IVAF) cultured in either M-CSF alone to maintain the macrophage phenotype or M-CSF and RANKL for 3 days to induce osteoclast formation (Figure 3A). As seen before, wild type BMMs cul-
tured with M-CSF and RANKL formed large, multinuclear, TRAP-positive osteoclasts, but both mutants did not (Figure 3B). Similarly, while RANKL treatment produced the established shift in CD68 molecular mass in wild type cells, the shift did not appear in either mutant (Figure 3C).

**CD68 alternative glycosylation is not dependent upon NFATc1**

In our and others’ previous studies of RANK’s signaling motifs, we found that, of the known signaling pathways activated through RANK, only nuclear factor of activated T-cells c1 (NFATc1) signaling is blunted by mutation of PVQET\(^{560-565}\), PVQEQG\(^{604-609}\), or IVVY\(^{535-538}\). Such reductions in NFATc1 activation were also seen in BMMs from P56 and IVAF mutant mice treated with M-CSF and RANKL for 3 days (Figure 4A). From this, we hypothesized that RANKL-induced alteration of CD68 is dependent upon NFATc1 signaling, and the failure of RANKL to cause a shift in CD68 migration rate in P56 and IVAF mutant BMMs is due to their inability to initiate robust NFATc1 signaling. To test this, we cultured cells in either M-CSF alone or M-CSF and RANKL for 3 days along with increasing concentrations of the calcineurin inhibitor cyclosporin A, which can block multiple pathways including NFATc1. As has been previously established, cyclosporin A treatment blocked differentiation of osteoclasts from BMMs cultured with M-CSF and RANKL (Figure 4B). Interestingly however, while cyclosporin A effectively blocked osteoclastogenesis, sub-toxic doses had no apparent effect on CD68 migration, and even a toxic dose of 8300nM only slightly affected CD68 alteration (Figure 4C).
An increase in ST3Gal1 expression correlates with CD68 altered glycosylation

Our group has previously performed microarray studies of IVAF mutant BMMs and found that the non-TRAF motif IVVY is involved in the RANKL regulated expression of multiple genes (Z. Shi et al., unpublished data). Most relevant to the findings of this study was an IVVY–dependent upregulation of ST3 beta-galactoside alpha-2,3-sialyltransferase 1 (ST3Gal1). This enzyme catalyzes the addition of sialic acid to terminal galctose residues via an α2-3 linkage. As an increase in α2-3 sialylation is a major modification of CD68 during osteoclastogenesis, ST3Gal1 is a strong candidate for a mediator of this change. To confirm and further explore our previous microarray findings as they relate to CD68 glycosylation, we collected RNA from wild type BMMs treated with M-CSF only, P56 and IVAF mutant BMMs treated with M-CSF and RANKL, and wild type BMMs treated with M-CSF, RANKL, and 1 μg/mL cyclosporin A for 3 days. Using real time quantitative PCR, we measured expression of ST3Gal1 mRNA relative to β-actin and found that RANKL treatment significantly increases ST3Gal1 expression in wild type, but not P56 or IVAF mutant, BMMs. Interestingly, while cyclosporin A treatment blocked osteoclast formation, it did not reduce RANKL-induced upregulation of ST3Gal1 (Figure 5). The expression of ST3Gal1 correlates with altered glycosylation of CD68 in that in instances where CD68 alteration is blocked, there is also no increase in ST3Gal1 expression.

Discussion

In this study, we determined that CD68 is alternatively glycosylated during the differentiation of BMMs to osteoclasts. This altered glycosylation is manifest primarily in
the form of reduced binding by GNA and PNA lectins and increased binding by MAA. This change in lectin affinity is the opposite of that which was reported in macrophages carrying out phagocytosis[7]. As it has been previously reported that BMMs express the phagocytic glycoform of CD68, the change in lectin affinity seen with RANKL treatment may reflect a switch from the phagocytic core 2 glycoform in BMMs to a PNA-inaccessible sialyl-core 1 glycoform in osteoclasts. PNA binding can be blocked by sialylation of the terminal galactose of the basic core 1 structure, and sialic acid moites are added to this galactose in an α2-3 pattern. What is not immediately consistent with an increase in sialylation is the apparent decrease in CD68 molecular mass. This can be resolved with understanding of the paradoxical relationship between mucin apparent molecular mass and sialic acid. In most cases, addition of a glycan to a protein will increase its molecular mass; addition of sialic acid residues, however, decrease apparent molecular mass likely by altering the electrical or structural nature of the protein[5,26,27]. Indeed, we found that α2-3 neuraminidase digestion increased both PNA binding and apparent molecular mass of CD68. Interestingly, while there was no detectible MAH binding of macrophage-derived CD68, α2-3 neuraminidase digestion enhanced PNA binding of this glycoform. This suggests that macrophage-derived CD68 contains additional PNA-recognized residues that are masked by sialic acid, but those sialic acid residues are not accessible to MAH possibly due to steric hinderence.

We further explored RANKL induced modification of CD68 glycosylation by examining the migration of CD68 extracted from BMMs expressing wild type or mutant RANK cultured with and without RANKL. As before, there was a shift in CD68 molecular mass when wild type BMMs were treated with RANKL, but CD68 did not shift at all
when either PVQET$_{560-565}$ → LLNDS$_{560-565}$-PVQEQG$_{604-609}$ → LLNDNA$_{609-604}$ or IVYY$_{535-538}$ → IVAF$_{535-538}$ mutants were treated with RANKL. This points to the importance of these three signaling motifs in the regulation of CD68 glycosylation during osteoclastogenesis. Interestingly, however, while both mutants fail to fully induce NFATc1 expression, inhibition of NFATc1 with cyclosporin A did not block alteration of CD68 migration rate shift. This supports the notion that while RANK motifs PVQET$_{560-565}$, PVQEQG$_{604-609}$, and IVYY$_{535-538}$ are essential to NFATc1 induction, which itself is essential to osteoclast formation, these motifs activate pathways unrelated to NFATc1 that contribute to osteoclastogenesis and CD68 glycosylation.

Finally, we assessed how RANK regulation of the enzyme ST3Gal1 may correlate with the altered glycosylation of CD68. We found that while RANKL treatment upregulated ST3Gal1 in wild type BMMs, treatment of both P56 and IVAF mutant BMMs, which do not show an alteration of CD68 with RANKL treatment, failed to do so. What was most compelling was that cyclosporin A treatment, which blocks osteoclastogenesis, did not blunt the RANKL upregulation of ST3Gal1. This indicates that ST3Gal1 expression correlates not with osteoclast formation, but with the RANKL-induced altered glycosylation of CD68. Taken together, these data suggest that RANKL upregulated ST3Gal1 may be responsible for the increased sialylation of CD68 seen in osteoclasts, but correlation is not causation, and future work should be done to determine whether the increased sialylation of CD68 in osteoclasts is dependent upon ST3Gal1. Furthermore, if alternative glycosylation of CD68 is dependent upon RANK but not NFATc1, the relevant signaling pathways remain to be determined.
The phenomenon of alternative glycosylation has become a major area of interest. In the immune system, in particular, the glycosylation pattern of individual cells reflects their level of maturation[15]. For example, as T cells develop, sialylation increases and affinity for the lectin PNA decreases. This trend is somewhat reversed, however, as mature T cells become activated; in this case, PNA binding partially returns. The alterations in glycosylation can have multiple effects in terms of T cell activation, matrix adhesion, and T cell receptor binding[16-20]. CD43, which is a T cell protein similar to CD68 in that it also has a large mucin-like stalk, has been shown to have both adhesive and anti-adhesive properties dependent upon its glycosylation state[21-25]. From this, it is clear that understanding a glycoprotein’s function is highly dependent upon being aware of its divergent glycosylation states and how alternative glycosylation can contribute to altered functionality.

As alterations in glycosylation have already been shown to have dramatic effects on the function of proteins, further understanding of the signaling pathways and enzymes that regulate CD68 glycosylation during osteoclastogenesis will undoubtedly shed light on the nature of this well-known, but poorly understood molecule.

Acknowledgements

This work was supported by National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS) grant number AR47830 (to XF) and a training fellowship (to JWA) from the University of Alabama at Birmingham’s Center for Metabolic Bone Disease (UAB CMBD) (T32 AR047512-10). We also thank the UAB CMBD for their ongoing material and intellectual support.
References


Figure 1. RANKL alters the migration rate and lectin binding of CD68. (A) M-CSF expanded BMMs were cultured with 44ng/mL M-CSF alone or 44ng/mL M-CSF + 100ng/mL RANKL. Whole cell lysates were subjected to SDS-PAGE and western blotting. With the addition of RANKL, the apparent molecular mass of CD68 decreases. Data is representative of 3 independent experiments. (B) Immunoprecipitated CD68 from macrophages and RANKL differentiated osteoclasts was subjected to SDS-PAGE and lectin blotting. +, lectin positive control (GNA: carboxypeptidase Y, SNA: transferrin, MAA: fetuin, PNA: asialofetuin, DSA: fetuin); Mφ, macrophage; OC, osteoclast. Osteoclast-derived CD68 demonstrated decreased GNA binding and increased PNA and MAA binding. Data are representative of 2 independent experiments.
Figure 2. RANKL increases CD68 terminal sialylation. (A) Immunoprecipitated CD68 from macrophages and osteoclasts was subjected to SDS-PAGE and chemiluminescent lectin blotting with or without α2-3 neuraminidase pre-digestion. α2-3 neuraminidase digestion enhanced PNA binding of both macrophage and osteoclast-derived CD68. MAA binding was increased in osteoclast-derived CD68, and this binding was reduced by α2-3 neuraminidase digestion. Data is representative of 2 independent experiments. (B) Whole cell lysates from macrophages and osteoclasts were subjected to SDS-PAGE with or without α2-3 neuraminidase pre-digestion and western blotted for CD68 and β-actin. α2-3 neuraminidase digestion attenuated RANKL-induced acceleration of CD68 migration rate. Data is representative of 2 independent experiments.
Figure 3. Mutation of RANK signaling motifs PTM5 and PTM6 or IVVY blocks osteoclastogenesis and RANKL induced alteration of CD68. (A) Schematic of relative position of RANK’s putative TRAF motifs (PTMs) and non-TRAF IVVY motif. P56 is a mutant RANK with signal-inhibiting point mutations in PTM5 (560 PVQET 565 → LLNDDS) and PTM6 (604 PVQEIQ 609 → LLNDNA). IVAF is a mutant RANK with signaling-inhibiting point mutations in RANK’s non-TRAF IVVY motif (535 IVVY 538 → IVAF). (B) WT, P56, IVAF BMMs were cultured with either 44ng/mL M-CSF alone or 44ng/mL + 100ng/mL RANKL for three days to induce osteoclastogenesis. With M-CSF and RANK, WT, but not P56 or IVAF, BMMs became multi-nuclear, TRAP-positive osteoclasts. (C) Whole cell lysates from WT, P56, and IVAF BMMs treated with either M-CSF alone or M-CSF + RANKL were submitted to SDS-PAGE and western blotted for CD68 and β-actin. P56 and IVAF BMMs did not demonstrate the shift in CD68 molecular mass seen in WT BMMs when treated with M-CSF and RANKL. Data are representative of 3 independent experiments.
Figure 4. RANKL induced alteration of CD68 is not dependent upon NFATc1. (A) Whole cell lysates from WT, P56, and IVAF BMMs treated with either 44ng/mL M-CSF alone or 44ng/mL M-CSF + 100ng/mL RANKL for 3 days were subjected to SDS-PAGE and western blotted for NFATc1 and β-actin. Neither P56 nor IVAF BMMs were able to reach the level of NFATc1 activation seen in WT. Data is representative of 2 independent experiments. (B) WT BMMs were treated with either M-CSF alone or M-CSF + RANKL with increasing concentrations of the NFATc1 inhibitor cyclosporin A for 3 days to induce osteoclastogenesis. Higher concentrations of cyclosporin A effectively blocked osteoclastogenesis. (C) Whole cell lysates from WT BMMs treated with either M-CSF alone or M-CSF + RANKL with increasing concentrations of cyclosporin A (CyA) for 3 days were subjected to SDS-PAGE and western blotted for CD68 and β-actin. Cyclosporin A treatment did not inhibit the RANKL-induced alteration of CD68 migration.
Figure 5. RANKL up-regulates ST3Gal1 expression dependent upon RANK motifs PTM5/PTM6 and IVVY, but not NFATc1. RNA extracted from WT BMMs treated with 44ng/mL M-CSF, WT, P56, and IVAF BMMs treated with 44ng/mL M-CSF + 100ng/mL RANKL, or WT BMMs treated with 44ng/mL M-CSF + 100ng/mL RANKL + 1μg/mL cyclosporin A for 3 days was subject to real time quantitative PCR to examine ST3Gal1 mRNA relative to β-actin mRNA. Expression of ST3Gal1 was significantly increased in WT BMMs treated with M-CSF + RANKL in both the absence and presence of cyclosporin A. No significant increase in ST3Gal1 was measured in either P56 or IVAF BMMs treated with M-CSF + RANKL. Data represents mean ± standard deviation. IVAF standard deviation not visible at this scale. *, p <0.005. Experiment was performed with biological triplicates of each treatment group.
4 - DEVELOPMENT OF CELL-BASED HIGH THROUGHPUT ASSAYS FOR THE IDENTIFICATION OF INHIBITORS OF RECEPTOR ACTIVATOR OF NUCLEAR FACTOR-KAPPA B SIGNALING

by

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Abstract

Bone loss due to metabolic or hormonal disorders and osteolytic tumor metastasis continues to be a costly health problem, but current therapeutics offer only modest efficacy. Unraveling of the critical role for the receptor activator of nuclear factor-kappa B (RANK) and its ligand, RANK ligand (RANKL), in osteoclast biology provides an opportunity to develop more effective antiresorptive drugs. The in vivo effectiveness of RANKL inhibitors demonstrates the potency of the RANKL/RANK system as a drug target. Here, we report the development of cell-based assays for high throughput screening (HTS) to identify compounds that inhibit signaling from two RANK cytoplasmic motifs (PVQEET$^{559-564}$ and PVQEQQ$^{604-609}$), which play potent roles in osteoclast formation and function. Inhibitors of these motifs’ signaling have the potential to be developed into new antiresorptive drugs that can complement current therapies. The cell-based assays consist of cell lines generated from RAW264.7 macrophages stably expressing an NF-κB-responsive luciferase reporter and a chimeric receptor containing the human Fas external domain linked to a murine RANK transmembrane and intracellular domain in which only one of the RANK motifs is functional. With these cells, specific RANK motif activation following chimeric receptor stimulation can be measured as an increase in luciferase activity. These assays demonstrated greater than 300% increases in luciferase activity following RANK motif activation and Z'-factor values over 0.55. Our assays will be used to screen compound libraries for molecules that exhibit inhibitory activity. Fol-
low up assays will refine hits to a smaller group of more specific inhibitors of RANK signaling.

Introduction

In normal physiology, bone homeostasis is maintained by the paired processes of bone resorption (carried out by osteoclasts) and bone formation (carried out by osteoblasts).\textsuperscript{1,2,3} This delicate homeostatic balance can be tipped in favor of the osteoclasts and bone resorption by several conditions. The chronic inflammation associated with rheumatoid arthritis leads to localized bone loss as does the osteolytic metastasis of some cancers.\textsuperscript{4,5} A more global loss of bone can be seen in osteoporosis, which is most commonly seen in postmenopausal women who have experienced a dramatic decrease in hormone (particularly estrogen) levels.\textsuperscript{6} Four major antiresorptive drugs (agents capable of inhibiting osteoclast formation and/or function) are currently available on the market: estrogen, selective estrogen receptor modulators (SERMs), bisphosphonates, and calcitonin.\textsuperscript{7,8,9,10,11} Nonetheless, these drugs either offer only modest efficacy or may cause adverse side effects in clinical management of various bone disorders.\textsuperscript{11,12,13,14} Thus, there is a need for development of more efficacious and safer antiresorptive drugs.

Currently, the most attractive target for antiresorptive therapy is the RANKL/RANK system. Together with the monocyte/macrophage colony stimulating factor (M-CSF), the interaction between RANK, located on the plasma membrane of bone marrow macrophages, and RANKL, present on the plasma membrane of bone stromal cells and osteoblasts and as an unbound, soluble variant, is both necessary and sufficient to induce differentiation into osteoclasts.\textsuperscript{15} In addition, the RANKL/RANK system also plays a potent
role in the function and survival of differentiated osteoclasts.\textsuperscript{16} Notably, denosumab, an anti-RANKL antibody developed by Amgen that functions to block the RANKL-RANK interaction, has shown great therapeutic potential in clinical trials.\textsuperscript{17,18,19} As potent and clinically effective as such a protein-based approach would be in reducing bone loss, the cost of manufacturing and the means of delivery may stand as barriers to its widespread application. Furthermore, since RANK has functions in biological processes beyond osteoclasts, global inhibition of the entirety of RANK’s signaling via the blockage of the RANKL-RANK interaction is likely to be accompanied by side-effects in other cells that utilize the RANK/RANKL system.\textsuperscript{20} As such, while targeting the RANKL-RANK interaction is a viable means for reducing bone resorption, a better approach would be to target individual RANK signaling pathways that are more specific to osteoclast formation and function.

RANK was identified as a member of the tumor necrosis factor receptor (TNFR) superfamily.\textsuperscript{21} As TNFR family members primarily employ TNF receptor associated factors (TRAFs) to transmit downstream signaling, numerous studies have been performed to characterize RANK’s TRAF-dependent signaling pathways, and these \textit{in vitro} biochemical studies have collectively identified six TRAF binding motifs (Motifs 1, 2, 3, 4, 5 and 6) in the RANK cytoplasmic domain (\textit{Fig. 1}).\textsuperscript{22,23,24,25,26,27,28,29} Our group has subsequently demonstrated that three of these TRAF-binding motifs (Motif 3: PFQEP\textsuperscript{369-373}, Motif 5: PVQEET\textsuperscript{559-564} and Motif 6: PVQEFG\textsuperscript{604-609}) play a functional role in osteoclast formation and function.\textsuperscript{30} Moreover, all three functional motifs activate the NF-κB signaling pathway in osteoclast precursors\textsuperscript{30}. Recently, we thoroughly evaluated the potential of these RANK functional motifs as therapeutic targets.\textsuperscript{31} Given that mutational inhibition
of either Motif 5- or Motif 6-mediated signaling pathways in osteoclast precursors results in a dramatic reduction in osteoclast formation and function. Motif 5- and Motif 6-mediated signaling pathways can serve as effective antiresorptive targets.\textsuperscript{30,31} Moreover, mutation of any TRAF-binding motif other than Motif 5 or 6 does not greatly impact osteoclastogenesis, though motifs other than 5 and 6 may contribute to other aspects of RANK signaling. Thus, pharmacological blockage of Motif 5- and/or Motif 6-initiated signaling pathways should effectively inhibit osteoclast formation and reduce bone resorption when applied in a clinical setting.\textsuperscript{31}

The potential for Motif 5 and Motif 6 to be highly effective antiresorptive targets prompted us to propose two cell-based assays (hFas-W5 and hFas-W6) for identifying compounds that block Motif 5- and/or Motif 6-mediated signaling pathways in osteoclast precursors (\textit{Fig. 1A}). Each assay consists of a cell line generated from the RAW264.7 macrophage line stably expressing an NF-κB-responsive luciferase reporter and one human Fas-RANK chimeric receptor.\textsuperscript{31} The chimeric receptor allows for specific activation of targeted RANK motifs without interference from endogenous RANK; the chimeric receptor is activated not by RANKL, but by an antibody (α-Fas) that specifically activates human (but not mouse) Fas (\textit{Fig. 2}). hFas-W5 cells express an hFas-RANK chimeric receptor in which all motifs except Motif 5 are mutated (\textit{Fig. 1A}). When hFas-W5 cells are treated with α-Fas, signaling through the chimeric receptor’s Motif 5 is initiated ultimately leading to an increase in NF-κB activation which can be measured as an increase in luciferase expression and activity. If hFas-W5 is treated with a compound that can block the signaling of Motif 5, the induction of luciferase activity and, thus, luminescence following antibody treatment will be reduced. Inhibitors of Motif 6 signaling can likewise
be identified using hFas-W6 cells (*Fig. 1A*). In addition, we have also generated two control assays (hFas-P1-6 and hFas-WT) (*Fig. 1B*).\(^{31}\) hFas-P1-6, in which all putative TRAF-binding motifs are mutated, serves as a negative control against which hFas-W5 and hFas-W6 baseline luciferase induction under different conditions can be measured. hFas-WT, in which all putative TRAF-binding motifs remain intact, can be used to assess the effects of varying assay conditions on unmodified RANK intracellular signaling.\(^{31}\)

Here, we report the development of four cell-based assays (*Fig. 1*) for HTS identification of small molecules that are capable of inhibiting TRAF-binding motif-specific RANK signaling.

**Materials and Methods**

*Chemical and Reagents*

Chemicals were purchased from Sigma (St. Louis, MO) unless indicated otherwise. NF-κB Activation Inhibitor II (Cat No 481408) and Bay 11-7082 (Cat No 196870) were purchased from EMD Chemicals (Gibbstown, NJ). DMEM (Cat No 10-013-CV), L-glutamine (Cat No 25-005-CI), tetracycline (Cat No 61-242-RG), G418 (Cat No 61-234-RF), hygromycin B (Cat No 30-240-CR), and puromycin (Cat No 61-385-RA) were purchased from Mediatech (Manassas, VA). Fetal bovine serum was purchased from Invitrogen (Carlsbad, CA) (Cat No 26140-079). Recombinant GST-RANKL was purified as previously described.\(^{32}\) Mouse M-CSF was prepared from a M-CSF-producing cell line, CMG14-12, which was constructed and kindly provided by Dr. Sunao Takeshita.\(^{33}\)
**Culturing of RAW264.7 Cells**

RAW264.7 (Cat No TIB-71) cells were purchased from American Type Culture Collection (Manassas, VA) and cultured at 37°C and 5% CO₂ in treated tissue culture plates with DMEM containing 10% heat inactivated fetal bovine serum, 2mM L-glutamine and 25 IU/mL penicillin/streptomycin. RAW264.7 cells were passaged by mechanical lifting with cell scrapers.

**Stable Transfection of RAW264.7 Cells with NF-κB-Luciferase Reporter Plasmid**

1×10⁶ RAW264.7 cells seeded into a 60mm tissue culture-treated dish and allowed to attach and grow for 24 hours. 8µg of pGL4.32[luc2P/NF-κB-RE/Hygro] vector (Cat No E8491) from Promega (Madison, Wisconsin) was transfected into seeded cells using Lipofectamine Plus™ from Invitrogen (Cat No 15338-100) according to the manufacturer’s instructions. 48 hours after transfection, cells were selected with 300µg/mL hygromycin B until resistant colonies appeared. Two colonies were isolated following emergence and the remaining colonies were allowed to expand into a mixed pool.

**Construction of hFas-RANK Chimeric Receptor Retroviral Vectors**

The TNFR external domain was cut out of five previously prepared TNFR-RANK chimeric receptor constructs using Xba I and Spe I.³⁰ The differences between RANK intracellular region constructs were as follows: one in which only Motif 5 is fully functional (W5), one in which only Motif 6 is fully functional (W6), one in which no motif is fully functional (P1-6), and one in which all of the motifs are fully functional (WT). The hFas external region was cloned out of a previously prepared hFas-TNFR chimeric recep-
tor construct using Xba I and Spe I and cloned into each of the previous plasmids between the Xba I and Spe I sites to generate the pBluescript-SK-hFas-(W5, W6, P1-6, and WT) constructs.\textsuperscript{34} The resulting chimeric hFas-RANK construct region was cloned out of pBluescript-SK using Xba I and BamH I and cloned between the Xba I and BamH I sites of pMX-Puro to generate the pMX-puro-hFas-(W5, W6, P1-6, and WT) retroviral vectors.

*Preparation of Retrovirus*

293GPG retrovirus packaging cells were kindly provided by Dr. Daniel S. Ory at Washington University and were cultured in DMEM containing 10% heat inactivated fetal bovine serum and supplemented with 500 µg/mL G418, 2mM L-glutamine, 25 IU/mL penicillin/streptomycin, 2 µg/mL puromycin and 1 µg/mL tetracycline as described.\textsuperscript{35} pMX-based retroviral vectors encoding the chimeric receptors were transiently transfected into 293GPG retrovirus packaging cells using the Lipofectamine Plus\textsuperscript{TM} from Invitrogen according to the manufacturer’s instructions. Following the transient transfection, cells were cultured in DMEM supplemented with 10% heat inactivated fetal bovine serum and 2mM L-glutamine. Virus-containing culture supernatant was collected at 48, 72, and 96 hours following transfection and filtered through 0.45µm low protein-binding filters. Supernatants were stored at -80°C.

*Retroviral Infections of RAW264.7 Cells*

RAW264.7 stable lines expressing the luciferase construct were seeded into 60mm treated culture dishes at a density of 3 × 10^5 cells per dish. 24 hours later, the growth me-
dium was aspirated from cells and cells were then infected with 1mL prepared virus-containing supernatants and 1mL growth medium in the presence of 8µg/mL polybrene for 24 hours. After the 24-hour infection, virus-containing medium was removed and cells were cultured with growth medium for additional 24 hours. Positively infected cells were then selected and maintained in growth medium containing 2µg/mL puromycin.

**Luciferase Assays**

2×10⁴ cells were seeded into the wells of clear (Costar® cell culture plate; Cat No 07-200-90; Corning; Lowell, MA) or white (CulturPlate™-96; Cat No 6005680; PerkinElmer; Waltham, MA) tissue culture-treated 96-well plates in 100µL culture medium and allowed to attach and grow for 16 hours. 100µL of culture medium containing 2× concentrations of either RANKL or α-Fas (CH11) (Millipore; Billerica, MA) (Cat No 05-201) were added to cells cultured in 100µL medium, and plates were incubated for varying times at 37°C. For experiments with NF-κB inhibitor, inhibitor was diluted to 500× its working concentration in DMSO, diluted 1/250 into culture medium, and then added to cultured cells (under 100µL culture medium) at a volume of 100µL for a final 1× concentration. When a “flash-type” substrate was used, the cells were washed once with PBS and lysed in 30µL passive lysis buffer (Cat No E1941; Promega). luciferase activity was measured by injecting 100µL luciferase reagent (Cat No E1483; Promega) directly into each well followed by a 1 second luminescence read using a microplate luminometer with automatic injector. When a “glow-type” substrate was used, 100µL BriteLite™ Plus (Cat No 50-904-9934; PerkinElmer) was added directly to each well with no washing, and plates were read at 1 second/well using a microplate luminometer. All treatments
were performed in triplicate unless otherwise noted. The steps of the assay procedure are summarized in Table 1.

### Table 1: Assay Protocol

<table>
<thead>
<tr>
<th>Step</th>
<th>Parameter</th>
<th>Value</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Plate cells</td>
<td>100µL</td>
<td>2 x 10^4 Assay Cells</td>
</tr>
<tr>
<td>2</td>
<td>Incubation time</td>
<td>16hrs</td>
<td>37°C, 5% CO₂</td>
</tr>
<tr>
<td>3</td>
<td>Reporter induction</td>
<td>100µL</td>
<td>Induc NF-κB Luciferase Reporter</td>
</tr>
<tr>
<td>4</td>
<td>Incubation time</td>
<td>6hrs</td>
<td>37°C, 5% CO₂</td>
</tr>
<tr>
<td>5</td>
<td>Remove medium</td>
<td>N/A</td>
<td>Remove via aspiration</td>
</tr>
<tr>
<td>6</td>
<td>Wash</td>
<td>100µL</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>7</td>
<td>Remove PBS</td>
<td>N/A</td>
<td>Remove via aspiration</td>
</tr>
<tr>
<td>8</td>
<td>Lyse cells</td>
<td>30µL</td>
<td>Passive lysis buffer</td>
</tr>
<tr>
<td>9</td>
<td>Reporter reagent</td>
<td>100µL</td>
<td>“Flash type” substrate</td>
</tr>
<tr>
<td>10</td>
<td>Assay Readout</td>
<td>N/A</td>
<td>Microplate luminometer</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Step</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Solid white tissue culture plates, tip dispense</td>
</tr>
<tr>
<td>3</td>
<td>Tip dispense, 200ng/ml α-Fas (final concentration 100ng/ml) + 2x assay concentration library compound in 100µL culture medium</td>
</tr>
<tr>
<td>10</td>
<td>1 second/well</td>
</tr>
</tbody>
</table>

**NB**: If a “glow type” substrate is used, omit steps 5-8. Add 100µL substrate directly to well.

**In vitro Osteoclastogenesis**

2×10⁵ cells were seeded into the wells of a 12-well plate with 100ng/mL RANKL. Medium with treatment was refreshed 48 hours later. Untreated cells were maintained in culture medium without RANKL. The osteoclastogenesis cultures were stained for TRAP expression with Leukocyte Acid Phosphatase Kit (Cat No 387-A) from Sigma 96 hours after seeding.

**Data Analysis**

Dose-response curves were drawn and IC50 values were calculated with a 4-parameter logistic fit using SigmaPlot 10. All other plots and statistical analyses were performed using Microsoft Excel 2007.

**Results**
RAW264.7 cells were chosen for the development of the cell-based assays because, among macrophage cell lines, RAW264.7 cells are unique in their ability to differentiate into osteoclast-like cells.\textsuperscript{36,37} This indicates that RAW264.7 cells retain all signaling components required for osteoclastogenic RANK signaling. Initially, multiple NF-κB reporters were examined, but ultimately Promega’s pGL4.32 reporter was chosen for use due to its low background activation and rapid luciferase response time (Data not shown). We obtained two clones stably expressing Promega’s pGL4.32 reporter (Clone 1 and Clone 2), which were derived from two isolated colonies that emerged from 3-weeks of hygromycin B selection following transfection. We also prepared a heterogeneous population of RAW264.7 cells stably expressing the reporter by expanding and pooling the remaining hygromycin B-resistant colonies (mixed pool).

In order to determine the responsiveness of the NF-κB reporter, we treated the mixed pool, Clone 1, and Clone 2 with 100µg/mL RANKL for 8 hours before measuring their induced luciferase activity. While the level of luciferase activity from the RANKL-treated mixed pool was over 10-fold higher than that of untreated cells, the luminescence counts were markedly lower than those measured from clone 1 and clone 2 cells (\textit{Fig. 3A}). Furthermore, clone 1 and clone 2 cells also demonstrated higher luciferase induction than the mixed population (\textit{Fig. 3B}). Treatment of clone 1 and clone 2 cells with RANKL for 2, 4, 6, and 8 hours revealed that a treatment time of 6 hours is sufficient for maximum luciferase induction (\textit{Fig. 3C}), and, while Clone 2 cells appeared to demonstrate a
higher induction fold than Clone 1 cells in a single time point experiment in the initial assay (Fig. 3B), the more thorough, multi-time point assessment revealed that Clone 1 consistently showed higher induction folds than Clone 2 (Fig. 3C). Because of this, Clone 1 was chosen for further assay development.

Construction and Initial Characterization of hFas-W5, hFas-W6, hFas-WT, and hFas-P1-6

Viruses encoding pMX-puro-hFas-(W5, W6, P1-6, and WT) were prepared by transiently transfecting the vectors into 293GPG packaging cells as described in Materials and Methods. Clone 1 cells were infected with these viruses and selected with puromycin. Cells infected with viruses encoding different chimeric receptors gave rise to hFas-W5, hFas-W6, hFas-WT, and hFas-P1-6 cells (Fig. 1). The infected cells were maintained in hygromycin and puromycin-containing medium to ensure continued presence of both the NF-κB reporter and chimeric receptor. To determine the responsiveness of each assay to α-Fas and to test whether the antibody stimulates a luciferase response through cross-reactions with receptors other than the chimeric receptor, parental Clone 1 cells and cells of hFas-W5, hFas-W6, hFas-WT, and hFas-P1-6 were treated with 50, 100, or 150ng/mL α-Fas antibody. Assay cell lines demonstrated a dose-dependent response to the α-Fas antibody, with hFas-WT cells showing the strongest luciferase response and hFas-P1-6 the weakest (Fig. 4A). Though, hFas-P1-6 cells demonstrated an increase in luciferase induction with increasing doses of antibody, hFas-W5, hFas-W6, and hFas-WT cells consistently exhibit higher inductions at all concentrations, with a marked increase in luciferase activity over untreated controls and hFas-P1-6 cells at 100ng/mL (Fig. 4B).
Because of this, we used 100ng/mL α-Fas throughout the remainder of the assay development process. Clone 1 cells showed an apparent response to the antibody, but this response was not commensurate with antibody concentration. Furthermore, when clone 1 cells were treated in a white plate, no increases in luciferase activation were observed (data not shown) indicating that the increases seen when the assay was previously performed were likely due to signal cross-talk from neighboring wells.

**Assay Cell Lines Retain Capacity for RANKL-Induced Osteoclastogenesis**

To determine whether the assay cell lines we have developed retain all the necessary components of RANK signaling and, thus, are appropriate for a RANK signaling inhibitor screen, we performed *in vitro* osteoclast formation assays with Clone 1, hFas-W5, hFas-W6, hFas-WT, and hFas-P1-6 cells. All cell lines were able to differentiate into large, multinuclear, tartrate-resistant acid phosphatase-positive (TRAP⁺) osteoclast-like cells when treated with 100ng/mL recombinant RANKL for 96 hours, while untreated Clone 1 cell remained mononuclear and TRAP⁻ (*Fig. 5*). This indicates that the cell line development process did not damage osteoclastogenic components of RANK signaling in any of the assay cell lines.

*NF-κB Inhibitors Reduce Induction of Luciferase by hFas-W5 and hFas-W6 Cells in a Dose-Dependent Manner*

To further assess whether hFas-W5 and hFas-W6 are suitable for use in an HTS setting, we addressed whether the induced reporter activity of hFas-W5 and hFas-W6 cells can be suppressed by established NF-κB inhibitors. To this end, we determined the effect
of two known small molecule NF-κB inhibitors (NF-κB Activation Inhibitor II and Bay 11-7082; Calbiochem) on α-Fas-induced luciferase induction. hFas-W5 and hFas-W6 cells were either untreated or treated with 100ng/mL α-Fas plus vehicle (DMSO) or an equal volume of DMSO containing increasing concentrations of inhibitor, and their luminescence was measured. Using this data, percent inhibition was calculated for both cell lines and both inhibitors at each concentration using the following equation (μDMSO, mean luminescence with α-Fas and vehicle treatment; ν[Inhibitor], luminescence from individual well treated with α-Fas and inhibitor):

\[
\text{%Inhibition} = 100 \times \left(1 - \frac{\nu_{[\text{Inhibitor}]}}{\mu_{\text{DMSO}}} \right)
\]

While α-Fas was still able to significantly activate expression of the reporter in the presence of DMSO in both hFas-W5 and hFas-W6 cells, addition of either NF-κB inhibitor reduced luminescence in a dose-dependent manner, and the inhibitory effect vs. concentration of both inhibitors followed a typical sigmoidal curve. Based on the dose-response curves (Fig 6A and B), IC50 values for each inhibitor were calculated for both hFas-W5 and hFas-W6. These values are reported in Table 2. IC50 values for both inhibitors and both cell lines were acceptably similar to published values and those documented by the manufacturer.39,40 These data further support the suitability of hFas-W5 and hFas-W6 cells for use in HTS.
Assessment of Well-to-Well Variability and Z’-factor Calculation

To evaluate whether hFas-W5 and hFas-W6 are suitable for use in an HTS setting, we first determined the robustness of these two assays. The Z’-factor analysis has been developed as a useful tool to determine the robustness of HTS assays. The Z’-test was run by dividing a white 96-well plate into four equal quadrants. Two quadrants contained hFas-W5 or hFas-W6 cells treated with 100ng/mL α-Fas (Treated), and the remaining two quadrants contained cells that were not treated with α-Fas (Untreated). Following treatment, cells were lysed and assessed luminometrically with either the “flash-type” luciferase substrate that we used previously during assay development or a “glow-type” substrate that is more commonly used in HTS. After measuring the luciferase values of each well the Z’-factor value was calculated using the following equation (σ, standard deviation; μ, mean luminescence):

\[
Z’-\text{Factor} = 1 - \frac{3(\sigma_{\text{Treated}} + \sigma_{\text{Untreated}})}{|\mu_{\text{Treated}} - \mu_{\text{Untreated}}|}
\]

This test quantifies the relationship between the assay’s induced and baseline reporter activation, variation among measured baseline signals, and variation among induced signals. Both hFas-W5 and hFas-W6 cells exhibited induction levels similar to those seen

<table>
<thead>
<tr>
<th>Sample</th>
<th>NF-κB Activation Inhibitor II IC50 (µM)</th>
<th>Bay 11-7082 IC50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hFas-W5</td>
<td>18.69</td>
<td>6.74</td>
</tr>
<tr>
<td>hFas-W6</td>
<td>26.47</td>
<td>7.11</td>
</tr>
<tr>
<td>Published Value</td>
<td>7.1</td>
<td>2-10</td>
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</table>
in previous experiments. $Z'$-factor values for both “flash-type” and “glow-type” substrates are reported in Table 3. The calculated $Z'$-factor values indicate that our assays are suitable for HTS and standard “glow-type” substrates are appropriate for use with our assays.

<table>
<thead>
<tr>
<th>Table 3: $Z'$-Factor Values</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Assay</strong></td>
</tr>
<tr>
<td>-----------------------------</td>
</tr>
<tr>
<td>hFas-W5</td>
</tr>
<tr>
<td>hFas-W6</td>
</tr>
</tbody>
</table>

Discussion

Despite continuous efforts to find new methods of clinical management, pathologic bone loss continues to present healthcare challenges. If a treatment is to be successful, it must meet three qualifications: (1) it must effectively provide relief from a pathologic state, (2) it must not have side effects severe enough to deter application, and (3) it must be simple, convenient, and affordable enough to ensure patient accessibility and compliance. No current antiresorptive therapy is capable of fulfilling all three requirements, highlighting a need for development of better antiresorptive drugs. Anti-RANKL antibodies have promise to display greater potency than bisphosphonates, but the problems of cost and safety remain to be addressed. Protein-based therapies are consistently several times higher in cost than small-molecule drugs. As such, there is a great impetus to develop new methods of discovering small molecule compounds that can be utilized in the development of affordable treatments which are not only effective, but also accessible.
Indiscriminate inhibition of the totality of RANK signaling also presents a potential for side-effects. Given that RANK signaling has been shown to be an important regulator of dendritic cell survival and activation, T-cell activation, and B-cell differentiation, the possibility that total RANK inhibition may perturb immune responses may preclude such an approach.\textsuperscript{41,42,20} Our goal is to identify small molecule inhibitors of downstream RANK signaling that is essential to osteoclastogenesis. Such inhibitors could have the potency of total RANK inhibition, but, because small molecules are relatively less expensive to produce than peptides, they would be more affordable than antibody therapy. More specific inhibition of RANK signaling by small molecules should also reduce the potential for side-effects on other RANK-utilizing cells.

As previously described, the assay system we have developed to exploit the potential specificity of RANK motifs \textit{PVQET}^{559-564} and \textit{PVQEQG}^{604-609} is based upon three components: the murine macrophage cell line RAW264.7, an NF-κB-responsive firefly luciferase reporter, and a chimeric receptor consisting of the extracellular region of the human Fas receptor linked to the transmembrane and intracellular regions of murine RANK (\textit{Fig. 2}).\textsuperscript{31} When a cell expressing both the luciferase reporter and chimeric receptor is treated with an activating antibody directed specifically against human Fas, the chimeric receptors on the plasma membrane activate signaling through the unmutated TRAF-binding motif that ultimately promotes an increase in NF-κB signaling (\textit{Fig. 2}, left side). The increased translocation of NF-κB to the nucleus stimulates the expression of luciferase via interaction with an NF-κB-responsive enhancer element. This increased expression of luciferase can then be quantified via luminometric means. The use of a chimeric receptor allows us to eliminate NF-κB activation by endogenous RANK; for
example, if we were to use a mutated RANK construct, we would be forced to use RANKL to activate signaling through the modified RANK’s unmutated TRAF-binding motif. This RANKL treatment would also activate endogenous RANK and make interpretation of motif activity difficult (Fig. 2, right side). The chimeric receptor allows us to specifically activate only the mutant receptor with an antibody that reacts only with human Fas, thus ensuring that NF-κB will be activated only by assay-specific motifs.

As was seen, assay cell lines demonstrate a different response to α-Fas treatment according to antibody concentration. It is not surprising that hFas-WT demonstrated the highest level of induction, where hFas-W5 and hFas-W6 showed lower levels of induction; the TRAF-binding motifs of hFas-WT are non-mutated and fully functional, where hFas-W5 and hFas-W6 bear inactivating mutations on all, but a single motif. It is important to note, however, that the inactivating mutations do not result in the absolute ablation of signaling from the mutated motif. The point mutations applied to the different chimeric receptors were designed to reduce the signaling from specific motifs without significantly impacting the functionality of neighboring motifs. The requirement that mutations of one motif not interfere with the signaling of another compelled us to choose careful point mutations that cannot completely inactivate motif signaling. As a consequence, hFas-P1-6 cells retain some luciferase induction in response to the α-hFas antibody (Fig. 4). Nevertheless, hFas-W5 and hFas-W6 cells demonstrate a greater induction of luciferase than hFas-P1-6 cells, and, at 100ng/mL, the difference in signal intensity between induced and non-induced hFas-W5 and hFas-W6 cells is high enough for screening purposes (Fig. 4B). In addition, at this concentration, hFas-W5 and hFas-W6 cells show increases over baseline that are more than double what is seen in hFas-P1-6 cells (Fig. 4B).
In comparison between hFas-W5, hFas-W6, and hFas-WT cells, it is clear that hFas-WT cells are capable of greater levels of luciferase induction than hFas-W5 or hFas-W6. This is not surprising, as hFas-WT retains full signaling functionality of all of its motifs. While it would be preferable to increase the luciferase induction of hFas-W5 and hFas-W6 cells, the levels measured during the assay development process reflect the biology of the chimeric receptors. Furthermore, for the purpose of screening compounds libraries, the inductions demonstrated by hFas-W5 and hFas-W6 coupled with their low well-to-well variability point to their sufficiency for identifying signaling inhibitors, and our experiments with NF-κB inhibitors indicate that the cells of hFas-W5 and hFas-W6 cells are sensitive enough to signaling inhibition that measurable decreases in signaling can be consistently detected.

The characterization of our cell-based assays supports their applicability towards HTS. It has been reported that if an assay's Z'-factor is ≥ 0.5, it is considered an excellent assay and generally a single test of a compound is sufficient.\textsuperscript{38} Given that the Z'-factor values for hFas-W5 and hFas-W6 are greater than 0.5 regardless of luciferase substrate used, our assays are well-suited for use in an HTS setting. Moreover, using known small molecule NF-κB inhibitors, we have also demonstrated that our assays are sensitive enough to identify signaling inhibitors (Fig. 6). Finally, the following factors support the cost-effectiveness of our assays: a) since RAW264.7 cells divide very rapidly and are easy to culture, large amounts of hFas-W5 and hFas-W6 cells for HTS assays can be obtained easily and rapidly; b) the cell lysis/luciferase reagents that are widely used for HTS are inexpensive (<10 cents/well); c) we have shown that 100ng/ml is a reasonable dose for the cell-based assays, and, at a cost of $379 for 50ug (enough for assays involving...
100 96-well plates in which each well contains 100μL medium, α-Fas will not contribute significantly to the cost of a screen.

In the early development of our assays we compared “flash type” and “glow type” luciferase substrate. In comparison with a “glow type” luciferase substrate, we found that, while induction folds were similar between “flash” and “glow” substrates, raw luminescence counts were nearly 10-fold higher in both hFas-W5 and hFas-W6 when a “flash type” reagent was used. For this reason, we used a “flash-type” reagent during much of the assay’s development. Nevertheless, “glow-type” reagents are more commonly used in HTS due to the convenience of adding the substrates directly to cultured cells and the stability of their signals. Thus, after developing our assay, we validated the reproducibility of the luciferase response by performing Z’ analyses using both the “flash-type” reagent we used throughout development and a more common “glow-type” substrate. We found that both substrates give similar Z’-factor values, indicating that either a “flash-type” or “glow-type” reagent may be used according to the needs of the screening process.

In this report, we have described the development of two cell-based assays for identifying inhibitors of RANK signaling. In the process we have determined that assays should be conducted in HTS standard white plates, and either a “flash-type” or “glow-type” luciferase substrate is suitable. It is likely that the assay will be scaled down to a 384- or 1536-well format prior to screening, and we have confidence in the flexibility of our assays to accommodate this change. In conclusion, throughout the development of these assays, we had three primary goals: to create an assay that was (1) capable of pro-
ducing a robust, measurable response to RANK motif signaling activation, (2) simple and fast enough to allow its adoption into HTS systems, and (3) inexpensive enough as to not be prohibitively expensive to screen large chemical libraries. We believe we have achieved these goals.

Acknowledgements

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Figure 1. Diagrams showing the key components of the cell-based screening assays (hFas-W5 and hFas-W6) and two control assays (hFas-WT and hFas-P1-6). (A) hFas-W5 and hFas-W6 are designed to observe the effects of compounds on the signaling of individual RANK TRAF-binding motifs. (B) hFas-WT and hFas-P1-6 were used to determine maximum and minimum signaling activation for a given assay configuration. The anti-human Fas activating antibody (\(\alpha\)-Fas) induces an oligomerization of the chimeric receptor which fully activates the signaling of motifs that are not mutated. This leads to an increase in NF-\(\kappa\)B translocation to the nucleus, and, as a consequence, an increase in luciferase gene activation. hFas, human Fas; Luc, luciferase; NF-\(\kappa\)B-RE, NF-\(\kappa\)B Responsive Element.
Figure 2. Rationale for use of a chimeric receptor consisting of human Fas external domain linked to the transmembrane and intracellular domains of mouse RANK for the cell-based assays. Using a chimeric RANK receptor (left side) allows for specific activation of only the motifs of interest, whereas a mutated RANK (right side) would require RANKL treatment which would also activate endogenous RANK. Endogenous RANK activation would make identification of motif-specific inhibitors difficult. ⊙, α-Fas; Δ, RANKL.
Figure 3. Responsiveness of NF-κB-luciferase reporter-expressing RAW264.7 cell to RANKL treatment. 2×10⁴ cells were seeded into the wells of clear tissue culture-treated 96-well plates in 100μL culture medium and allowed to attach and grow for 24 hours. 100μL of culture medium containing 200ng/mL RANKL added to the cells for a final concentration of 100ng/mL, and cells were incubated for 8 hours at 37°C at 5% CO₂. (A) Clone 1 and Clone 2 displayed greater luminescence intensity than the mixed pool. (■, untreated; □, 100ng/mL RANKL) (B) Clone 1 and Clone 2 also displayed greater luciferase inductions than the mixed pool. (C) Time-dependent fold inductions for Clone 1 and Clone 2 (●, clone 1; ■, clone 2). All treatments were performed in triplicate; error bars = standard deviation. Experiment was repeated 3 times; representative data is shown.
**Figure 4.** The responsiveness of parental Clone 1, hFas-WT, hFas-W5, hFas-W6, and hFas-P1-6 to α-Fas treatment. 2×10⁴ cells were seeded into the wells of clear 96-well plates in 100µL culture medium and allowed to attach and grow for 24 hours. 100µL of culture medium containing various 2× concentrations of α-Fas added to the cells, and cells were incubated for 6 hours at 37°C at 5% CO₂. (A) Luminescence displayed by parental Clone 1, hFas-WT, hFas-W5, hFas-W6, and hFas-P1-6 cells in response to increasing concentrations of α-Fas. (■, 0ng/mL; □, 50ng/mL; ▲, 100ng/mL; ▴, 150ng/mL) (B) Percent increase in luciferase activity of treated hFas-P1-6, hFas-W5, hFas-W6, and hFas-WT cells over that of their untreated counterparts. (■, hFas-P1-6; □, hFas-W5; ▲, hFas-W6; ▴ hFas-WT). All treatments were performed in triplicate; error bars = standard deviation. Experiment was repeated 2 times. Representative data is shown.
Figure 5. RANKL-induced osteoclastogenesis in parental Clone 1, hFas-WT, hFas-W5, hFas-W6, and hFas-P1-6 cells. 2×10^5 cells seeded into the wells of a 12-well plate with 100ng/mL RANKL. Medium was refreshed 48 hours later. Cells were TRAP stained 96 hours after seeding. All cell lines were capable of forming large, multinuclear, and TRAP^+ osteoclasts indicating that all assay cell lines retain osteoclast-critical RANK signaling components. Untreated Clone 1 cells were mononuclear and TRAP^−.
Figure 6. Effect of a known NF-κB inhibitors on induction of luciferase by hFas-W5 and hFas-W6 cells. 2×10^4 cells were seeded into the wells of white tissue culture-treated 96-well plates in 100µL culture medium and allowed to attach and grow for 24 hours. 100µL 200ng/mL α-Fas and varying 2× concentrations (DMSO, 1.5625, 3.125, 6.25, 12.5, 25, 50, 100 µM) of either NF-κB Activation Inhibitor II or Bay 11-7084 added to cells and incubated for 6 hours at 37°C at 5% CO₂. Plots compare inhibitory effects of NF-κB Activation Inhibitor II (●) and Bay 11-7082 (○) on (A) hFas-W5 and (B) hFas-W6. IC50 values are reported in Table 2 alongside published values.⁹⁰ Plotted data points are means +/- standard deviation. All treatments were performed in triplicate.
5 - GENERAL DISCUSSION AND FUTURE DIRECTIONS

In this dissertation, three major research directions were pursued: (1) investigation of the expression of CD68 in BMMs and osteoclasts and the consequences of its genetic ablation, (2) exploration of how CD68 glycosylation is modified by RANKL/RANK signaling, and (3) development of a cell-based assay that can identify motif-specific RANK signaling inhibitors. To address the first aim, we used homologous recombination techniques to replace exon 1 and part of exon 2 of the CD68 gene with a neomycin phospho-transferase expression cassette. This targeted insertion resulted in a mutant allele that, when homozygous, resulted in complete deletion of CD68 expression in the entire animal. The decision to generate a CD68 global knockout mouse rather than a myeloid or osteoclast specific knockout using lysosome M or cathepsin K promoter driven Cre recombinase approach was made for two reasons. First, given the primarily myeloid lineage restriction of CD68 expression, we did not anticipate a lethal phenotype would result due to loss of CD68 expression in tissues outside our interest. Furthermore, specifically deleting CD68 from myeloid lineage cells when these are the primary CD68-expressors would result in a de facto global knockout because significant levels expression would not likely occur elsewhere; this would render the extra effort largely redundant. Second, as we suspected CD68 would play a role in the formation or function of osteoclasts, we were interested in an opportunity to observe any severe phenotypes that would preclude potential application of CD68 as a therapeutic target in the treatment or prevention of bone loss.
Upon generation of homozygous CD68 knockout mice, we were pleased to find that these mice were healthy, active, and fertile. These observations were also made by Li et al., who found no apparent immunological defects from CD68 knockout, which they generated, as we did, by inserting a neomycin phosphotransferase expression cassette into the CD68 gene[135]. Furthermore, Li et al. also found no defects in macrophage phagocytosis, inflammatory activation, or M1/M2 polarization with CD68 deletion. This group did, however, find that CD68 knockout resulted in increased macrophage surface expression of both the T-cell co-stimulatory molecule CD86 and the major histocompatibility complex class II. They concluded that CD68 may play a role in internalization and trafficking of these molecules. Despite this, Li et al. found no increased proclivity towards autoimmunity as the mice aged. Working with in vitro differentiated osteoclasts, we found that CD68 knockout osteoclasts have an aberrant morphology marked with intracellular accumulation of vacuole-like structures and reduced attachment to tissue culture substrates. The reduction in adhesion may point to a direct role for CD68 in the attachment of osteoclasts, but weakened attachment can also be caused by intracellular dysfunctions such as defective vesicular trafficking and cytoskeletal organization[137,138]. The cells also have a reduced ability to resorb bone in vitro. Our analysis of the bone phenotype of 6-month-old female CD68 knockout mice revealed an increase trabecular bone that, nevertheless, had a slightly lower tissue mineral density than that of CD68-expressing animals. Static histomorphometric analysis revealed no significant change in osteoclast or osteoblast numbers. Dynamic histomorphometry of calcein double labeled bones, however, showed a significant increase in the mineral apposition rate in CD68 knockout mice. This increase in mineralization rate may explain the decrease in overall
mineralization as rapidly bone-forming osteoblasts may not sufficiently mineralize a layer of bone before adding a new layer on top of it. This could point to a role for CD68 in the coupling of bone resorption to bone formation or it may be a consequence of dysfunctional osteoclasts continuing to stimulate osteoblast activity as is seen in some forms of osteoclast inhibition[64,139,140]. Interestingly, bone parameters measured via μCT in 6-week old mice did not reveal a significant difference between CD68-expressing and CD68 knockout mice. This indicates that, while CD68 knockout osteoclasts have reduced resorption abilities, these defects more likely result in a slowing of age-related bone loss than an increase in bone in young, healthy animals. As such, the role of CD68 in osteoclasts may be greater in pathologic rather than physiologic conditions.

In the future, we plan to further investigate the mechanistic role of CD68 in osteoclast function. Given that we see abnormal vesicles in CD68 knockout osteoclasts, a role for CD68 in vesicular trafficking is a possibility. Experiments that define the origin and contents of those abnormal vesicles will provide further evidence for a role for CD68 in trafficking and give clues as to what intracellular components may depend upon CD68 for their appropriate delivery. The increase in CD68 localization to the periphery of the osteoclasts and its heavy glycosylation also suggests a potential role in isolating the sealing zone. The sugars of CD68 may form a barrier that helps to prevent the leak of protons and enzymes from Howship’s lacuna. If this is the case, measurement of medium pH from CD68 knockout osteoclasts should reveal an increase in acidification as protons leak from an incomplete sealing zone. Such experiments should be carefully designed with appropriate controls as sources other than proton leak, such as glycolysis, can contribute to medium acidification.
The potential for CD68 to serve as an attachment molecule also remains. CD68 can be detected on the cell surface, and the presence of CD68 in the cell periphery provides it the opportunity to assist in attachment. It is apparent that CD68 knockout osteoclasts have reduced adhesion to tissue culture plates. Assessing osteoclast adhesion to culture plates coated with bone components such as hydroxyapatite, collagen, and non-collagenous matrix proteins will be informative. The measurement of cell attachment, however, is a difficult endeavor, and multiple defects not directly related to attachment can result in reductions in cell adhesion. It will likely be necessary to perform in vitro molecular interaction studies between purified CD68 and potential substrates such as hydroxyapatite or type 1 collagen to determine whether CD68 itself has a significant affinity for any of these substrates. These studies can be followed by work with mutant CD68 molecules to determine what region(s), if any, contribute to this affinity. At that point, mutants with low substrate affinity can be re-expressed in CD68 knockout cells with the purpose of assessing adhesion strength in the absence of potentially confounding intracellular defects seen in the CD68 knockout osteoclast. This represents an incredibly difficult and time-consuming process, but if a potential direct role for CD68 in osteoclast adhesion is to be fully explored, this is a systematic approach. Figure 1 depicts the above-described potential roles for CD68 in the osteoclast.

In addition to mechanistic studies, the effects of CD68 knockout on whole mice should be further explored. In this dissertation, the baseline phenotype of mice lacking CD68 was described. One ultimate goal of our study of CD68 is to determine its potential as a new therapeutic target for bone disease. Future studies of these animals should focus on experimental models of bone loss. Post-menopausal osteoporosis can be mimicked by
ovariectomizing female mice[141]. The resulting loss in estrogen can promote a bone-loss phenotype very similar to postmenopausal osteoporosis, and, in this context, the protective effect of CD68 loss can be examined. Other models of bone loss such as glucocorticoid-induced osteoporosis, collagen-induced arthritis, and osteolytic cancer metastasis can also be explored using these CD68 knockout mice[142-144]. Just as CD68 knockout has produce modest increases in bone in untreated animals, loss of CD68 may prevent bone loss in many of the above-mentioned disease models.

Figure 1. Potential roles for CD68 in the osteoclast

As more mechanistic studies into CD68 function are performed, its glycosylation will likely be a major complicating factor. As with other highly glycosylated molecules, the particular functions of CD68 are likely to be influenced by its glycosylation state[145-149]. This is particularly relevant in studies of BMMs and osteoclasts because,
as explored earlier in this dissertation, these two cell types express different glycoforms of CD68. We have found that RANK signaling initiated through intracellular motifs PVQEET\textsubscript{560-565}, PVQEQG\textsubscript{604-609}, and IVVY\textsubscript{535-538} induces a change in CD68 glycosylation that is manifest primarily in four forms: (1) the migration of CD68 through a polyacrylamide gel is farther resulting in a change in apparent molecular mass from 95-120kDa to 80-100kDa, (2) complete elimination of recognition by the core 1 galactose-binding lectin peanut agglutinin, (3) a strong increase in binding by the sialic acid recognizing lectin \textit{Maackia Amurensis} agglutinin, and (4) a roughly 50% reduction in recognition by the mannose-binding lectin \textit{Galanthus nivalis} agglutinin.

The changes related to the migration of CD68 are dependent upon PVQEET\textsubscript{560-565}, PVQEQG\textsubscript{604-609}, and IVVY\textsubscript{535-538}. Surprisingly, however, while PVQEET\textsubscript{560-565}/PVQEQG\textsubscript{604-609} and IVVY\textsubscript{535-538} mutants fail to efficiently induce NFATc1 activation, inhibition of this signaling factor does not affect RANKL induced alteration of CD68.

The situation is similar for RANKL up-regulation of a sialating enzyme called ST3Gal1. While RANKL mutants do not up-regulate expression of this enzyme, NFATc1 inhibition shows no blockage of its up-regulation in the presence of RANKL. ST3Gal1 catalyzes the addition of sialic acid in an \(\alpha2-3\) linkage to galactose residues. Such a reaction would generate both the CD68 lectin binding patterns seen in RANKL treated BMMs and the downward shift in CD68 molecular mass as addition of sialic acid residues paradoxically accelerates protein migration through polyacrylamide gels and, thus, reduces their apparent molecular mass[119]. Nevertheless, the evidence for ST3Gal1’s involvement in the glycosylation of CD68 remains correlative, and more direct studies are necessary to determine whether ST3Gal1 is a mediator of CD68 RANKL-induced alternative glycosylation.
tion. That the alteration of CD68 is dependent upon PVQEET$^{560-565}$ and PVQEQG$^{604-609}$, but not the NFATc1 signaling they initiate is compelling and points to the existence of unexplored RANK signaling pathways that are essential to normal osteoclast formation and/or function. Such pathways would be attractive targets for therapeutic inhibition.

As there is no single perfect anti-resorptive, new targets and new treatment modalities are under continuous investigation. The osteoclast-inhibiting potency of denosumab demonstrates the potential utility of targeting RANK signaling. Though global inhibition of the totality of RANK signal may have drawbacks, more selective inhibition could still prove valuable. Investigation of RANK’s intracellular signaling motifs has revealed multiple new targets, and further exploration of these motifs may serve as a basis for future therapies[112,114,116,150]. As previously described, the vacuolar ATPase and cathepsin K are both important mediators of osteoclast function, and, as such, inhibitors to both molecules are under investigation[151-153]. The preliminary effectiveness of these inhibitors and the dramatic osteopetrosis cases that result in loss of the components they target points to the potential value of identifying and characterizing molecules utilized by osteoclasts.

Nevertheless, the importance of RANK signaling in general and RANK PVQEET$^{560-565}$, PVQEQG$^{604-609}$, and IVVY$^{535-538}$ motif signaling in particular in osteoclast function continues to drive interest in its therapeutic inhibition[116,117,150,154]. That RANKL-induced alternative glycosylation of CD68 is dependent upon signaling from PVQEET$^{560-565}$ and PVQEQG$^{604-609}$ further highlights the importance of these motifs in the osteoclast. We have previously shown that mutations in these motifs block osteoclastogenesis without affecting known RANK signaling pathways other than NFATc1. We
previously suspected that these motifs activate as yet unidentified signaling pathways that contribute to osteoclast formation, and our findings that NFATc1 inhibition does not block alterations in CD68 glycosylation following RANKL treatment supports this suspicion. If this is so, these RANK motifs and the signaling they initiate may be valuable targets for anti-resorptive therapy.

To aid in the identification of RANK motif signaling inhibitors, we developed cell-based assays capable of screening compound libraries for inhibitors of signaling from PVQEET$^{560-565}$ and PVQEQG$^{604-609}$. These assays exploit the macrophage-like RAW264.7 cell line, which can differentiate into osteoclast-like cells with the addition of RANKL, and the ability of PVQEET$^{560-565}$ and PVQEQG$^{604-609}$ to activate NF-κB signaling. RAW264.7 cells were stably transfected with an NF-κB-responsive Luciferase reporter construct. The most responsive clone from the transfection pool was transduced with a viral vector that delivered an expression cassette encoding a chimeric receptor consisting of the extracellular, ligand-binding domain of human Fas linked to the intracellular, signaling domain of mouse RANK. Different chimeric receptor constructs bore inactivating mutations on different signaling motifs. For example, a receptor used to identify inhibitors of signaling from PVQEET$^{560-565}$ has inactivating mutations in every known TRAF signaling motif except PVQEET$^{560-565}$. Since the mutant chimeric receptors contain the ligand binding domain of human Fas, they can be activated by an oligomerizing IgM antibody that specifically recognizes human Fas. This allows us to bypass the endogenous RANK expressed by RAW264.7 cells and measure signaling activation of single RANK motifs.
While our aim is not to identify NF-κB inhibitors, NF-κB can still be used as a marker of motif activation as any compound that blocks events upstream of NF-κB should result in a decrease in reporter activation. Both the PVQEET\textsuperscript{560-565} and PVQEQQ\textsuperscript{604-609} screening assays have proven to be robust in validation studies are well-suited to high-throughput screening of compound libraries. The molecules identified using these assays will be assessed functionally for their potential osteoclast-inhibiting qualities as well as molecularly to determine what aspects of RANK signaling they inhibit.

While much has been revealed over the recent decades in the field of osteoclast biology, there still remain many mysteries regarding how this unique cell forms and functions. The discovery of the RANKL/RANK system was an important milestone, and has been foundational to almost every osteoclast study performed since - including the work described in this dissertation. However, the RANKL/RANK system itself and the signaling it activates is not yet fully understood. CD\textsuperscript{68} is not merely an important piece of the osteoclast’s functional machinery; questions regarding the regulation of its glycosylation are at the heart of RANK signaling. It is tempting to speculate that the heretofore unidentified RANK signaling pathways responsible for alternative glycosylation of CD\textsuperscript{68} during osteoclastogenesis are the same pathways that drive osteoclastogenesis itself. Progress is not built on speculation alone, however; there are many more experiments to be done before the osteoclast is completely understood, and this dissertation represents a small step in that direction. In summary, the work presented in this dissertation has introduced the importance of CD\textsuperscript{68} in osteoclast function, opened new pathways of research into its regulation, and developed a tool for identifying inhibitors of RANK signaling that
is essential not only to CD68 glycosylation but, perhaps, to osteoclast formation and function itself.


procedures: the Women's Health Initiative randomized trial. JAMA 290: 1739-1748.


APPENDIX: IACUC APPROVAL FORMS
NOTICE OF APPROVAL

DATE: September 1, 2009

TO: Feng, Xu
   VH G046B 0019
   975-0990

FROM: 

Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Investigation of the Role of Macrolsatin/COD68 as a Mediator of Cellular Attachment to Bone
   Sponsor: Internal
   Animal Project Number: 090608911

On September 1, 2009, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

<table>
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<th>Species</th>
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<th>Number in Category</th>
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<tbody>
<tr>
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<td>A</td>
<td>120</td>
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Animal use is scheduled for review one year from September 2009. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 090608911 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7602.
MEMORANDUM

DATE: September 1, 2009

TO: Feng, Xu
VH 00468 0010
975-0990

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.

The following application was reviewed and approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on September 1, 2009.

Title: Investigation of the Role of Macrophilin/CD68 as a Mediator of Cellular Attachment to Bone
Sponsor: Internal

This institution has an Animal Welfare Assurance on file with the Office for Protection from Research Risks (Assurance Number A3255-01) and is registered as a Research Facility with the United States Department of Agriculture. The animal care and use program is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International).
NOTICE OF APPROVAL

DATE: September 16, 2010

TO: Feng, Xu
    VH G0468 0019
    975-0990

FROM: Judith A. Kapp, Ph.D., Chair
       Institutional Animal Care and Use Committee

SUBJECT: Title: Investigation of the Role of Macrolalin/CD68 as a Mediator of Cellular Attachment to Bone
       Sponsor: Internal
       Animal Project Number: 100908911

On September 26, 2010, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

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<td>Mice</td>
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Animal use is scheduled for review one year from September 2010. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 100908911 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7892.
MEMORANDUM

DATE: September 16, 2010

TO: Feng, Xu
    VH G046B 0019
    975-0990

FROM: 
    Judith A. Kapp, Ph.D., Chair
    Institutional Animal Care and Use Committee

SUBJECT: NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.

The following application was reviewed and approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on September 26, 2010.

Title: Investigation of the Role of Macrophilin/CD68 as a Mediator of Cellular Attachment to Bone

Sponsor: Internal

This institution has an Animal Welfare Assurance on file with the Office for Protection from Research Risks (Assurance Number A3255-01) and is registered as a Research Facility with the United States Department of Agriculture. The animal care and use program is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International).
NOTICE OF RENEWAL

DATE: August 10, 2011
TO: XU FENG, Ph.D.
     VH-G046 0019
     FAX: (205) 934-1775

FROM: Judith A. Kapp, Ph.D., Chair
       Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: Investigation of the Role of Macrophalin/CD68 as a Mediator of Cellular Attachment to Bone
         Sponsor: Internal
         Animal Project Number: 110908911

As of September 26, 2011, the animal use proposed in the above referenced application is renewed. The University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approves the use of the following species and numbers of animals:

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Animal use must be renewed by August 31, 2012. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 110908911 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7692.

Institutional Animal Care and Use Committee
CH19 Suite 403
933 19th Street South
205.934.7692
FAX 205.934.1186

Mailing Address:
CH19 Suite 403
1530 3RD AVE S
BIRMINGHAM, AL 35294-0019
THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: August 19, 2011
TO: XU FENG, Ph.D.  
YH -0346 0019  
FAX: (205) 994-1775

FROM: Judith A. Kapp, Ph.D., Chair  
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.

The following application was renewed by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on August 19, 2011.

Title of Application: Investigation of the Role of Macrophage/CD68 as a Mediator of Cellular Attachment to Bone  
Fund Source: Internal

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).