

THE ROLE OF THE BONE MARROW MICROENVIRONMENT IN THE
ESTABLISHMENT AND PROGRESSION OF
MULTIPLE MYELOMA

By

Jessica Aaron Fowler

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Approved:

Gregory Mundy

Claire Edwards

Florent Elefteriou

Barbara Fingleton

Simon Hayward

For my father,
for being my first teacher
and for igniting my inquisitive nature

and

For my late mentor, Dr. Gregory Mundy,
for helping me
appreciate my abilities as a scientist

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LIST OF ABBREVIATIONS

AMPK	AMP-activated protein kinase
AP-1	Activator protein-1
BMP-2	Bone morphogenetic protein
BMSCs	Bone marrow stromal cells
BSA	Bovine serum albumin
COX-2	Cyclooxygenase-2
DC	Dendritic cell
Dkk1	Dickkopf-related protein 1, Wnt-signaling antagonist
GFP	Green fluorescent protein
GSK-3	Glycogen synthase kinase-3
GTPase	Guanosine transforming protein
HDAC	Histone deacetylase
HGF	Hepatocyte growth factor
HMW	High molecular weight
HSP	Heat shock protein
IFN- γ	Interferon- γ
IGF-1	Insulin-like growth factor-1
IKK	I κ B kinase
IL-1 (-3 or -6)	Interleukin-1, -3, -6
JAK	Janus kinase
MAPK	Mitogen-activated protein kinase

M-CSF	Macrophage colony-stimulating factor
MGUS	Monoclonal gammopathies of undetermined significance
MIP-1 α	Macrophage inflammatory protein-1 α
MM	Multiple myeloma
MMP-9	Matrix metalloproteinase-9
MSCs	Mesenchymal stem cells
NF κ B	Nuclear factor κ B
NK	Natural killer
OPG	Osteoprotegerin, RANKL decoy receptor
PBMCs	Peripheral blood mononuclear cells
PI3K	Phosphoinositide 3-kinase
PGE ₂	Prostaglandin E ₂
PTHrP	Parathyroid hormone-related protein
RAG-2	Recombinase activating gene-2
RANKL	Receptor activator of nuclear factor κ B ligand
sFRP-2	Soluble frizzled related protein-2
STAT	Signal transducer and activator of transcription
TAMs	Tumor-associated macrophages
TGF- β	Transforming growth factor-beta
Thal	Thalidomide
TNF	Tumor necrosis factor
TRAP	Tartrate-resistant acid phosphatase
VEGF	Vascular endothelial growth factor

CHAPTER I

INTRODUCTION

Multiple myeloma

Multiple myeloma is a fatal hematological malignancy that develops within the bone marrow microenvironment. The American Cancer Society estimated that approximately 20,000 new multiple myeloma diagnoses and 10,800 myeloma deaths were expected in 2007 in the United States alone, making it the second most common hematological malignancy (Jemal, Siegel et al.). Myeloma is characterized by the uncontrolled clonal proliferation of malignant plasma cells within the bone marrow, resulting in numerous pathological features. One of these features unique to multiple myeloma, in contrast to other hematological malignancies, is the destructive osteolytic bone disease that develops in the majority of patients. This myeloma bone disease often results in lytic lesions (Figure 1A), pathological fractures (Figure 1B), and spinal cord compression (Figure 1C). Other clinical features that manifest in patients include anemia, renal failure, immune suppression, and hypocalcaemia. The mechanisms involved in the development of myeloma are not well understood; therefore, despite many advances in the treatment of multiple myeloma, it still remains an incurable and fatal malignancy.

Plasma cells are terminally differentiated B cells derived in the germinal centers following T cell-mediated activation. Following activation, germinal center B cells become either memory B cells or antigen-producing plasma cells

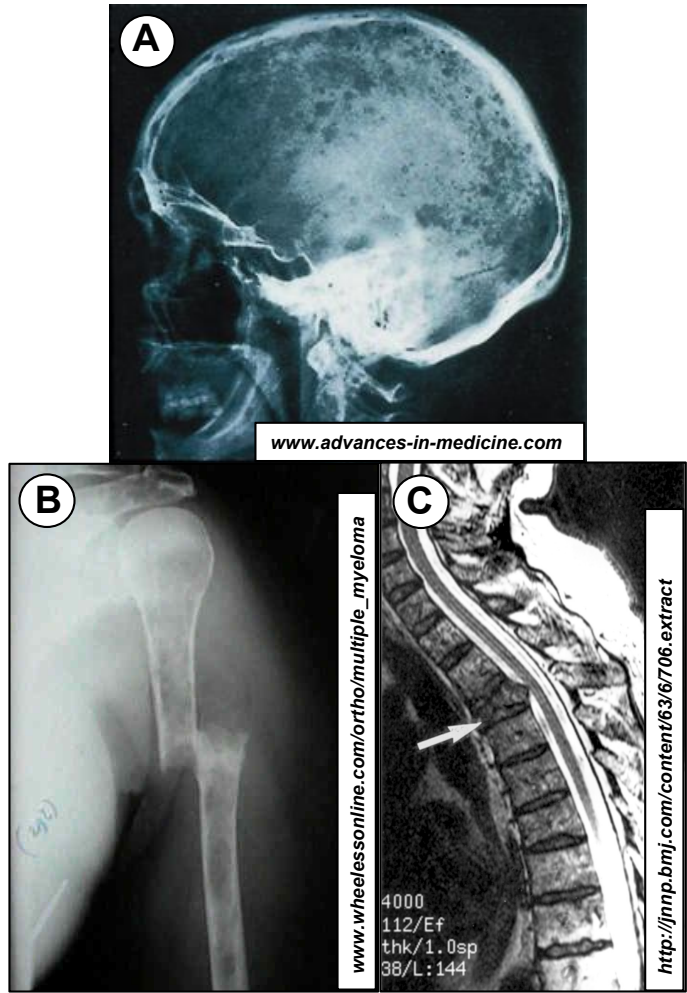


Figure 1. Clinical features of myeloma bone disease. A) Radiograph displaying lytic bone lesions in the skull of a myeloma patient. B) Pathological fracture in humerus of myeloma patient, demonstrated by radiograph. C) Tumor cells present in the spines of myeloma patients often cause spinal cord compression resulting from the collapse of vertebral bodies (white arrow).

(Honjo, Alt et al. 2004). Differentiating B cells undergo a process called somatic hypermutation, where the immunoglobulin genes undergo mutations in order to create antibodies specific for particular antigens (Janeway 2005). In pathological conditions, malignant plasma cells can have abnormal antibody production and accumulate in the bone marrow.

Monoclonal gammopathies of undetermined significance (MGUS) is considered a precursor condition to multiple myeloma (Waldenstrom 1960; Axelsson 1986). Clinical diagnosis of MGUS is classically characterized as abnormal protein production by plasma cells (<10% of plasma cells), in the absence of bone disease, anemia, and hypocalcaemia (Kyle, Therneau et al. 2006). On average, approximately 1% of patients with MGUS progress to multiple myeloma every year (Kyle and Rajkumar 2007); however the reasons for this progression are unknown. Some clinical evidence suggests the progression is due to changes that occur to the bone marrow microenvironment, but there are no reliable predictors for disease progression (Blade, Rosinol et al. 2008). It is critical to identify novel predictors of malignant transformation to determine what patients are at risk. Myeloma progression and the development of osteolytic bone disease are inextricably linked and dependent upon cellular interactions within the bone marrow microenvironment. Therefore, the study of the bone marrow microenvironment in myeloma is critical for both understanding of mechanisms involved in disease progression, and the identification of novel therapeutic targets.

***In vivo* models of multiple myeloma**

Advances in the treatment of myeloma are limited due to the number of clinically relevant animal models that allow for the *in vivo* study of myeloma development in the context of a bone marrow microenvironment. The current animal models for myeloma include the murine Radl 5T model, the SCID-hu/rab xenograft model, and models generated by genetic manipulation, such as overexpression of the transcription factor X-box binding protein. Of these models, the murine Radl 5T model, which shares a number of clinical, histological, immunological and cytogenetic features with human myeloma bone disease, is one of the most promising. The Radl 5T murine model of myeloma was originally identified as occurring spontaneously in aging mice of the C57BL/6 substrain, C57Bl/KaLwRij (Radl, De Gloppe et al. 1979; Radl, Croese et al. 1988). Several 5T cell lines have been developed from this model, which include 5T2, 5T33 and 5TGM1, all of which result in tumor growth within bone and osteolytic bone disease when cells are inoculated into the syngeneic C57Bl/KaLwRij strain or bg/Nu/Xid mice (Garrett, Dallas et al. 1997). In contrast, myeloma does not develop when cells are inoculated into closely related C57Bl/6 mice. The genetic mutation that defines C57Bl/KaLwRij mice is unknown. The SCID-hu xenograft model provides a system where primary human myeloma cells can be injected into either a fetal human or rabbit bone that is implanted subcutaneously into immunocompromised mice (Yaccoby, Barlogie et al. 1998). Both of these models allow the study of tumor growth and myeloma bone disease, and have proven to be effective preclinical models to test novel therapeutic approaches for

the treatment of myeloma bone disease. A major limitation of both models is that manipulation of the bone marrow microenvironment independent of the tumor is limited to systemic pharmacological reagents, rendering it impossible to elucidate specific cellular and molecular mechanisms of myeloma bone disease within the bone marrow microenvironment. Current research demonstrates the critical role that the tumor microenvironment plays in disease progression, however the existing animal models for the study of the tumor microenvironment in myeloma severely impair both clinical and basic research in this field.

Tumor-host cell interactions in myeloma

The bone marrow microenvironment consists of a complex network of cellular interactions and exposure of numerous secreted factors. Cell types within this microenvironment include stromal cells, osteoclasts, osteoblasts, hematopoietic stem cells, B and T lymphocytes, macrophages, and various other immune cells. Myeloma and the associated bone disease can be perpetuated by alterations to resident bone marrow cells and surrounding stroma found within this microenvironment.

Osteolytic bone disease

Osteolytic bone disease is a major clinical complication that arises in patients with multiple myeloma. Multiple myeloma, and other cancers that metastasize to the bone marrow, create an interdependent relationship between tumor cells and cells of the bone marrow microenvironment which promotes both

tumor growth and bone destruction (Edwards, Zhuang et al. 2008). This bone disease is typically associated with increased osteoclast number and activity (Figure 2A), decreased osteoblast formation and function, decreased overall trabecular bone volume (Figure 2B), and lytic lesions through the cortical bone (Figure 2C). In the normal bone marrow microenvironment, bone is constantly undergoing remodeling with a delicate balance between osteoclastic bone resorption and osteoblastic bone formation. The initial stages of normal bone remodeling involve the systematic recruitment and activation of osteoclasts, which are mediated by the interaction of RANKL- and M-CSF- expressing osteoblasts and BM stroma with osteoclast precursors expressing the receptor RANK (Roodman 1999; Yavropoulou and Yovos 2008). Following exposure to RANKL, these mononuclear osteoclast precursors fuse to form functionally mature multinucleated osteoclasts. These mature osteoclasts ultimately bind to the exposed bone matrix where they form resorbing compartments and secrete matrix-degrading enzymes such as tartrate-resistant acid phosphatase (TRAP), cathepsin K, and matrix metalloproteinase-9 (Delaisse, Andersen et al. 2003). The digestion of the bone matrix is followed by osteoclast apoptosis and a transition to osteoblastic bone formation. The signalling involved in this transition is unknown, however it has been suggested to be mediated by osteoclasts (Martin and Sims 2005). Mesenchymal stem cell differentiation into osteoblasts is mediated by the Wnt/ β -catenin pathway (Logan and Nusse 2004). Mature osteoblasts synthesize and regulate mineralization of new bone matrix resulting in increased bone formation and volume (Anderson 2003).

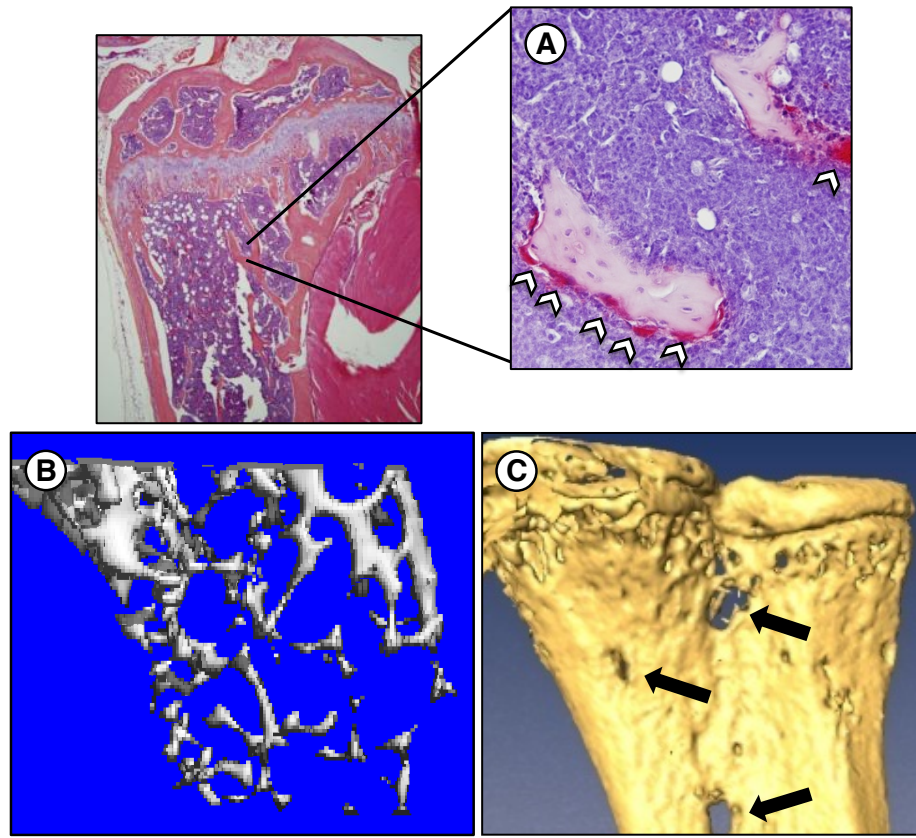


Figure 2. Characteristics of myeloma bone disease. Myeloma bone disease is characterized by A) an increase in TRAP-positive osteoclasts (white arrowheads) and their function, B) a decrease in trabecular bone volume, and C) formation of osteolytic lesions (black arrows) through the cortical bone.

Enhanced osteoclastic bone resorption

Within the myeloma microenvironment, there is a dysregulation in normal remodeling that results in decreased bone formation and enhanced osteoclast function. Myeloma cells play an active role in creating this imbalanced pathological process. This dysregulated process was initially described as a reciprocal relationship between tumor growth and osteoclastic bone resorption, where myeloma cells release “osteoclast activating factors” and the process of resorbing bone further promotes tumor growth and survival (Figure 3A) (Mundy, Raisz et al. 1974). Among some of these “osteoclast activating” cytokines produced by myeloma cells are IL-1, IL-3, IL-6, TNF, macrophage inflammatory protein-1 α (MIP-1 α), hepatocyte growth factor (HGF), and parathyroid hormone-related protein (PTHrP) (Cozzolino, Torcia et al. 1989; Bataille, Chappard et al. 1992; Caligaris-Cappio, Gregoretti et al. 1992; Hjertner, Torgersen et al. 1999; Callander and Roodman 2001; Lee, Chung et al. 2004). Several studies were able to show myeloma cells in close proximity to sites of bone resorption (Mundy, Luben et al. 1974; Mundy, Raisz et al. 1974; Valentin-Opran, Charhon et al. 1982); therefore supporting their role in stimulating osteoclast formation and activity. The last decade of myeloma research has provided extensive evidence implicating two pathways, NF- κ B and MIP-1 α , as having major roles as “osteoclast activating factors” and as being key components in myeloma bone disease.

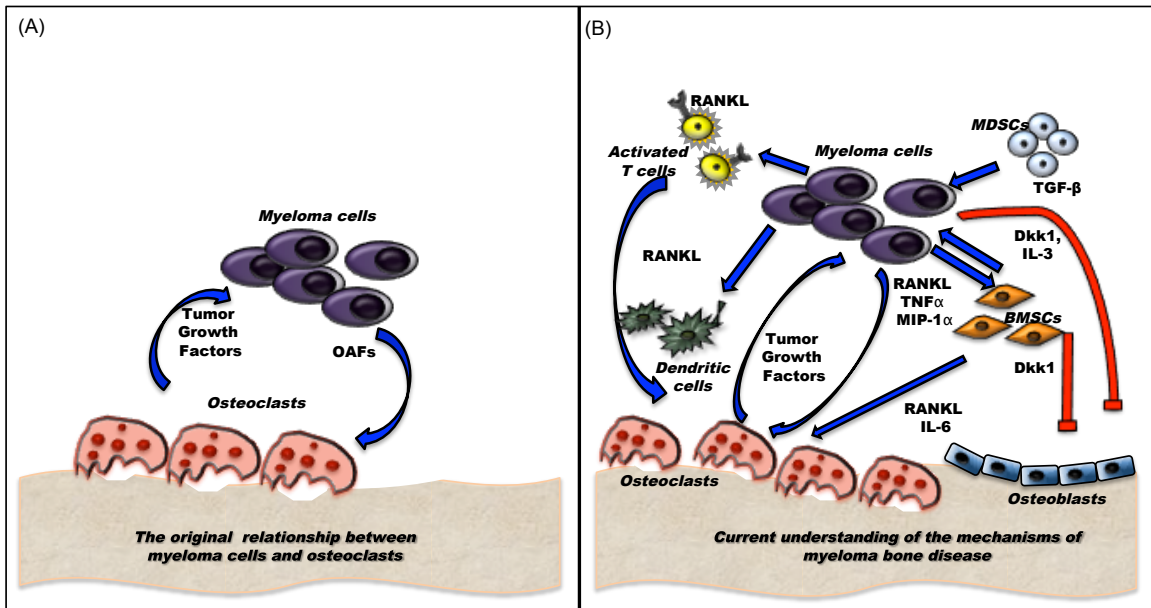


Figure 3. Progression of our understanding of the complex cellular relationships in myeloma bone disease. (A) The original studies first described the relationship between myeloma cells and osteoclasts, whereby myeloma cells released “osteoclast activating factors’ (OAFs) that stimulated osteoclastic bone resorption which in turn released growth factors which promoted myeloma cell growth and survival. (B) Identification of many more cell types and factors has advanced our current knowledge of contributors to disease progression, although the original concepts of tumor cells promoting bone destruction which in turn promote tumor growth remain the fundamental aspects of this increasingly complex network of interactions.

RANKL

Three proteins that belong to the tumor necrosis factor (TNF) receptor family were identified as critical components for normal osteoclastogenesis and bone remodeling. These factors are receptor activator of nuclear factor κ B (RANK) (Anderson, Maraskovsky et al. 1997), its ligand RANKL (Anderson, Maraskovsky et al. 1997; Wong, Rho et al. 1997; Lacey, Timms et al. 1998; Yasuda, Shima et al. 1998); and the RANKL decoy receptor osteoprotegerin (OPG) (Simonet, Lacey et al. 1997; Tsuda, Goto et al. 1997; Yasuda, Shima et al. 1998). The interaction between the transmembrane receptor RANK and the membrane-bound protein RANK ligand is part of normal and pathologic bone remodeling.

In the context of myeloma, early studies demonstrated that bone marrow from patients had increased RANKL expression (Giuliani, Bataille et al. 2001; Pearce, Sordillo et al. 2001). Additionally, myeloma cells decrease expression of OPG expressed by bone marrow stromal cells and osteoblasts (Shipman and Croucher 2003). Some studies have demonstrated that myeloma cells can directly stimulate osteoclastogenesis, independent of osteoblasts, through their own expression of RANKL (Croucher, Shipman et al. 2001; Sezer, Heider et al. 2002). Furthermore, other resident bone marrow cell types contribute to increase RANKL expression. RANKL is upregulated in T cells following antigen stimulation (Anderson, Maraskovsky et al. 1997; Wong, Rho et al. 1997) that can occur from exposure to numerous infectious agents. Any activation of T cells under inflammatory or even pathologic conditions could potentially augment osteoclast formation and activity (Walsh and Choi 2003; Rho, Takami et al. 2004;

Takayanagi 2005). Giuliani et al. showed that myeloma cells have effects on T cells and their function (Giuliani, Colla et al. 2002). The authors demonstrated that RANKL expression in T cells was increased following co-culture with human myeloma cell lines and this soluble RANKL could enhance osteoclastogenesis. These studies found that IL-6 secretion by the myeloma cells was largely responsible for the increased RANKL expression. These authors, as well as others (Colucci, Brunetti et al. 2004), have shown T cells from multiple myeloma patients with osteolytic bone disease expressed RANKL. Given the numerous sources of RANKL within the myeloma bone marrow microenvironment, RANKL serum concentrations are elevated while OPG concentrations are decreased in patients with multiple myeloma (Terpos, Szydlo et al. 2003).

Various *in vivo* models of multiple myeloma and therapeutic studies support the importance of inhibiting RANKL in myeloma bone disease. Treatment of 5T2 myeloma-bearing mice with recombinant OPG was shown to decrease osteoclastogenesis, resulting in an overall reduction of bone loss and osteolytic bone lesions (Croucher, Shipman et al. 2001). Additionally, several groups demonstrated that the over-expression of OPG by either ARH-77 myeloma cells or bone marrow stromal cells resulted in inhibition of myeloma bone disease (Doran, Turner et al. 2004; Rabin, Kyriakou et al. 2007).

Macrophage inflammatory protein-1 α (MIP-1 α)

The second pathway critical for activating osteoclasts is MIP-1 α . MIP-1 α is first implicated in myeloma bone disease in studies by Choi et al, when it was

detected at elevated levels in the bone marrow of myeloma patients (Choi, Cruz et al. 2000). These elevated levels of MIP-1 α were later shown to have correlation with the development of myeloma bone disease (Terpos, Politou et al. 2003). The effects of MIP-1 α on osteoclast formation and activation have been reported to be both dependent (Abe, Hiura et al. 2002; Oyajobi, Franchin et al. 2003) and independent of RANKL (Han, Choi et al. 2001); therefore this function remains unclear. Not only has MIP-1 α been shown to stimulate osteoclasts, but it also can activate signaling pathways in myeloma cells that are important for survival and growth (Lentzsch, Gries et al. 2003).

Osteoblast suppression and decreased bone formation

The uncoupling of normal bone remodeling not only involves enhanced osteoclastic bone resorption but also the suppression of new bone formation. Despite the effectiveness of bisphosphonates, patients with myeloma still develop skeletal related events (Levy and Roodman 2009) and the existing damage to the bone remains unrepaired. This has led to research focused upon preventing the suppression of bone formation and stimulating repair.

New bone formation is inhibited in two ways in the context of myeloma. Firstly, the activity of already existing osteoblasts is suppressed (Bataille, Chappard et al. 1986; Evans, Galasko et al. 1989; Bataille, Delmas et al. 1990). Secondly, differentiation of mesenchymal stem cells (MSCs) into mature osteoblasts is impaired (Bataille, Chappard et al. 1986; Bataille, Chappard et al. 1990; Bataille, Chappard et al. 1991). In addition to overall suppression of bone

formation, this block in differentiation exacerbates the osteolytic bone disease experienced in these patients as immature osteoblasts provide a rich source of RANKL ligand (Atkins, Kostakis et al. 2003), a critical factor for osteoclastogenesis. The molecular mechanisms responsible for the inhibition of osteoblast differentiation are only now becoming clear.

Wnt signaling Pathway

Investigations of myeloma-induced osteoblast suppression have largely focused on the Wnt/ β -catenin signaling pathway because of its critical role in normal bone physiology. The first evidence for a role for the Wnt signaling pathway, and specifically the Wnt-signaling antagonist Dickkopf-related protein 1 (Dkk1), in myeloma bone disease came from a study by Tian and colleagues, who demonstrated that patients with multiple myeloma had increased expression of Dkk1 which correlated with the extent of the osteolytic bone disease (Tian, Zhan et al. 2003). Subsequent studies have also observed a significant increase in Dkk1 expression in patients with myeloma, a correlation between Dkk1 expression and osteolytic bone lesions, and a reduction in serum Dkk1 concentrations following anti-myeloma treatment (Politou, Heath et al. 2006; Haaber, Abildgaard et al. 2008; Kaiser, Mieth et al. 2008). In contrast to these findings, Oshima and colleagues demonstrated a role for myeloma cell-derived soluble frizzled related protein-2 (sFRP-2), another antagonist of Wnt signaling, in the suppression of bone formation (Oshima, Abe et al. 2005).

In vitro investigations by Tian et al. demonstrated that osteoblast differentiation was blocked by bone marrow serum from patients with myeloma, and the inhibitory effect was found to be due to the presence of Dkk1 (Tian, Zhan et al. 2003). Dkk1 was found to inhibit Wnt-3A-induced β -catenin accumulation and bone morphogenetic protein (BMP-2) mediated osteoblast differentiation. In contrast to these studies, Giuliani and colleagues found that although myeloma cells or bone marrow plasma from myeloma patients could inhibit canonical Wnt signaling in murine osteoprogenitor cells, and express high concentrations of soluble Wnt antagonists, that they did not block canonical Wnt signaling in human mesenchymal stem cells or osteoprogenitor cells (Giuliani, Morandi et al. 2007). In addition to direct effects on myeloma bone disease, Gunn et al. have reported that conditioned media from mesenchymal stem cells can promote myeloma cell proliferation and increase expression of Dkk1 by myeloma cells. Dkk1 then acts back on the mesenchymal stem cells to prevent their osteoblastic differentiation and maintain them in an immature state. Immature osteoblasts express higher levels of IL-6 and therefore have greater potential to stimulate myeloma cell proliferation. This interdependent relationship between mesenchymal stem cells and myeloma cells results in stimulation of myeloma cell proliferation and decreased osteoblastogenesis (Gunn, Conley et al. 2006).

Until recently, the major focus has been on Dkk1 derived from myeloma cells; however there is increasing evidence to suggest that myeloma cells may not be the sole source for Dkk1 within the myeloma bone marrow microenvironment. Several studies have identified an increase in Dkk1 in

mesenchymal stem cells isolated from patients with multiple myeloma (Corre, Mahtouk et al. 2007; Garderet, Mazurier et al. 2007). Drake and colleagues recently demonstrated that serum Dkk1 was significantly increased in patients with MGUS, as compared to controls. Furthermore, these increases were associated with bone loss and changes in skeletal microstructure, quantitated by high resolution pQCT (Drake, Ng et al. 2009). In support of a role for bone marrow stromal cell derived Dkk1 in myeloma bone disease, our data presented in this dissertation demonstrate that myeloma-associated fibroblasts, which are capable of promoting myeloma growth *in vivo*, can induce osteoblast suppression *in vivo* with no requirement for the presence of myeloma cells, and that this effect may be mediated, at least in part, via secretion of Dkk1 (Fowler, Mundy et al. 2009).

Targeting the Wnt signaling pathway in myeloma bone disease

Preclinical studies using murine models of myeloma strongly support targeting the Wnt signaling pathway for the treatment of myeloma bone disease. Inhibition of Dkk1, using neutralizing antibodies, has proven to be effective in several murine models of myeloma, with a significant reduction in myeloma bone disease and tumor burden (Yaccoby, Ling et al. 2007; Fulciniti, Tassone et al. 2009; Heath, Chantry et al. 2009). In addition to directly targeting Dkk1, several studies have investigated targeting other components of the Wnt signaling pathway. Sukhedo et al. used a novel small molecule inhibitor, which acts to disrupt the interaction between β -catenin and TCF and so inhibit Wnt signaling

(Sukhdeo, Mani et al. 2007). Inhibition of Wnt signaling was found to inhibit tumor growth and prolong survival in a xenograft model of myeloma, however the effects of this small molecule have not been evaluated in models of myeloma bone disease. Edwards et al. used a systemic pharmacological approach, by treatment with lithium chloride, which acts to inhibit glycogen synthase kinase 3 β (GSK-3 β) and so activate β -catenin (Edwards, Edwards et al. 2008). Lithium chloride was found to significantly prevent myeloma bone disease and reduce tumor burden within bone in the 5TGM1 murine model of myeloma. In support of this, a small molecule inhibitor of GSK-3 has been shown to prevent myeloma bone disease in the 5T2 myeloma model (Abdul, Stoop et al. 2009). Qiang et al. have also demonstrated that systemic Wnt3A treatment could prevent the development of myeloma bone disease and reduce tumor burden in a SCID mouse model of myeloma (Qiang, Shaughnessy et al. 2008).

Although there is compelling evidence that targeting Dkk1 and Wnt signaling prevents myeloma bone disease in experimental models, concern has been raised over the implications for tumor growth. Activation of the Wnt signaling pathway through β -catenin plays a critical oncogenic role in many human malignancies and expression of β -catenin has been demonstrated in myeloma cell lines and in malignant plasma cells from patients with multiple myeloma (Derksen, Tjin et al. 2004; Giuliani, Morandi et al. 2007). Currently, published data are conflicting as to the role of Wnt signaling in myeloma cells (Qiang, Endo et al. 2003; Derksen, Tjin et al. 2004; Edwards, Edwards et al. 2008; Qiang, Shaughnessy et al. 2008). Importantly, in all studies, when the

tumor cells were present within the bone marrow microenvironment, activation of Wnt signaling resulted in a reduction in tumor burden and prevention of myeloma bone disease (Edwards, Edwards et al. 2008; Qiang, Shaughnessy et al. 2008). These data highlight the importance of interactions in the local microenvironment and demonstrate that, despite potential direct effects to increase tumor growth at extraosseous sites, increasing Wnt signaling in the bone marrow microenvironment can prevent the development of myeloma bone disease. Overall, targeting the Wnt signaling pathway represents an attractive therapeutic approach for the treatment of myeloma bone disease. However, further work needs to be undertaken to establish the effects of blocking Dkk1 and promoting Wnt signaling on myeloma growth and survival in both intra-osseous and extramedullary sites.

Alternative contributions to myeloma bone disease by the bone marrow microenvironment

In addition to osteoclast stimulation and suppression of osteoblasts, myeloma bone disease can be perpetuated by alterations to surrounding stroma and other cell types found within the bone marrow (Figure 2B). Only recently has myeloma research begun to focus on how other cell types within the bone marrow microenvironment contribute to myeloma pathogenesis. The limited study of the host microenvironment in myeloma has been due to the availability of appropriate mouse models for myeloma and its associated bone disease. However, we have recently developed a mouse model of myeloma that utilizes

mice deficient in the recombinase activating gene-2 (RAG-2) (Fowler, Mundy et al. 2009). These mice possess a host microenvironment that allows for growth of the 5T murine myeloma cells. The RAG-2 deficient mice are easy to breed with other genetically modified mice and so permit examination of the host microenvironment in myeloma *in vivo*. These studies will be described in more detail in Chapter III. The ability to target specific factors and cell types within the bone marrow microenvironment will both enhance our understanding of the cellular and molecular mechanisms that contribute to myeloma bone disease and identify and validate novel therapeutic approaches.

Bone marrow mesenchymal stem cells/Bone marrow stromal cells/fibroblasts

Recently Todoerti et al, performed gene expression analysis of MSCs and osteoblasts from normal donors, and patients with monoclonal gammopathy of undetermined significance (MGUS) or myeloma. For those patients with myeloma they also compared those with or without osteolytic bone lesions (Todoerti, Lisignoli et al.). There were no differences in the phenotype, in terms of cell proliferation, between MSCs and osteoblasts from any of the groups examined. These studies demonstrated that Dkk1 expression is higher in MSCs from myeloma patients with osteolytic bone disease than without bone disease. Overall, the microarray analysis revealed that MSCs in myeloma patients are greatly altered in regards to gene expression, compared to osteoblasts. Prior to the gene expression studies performed by Todoerti et al., there were expression studies by Bourin and colleagues examining bone marrow mesenchymal stem

cells in normal, MGUS, and myeloma patients (Corre, Mahtouk et al. 2007). In contrast to the more recent study, the investigation by this group used bone marrow cells that were in culture for an extended period of time. A concern with these expression studies is that the expression profiles in this population of cells could be dramatically altered given the plasticity of these cells. Among the genes that were identified as differentially expressed in myeloma BM MSCs compared to normal MSCs were Dkk1, IL-6, and IGF-1. These factors are known to play an important role in osteoblast differentiation (Tian, Zhan et al. 2003), osteoclastogenesis (Wang, Nishida et al. 2006), and support of myeloma growth and survival (De Bruyne, Bos et al. ; Abe, Hiura et al. 2004). Additionally, the authors found that MSCs from myeloma patients were less capable of forming mineralized nodules, indicative of mature osteoblasts, during *in vitro* differentiation studies (Corre, Mahtouk et al. 2007). Even though these studies focused on the MSC populations specifically, they stimulate the intriguing question of the extent to which other cell types contribute to disease progression and development of osteolytic bone disease, and whether these changes are a cause or consequence of the presence of tumor and bone disease.

Myeloma growth and survival

The interaction of myeloma cells with the bone marrow microenvironment is not only important for growth and survival of these cells, it is also critical in terms of drug resistance using clinical treatments. Interactions with bone marrow stromal cells can activate proliferative and anti-apoptotic signaling cascades in

myeloma cells, such as adhesion-mediated nuclear factor- κ B (NF- κ B) activation and growth factor-induced activation of MAPK, JAK/STAT, and PI3K/Akt signaling pathways (Chauhan and Anderson 2003; Hideshima, Mitsiades et al. 2007). Bone marrow stromal cells also activate NF- κ B transcription and secrete cytokines, like interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF) in response to these cellular interactions, which further enhance myeloma pathogenesis and promote drug-resistance (Chauhan, Uchiyama et al. 1996; Gabrilovich, Chen et al. 1996). Additionally, NF- κ B activation can result in increased expression of adhesion molecules present on both myeloma cells and bone marrow stroma (Hideshima, Chauhan et al. 2001). Myeloma cells adhere to resident bone marrow cells and also to matrix proteins. Interaction of myeloma cells with fibronectin present in the local matrix via the α 4 and α 5 β 1 integrins results in MM cell drug-resistance (Damiano, Cress et al. 1999; Shain, Landowski et al. 2002). Current therapies must target not only the cancer cells themselves, but also the microenvironment that provides a protective milieu for continued survival.

Current therapeutic approaches

The chemotherapeutic drug melphalan and the synthetic corticosteroid prednisone have historically been the main treatment for myeloma (Palumbo and Rajkumar); however there is evidence that these therapies themselves may have deleterious effects on bone (Arrington, Fisher et al. ; Hu, Lu et al.). The introduction of autologous stem-cell transplantation has dramatically improved

myeloma patient outcomes following conventional chemotherapy treatment (Attal, Harousseau et al. 1996; Child, Morgan et al. 2003). Unfortunately, this therapeutic approach is limited to younger myeloma patients because of increased risk with age (Palumbo and Rajkumar). Therapeutic regimens for the treatment of multiple myeloma ideally must provide a double advantage for patients—elimination of cancer cells and benefit to the associated osteolytic bone disease. Recent studies in myeloma treatment have started to appreciate how myeloma growth is intrinsically linked to the bone marrow microenvironment; therefore treatments also target the surrounding microenvironment to make it less hospitable to myeloma cells.

Anti-myeloma therapies

Proteasome inhibitors

A recent success in the treatment of myeloma is the proteasome inhibitor bortezomib, commonly known as Velcade. This therapeutic agent has been shown to induce apoptosis and reduce proliferation in numerous malignant cell types and tumors (Adams, Palombella et al. 1999; Lun, Zhang et al. 2005; Zhu, Zhang et al. 2005). Some of the most pronounced anti-tumor effects of the proteasome inhibitors are observed in myeloma and other hematologic malignancies (Zheng, Georgakis et al. 2004). These therapeutic agents inhibit the degradation of important cell cycle regulatory proteins that are ubiquitinated and targeted for destruction by the proteasome (King, Deshaies et al. 1996). More importantly, the use of proteasome inhibitors in the treatment of multiple

myeloma is believed to correlate with its ability to block the degradation of the $\text{I}\kappa\text{B}\alpha$, a regulator and inhibitor of NF- κB (Dai, Rahmani et al. 2003; Lun, Zhang et al. 2005). The NF- κB signaling pathway is a major pathway involved in the development of multiple myeloma because its constitutive activity inhibits apoptosis leading to various cancers. A variety of stimuli can activate the inactive NF- κB / $\text{I}\kappa\text{B}\alpha$ complex causing the initiation of various signal transduction pathways (Karin and Ben-Neriah 2000). Despite suggestive evidence for the NF- κB pathway being a potential target, disappointing clinical trial results using NF- κB -specific inhibitors (Bentires-Alj, Hellin et al. 1999) suggest that the NF- κB pathway is necessary but not sufficient for disease progression.

In addition to the anti-tumor effects, proteasome inhibitors have demonstrated benefits to bone disease. Proteasome inhibition in osteoblasts is necessary for bone formation as transcription factors critical for this process are regulated by proteolytic processing (Muller and Basler 2000). When proteasome inhibitors are given systemically to mice, there is evidence of increased bone formation rates and overall bone volume (Garrett, Chen et al. 2003); however there are no clinical studies indicating osteoblast stimulation in the context of myeloma bone disease. Additionally, proteasome inhibitors also act to inhibit osteoclastogenesis via the inhibition of p38, AP-1, and NF- κB activation (Ahn, Sethi et al. 2007; von Metzler, Krebbel et al. 2007). The inhibition of osteoclasts has been demonstrated clinically where peripheral blood from myeloma patients that received bortezomib showed a reduction in osteoclast formation and function (Hongming and Jian 2009). Despite the success of proteasome inhibitors in

myeloma treatment, there are many other cellular processes that are regulated by the proteasome. There is the need for a more specific therapy that could benefit both sides of multiple myeloma.

HDAC inhibitors

Histone deacetylases are enzymes critical for prevention of DNA transcription by acting to remove acetyl groups on histone, resulting in condensed DNA (Zupkovitz, Tischler et al. 2006; Choudhary, Kumar et al. 2009). HDAC overexpression in myeloma cells often results in loss of tumor suppressor gene expression (Ocio, Mateos et al. 2008). HDAC inhibitors inhibit proliferation and promote myeloma cell apoptosis despite presence of BMSCs and IL-6, by altering gene transcription that benefits myeloma progression (Mitsiades, Hideshima et al. 2009). Similar to the proteasome inhibitors, HDAC inhibitors prevent transcription of genes in the ubiquitin/proteasome pathway (Mitsiades, Mitsiades et al. 2004).

Treatments for the associated bone disease

Bisphosphonates

Bisphosphonates are thought of as the most fundamental therapy for multiple myeloma bone disease. Bisphosphonates are analogs of inorganic pyrophosphate that contain stable P—C—P bonds and are nonhydrolyzable (Rogers, Frith et al. 1999). The two phosphonate groups of these drugs allow for strong binding to calcium-rich bone mineral and antiresorptive potency (Russell,

Muhlbauer et al. 1970). Bisphosphonates inhibit osteoclastic bone resorption by acting directly on osteoclasts to inhibit intracellular signaling pathways and induce apoptosis (Yeh and Berenson 2006). Considering the high-affinity of this reagent for bone mineral, bisphosphonates home to sites of bone resorption where osteoclasts are exposing mineral through the process of resorption (Azuma, Sato et al. 1995). Osteoclasts are endocytic therefore it is likely that localized bisphosphonates at sites of resorption are internalized. Nitrogen-containing bisphosphonates cause the dysregulation of small GTPases resulting in a decrease in osteoclast attachment to bone and ultimately osteoclast inactivation (Luckman, Hughes et al. 1998). Osteoclast inactivation by these bisphosphonates functions through inhibition of the mevalonate pathway, resulting in inhibition of FFP synthase and preventing prenylation of small GTPases. Prenylation is required for post-translational modification and proper function of small GTP-binding proteins, such as Ras, Rho, and Rac, that are necessary for cytoskeleton structure and intracellular trafficking (Luckman, Hughes et al. 1998). The disruption of the cytoskeleton through reduction in functional small GTPases could explain the morphological changes of osteoclasts following bisphosphonate treatment (Sato and Grasser 1990; Murakami, Takahashi et al. 1995) as well as lack of cell survival. Previous clinical studies have shown that treatment with bisphosphonates results in the reduction of myeloma bone disease. These reagents prevent further accelerated bone resorption in the disease by inhibiting osteoclasts, but they cannot repair

already existing bone lesions; therefore representing a major disadvantage in the treatment of osteolytic bone disease.

Therapies targeting the bone marrow microenvironment

Thalidomide and lenalidomide

In recent years, thalidomide (Thal) has proven to have strong anti-myeloma capabilities (Mitsiades, Hideshima et al. 2009). Despite the use of this therapeutic agent to inhibit angiogenesis, clinical evidence suggests the anti-myeloma effects of thalidomide are not due to decreases in bone marrow angiogenesis in multiple myeloma patients. Examination of Thal and Thal analogs have demonstrated immunomodulatory effects, including direct inhibition of myeloma cell proliferation (Hideshima, Chauhan et al. 2000) and acting as a costimulatory signal to T cells (Haslett, Corral et al. 1998). In previous studies, Thal could only increase T cell proliferation in the presence of anti-CD3 or dendritic cells (Haslett, Corral et al. 1998). Anderson and colleagues further examined Thal action on the immune system indicating specific effects on NK cell function (Davies, Raje et al. 2001). In multiple myeloma patients and in healthy individuals, there was an increase in CD3+ T cell proliferation together with IFN- γ and IL-2 secretion. Additional T cell subsets, including CD4+ and CD8+ T cells, also demonstrated an increase in proliferation in response to Thal and Thal analog treatment. Thal treatment of PBMCs in combination with IL-2 resulted in increased myeloma cell lysis. Additionally, depleted PBMC cultures containing CD56+ NK cells still had enhanced myeloma cell lysis in the presence

of Thal; however, CD56+ depletion showed reduced lysis. The increase in NK-mediated lysis was not dependent on the upregulation of NK activation markers. In PBMCs from multiple myeloma patients, Thal or Thal analog treatment, following IL-2 treatment, resulted in enhanced lysis of myeloma cells. In treated multiple myeloma patients, there were increases in NK function and number; however, there were no differences in other T cell subsets. The increase in CD56+ NK cells correlated with patients who responded to Thal treatment.

Many of the therapies for the treatment of multiple myeloma must consider both the inhibition of tumor growth and benefit the associated osteolytic bone disease. Lenalidomide, a thalidomide analog, not only has immunomodulatory effects, but studies have also show it to have specific effects on osteoclasts. *In vitro* studies revealed that lenalidomide could dose-dependently decrease osteoclast formation and resorption activity (Breitkreutz, Raab et al. 2008). The inhibition of osteoclast differentiation and function were due to a decrease in $\alpha V\beta 3$ integrin and cathepsin K expression following lenalidomide treatment. The authors determined that lenalidomide inhibits osteoclastogenesis during stages of differentiation, indicated by the inhibition of both ERK activation and the transcription factor, PU.1. Lenalidomide treatment of osteoclast cultures resulted in decreased secretion of the cytokines MIP-1 α , BAFF, and APRIL, which are all important for osteoclast and myeloma cell survival and growth. Additionally, bone marrow stromal cells treated with lenalidomide showed a reduction in RANKL secretion, further enhancing the inhibition of osteoclastogenesis. Multiple myeloma patients with osteolytic bone disease often have a high ratio of

RANKL/OPG concentrations present in their serum. Myeloma patients treated with lenalidomide had an increase in OPG serum concentrations while RANKL concentrations were significantly reduced.

MAP kinase inhibitors

MAP kinase inhibitors have demonstrated dramatic effects on the myeloma microenvironment. Following treatment with MAPK inhibitors there was a decrease in myeloma cell-mediated vessel formation through the inhibition of VEGF secretion (Giuliani, Lunghi et al. 2004). Neovascularization within the bone marrow cavity is one of the hallmark features in patients with myeloma. Myeloma cells are not the only source of VEGF in the myeloma microenvironment. Tumor-associated macrophages (TAMs) contribute a rich source of proangiogenic factors and cytokines. Scavelli and colleagues have shown that macrophages from multiple myeloma patients can display “vasculogenic mimicry” (Scavelli, Nico et al. 2008). Regulating pathological angiogenesis is one way to control myeloma progression.

The effects of MAPK inhibitors on resident BMSCs may be critical for combination therapy for drug-resistant myelomas. Myeloma cell proliferation was inhibited indirectly because of the effects on BMSC-derived IL-6, a known stimulatory factor of myeloma cells (Giuliani, Lunghi et al. 2004; Nguyen, Stebbins et al. 2006). Proteasome inhibitor cytotoxicity against myeloma cells was enhanced via upregulated pro-apoptotic signaling, with the addition of these kinase inhibitors (Hideshima, Podar et al. 2004).

Dhodapkar and colleagues demonstrated that myeloma cells have effects on dendritic cells (DCs) and have the ability to promote differentiation of these cells into osteoclasts (Kukreja, Radfar et al. 2009). Antigen-presenting cells, such as DCs, produce various cytokines that are responsible for induction and differentiation of T cells. Recent findings by Anderson and colleagues have shown a role for a specific population of DCs in immunosuppression, often associated with myeloma. These studies demonstrated pDCs from patients with multiple myeloma had impaired ability to stimulate allogeneic T cell response despite elevated numbers of these cells present in their bone marrow, in comparison to pDCs from normal donors (Chauhan, Singh et al. 2009). MAPK inhibitors can restore myeloma-altered DCs to normal function, characterized by cytokine secretion and stimulation of T cell responses (Wang, Yang et al. 2006).

IKK inhibitors

NF- κ B activation is evident in both myeloma cells and from the interactions between myeloma cells and the bone marrow microenvironment. Recent gene expression profiling studies by Annunziata and colleagues demonstrated specific signatures indicative of dependence on NF- κ B signaling (Annunziata, Davis et al. 2007). Among the genes found within the expression signature were *NIK* and *TRAF3*, which encode for key regulators of both alternative and classical signaling of NF- κ B (Woronicz, Gao et al. 1997; O'Mahony, Lin et al. 2000; Xiao and Sun 2000; Yin, Wu et al. 2001; Claudio, Brown et al. 2002; Coope, Atkinson et al. 2002; Liao, Zhang et al. 2004;

Ramakrishnan, Wang et al. 2004). Patients with multiple myeloma had high expression of the NF- κ B activating kinase, NIK, and low expression of TRAF3, a known negative regulator of NIK, in their plasma cells in comparison to normal patients (Annunziata, Davis et al. 2007). Studies with NIK overexpression or disrupted NIK/TRAF3 interactions can cause B cell hyperplasia by either amplifying B cell-activating factor of the TNF family (BAFF or BLyS) -induced alternative NF- κ B signaling or inducing independent mechanisms (Sasaki, Calado et al. 2008). NF- κ B activation in immune cells, specifically T cells within the myeloma microenvironment, can contribute to the resulting pathological bone disease as discussed in a previous section of this chapter.

I κ B kinase (IKK) is responsible for phosphorylating the inhibitory I κ B α protein, which results in the dissociation and activation of NF κ B signaling cascades (Karin 1999). A combination of IKK inhibitors can block both the canonical and non-canonical NF κ B pathways resulting in myeloma growth inhibition (Hideshima, Chauhan et al. 2009). IKK inhibitors offer some level of specificity for inhibiting myeloma cells, as Jourden et al found that one of these inhibitors did not alter survival of other mononuclear cells within the bone marrow microenvironment (Jourdan, Moreaux et al. 2007). Despite having little effect on growth and survival of non-myeloma cells, IKK inhibitors can alter IL-6 and IGF-1 secretion from BMSCs that indirectly contribute to myeloma growth inhibition (Hideshima, Neri et al. 2006). These studies demonstrate how inhibition of NF κ B pathways can eliminate both the growth advantage and the protective benefits that BMSCs provide to myeloma cells.

Heat shock protein 90 inhibition

Heat shock proteins, specifically 90 kDa (HSP-90), are overexpressed in many types of cancer (Whitesell and Lindquist 2005). The overexpression of these proteins is thought to be a requirement for cancer cell survival; therefore the inhibition of HSP-90 is thought to be a viable therapeutic option for the treatment of various cancers. Myeloma cells have also been responsive to HSP-90 inhibition both *in vitro* and in *in vivo* models of myeloma (Mitsiades, Mitsiades et al. 2006). HSP-90 inhibition in myeloma cells results in a disruption of protein chaperoning and an accumulation of misfolded proteins, ultimately resulting in myeloma cell death (Mitsiades, Hideshima et al. 2009). Ansamycin-based compounds, such as 17-allylamino-17-demethoxygeldanamycin (17-AAG), are used for the inhibition of HSP-90s and are currently being assessed in clinical trials for multiple myeloma. Cippitelli and colleagues investigated the mechanism of action by which HSP-90 inhibitors induce myeloma cell death (Fionda, Soriani et al. 2009). Considering the immunomodulatory capabilities of multiple myeloma therapies thalidomide and lenalidomide, these authors examined whether HSP-90 inhibitors had the ability to stimulate NK cell cytotoxic function by regulating the expression of NK-activating ligands present on myeloma cells. The authors found HSP-90 inhibitors, 17-AAG and radicicol, upregulated the expression of NK-activating ligands MICA/B, at both the protein and mRNA level. Their studies showed the unfolded protein response (UPR) was not responsible for the upregulation of these ligands. The activation and binding of the transcription factor HSP-1 to MICA and MICB promoters results from HSP-90

inhibition. Treatment with HSP-90 inhibitors also increased NK degranulation in response to enhanced NK-activating ligands.

Current therapies for multiple myeloma do not offer a cure for either the B-cell malignancy itself or the accompanied bone disease. The major treatment regimens for patients with multiple myeloma must involve combination therapy, which can target all the components of the disease. Even though extensive use of these therapies is seen in the clinic, there is a significant gap in understanding the mechanisms of action of these reagents. Additionally, there are few therapeutic reagents that can act on both the malignant plasma cells and the associated bone disease. Despite the advancements in the treatment of myeloma with reagents like proteasome inhibitors, thalidomide, and lenalidomide that improve survival, none of these therapies are curative. The investigation of new treatment options is critical for enhancing patient survival and quality of life.

Goals of dissertation

The goal of my dissertation research was to investigate alterations to the bone marrow that make this microenvironment susceptible as a myeloma cell niche. Despite the use of the C57Bl/KaLwRij strain of mice in the field of myeloma for many years, the exact genetic mutation(s) and/or modifications to the bone marrow that constitute the myeloma cell niche are not known. In the following chapters, I sought to identify bone marrow microenvironmental changes that create the unique and permissive milieu for myeloma growth and progression. Initially, these changes were identified in the C57Bl/KaLwRij mice since these mice share many of the same features of human myeloma and the associated bone disease. After the initial identification of these factors in the C57Bl/KaLwRij mice, findings in patients, either from the literature or by direct examination of specimens, provide strong support that these changes have clinical relevance. In addition to identifying critical changes within the bone marrow microenvironment, this research also discovered a new *in vivo* tool for the study of specific host-derived factors (chapter III) and was utilized in several studies in this dissertation.

The ultimate goal of this dissertation was to gain a better understanding of the mechanisms that precede myeloma bone disease. The investigation of strains of mice that are permissive for myeloma growth may provide clues to human disease development. Specific to the adiponectin studies, the future of these studies would be to advance the screening process for MGUS patients and potentially a new treatment option for both MGUS and myeloma patients.

CHAPTER II

MATERIALS AND METHODS

Cell culture

The 5TGM1-GFP myeloma (MM) cell line was cultured as previously described (Dallas, Garrett et al. 1999). For primary BMSCs, bone marrow was flushed from the tibia and femur of age- and sex-matched C57Bl/KaLwRij and C57Bl6 mice to isolate primary bone marrow stromal cells. Cells were used to establish long-term Dexter type marrow cultures. Briefly, cells were plated at 1×10^7 /mL in Alpha DMEM supplemented with 10% FCS. Adherent cells were washed and culture media replaced after 3-4 days, to remove red blood cells. Cells were cultured for approximately 3 weeks, until confluent. Non-adherent cells were removed, and adherent cells used as a source of primary stromal cells. RNA and protein was isolated from C57Bl/KaLwRij and C57Bl6 bone marrow stromal cells.

***In vivo* 5TGM1 myeloma studies**

Studies were performed using 8- to 10-week-old female C57Bl6 (Harlan U.S., Indianapolis, Indiana), C57Bl/KaLwRijHsd (Harlan Netherlands, Horst, The Netherlands), RAG-2^{-/-}, RAG-2^{-/-}/MMP-9^{-/-}, or RAG-2^{-/-}/Adiponectin^{-/-} mice. Studies were approved by the Institution of Animal Care and Use Committee at Vanderbilt University and conducted in accordance with the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals (National

Research Council. Guide for the Care and Use of Laboratory Animals 1985; Washington, DC National Academy Press pp. 86–23 NIH publication no.). Myeloma in these animals was propagated by the intravenous inoculation of 5×10^5 5TGM1-GFP tagged MM cells in 100 μ L of phosphate buffered saline (PBS).

Assessment of tumor burden by ELISA

Retro-orbital blood was taken weekly from mice used in the *in vivo* studies to obtain serum. Tumor burden was assessed by serum analysis of the myeloma-specific immunoglobulin IgG2b κ , as described previously (Dallas, Garrett et al. 1999). A high binding ELISA plate was coated overnight at 4°C with 2 μ g/mL of IgG2b κ antibody (Research Diagnostics, Fitzgerald Industries). An 8-point standard curve was generated from recombinant mouse IgG2b κ serially diluted in PBS/0.3% BSA. Serum was diluted 1:20,000 for baseline, week 1, and week 2 time points and 1:40,000 for week 3 and endpoint. The substrate reagent used for detection was O-phenylene-diamine tablets (Sigma, St. Louis, MO) with 0.05% H₂O₂ in H₂O. Upon color development (~15min), ELISA plates were read at 450nm OD.

Assessment of tumor burden by flow cytometry

Bone marrow was flushed from the tibia and femur of 5TGM1 MM-bearing mice. Splenic cells from myeloma-bearing mice were obtained by homogenization in tissue culture media. Cell suspensions from both organs were filtered through a

70 μm filter followed by analysis for GFP fluorescence using a 3 laser BD LSRII (Becton Dickinson, San Jose, CA).

Bone histomorphometry analysis

Histomorphometric analysis was performed to quantify bone volume, osteoclast and osteoblast number and surface, trabecular number and trabecular spacing. Tibia and femur were formalin-fixed, decalcified in 14% EDTA, paraffin-embedded, sectioned along the mid-sagittal plane in 4- μm -thick sections. Sections were stained with haematoxylin and eosin and for tartrate-resistant acid phosphatase (TRAP) activity to stain osteoclasts. Three non-consecutive sections were evaluated using Osteomeasure histomorphometry software as previously described (Edwards, Edwards et al. 2008). Briefly, trabecular bone surface area, within the cortical bone, at the proximal and distal metaphyses of the long bones was traced manually using this software. Additionally, the number of TRAP-positive multinucleated osteoclasts and mononuclear cuboidal osteoblasts present on the trabecular bone surface were quantified.

Microcomputed tomography (microCT) analysis

Cortical bone lesions were measured using microCT analysis on the proximal tibia. Bones were fixed in formalin and scanned at an isotropic voxel size of 12 μm using a microCT40 (SCANCO Medical, Bassersdorf, Switzerland). For analysis of cortical bone lesions, cross-sectional images of the entire metaphysis including the cortices and extending 0.25 mm from the growth plate were

exported in tiff format then imported into AMIRA 3-D graphics software (Mercury Computer Systems, Chelmsford, MA). AMIRA software generated a 3-D reconstruction of the metaphyses using a consistent threshold. The number of osteolytic lesions that completely penetrate the cortical bone seen in the virtual reconstruction were counted. MicroCT analysis was also performed on the trabecular bone to assess overall volume and structural characteristics of the trabeculae. Contours were drawn within the cortices of the proximal tibia using the microCT40. The analysis provided a ratio measurement of bone volume to total tissue volume within the cortical bone.

Mesenchymal lineage differentiation studies

Differentiation of 14M1 and ST2 BMSCs into mature adipocytes and osteoblasts was induced by culture in either adipogenic or osteogenic media, respectively. Adipogenic differentiation media consisted of alpha-MEM with 5 $\mu\text{g}/\text{mL}$ insulin (Sigma, St. Louis, MO) and 10nM dexamethasone (Sigma, St. Louis, MO). Osteogenic media consisted of alpha-MEM with 50 $\mu\text{g}/\text{mL}$ ascorbic acid (Sigma, St. Louis, MO), 10 mM β -glycero-phosphate (Sigma, St. Louis, MO), and 10ng/mL BMP-2 (R&D Systems, Minneapolis, MN). Cells were fixed with 10% formalin for 10 minutes and assessed for either adipocytes or osteoblast differentiation. Adipocytes were visualized with Oil Red-O staining following 10 days of culture. Osteoblasts were visualized by von Kossa staining following 15 days of culture.

BMSC co-inoculation *in vivo* studies

Age- and sex-matched non-permissive C57Bl6 and permissive C57Bl/KaLwRij mice were intravenously inoculated with either 10^6 5TGM1-GFP MM cells alone, 10^6 dsRed2 14M1 BMSCs alone, ST2 BMSCs alone, 5×10^5 5TGM1-GFP + 5×10^5 14M1 BMSCs, or 5×10^5 5TGM1-GFP MM cells + 5×10^5 ST2 BMSCs. Mice were inoculated with PBS alone to serve as a control. The permissive C57Bl/KaLwRij mice were inoculated with 5×10^5 5TGM1-GFP cells alone to serve as a positive control for myeloma development. To determine the contribution of 14M1 BMSC-derived Dkk1, Dkk1 expression was stably knocked down using 500 ng/mL of mouse Dkk1 shRNA plasmid in addition to a control scrambled shRNA sequence (Santa Cruz Biotechnology, Santa Cruz, CA). Stable Dkk1 knockdown cells were selected with 10 μ g/mL of puromycin in cell culture media. The efficiency of Dkk1 silencing was determined by measurement of Dkk1 in conditioned media by ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Bone marrow comparison by microarray

RNA was isolated from whole bone marrow of age- and sex-matched C57Bl6 and C57Bl/KaLwRij mice for microarray gene expression analysis. Bone marrow was flushed from three separate mice of each strain. Red blood cells were eliminated using a lysis buffer consisting of NH_4Cl , KCO_3 , and Na_2EDTA . RNA was isolated from the remaining cells using a monophasic isolation reagent, TRIzol (Invitrogen, Carlsbad, CA). Following RNA precipitation, the RNA pellet

was treated with DNase I on-column and cleaned using RNeasy kit (Qiagen, Germantown, MD) to ensure high quality RNA. Pooled RNA samples from each strain were submitted to the Vanderbilt Functional Genomics Shared Resource (FGSR) for hybridization using the Affymetrix GeneChip exon expression array. Differentially expressed genes were based on changes of 2 fold or more between C57Bl6 and C57Bl/KaLwRij bone marrow.

Reverse transcription-PCR

RNA from both cell lines and primary BMSCs was isolated using the RNeasy kit (Qiagen, Germantown, MD). The cDNA was generated using the SuperScript III First Strand Synthesis SuperMix kit (Invitrogen, Carlsbad, CA). Mouse hydroxyprostaglandin dehydrogenase 15 (NAD), glycerophosphodiester phosphodiesterase domain containing 3, and adiponectin were detected using Taqman Gene Expression assay primer sets (Applied Biosystems, Carlsbad, CA). Relative gene expression of adiponectin was normalized to the Taqman Gene Expression assay for GAPD (Applied Biosystems, Carlsbad, CA).

PCR primers for mouse adiponectin receptor 1 and adiponectin receptor 2:

5'-ACGTTGGAGAGTCATCCCGTAT-3', mouse adiponectin receptor 1 (forward);

5'-CTCTGTGTGGATGCGGAAGAT-3', mouse adiponectin receptor 1 (reverse);

5'-GCCCAGCTTAGAGACACCTG-3', mouse adiponectin receptor 2 (forward);

5'-GCCTTCCCACACCTTACAAA-3', mouse adiponectin receptor 2 (reverse).

Generation of RAG-2^{-/-}Adiponectin^{-/-} mice

To order to determine the effect of host-derived adiponectin on myeloma development *in vivo* using a genetic model, 5T myeloma cell growth in adiponectin deficient mice was assessed. As will be discussed in chapters of this dissertation, the genetic mutation that defines C57Bl/KaLwRij mice of the 5T Radl model of myeloma is unknown. Additionally, 5T myeloma cells will not grow in the closely related C57BL/6 mice; therefore genetic manipulation in order to study the role of specific host-derived factors is limited. It was determined through the studies discussed in chapter III that mice deficient in recombinase activating gene-2, or RAG-2, are permissive for 5T myeloma cell growth. These mice can be successfully bred with other strains that are genetically modified; therefore these mice were utilized for the adiponectin studies discussed in chapter V to generate double deficient mice. Adiponectin deficient mice had been generated and used by others groups previously (Maeda, Shimomura et al. 2002; Summer, Little et al. 2008) and were obtained as a gift from Dr. Ken Walsh of Boston University for our studies. The breeding scheme is outlined in Figure 4. These adiponectin deficient mice were bred with RAG-2 deficient mice (cross 1) to generate 100% RAG-2^{+/-}Adiponectin^{+/-} (heterozygous for both RAG-2 and adiponectin). These heterozygous mice were crossed with RAG-2^{-/-} mice (cross 2) to generate a second generation with 25% RAG-2^{-/-}Adiponectin^{+/-}. RAG-2^{-/-}Adiponectin^{+/-} mice were bred with each other (cross 3) to produce mice that were all deficient in RAG-2 but littermates were Adiponectin^{+/+} (WT—25%),

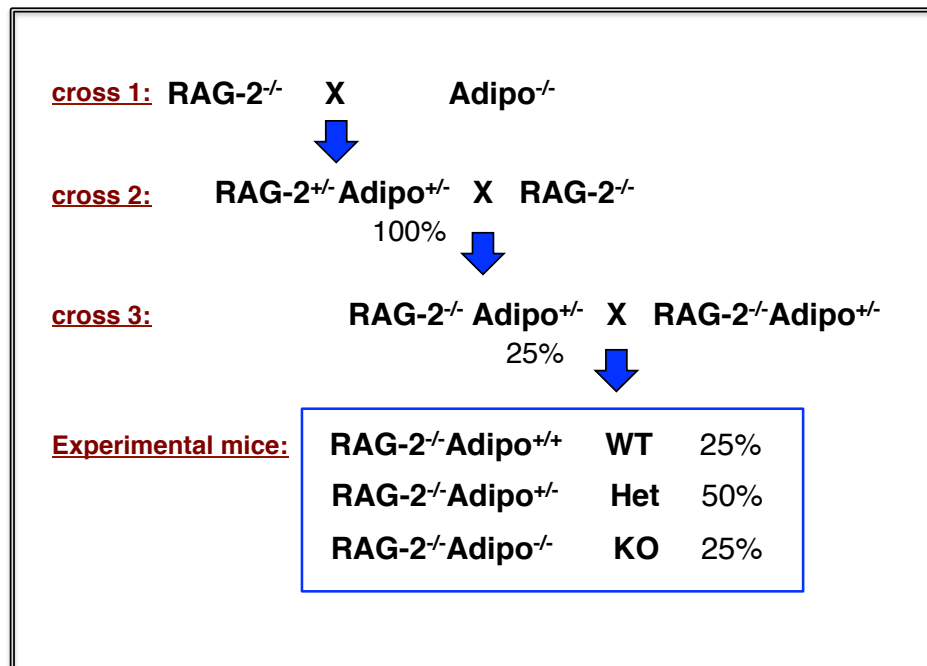


Figure 4. Mouse breeding scheme for host-derived adiponectin *in vivo* studies. The percentage of each genotype generated from each cross is indicated. As shown, it will take three crosses to generate littermates of WT and KO genotypes to be used in adiponectin studies. This breeding scheme is the most efficient way to generate greater numbers of each genotype that are deficient for RAG-2.

Adiponectin^{+/-} (Het—50%), or Adiponectin^{-/-} (KO—25%). Mice were genotyped from tail DNA by PCR amplification for adiponectin and RAG-2 genes.

PCR primers used to genotype adiponectin wildtype, heterozygous, and knockout mice: 5'-TGGATGCTGCCATGTTCCCAT-3', wildtype adiponectin (forward);

5'-CTTGTGTCTGTGTCTAGGCCTT-3', wildtype adiponectin (reverse);

5'-CTCCAGACTGCCTTGGGA-3', mutant adiponectin (reverse).

Immunoblotting

Total adiponectin and the three different isoforms of adiponectin (high, HMW; middle, MMW; and low, LMW, molecular weight forms) were detected in the serum of mice using an adiponectin antibody (Abcam, Cambridge, MA). To detect the various isoforms, the samples were loaded in a non-reducing/non-denaturing gel without boiling the protein samples with a reducing agent. For adiponectin treatment studies, AMPK and p38 activation was detected with antibodies for phosphorylated AMPK (Cell Signaling, Danvers, MA), total AMPK (Cell Signaling, Danvers, MA), phosphorylated p38 (Santa Cruz Biotechnology, Santa Cruz, CA) and total p38 (Santa Cruz Biotechnology, Santa Cruz, CA). Apoptotic downstream signaling was detected with antibodies for cleaved caspase-3 and cleaved PARP-1 (Cell Signaling, Danvers, MA).

ELISAs

Tumor burden in myeloma-bearing mice was assessed by ELISA of the myeloma-specific immunoglobulin IgG2b κ (Research Diagnostics Inc., Division of Fitzgerald Industries International), as described in a previous section. ELISAs for mouse adiponectin and Dkk1 (R & D Systems, Minneapolis, MN) were used to measure total adiponectin and Dkk1 serum concentrations in mice according to the manufacturer's instructions.

Adiponectin treatment and apoptosis

5TGM1 MM cells, ST2, and primary C57Bl6 and C57Bl/KaLwRij BMSCs were cultured for 48 hours with 5, 10, and 15 μ g/mL of recombinant adiponectin (ProSpec, Rehovot, Israel).

Following treatment with either recombinant adiponectin or vehicle for 48 hours, the percentage of viable, apoptotic, and necrotic cells was determined by annexin V and SYTOX AADvanced cell staining for flow cytometry analysis (Molecular Probes, Invitrogen, Carlsbad, CA). Cells positive for both annexin V and SYTOX AADvanced are considered necrotic cells, cells positive for only annexin V are apoptotic, and cells negative for both are viable cells.

L-4F treatment *in vitro*

BMSCs and 5TGM1 MM cells were plated at 5×10^4 cells/mL and allowed to adhere (~4-5hrs) prior to being treated with L-4F. Once attached, BMSCs were washed with PBS before replacing with L-4F containing media. Cells were

treated with vehicle, 20, or 40 $\mu\text{g}/\text{mL}$ of L-4F. L-4F vehicle is ABC diluent consisting of 50mM ammonium bicarbonate and 0.1 mg/mL Tween-20.

L-4F treatment *in vivo*

L-4F or vehicle was administered daily by intra-peritoneal injection at a concentration of 200 $\mu\text{g}/100\text{g}$ prior to tumor cell inoculation until treated mice showed an increase in adiponectin expression present in the serum. 5TGM1 myeloma cells were inoculated once increased adiponectin expression was detected and mice continued to receive daily treatment with L-4F. To determine whether L-4F treatment increased overall survival in myeloma-bearing mice, we performed a survival study. The survival study was performed as above except animals were sacrificed at the time when mice first became paraplegic. Time to paraplegia was used as a surrogate for survival.

Immunohistochemistry and TUNEL

For MMP-9 and tartrate resistant acid phosphatase (TRAcP) localization, the following technique was employed. Sections were rehydrated through a series of ethanols and then rinsed in Tris buffered saline (TBS; 10mM Tris at pH 7.4, 150mM NaCl) with Tween-20 (0.05%). For antigen retrieval, slides were immersed in a 20 $\mu\text{g}/\text{ml}$ solution of proteinase K according to the manufacturer's instructions for 10 minutes at room temperature. Following washing in TBS, tissue sections were blocked using standard blocking criteria for 1 hour at room temperature. MMP-9 (Oncogene, Cat. No. AB3-IM37L) antibodies at a dilution of

1:100 were added in blocking solution overnight at 4°C. Slides were washed extensively in TBST prior to the addition of a species-specific fluorescently labeled secondary antibody (Alexafluor 568nm, Invitrogen) diluted 1:1,000 in blocking solution for 1 hour at room temperature. Slides were washed in TBS and then equilibrated in an acetate buffer as described (Filgueira 2004). The ELF97 TRAcP stain (Invitrogen, Cat. No. 6601) was diluted 1:1,000 in acetate buffer and slides were incubated for 15 minutes at room temperature. Following washing, slides were aqueously mounted in media (Biomedica Corp, Foster City, CA) containing 2µM DAPI (4', 6 diamidino-2-phenylindole) for nuclear localization.

Tibia sections adjacent to those used to assess bone disease were stained for TUNEL (AP kit, Roche) for quantification of apoptotic myeloma cells and phosphorylated histone H3 to measure proliferating myeloma cells. Sections were rehydrated and rinsed as mentioned above. For antigen retrieval, slides were then incubated in 100mM citrate buffer for 1-2 minutes in the microwave. After washes, sections were blocked in solution containing 3% BSA and 20% FCS for TUNEL or in 10% normal goat serum for histone H3, for 1 hour at room temperature. Histone H3 antibody was diluted 1:500 in blocking solution and incubated overnight at 4°C. Following washes, histone H3 sections were incubated at room temperature for 1 hour in species-specific biotinylated secondary antibody diluted 1:500 in appropriate blocking solution. For TUNEL stained sections, the manufacture's protocol were followed accordingly. TUNEL reaction mixture was prepared fresh and sections incubated in mixture for 1 hour

at 37°C. Slides were washed following incubation and then incubated with Converter-AP for 30 minutes at 37°C. The substrate solution was applied for 10 minutes at room temperature and then mounted as mentioned above. Apoptotic or proliferating myeloma cells were quantified using MetaMorph (Molecular Devices) computer software.

Human serum specimens

All serum samples were age-, sex-, and BMI-matched from patients with multiple myeloma, MGUS patients with progression to myeloma, MGUS patients with no progression to myeloma. These and the respective matched control serum samples were obtained through collaborations with Matthew T. Drake, M.D. at the Mayo Clinic, Rochester, MN. Prior to receiving these specimens, we obtained IRB approval to measure serum concentrations of adiponectin in these samples. ELISAs for human total adiponectin and high molecular weight adiponectin (Millipore) were used to measure the serum concentrations in myeloma and MGUS patients.

Statistical analysis

Statistical significance was determined using a Mann-Whitney *U* test for nonparametric data and considered significant given *P* less than or equal to .05. Oneway ANOVA and Tukey–Kramer method tests were used for analysis of multiple groups in both *in vivo* and *in vitro* studies. Data are presented as means (\pm S.E.M) unless otherwise stated.

CHAPTER III

DEVELOPING A MURINE MODEL OF MYELOMA THAT ALLOWS GENETIC MANIPULATION OF THE HOST MICROENVIRONMENT

Summary

Multiple myeloma, and the associated osteolytic bone disease, is critically dependent upon cellular interactions within the bone marrow microenvironment. A major limitation of existing myeloma models is the requirement for a specific host strain of mouse, preventing molecular manipulation of the bone marrow microenvironment. The aim of the current study was to develop a model of myeloma in which the host microenvironment could be genetically modified. The Rad1 5T murine model of myeloma is well characterized and closely mimics human myeloma. In the current study, we demonstrate 5T myeloma establishment in recombinase activating gene-2 (RAG-2) deficient mice, which have improper B and T cell development. Importantly, these mice can be easily bred with genetically modified mice to generate double knockout mice; allowing manipulation of the host microenvironment at a molecular level. Inoculation of 5TGM1 myeloma (MM) cells into RAG-2^{-/-} mice resulted in myeloma development, associated with tumor within bone and an osteolytic bone disease, as assessed by microCT, histology and histomorphometry. Myeloma-bearing RAG-2^{-/-} mice displayed many features similar to both human myeloma and the original 5T Rad1 model. To demonstrate the use of this model, we have examined

the effect of host derived MMP-9 in the development of myeloma *in vivo*. Inoculation of 5TGM1 myeloma cells into mice deficient in RAG-2 and MMP-9 resulted in a reduction in tumor burden and osteolytic bone disease as compared with RAG-2 deficient wild-type myeloma-bearing mice. The establishment of myeloma in RAG2^{-/-} mice permits molecular examination of the host contribution to myeloma pathogenesis *in vivo*.

Introduction

Myeloma progression and the development of the osteolytic bone disease are inextricably linked and dependent upon cellular interactions within the bone marrow microenvironment. Therefore, the study of the bone marrow microenvironment in myeloma is critical for both our understanding of mechanisms involved in disease progression, and the identification of novel therapeutic targets. The advances in the treatment of myeloma are limited due to the number of clinically relevant animal models that allow for the *in vivo* study of myeloma development in the context of a bone marrow microenvironment.

The current animal models for myeloma include the SCID-hu/rab xenograft model, a conditional mouse model dependent upon MYC activation in germinal center B cells and the Rad1 5T model. The SCID-hu/rab xenograft model provides a system where primary human myeloma cells can be injected into either a fetal human or rabbit bone that is implanted subcutaneously into immunocompromised mice (Yaccoby, Barlogie et al. 1998) (Yaccoby, Ling et al. 2007). The Rad1 model utilizes 5T MM cells that spontaneously arose in aged, in-

bred C57Bl/KaLwRijHsd mice and is propagated by the inoculation of these myeloma cells into syngeneic mice (Radl, de Glopper et al. 1979; Radl, Croese et al. 1988; Garrett, Dallas et al. 1997). Both of these models allow the study of tumor growth and myeloma bone disease, and have proven to be effective preclinical models to test novel therapeutic approaches for the treatment of myeloma bone disease (Dallas, Garrett et al. 1999; Croucher, Shipman et al. 2001; Croucher, De Hendrik et al. 2003; Oyajobi, Franchin et al. 2003; Yaccoby, Wezeman et al. 2004; Edwards, Mueller et al. 2007; Yaccoby, Ling et al. 2007; Edwards, Edwards et al. 2008). Activation of MYC under the control of the kappa light chain regulatory elements results in the development of myeloma with features similar to human multiple myeloma (Chesi, Robbiani et al. 2008). A major limitation of all existing models is that manipulation of the bone marrow microenvironment independent of the tumor is limited to systemic pharmacological reagents, rendering it impossible to elucidate specific cellular and molecular mechanisms of myeloma bone disease within the bone marrow microenvironment. Current research has demonstrated the critical role that the tumor microenvironment plays in disease progression, but the existing animal models for the study of the tumor microenvironment in myeloma severely impair both clinical and basic research in this field.

The aim of the study presented in this chapter was to develop a murine model of myeloma in which the host microenvironment could be genetically modified, thus enabling molecular studies of the host contribution to multiple myeloma progression *in vivo*. The Radl 5T murine model of myeloma was

originally identified as occurring spontaneously in aging mice of the C57Bl/KaLwRij strain. Several 5T MM cell lines have been developed from this model, which include 5T2 and 5TGM1, all of which result in tumor growth within bone and osteolytic bone disease when cells are inoculated into the syngeneic C57Bl/KaLwRij strain or bg/Nu/Xid mice (Garrett, Dallas et al. 1997; Asosingh, Radl et al. 2000). In contrast, myeloma does not develop when cells are inoculated into C57Bl6 mice. The genetic mutation that defines C57Bl/KaLwRij mice is unknown, and the deleterious effects of the bg/nu/Xid mutation on breeding and lifespan mean that neither of these strains of mice can be crossed with genetically modified mice in order to modify the host microenvironment in mice, which are permissive to myeloma growth. In the current study, we investigated the establishment of 5TGM1 MM in immunocompromised recombinase activating gene-2 (RAG-2) deficient mice on a C57Bl6 background. These mice have a targeted disruption of the RAG-2 gene resulting in the absence of functional recombinases leading to improper B and T cell development (Shinkai, Rathbun et al. 1992). Importantly, these mice can be easily bred with genetically modified mice to generate double knockout mice; therefore greatly improving our ability to genetically manipulate the host microenvironment.

Results

RAG-2^{-/-} mice develop characteristic myeloma tumor burden

RAG-2 deficient mice on a C57Bl6 background were inoculated with 10^6 GFP-tagged 5TGM1 MM cells by intravenous tail vein injection. Tumor burden was measured by serum IgG2bk ELISA, histomorphometric analysis of tumor burden in bone, and flow cytometric analysis of tumor burden in bone marrow and spleen. The development of myeloma in RAG-2 deficient mice was compared with the syngeneic C57Bl/KaLwRij mice, C57Bl6 mice, bg/Nu/Xid mice, and T cell-deficient athymic nude mice.

Following intravenous inoculation of 5TGM1 MM cells, the RAG-2 deficient mice developed myeloma at the same rate as that observed with the syngeneic C57Bl/KaLwRij mice of the 5T model. Tumor burden of the RAG-2 deficient mice increased over time as determined by measurement of serum levels of myeloma-specific immunoglobulin IgG2b (Figure 5A). The increase of IgG2b levels in the RAG-2 deficient mice was comparable to the tumor burden found in the myeloma-bearing C57Bl/KaLwRij mice (Figure 5A). Inoculation of 5TGM1 MM cells into immune-competent C57Bl6 mice did not result in myeloma development. Tumor burden was also assessed by measuring the percent of GFP-positive myeloma cells present in the bone marrow and spleen. The myeloma-bearing RAG-2 deficient mice showed significant accumulation of GFP-positive myeloma cells in both the bone marrow and spleen (Figure 5B) and this burden was comparable to that observed in the C57Bl/KaLwRij mice. Therefore, the development of multiple myeloma in RAG-2 deficient mice occurs in an

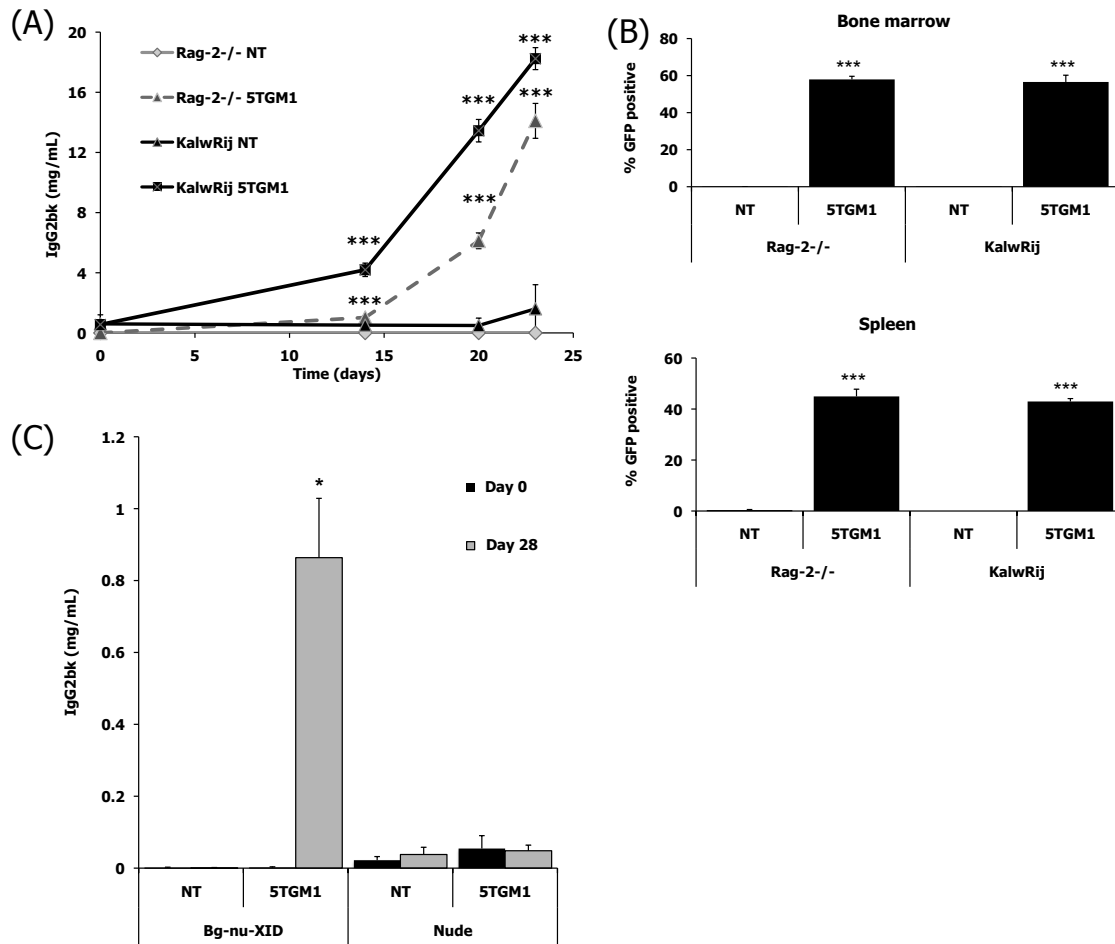


Figure 5. RAG-2^{-/-} mice develop characteristic myeloma tumor burden. Intravenous inoculation of 5TGM1 MM cells into RAG-2^{-/-} mice, which have improper B and T cell development. A) Tumor burden represented by myeloma-specific immunoglobulin levels, IgG2bκ, present in the serum of 5TGM1 myeloma bearing and non-tumor (NT) mice. B) Tumor burden represented by GFP positive 5TGM1 myeloma cells within the bone marrow and spleen, measured by flow cytometry. (NT n=8, MM n=12) C) Serum IgG2b levels in non-tumor and 5TGM1 MM-bearing Bg-nu-XID (deficient in B, T, and natural killer cells) and Nude (T-cell deficient) mice at day 0 and day 28 following tumor cell inoculation (n=5 per group). Data are shown as mean ± S.E.M. Significant differences are indicated by * p<0.05, ** p< 0.01, *** p<0.001 as compared with NT mice (One-way ANOVA).

identical manner, both with respect to time for tumor development and extent of tumor burden, in RAG-2 deficient mice as compared with C57Bl/KaLwRij mice.

In contrast to the accumulation of myeloma cells observed in RAG-2 deficient mice and in bg/nu/Xid mice, when 5TGM1 MM cells were inoculated into T cell deficient athymic nude mice, measurement of the myeloma-specific immunoglobulin levels in the serum demonstrated that there was no increase in IgG2b levels in 5TGM1-bearing athymic nude mice (Figure 5C). This demonstrates that a lack of T cells is not sufficient to permit myeloma development *in vivo*.

RAG-2^{-/-} mice develop myeloma-associated bone disease

In addition to indices of tumor burden, we also evaluated the myeloma-associated osteolytic bone disease in RAG-2 deficient mice in comparison to the well-characterized bone disease of the C57Bl/KaLwRij mice. Trabecular bone volume and osteolytic lesions were analyzed by microCT, osteoclast number and osteoblast number were determined by bone histomorphometry. Myeloma-bearing RAG-2 deficient mice were found to have characteristic features of myeloma bone disease, both identical to those seen in C57Bl/KaLwRij mice and strikingly similar to human multiple myeloma. The myeloma-bearing RAG-2 deficient mice had a significant number of osteolytic lesions found within the cortical bone, whereas the non-tumor mice had no lesions (Figure 6A and B). Histological analysis confirmed areas where the cortical bone had been destroyed with tumor cells expanding through the cortices, leading to the

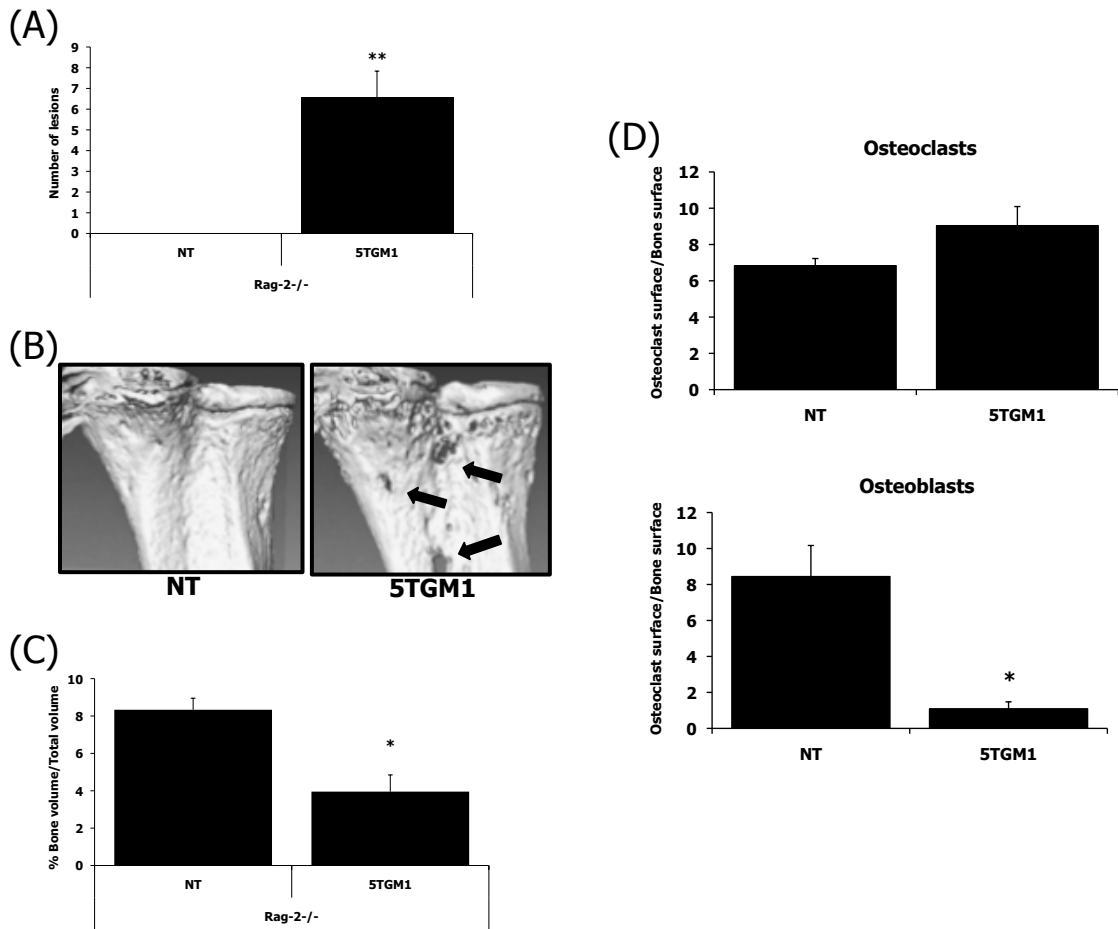


Figure 6. RAG-2^{-/-} mice develop myeloma-associated bone disease. Myeloma-associated bone disease assessed by microCT analysis, histomorphometry and histology. A) microCT analysis of osteolytic bone lesion through the cortical bone B) representative microCT images of cortical bone lesions (black arrows) C) microCT analysis of trabecular bone volume. D) Histomorphometry analysis of osteoclast and osteoblasts per bone surface (mm²/mm³) in RAG-2^{-/-}. Data are shown as mean ± S.E.M. * p<0.05, ** p< 0.01, *** p<0.001 as compared with NT bearing mice (Mann-Whitney *U* test). (NT n=4, MM n=5)

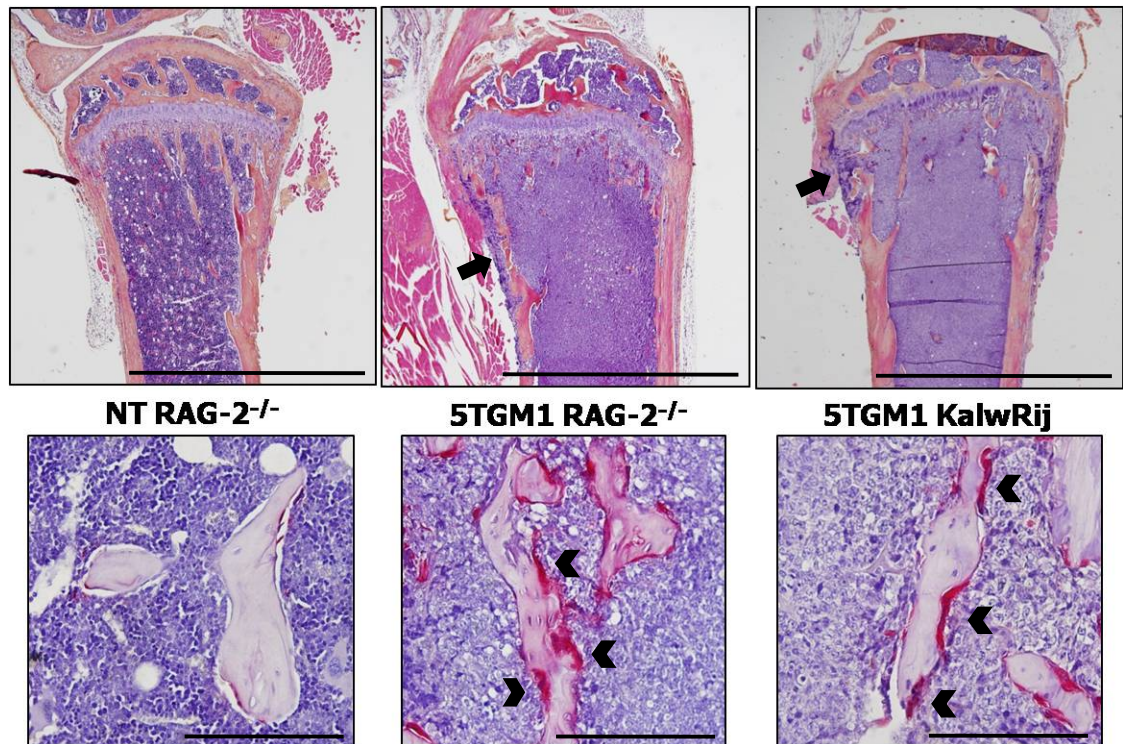


Figure 7. $RAG-2^{-/-}$ mice develop characteristic pathology typical of clinical myeloma. The pathology present in myeloma-bearing $RAG-2$ deficient mice is similar to that seen in the well-established 5T Radl murine model of myeloma in C57Bl/KaLwRij mice. (Top) Myeloma cell growth within the bone marrow cavity and osteolytic lesions through the cortical bone (black arrows). Bars, 2mm (Bottom) Myeloma-bearing mice display characteristic increase in TRAP-positive osteoclasts. (black arrowheads). Bars, 200 μ M.

development of discrete osteolytic lesions (Figure 7). We found the myeloma-bearing RAG-2^{-/-} deficient mice had a significant decrease in the overall trabecular bone volume when compared to the non-tumor control mice (Figure 6C and Figure 7). Histomorphometric analysis of the RAG-2 deficient myeloma-bearing mice demonstrated other features characteristic of myeloma-associated bone disease, such as an increase in bone-resorbing osteoclasts and a decrease in bone-forming osteoblasts (Figure 6D and Figure 7). Histological analysis demonstrated a striking similarity both in terms of tumor expansion within the bone marrow cavity and development of myeloma bone disease in 5TGM1 myeloma-bearing RAG-2^{-/-} mice as compared with the myeloma-bearing syngeneic C57Bl/KaLwRij mice (Figure 7).

Matrix metalloproteinase-9 deficiency decreases tumor burden and severity of associated osteolytic bone disease

The MMP family of proteolytic enzymes has been extensively studied for their role in extracellular matrix degradation resulting in cancer progression in various tumor cell types including myeloma (Barille, Akhoundi et al. 1997; Vacca, Ribatti et al. 1998; Barille, Bataille et al. 1999; Vacca, Ribatti et al. 1999). Previous studies have demonstrated a role for tumor-derived matrix metalloproteinase-9 (MMP-9) in myeloma progression, but also revealed the presence of host-derived MMP-9 within the bone marrow microenvironment (Van Valckenborgh, Mincher et al. 2005). In order to demonstrate the use of this model of myeloma, we chose to investigate myeloma development in mice deficient in

MMP-9. MMP-9 expression in the bone marrow of C57Bl/KaLwRij mice was demonstrated by immunohistochemistry in TRAP-positive multi-nucleated osteoclasts on the surface of trabecular bone (Figure 8A). Similar expression was observed in RAG-2 deficient mice. Mice deficient in both RAG-2 and MMP-9, in addition to mice deficient for RAG-2 alone, were inoculated intravenously with 5TGM1 MM cells to determine how MMP-9 deficiency would affect tumor burden and the associated bone disease. Tumor burden, as indicated by IgG2b serum levels, in myeloma-bearing mice deficient in both RAG-2 and MMP-9 was significantly decreased at 14 and 21 days following tumor inoculation as compared with mice deficient only for RAG-2 (Figure 8B). Mice deficient in both RAG-2 and MMP-9 showed a significant decrease in the proportion of GFP-positive 5TGM1 MM cells present in the bone marrow, as compared to myeloma-bearing mice RAG-2 deficient mice, however there was no significant difference in the proportion of GFP-positive myeloma cells in the spleen (Figure 8C). These results suggest that MMP-9 present in the bone marrow may have a more important role in myeloma progression. The contribution of host-derived MMP-9 from the osteoclasts within the microenvironment also had significant effects on myeloma bone disease. The number of lesions present through the cortical bone of myeloma-bearing mice deficient in both RAG-2 and MMP-9 was significantly decreased as compared to myeloma-bearing RAG-2 deficient mice (Figure 8D). Additionally, the overall bone loss in myeloma-bearing double deficient mice was significantly less as compared to the control RAG-2 deficient mice, as indicated by microCT analysis of trabecular bone volume (Figure 8E). Histomorphometric

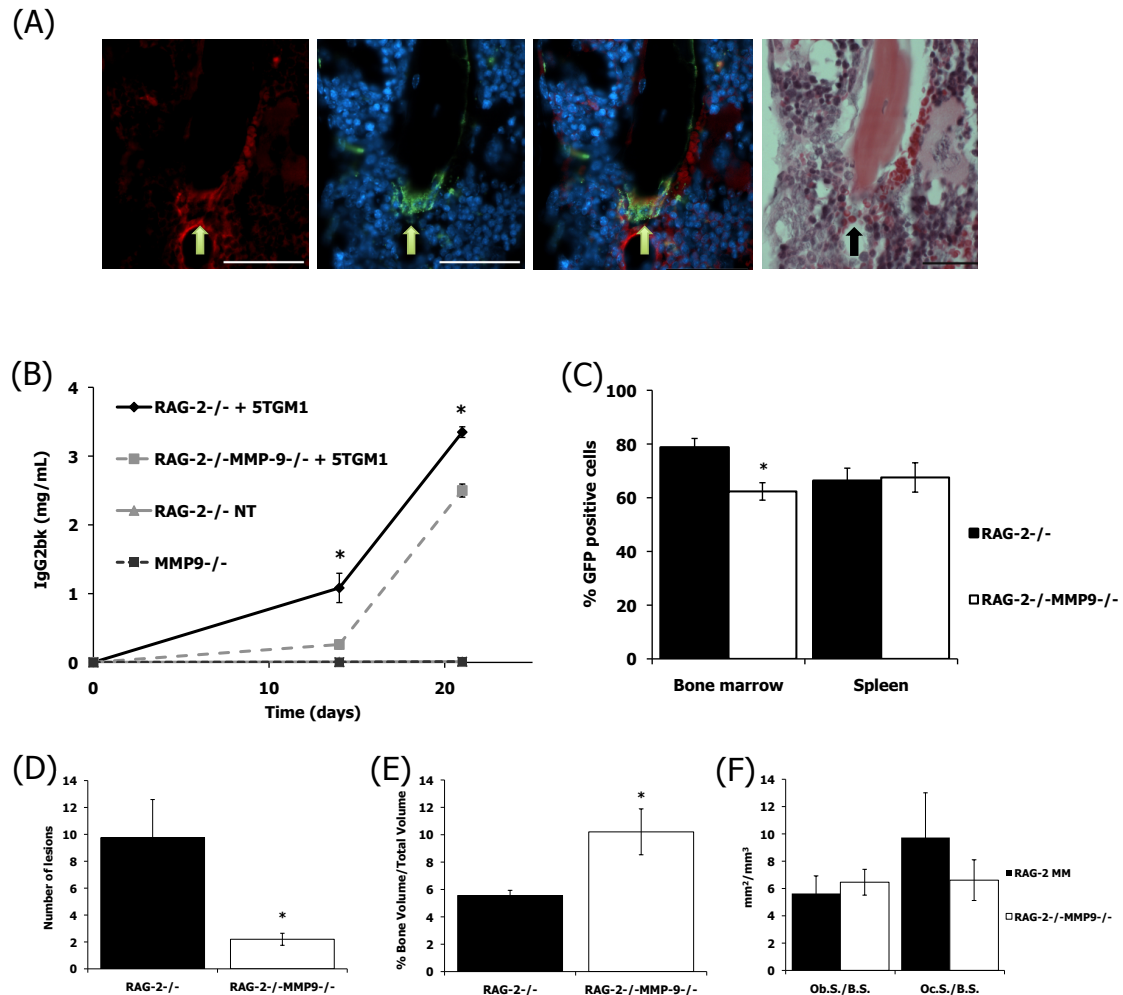


Figure 8. Lack of host-derived MMP-9 significantly reduces tumor burden and myeloma bone disease *in vivo*. Intravenous inoculation of 5TGM1 cells into either RAG-2^{-/-} mice or mice deficient in both RAG-2 and MMP-9 followed by assessment of tumor burden. A) MMP-9 localization in KaLwRij bone marrow. Fluorescent TRAcP staining (green) was used to localize osteoclasts (arrows) while immunofluorescence was used to localize MMP-9. DAPI (blue) was used as a nuclear stain. Murine IgG was used as a negative control. Scale bars are 50 μ M. B) Tumor burden represented by IgG2bk levels present in the serum of 5TGM1 myeloma bearing and NT mice. C) Tumor burden represented by GFP positive 5TGM1 myeloma cells within the bone marrow, measured by flow cytometry. Myeloma bone disease was assessed by microCT analysis, histomorphometry and histology D) microCT analysis of osteolytic bone lesions through the cortical bone, E) microCT analysis shows a loss of trabecular bone volume, both indicative of bone disease. F) Histomorphometric analysis of osteoclast number per bone surface. Data are shown as mean \pm S.E.M. * $p < 0.05$ as compared with tumor-bearing RAG-2^{-/-} mice (Mann-Whitney *U* test). (RAG-2^{-/-} n=4, RAG-2^{-/-}MMP-9^{-/-} n=6)

analysis demonstrated a trend towards a reduction in osteoclasts in myeloma-bearing MMP-9 deficient mice as compared to myeloma-bearing RAG-2 deficient mice (Figure 8F). No difference in osteoblast number was observed (Figure 8F).

Conclusions

The present study demonstrates a new *in vivo* system for the examination of the host tumor microenvironment and the contributions of this specialized niche to myeloma development. Despite many therapeutic advancements in the treatment of myeloma using existing mouse models, the field of myeloma research has long been limited by the inability of these models to permit specific investigation of the tumor microenvironment. The results from the current study demonstrate that myeloma development in RAG-2 deficient mice shares many of the clinical and histological features of human myeloma and the associated osteolytic bone disease that is also demonstrated in the established Radl 5T model. Myeloma-bearing RAG-2 deficient mice displayed extensive tumor burden within the bone marrow, an increase in osteoclasts, a decrease in osteoblasts and the development of destructive lytic lesions and overall bone loss. In the 5TGM1 model of myeloma, inoculation of myeloma cells results in homing of myeloma cells to both the bone marrow and the spleen, with homing to the spleen a result of the hematopoietic nature of this organ in mice. The growth of myeloma cells in bone and non-bone sites is a useful tool for elucidating the role of the bone marrow microenvironment, and this important feature was also observed in myeloma-bearing RAG-2 deficient mice, with an accumulation of

myeloma cells within the bone marrow and spleen. The use of RAG-2 deficient mice in a myeloma model is an extremely important advance for myeloma research as gene expression in the host compartment of the tumor microenvironment can be more specifically manipulated.

The use of the RAG-2 deficient mice in a model of multiple myeloma creates many opportunities to improve current therapies by increasing our understanding of specific mechanisms within the host tumor microenvironment. The use of this new animal model will allow for the specific manipulation of the host tumor microenvironment through genetic mutation. An example of this was demonstrated here where the use of this model system allow for the specific examination of host-derived MMP-9 and how it contributes to myeloma progression. MMPs are known to play important roles in tumor progression, however, it is has been difficult to discern their specific contributions due to the lack of specificity of MMP inhibitors. The ability to inhibit specific MMP expression in the host microenvironment using MMP deficient mice permits the investigation of the specific roles of individual MMPs in myeloma pathogenesis. In a previous study, Van Valckenborgh, et al. used an MMP-9 pro-drug to specifically target tumor cells within the bone marrow microenvironment. MMP-9 activity was higher in myeloma-bearing mice compared to non-tumor-bearing mice (Van Valckenborgh, Mincher et al. 2005). However, cells in the bone marrow of non-tumor-bearing mice still showed elevated levels of MMP-9 expression, suggesting the presence of MMP-9 in the bone marrow of C57Bl/KaLwRij mice. Our studies confirmed this, using immunofluorescence to

demonstrate expression of MMP-9 in osteoclasts within both C57Bl/KaLwRij and RAG-2 bone marrow. By investigating the development of 5TGM1 MM in mice deficient in RAG-2 and MMP-9, we were able to demonstrate a significant reduction in both tumor burden and the associated osteolytic bone disease in MMP-9 deficient mice. This both identifies a role for host-derived MMP-9 in myeloma pathogenesis, and illustrates the potential for this new model of myeloma to study the host microenvironment in myeloma.

There are many important questions in myeloma research regarding the relative contribution of host-derived versus tumor-derived factors, such as Receptor Activator for Nuclear Factor κ B Ligand (RANKL) and Dickkopf homolog 1 (DKK1), which are known to be expressed by both tumor cells and other cells within the bone marrow microenvironment, including stromal cells. The study of myeloma growth *in vivo* combined with genetic modification of the host microenvironment offers a novel molecular approach to elucidate such specific host-tumor interactions. The development of this *in vivo* tool was critical for this dissertation research, specifically to determine the role of host-derived adiponectin in myeloma growth and progression described in chapter V. Overall, the establishment of multiple myeloma in RAG-2 deficient mice and the resultant ability to study myeloma growth and the associated bone disease in a genetically modified host microenvironment is a major advance in myeloma research and provides a critically important tool for the myeloma research community.

CHAPTER IV

BONE MARROW STROMAL CELLS PROMOTE THE INITIAL ESTABLISHMENT AND PROGRESSION OF MYELOMA VIA STROMAL-DERIVED DKK1

Summary

The contributions of the bone marrow microenvironment to the early stages of myeloma development are poorly understood. To investigate this, we have utilized the 5T Radl myeloma model, where transplantation of 5T myeloma cells into mice of the specific C57Bl/KaLwRijHsd (KaLwRij) substrain, but not closely related C57Bl6 mice, results in the propagation of myeloma with many features of the human disease. Co-inoculation of 5TGM1 myeloma (MM) cells and a BMSC line isolated from KaLwRij mice (14M1 BMSCs) resulted in myeloma development in non-permissive C57Bl6 mice, associated with tumor growth within the bone marrow and osteolytic bone disease. 14M1 BMSCs inoculated alone induced osteoblast suppression and increased circulating Dkk1 concentrations. Dkk1 was over-expressed in myeloma-permissive KaLwRij bone marrow, and knockdown of Dkk1 expression in 14M1 BMSCs decreased their effect to promote myeloma development in C57Bl6 mice. Collectively, our results demonstrate a novel role of BMSCs to promote myeloma development in a non-permissive microenvironment and of BMSC-derived Dkk1 in the pathogenesis of multiple myeloma.

Introduction

Much of current cancer research has been dedicated to the study of the tumor microenvironment and how host cells within this microenvironment contribute to cancer progression. Evidence in many solid tumor cancers has demonstrated that cancer cells can directly alter surrounding stroma to form a permissive and supportive environment for tumor progression. Some of these alterations in the surrounding stroma include changes in extra-cellular matrix composition, angiogenesis, and altered fibroblasts (De Wever and Mareel 2003). Despite the somewhat extensive study of the altered tumor microenvironment in solid tumors, there has been limited research in non-solid tumor or hematological malignancies.

The bone marrow tumor microenvironment has a critical role in myeloma cell growth and survival, as well as the development of a destructive osteolytic bone disease; however the contributions of this microenvironment during early stages of the disease are poorly understood. The Radl 5T model of myeloma utilizes 5T MM cells originally derived from myeloma-bearing C57Bl/KaLwRij mice. The transplantation of these 5T MM cells results in consistent myeloma development similar to human disease in syngeneic C57Bl/KaLwRij mice. Interestingly, myeloma does not develop in closely related C57Bl6 mice. The emerging role of the microenvironment in cancer progression and this unique feature of the C57Bl/KaLwRij mice suggest that there are critical differences in this bone marrow milieu.

In the study presented in this chapter, we hypothesized that BMSCs from myeloma-permissive C57Bl/KaLwRij mice may promote myeloma development in a host microenvironment usually not permissive to 5T MM cell growth. We found that non-permissive C57Bl6 mice developed characteristic myeloma when co-inoculated with 5TGM1 MM cells and BMSCs from myeloma-bearing C57Bl/KaLwRij mice (14M1 BMSCs). There is increasing evidence that patients with the pre-myeloma condition MGUS have elevated Dkk1 (Todoerti, Lisignoli et al. ; Corre, Mahtouk et al. 2007), which suggests a role for host-derived Dkk1 in early stages of myeloma development. These studies demonstrate that C57Bl/KaLwRij BMSCs create a myeloma-permissive microenvironment through the secretion of Dkk1 and this has important implications in myeloma pathogenesis.

Results

C57Bl/KaLwRij BMSCs promote myeloma in non-permissive mice

To investigate the role of bone marrow stromal cells (BMSCs) in myeloma pathogenesis, we utilized 14M1 BMSCs that were originally isolated from myeloma-bearing C57Bl/KaLwRij mice. The C57Bl/KaLwRij mice of the 5T Radl model for multiple myeloma possess a unique bone marrow microenvironment in that they are permissive for 5T MM cell growth in contrast to C57BL6 mice (Radl, De Glopper et al. 1979; Radl, Croese et al. 1988; Garrett, Dallas et al. 1997; Fowler, Mundy et al. 2009). After initial characterization of the 14M1 BMSCs, we determined that 14M1 BMSCs did not possess osteogenic or adipogenic

differentiation potential, commonly seen in BMSCs (Figure 9A). 14M1 BMSCs expressed both vimentin and fibroblast-specific protein-1 (S100A4), indicative that these cells are fibroblasts (Figure 9B). These traits combined suggest that 14M1 BMSCs are a more differentiated fibroblast-like cell population.

To investigate the contribution of these BMSCs to myeloma cell growth, non-permissive C57Bl6 mice were inoculated with 5TGM1 MM cells alone, 14M1 BMSCs alone, or 5TGM1 and 14M1 cells combined. 4 weeks following cell inoculation, C57Bl6 mice inoculated with either 5TGM1 MM cells alone or 14M1 BMSCs alone did not develop myeloma. However, C57Bl6 mice inoculated with 5TGM1 and 14M1 cells combined developed myeloma, characterized by a significant increase in tumor burden as assessed by measurement of myeloma-specific immunoglobulin concentrations present in the serum and a significant increase in GFP positive myeloma cells present in the bone marrow and spleen (Figure 10A and B). At the experimental endpoint, flow cytometric analysis of dsRed2 positive cells showed that approximately 13% of the BM was composed of these BMSCs (Figure 9D). The rate of tumor growth and the features of myeloma development were identical to myeloma development in the C57Bl/KaLwRij mice of the Radl model.

Histological examination of the co-inoculated C57Bl6 mice demonstrated pathology similar to that seen in myeloma-bearing C57Bl/KaLwRij mice (Figure 11). Histology of co-inoculated C57Bl6 mice demonstrated myeloma cells filling the BM cavity and the presence of osteolytic lesions through the cortical bone, respectively (Figure 11). The single-cell inoculated mice, either 5TGM1 or 14M1

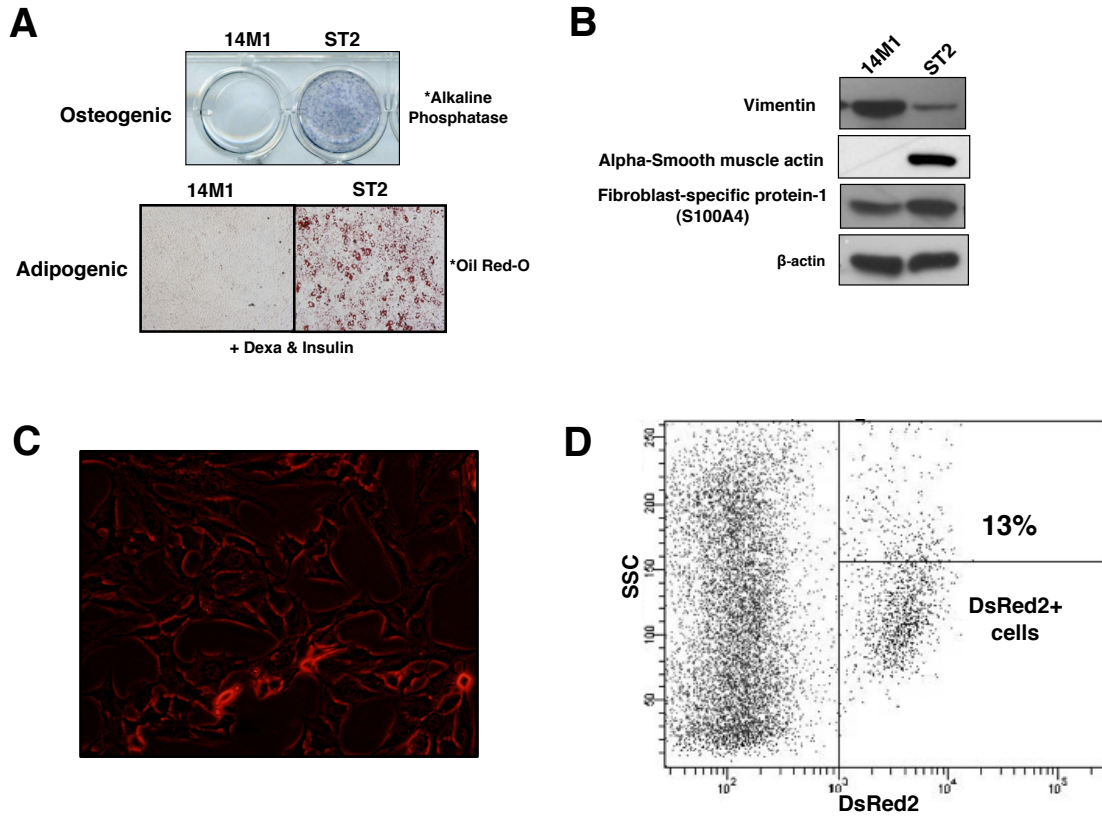


Figure 9. Characterization of 14M1 bone marrow stromal cells (BMSCs). A) 14M1 BMSC characterization by mesenchymal stem cell differentiation studies into osteoblasts and adipocytes. Mature osteoblasts indicated by alkaline phosphatase staining following differentiation. Mature adipocytes indicated by Oil Red O staining following differentiation. B) 14M1 characterization by Western blot analysis of fibroblast-specific proteins. C) Fluorescent microscope image of dsRed2 positive 14M1 BMSCs. D) Flow cytometric analysis of dsRed2 positive BMSCs in the bone marrow at experimental endpoint.

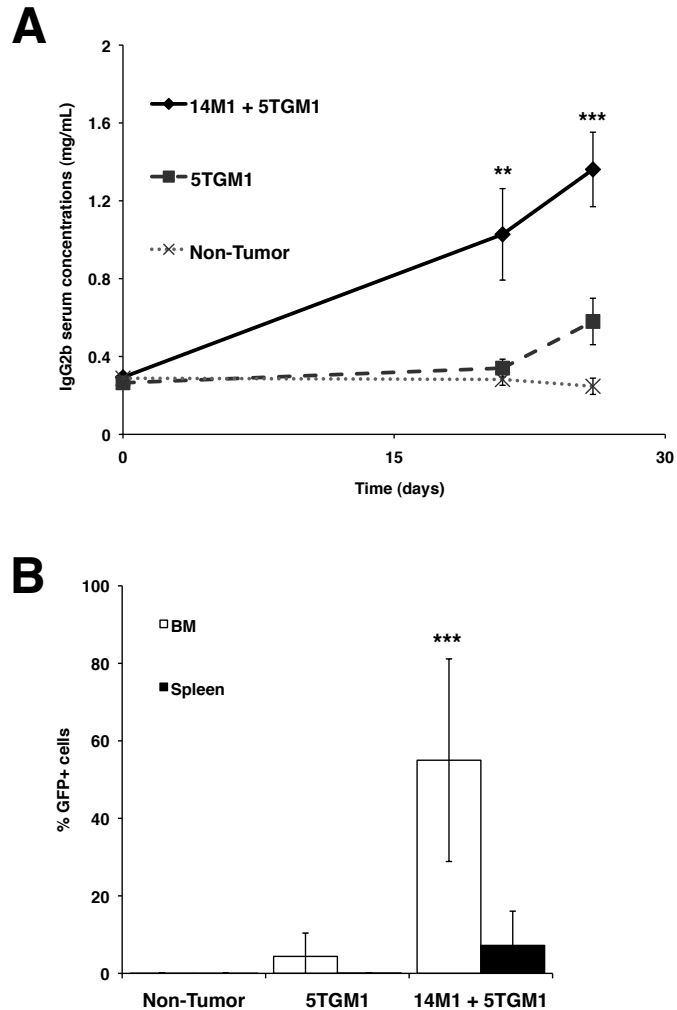


Figure 10: C57Bl/KaLwRij BMSCs promote myeloma cell growth in non-permissive mice. Non-permissive C57Bl6 mice co-inoculated with 14M1 BMSCs and 5TGM1 MM cells development characteristic myeloma tumor burden represented by A) IgG2b κ serum concentrations and B) GFP positive myeloma cells present in the bone marrow and spleen. Data are shown as mean \pm S.E.M. ** $p < 0.01$, *** $p < 0.001$ as compared to 5TGM1 (One-way ANOVA). (n=5 per group)

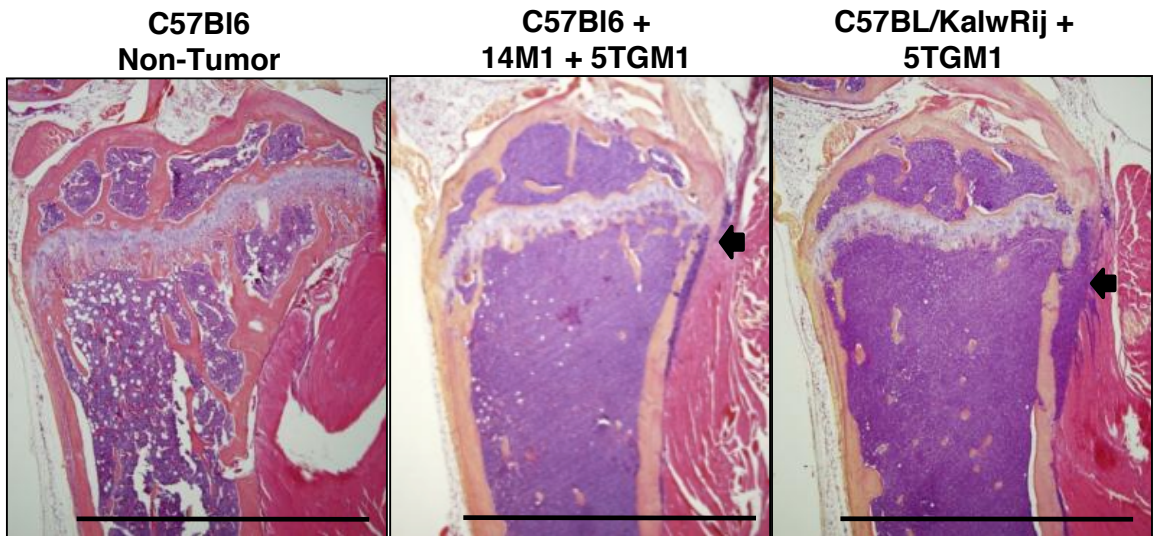


Figure 11. C57Bl/KalwRij BMSCs promote pathology similar to myeloma-bearing C57Bl/KalwRij mice of the Radl model. Histological sections of bone marrow from C57Bl6 non-tumor and 14M1 BMSC co-inoculated mice, compared to section from MM-bearing C57/KalwRij mice. Lesions through the cortical bone are typical of myeloma bone disease (black arrows). Bars, 2mm.

BMSCs alone appeared similar to the non-tumor control mice. MicroCT analysis also indicated the presence of cortical bone lesions in the C57Bl6 mice co-inoculated with 5TGM1 MM cells and 14M1 BMSCs (Figure 12A). Mice co-inoculated with 14M1 BMSCs also had a significant decrease in trabecular bone (Figure 12B) and osteoblasts (Figure 12C), with a significant increase in bone resorbing osteoclasts (Figure 12C). Importantly, co-inoculation of mice with 5TGM1 MM cells and normal ST2 BMSC had no significant difference in tumor burden compared to mice with 5TGM1 MM cells alone (31.5% difference, 1.58 ± 1.06 for 5TGM1 alone and 2.31 ± 1.01 for ST2 with 5TGM1 MM cells in IgG2b κ serum concentrations, $p=0.64$). These studies suggest that BMSCs from myeloma-permissive C57Bl/KaLwRij mice can promote myeloma development in C57Bl6 mice, which is an effect specific to 14M1 BMSCs and not a general effect of all BMSCs.

We wanted to determine whether these cells simply enhanced 5TGM1 MM cell growth in order for them to grow in the non-permissive C57Bl6 mice. Considering the C57Bl/KaLwRij mice of the Radl model are already permissive to 5T MM cell growth, we decided to co-inoculate these mice with 14M1 BMSCs. Interestingly, C57Bl/KaLwRij mice co-inoculated with 14M1 BMSCs did not have an increase in tumor burden compared to when 5TGM1 myeloma cells were inoculated alone (Figure 13A and B). The myeloma-promoting effect of these cells together with the results in the C57Bl/KaLwRij mice suggests that these BMSCs do not merely enhance myeloma cell growth but may alter the bone marrow microenvironment, making it favorable for myeloma.

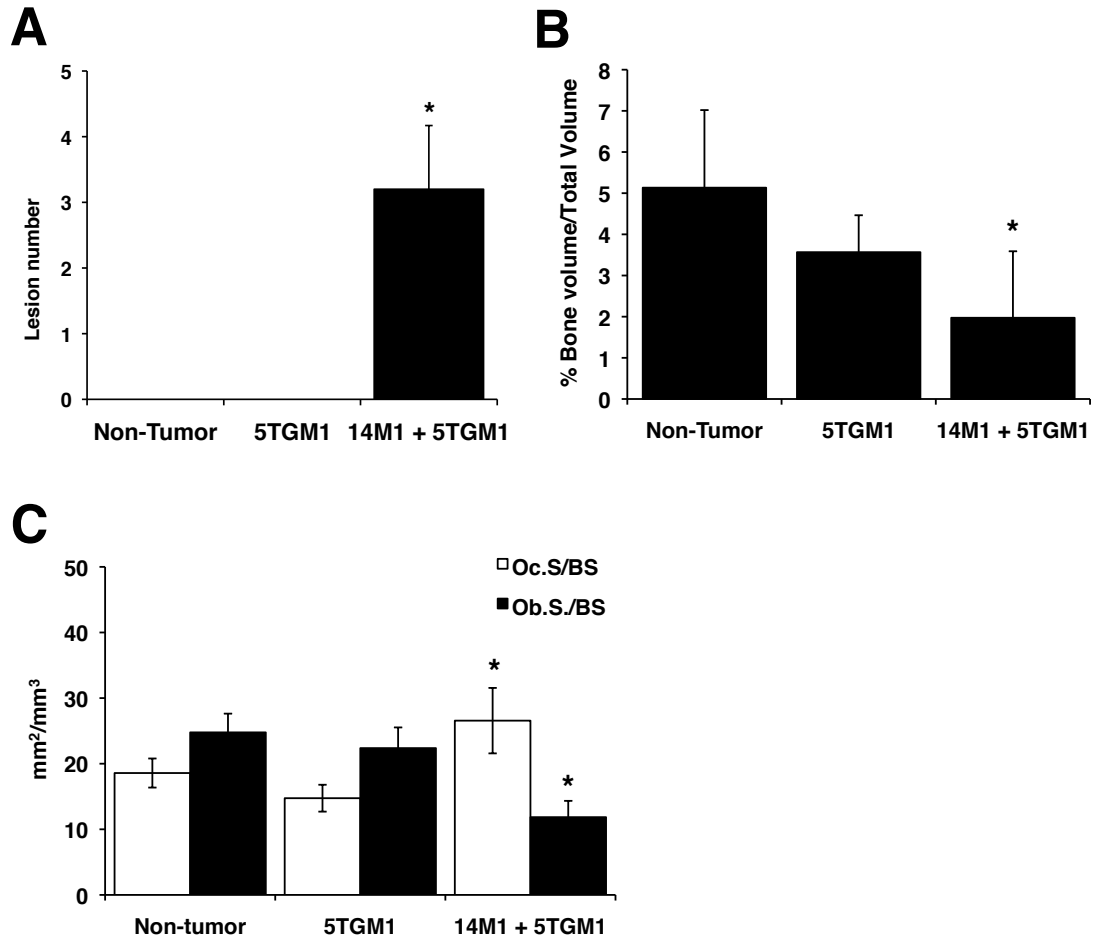


Figure 12. C57Bl/KaLwRij BMSCs promote myeloma bone disease in non-permissive mice. A) Osteolytic lesions through the cortical bone, measured by microCT analysis. B) Trabecular bone volume to total volume ratio, measured by microCT. C) Osteoblast surface to bone surface (Ob.S/BS) and osteoclast surface to bone surface (Oc.S/BS) ratios, measured by histomorphometry. Data are shown as mean \pm S.E.M. * $p < 0.05$ as compared to 5TGM1 (One-way ANOVA & Tukey-Kramer tests). (n=5 per group)

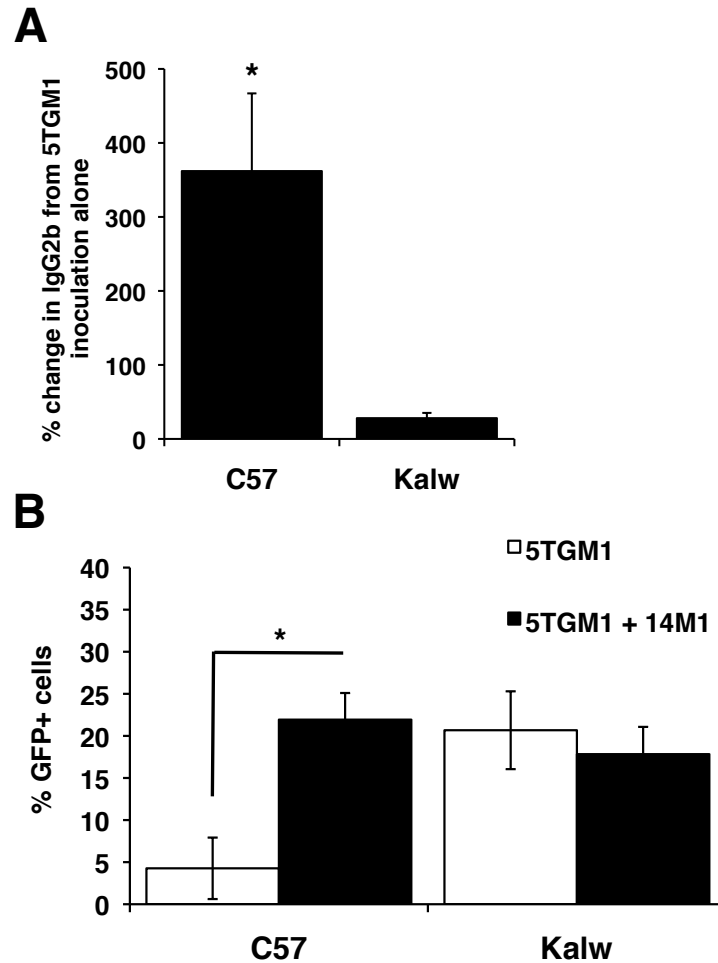


Figure 13. C57BI/KaLwRij BMSCs do not enhance myeloma cell growth in myeloma-permissive C57BI/KaLwRij mice. A) Tumor burden represented by measuring myeloma-specific immunoglobulin concentrations present in the serum of co-inoculated C57BI6 and C57BI/KaLwRij mice. B) Tumor burden represented by measuring the GFP positive myeloma cells present in the bone marrow. Data are shown as mean \pm S.E.M. * $p < 0.05$ as compared to 5TGM1. (n=5 per group)

C57Bl/KaLwRij BMSCs create a permissive microenvironment independent of tumor cell presence

We next wanted to examine whether these BMSCs could alter the microenvironment independent of tumor cell presence. To investigate this, C57Bl6 mice were inoculated with 14M1 BMSCs alone. Following a 4-week timeframe similar to the 5T myeloma model, we assessed the bone parameters by microCT and histomorphometry. Assessment of the bone in the BMSC-inoculated mice showed a 23.4% decrease in trabecular bone volume, determined by microCT (control mice, 7.38 ± 1.50 compared to 14M1 inoculated, 5.65 ± 2.13 ; mean \pm standard error). Histomorphometry demonstrated that mice BMSCs alone also had a significant decrease in osteoblasts present on the trabecular bone surface and in bone formation rates; however there was no change in osteoclast number (Figure 14A and B). Mice inoculated with ST2 BMSCs alone did not show a significant change in osteoblasts (31.75 ± 2.658 compared to control at 36.95 ± 3.569) or osteoclasts (18.75 ± 1.315 compared to control at 14.63 ± 2.300).

Since 14M1 BMSCs promote myeloma in mice that normally do not develop 5T myeloma, we sought to determine whether these cells modify the host microenvironment in order to make it permissive for myeloma cell growth. Considering 14M1 BMSCs can suppress osteoblasts *in vivo* and a major mediator of osteoblast suppressor in myeloma bone disease is the Wnt antagonist Dickkopf-1 (Dkk1), we examined the expression of Dkk1 in these cells. *In vitro* characterization of the myeloma-promoting 14M1 BMSCs

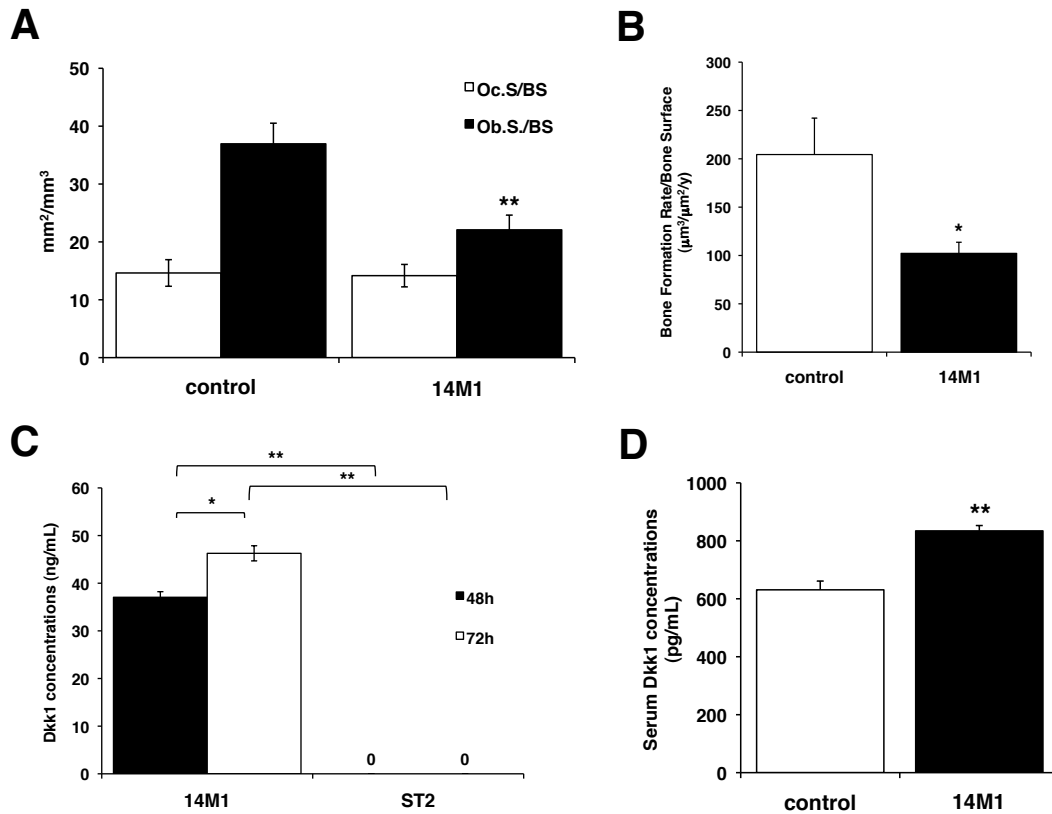


Figure 14. C57Bl/KaLwRij BMSCs create a permissive microenvironment independent of tumor cell presence. A) Histomorphometry of osteoblasts and osteoclasts in C57Bl6 inoculated with 14M1 BMSCs alone. B) Histomorphometry of bone formation rates per year (BFRs) in C57Bl6 mice inoculated with 14M1 BMSCs alone. C) ELISA measurement of Dkk1 concentrations present in the conditioned media of 14M1 BMSCs in comparison to ST2 BMSC line. D) ELISA measurement of Dkk1 concentrations present in the serum of C57Bl6 mice inoculated with 14M1 BMSCs alone. Data are shown as mean \pm S.E.M. * $p < 0.05$. ** $p < 0.01$ as compared to control (Mann-Whitney *U* test). (n=5 per group)

demonstrated strong expression and secretion of Dkk-1 (Figure 14C). We measured Dkk1 concentrations in the serum of mice inoculated with 14M1 BMSCs alone and found that there was a significant increase in Dkk1 serum concentrations, compared to control mice (Figure 14D). Additionally, mice inoculated with ST2 BMSCs do not show this effect. In support of increased Dkk1 concentrations in myeloma-permissive microenvironments, C57Bl/KaLwRij mice have a significant reduction in trabecular bone volume, in addition to elevated Dkk1 as compared to C57Bl6 mice (Figure 15A and B). These results combined supported further investigation to determine the contribution of BMSC-derived Dkk1 in myeloma pathogenesis.

C57Bl/KaLwRij BMSCs modify the BM microenvironment via Dkk1

Finally, we wanted to investigate the contribution of 14M1 BMSC-derived Dkk1 in creating a permissive microenvironment. To do this, Dkk1 expression was stably knocked down using a mouse Dkk1 shRNA (Santa Cruz). Dkk1 secretion by the 14M1 BMSCs was significantly knocked down (KD) in comparison to conditioned media from control scrambled 14M1 BMSCs (Figure 16A). To determine whether Dkk1 KD would abolish myeloma-promoting effect, C57Bl6 mice were co-inoculated with 5TGM1 MM cells and either 14M1 control BMSCs or 14M1 Dkk1 KD BMSCs. We found that mice co-inoculated with 14M1 Dkk1 KD BMSCs had a significant reduction in tumor burden, compared to mice co-inoculated with control 14M1 BMSCs (Figure 16B). Additionally, mice co-inoculated with 14M1 Dkk1 KD BMSCs did not show a reduction in trabecular

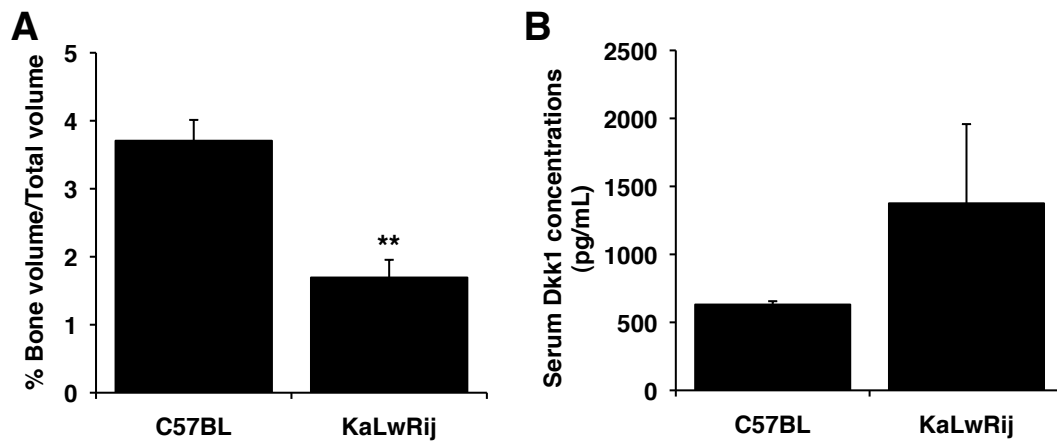


Figure 15. Myeloma-permissive C57Bl/KaLwRij mice have decreased bone volume and elevated Dkk1. A) Trabecular bone volume measured by microCT analysis in age- and sex-matched C57Bl6 and C57Bl/KaLwRij mice. B) Elevated Dkk1 serum concentrations in C57Bl/KaLwRij mice. Data are shown as mean \pm S.E.M. ** $p < 0.01$, compared to control (Mann-Whitney U test). (n=5 per group)

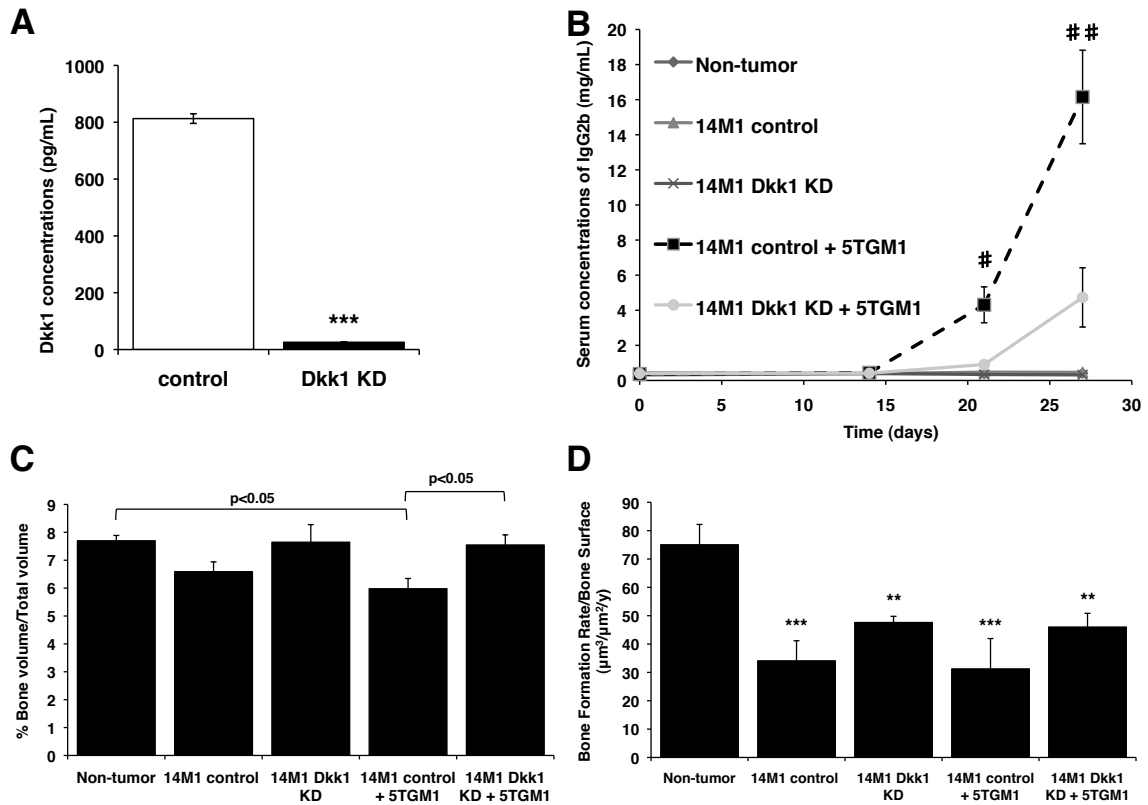


Figure 16. C57Bl/KaLwRij BMSCs modify the BM microenvironment via Dkk1. A) ELISA measurement of Dkk1 concentrations in conditioned media of 14M1 BMSCs transfected with scrambled control shRNA or with Dkk1 KD shRNA. B) Tumor burden assessed by measurement of IgG2bk serum concentrations. C) Trabecular bone volume assessed by microCT analysis. D) Bone formation rates assessed by histomorphometric analysis. Data are shown as mean \pm S.E.M. # $p < 0.05$, ## $p < 0.01$ as compared to 14M1 control + 5TGM1 (Mann-Whitney *U* test). ** $p < 0.01$, *** $p < 0.001$ as compared to control (One-way ANOVA and Tukey-Kramer). (n=5 for single cell-inoculated mice, n=10 for co-inoculated mice)

bone volume while co-inoculation of control 14M1 BMSCs resulted in a significant reduction in bone volume (Figure 16C). Interestingly, it appears in contrast to mice inoculated with 14M1 control BMSCs, that mice inoculated with 14M1 Dkk1 KD BMSCs had little to no decrease in bone volume (Figure 16C). Histomorphometric analysis revealed that mice inoculated with 14M1 Dkk1 KD BMSCs had less of a decrease in osteoblast activity than 14M1 control BMSCs (Figure 16D). These results raise the possibility that osteoblasts within the BM microenvironment may be protective against myeloma growth and progression.

Conclusions

There is increasing evidence to suggest that cells of the BM microenvironment are altered in the pre-myeloma state, MGUS, and that Dkk1 is increased in these patients along with a generalized bone loss (Drake, Ng et al 2009). In support of this, these data demonstrate that BMSC derived-Dkk1 from myeloma-permissive C57Bl/KaLwRij mice were able to alter the BM microenvironment directly by promoting a generalized bone loss seen similar to that seen in MGUS and myeloma patients. These studies also support the concept that myeloma cells are not the only source of Dkk1 in the context of myeloma. The secretion of Dkk1 by BMSCs suggests that alterations in BM stroma may be important in early stages of myeloma before significant populations of myeloma cells are present in the BM cavity (Figure 17). As depicted in figure 17, these studies suggest that contributions of Dkk1 from other resident cells within the microenvironment may be important for myeloma

progression. Our studies with altered BMSCs from C57Bl/KaLwRij mice also support this notion, as the 5TGM1 MM cells used in our system do not secrete Dkk1 themselves.

The creation of a myeloma permissive microenvironment through osteoblast suppression also generates interesting questions for future examination. It is necessary to question whether the myeloma promoting effect is due specifically to Dkk1 secretion resulting in osteoblast suppression or whether other methods of osteoblast suppression would be sufficient to promote myeloma growth. Osteoblasts play a critical role in maintaining bone mass, but also in regulating normal and (more recently) pathological hematopoiesis. The involvement of osteoblasts in this process was made evident in studies where osteoblast ablation resulted in dramatic alterations in cell populations resident in the bone marrow, including decreases in myeloid and lymphoid populations (Visnjic, Kalajzic et al. 2004). Distinguishing the role of osteoblast suppression from a generalized bone loss is necessary for understanding the mechanisms involved in creating a myeloma-permissive microenvironment. Recent studies have implicated alterations of the bone marrow microenvironment, associated with bone loss, as having an active role in initiation of hematological malignancies (Raaijmakers, Mukherjee et al. ; Walkley, Olsen et al. 2007; Walkley, Shea et al. 2007).

Furthermore, the 14M1 Dkk1 KD studies highlight important contributions of osteoblast and stromal-derived factors. Mice inoculated with the Dkk1 KD BMSCs appeared to be protected against bone loss, in comparison with mice

inoculated with 14M1 control BMSCs. These results provoke an interesting question as to whether the BMSCs are themselves protective or alternatively if functional osteoblasts within the BM microenvironment are protective against myeloma progression and subsequent bone loss. There is other evidence in the literature suggesting that the presence of osteoblasts within the bone marrow microenvironment provides protection against myeloma progression. Takeuchi et al demonstrated that terminally differentiated osteoblasts have the ability to suppress myeloma cell growth and survival (Takeuchi, Abe et al.). Additionally, decorin was recently identified as an osteoblast-derived factor that can suppress myeloma cell growth and survival. However, there are currently a limited number of studies investigating osteoblast-derived factors responsible for myeloma suppression.

Of additional interest is that the 14M1 BMSCs were derived from myeloma-bearing C57Bl/KaLwRij mice. The origin of these cells raises the obvious question as to whether the myeloma-promoting effect of these cells is due to the altered microenvironment of the C57Bl/KaLwRij mice or because they are cancer-associated. Mesenchymal stem cells are pluripotent progenitor cells that reside primarily in the bone marrow. It is now well accepted that cancer cells can directly alter their adjacent stroma, which in turn forms a permissive and supportive environment for tumor progression and metastasis (Karnoub, Dash et al. 2007). The metastatic potential of human breast cancer cells was greatly increased when the cancer cells were mixed with bone marrow-derived mesenchymal stem cells. These studies suggest that aggressive and metastatic

characteristics can be acquired through interactions between tumor cells and the surrounding stroma. This study also strongly supports our overall hypothesis that changes in the bone marrow microenvironment promote the development of multiple myeloma *in vivo*. Examination of cancer-associated fibroblasts has not been investigated in the context of non-solid tumors; therefore research is needed in this area. The direct effects of cancer-associated fibroblasts on osteolytic bone disease are unknown. A detailed investigation of primary cancer-associated fibroblasts in future studies will be discussed in chapter VI.

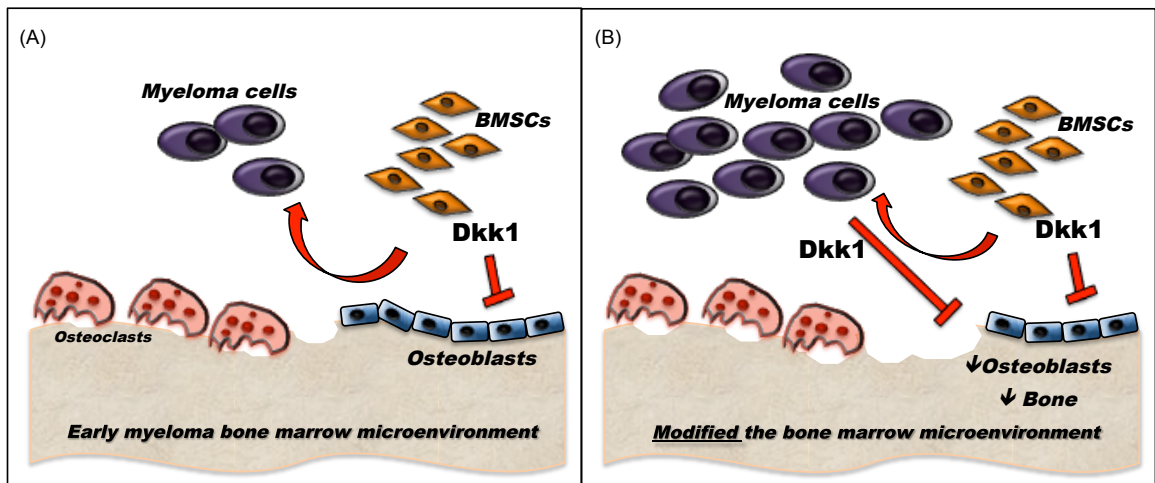


Figure 17. Contributions of altered BMSCs in the bone marrow microenvironment early in myeloma development. (A) In the early myeloma microenvironment, altered BMSCs secrete Dkk1 prior to the accumulation of myeloma cells within the bone marrow cavity. (B) BMSC-derived Dkk1 modifies the BM microenvironment by suppressing osteoblast activity resulting in decreased bone, which creates a permissive environment for myeloma cell growth.

CHAPTER V

DECREASED HOST-DERIVED ADIPONECTIN CONTRIBUTES TO MYELOMA PATHOGENESIS

Summary

The contributions of the host microenvironment to the early stages of myeloma pathogenesis are poorly understood. Microarray analysis identified a decrease in adiponectin in mice permissive for myeloma development, as compared to mice that were not permissive to myeloma. Clinical evidence demonstrates that MGUS patients who progress to myeloma have a decrease in serum adiponectin concentrations, as compared to normal controls or MGUS patients that do not progress to myeloma. Adiponectin induced caspase-dependent apoptosis in myeloma cells. Myeloma pathogenesis was exacerbated in adiponectin-deficient mice with increased tumor burden and osteolytic bone disease. Increasing circulating adiponectin *in vivo* resulted in reduced tumor burden, the prevention of myeloma bone disease, and increased overall survival of myeloma-bearing mice. These data demonstrate that decreased adiponectin contributes to myeloma pathogenesis, and establishes the potential therapeutic benefit of increasing adiponectin in the treatment of both MGUS and myeloma patients.

Introduction

Adiponectin is among a group of proteins called adipokines originally identified as a protein secreted by the adipose tissue into the bloodstream. More recent studies have found it to be expressed by multiple other cell types, including bone marrow-derived osteoblasts and fibroblasts (Berner, Lyngstadaas et al. 2004). There are three circulating forms of adiponectin, all of which differ in their biological activities and tissue specificities (Waki, Yamauchi et al. 2003). The variation in biological activity may be due to the differences in expression patterns of adiponectin receptors (Yamauchi, Kamon et al. 2003). Adiponectin has two receptors (AdipoR1 and AdipoR2) with seven transmembrane domains. These domains are similar to G-protein-coupled receptors (GPCRs); however their topology is the opposite with the amino (N)-termini intracellular (Yamauchi, Kamon et al. 2003). Stimulation with adiponectin results in an increase in the phosphorylation of both p38 mitogen-activated protein kinase (MAPK) and AMP-activated protein kinase (AMPK) in various cell types (Yamauchi, Kamon et al. 2002; Tsao, Tomas et al. 2003; Mao, Kikani et al. 2006; Cheng, Lam et al. 2007). Only recent research has begun to study adiponectin signaling and its downstream mediators; therefore the physiological signaling pathways mediated through these receptors have not been fully elucidated. One major role of adiponectin is the regulation of insulin sensitivity (Beltowski 2003).

Adiponectin deficiency (hypoadiponectinaemia) is thought to have a role in obesity, cardiovascular disease and diabetes; however the exact physiological functions of adiponectin are currently unclear. Adiponectin levels are inversely

correlated with body fat percentage; in that adiponectin serum levels are reduced in patients with obesity and diabetes (Hu, Liang et al. 1996; Arita, Kihara et al. 1999; Hotta, Funahashi et al. 2000). Adiponectin also has potent anti-inflammatory activities. These activities include its ability to reduce TNF- α production and activity (Maeda, Shimomura et al. 2002), inhibit pro-inflammatory IL-6, and induce anti-inflammatory cytokines IL-10 and IL-1 receptor antagonist (Kumada, Kihara et al. 2004; Wolf, Wolf et al. 2004; Wulster-Radcliffe, Ajuwon et al. 2004). Recently, low adiponectin serum levels have been associated with a high incidence of obesity-related cancer, including endometrial, breast, prostate, and gastric cancers. Hypoadiponectinaemia is known to be an independent risk factor for breast cancer, and is associated with more aggressive phenotypes (Miyoshi, Funahashi et al. 2003; Schaffler, Scholmerich et al. 2007).

The condition known as monoclonal gammopathies of undetermined significance is defined by elevated protein production by plasma cells resulting in hypercalcaemia with minor bone involvement (Berenson, Anderson et al. ; Kyle, Therneau et al. 2006; Berenson and Yellin 2009). In recent years, MGUS has been demonstrated to consistently precede myeloma (Landgren, Kyle et al. 2009; Weiss, Abadie et al. 2009). On average, approximately 16% of MGUS patients progress to myeloma (Kyle and Rajkumar 2007); however the reasons for this progression are unknown despite investigation of potential risk factors. Some clinical evidence suggests the progression is due to changes that occur to the bone marrow microenvironment, but there are no reliable predictors for

disease progression (Blade, Rosinol et al. 2008). It is critical to identify novel predictors of malignant progression to determine what patients are at risk.

The study of the bone marrow microenvironment in the initial establishment myeloma is critical for both our understanding of mechanisms involved in disease progression, and the identification of novel therapeutic targets. To elucidate the necessary features and mechanisms that contribute to myeloma development in humans, we used the myeloma-permissive C57Bl/KaLwRij mice of the Radl 5T model of myeloma as a tool to provide insights into the changes that occur to the bone marrow microenvironment that are critical for disease progression. An important feature of this model is that 5T MM cells will not grow in the closely related, immune-competent C57Bl6 strain of mice (Radl, De Glopper et al. 1979; Radl, Croese et al. 1988; Garrett, Dallas et al. 1997; Fowler, Mundy et al. 2009), which suggests a critical role for the host bone marrow microenvironment. In this chapter, we examined the differences between a myeloma permissive and non-permissive bone marrow microenvironment using the C57Bl/KaLwRij mice and determined that these mice have a significant decrease in adiponectin expression. Using mice deficient in adiponectin, we further demonstrate that decreased adiponectin production is critical for myeloma establishment and progression. Finally, we determined the therapeutic benefit of increasing adiponectin concentrations *in vivo* on myeloma development and progression.

Results

Adiponectin is decreased in the host microenvironment of mice permissive for multiple myeloma

The BM microenvironment is known to play a critical role in tumor growth and the development of myeloma bone disease. The C57Bl/KaLwRij strain of mice of the well-established Radl model of multiple myeloma is unique in that 5T MM cells will only grow in syngeneic C57Bl/KaLwRij mice, and not in closely related C57Bl6 mice (Figure 18) (Fowler, Mundy et al. 2009). This suggests an important role for the C57Bl/KaLwRij host microenvironment in the establishment of multiple myeloma. Additionally, the similarities between human myeloma and that observed in the C57Bl/KaLwRij mice lead us to hypothesize that differences between a myeloma-permissive and non-permissive microenvironment may provide valuable insights into human disease.

Given that the C57Bl/KaLwRij microenvironment is permissive for myeloma establishment and progression, we investigated factors that were differentially expressed between the C57Bl/KaLwRij and C57Bl6 BM microenvironment. We performed a microarray analysis on pooled whole BM from C57Bl/KaLwRij and C57Bl6 mice. The microarray revealed a short list of genes that were differentially expressed between the BM of the two strains of mice (Table 1). Several of the expression profiles found in the microarray were verified by quantitative real-time PCR, including a significant reduction in hydroxypostaglandin dehydrogenase 15, a significant increase in glycerophosphodiesterase domain containing 3 and a significant reduction in

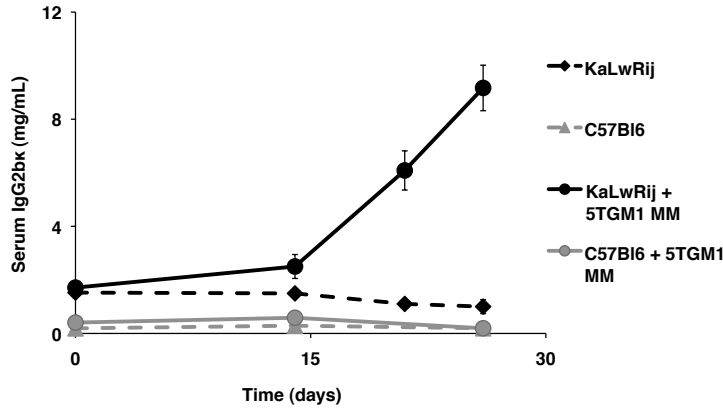


Figure 18. Myeloma permissive and non-permissive mice. Tumor burden in non-permissive C57Bl6 (C57) and myeloma-permissive C57Bl/KaLwRij (KaLwRij) mice, determined by measuring myeloma-specific IgG2bκ concentrations. Data are shown as mean ± S.E.M. *** $p < 0.005$ KaLwRij+MM compared to C57Bl6+MM (One-way ANOVA). (n=8 per group)

Gene	Gene Description	Fold change*
Abhd6	abhydrolase domain containing 6	2.01 increase
Adipoq	adiponectin, C1Q and collagen domain containing	2.08 decrease
Abca13	ATP-binding cassette, sub-family A (ABC1), member 13	2.15 increase
C5ar1	complement component 5a receptor 1	2.06 increase
Cysltr2	cysteinyl leukotriene receptor 2	2.98 decrease
Gdpd3	glycerophosphodiester phosphodiesterase domain containing 3	32.7 increase
Gnal	guanine nucleotide binding protein, alpha stimulating, olfactory type	2.05 decrease
Grap2	GRB2-related adaptor protein 2	2.38 decrease
Hist1h2bc	histone cluster 1, H2bc	2.27 decrease
Hist1h4c	histone cluster 1, H4c	3.18 decrease
Hpgd	hydroxyprostaglandin dehydrogenase 15 (NAD)	5.05 decrease
Mgl1	macrophage galactose N-acetyl-galactosamine specific lectin 1	2.19 decrease
Mrgpra2	MAS-related GPR, member A2	2.08 increase
Mc2r	Melanocortin 2 receptor	2.06 increase
Olfm4	olfactomedin 4	2.21 increase
Plxdc2	plexin domain containing 2	2.62 decrease
Samsn1	SAM domain, SH3 domain and nuclear localization signals, 1	6.82 decrease
Sirpb1	signal-regulatory protein beta 1	2.17 increase
Snord61	small nucleolar RNA, C/D box 61	2.31 decrease
Stfa1	stefin A1	2.28 increase
Stfa2	stefin A2	2.55 increase
Stfa3	stefin A3	2.49 increase
Tcte3	t-complex-associated testis expressed 3	2.21 decrease
Tnfrsf26	tumor necrosis factor receptor superfamily, member 26, mRNA	5.08 decrease
Trdn	Triadin	2.29 increase

*Change in expression in KaLwRij bone marrow as compared to C57Bl6 bone marrow.

Table 1. List of differentially expressed genes identified in microarray comparison of pooled whole bone marrow from permissive C57Bl/KaLwRij and non-permissive C57Bl6 mice (2-fold or more change).

adiponectin expression (Figure 19A-C). Myeloma is often associated with a destructive bone disease; therefore one gene, adiponectin, drew particular interest based on its association with both cancer and bone biology. The adipokine adiponectin was significantly decreased in the BM of the myeloma-permissive C57Bl/KaLwRij mice (Figure 19C). Since adiponectin is a secreted factor, we also assessed the serum concentrations of adiponectin in both non-permissive C57Bl6 and permissive C57Bl/KaLwRij mice. We found that the permissive C57Bl/KaLwRij mice of the myeloma model had significantly lower serum concentrations of total adiponectin, compared to non-permissive C57Bl6 (Figure 19D). There was also a decrease in the high molecular weight (HMW) form of adiponectin (Figure 19E).

Although adiponectin was originally identified as an adipocyte-specific factor, it is now known that adiponectin is also secreted other cell types, including BM stromal cells and osteoblasts. To identify the source of differential adiponectin expression, we determined adiponectin expression in a panel of BMSCs, including populations from the permissive C57Bl/KaLwRij and the non-permissive C57Bl6 mice. Adiponectin was strongly expressed in the normal ST2 BM stromal cell line and primary BMSCs isolated from non-permissive C57Bl6 mice. In contrast, primary BMSCs from myeloma permissive C57Bl/KaLwRij mice did not express adiponectin (Figure 19F), providing evidence for decreased adiponectin in the myeloma-permissive C57Bl/KaLwRij host microenvironment. We also showed that adiponectin was not expressed by our 5TGM1 myeloma cells (Figure 19F). These data demonstrate that circulating and local

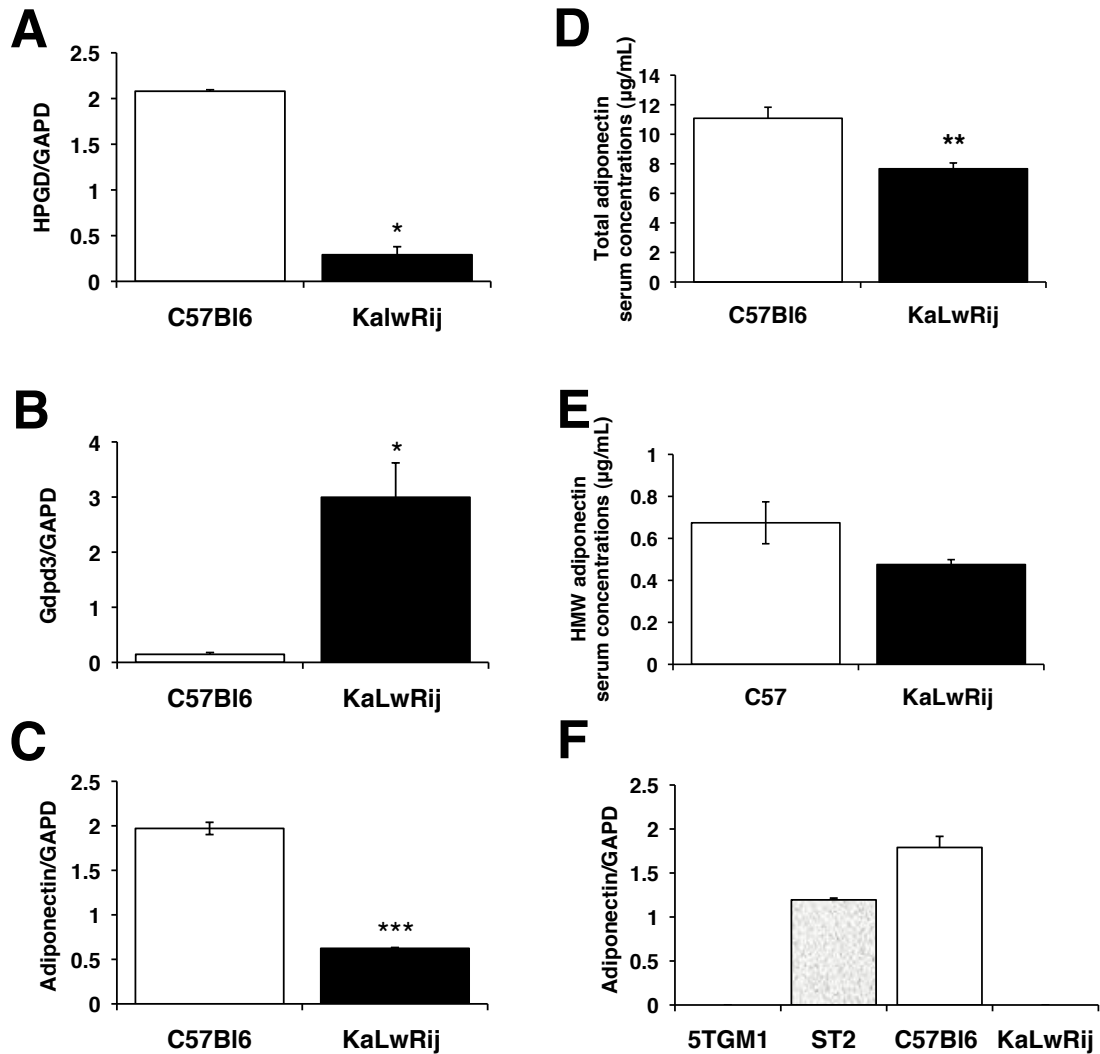


Figure 19. Strain comparison of permissive and non-permissive BM microenvironments by microarray analysis. (A-C) QPCR confirming differential expression of genes identified in the microarray comparing pooled whole BM from C57Bl/6 and C57Bl/KaLwRij mice. Results show a decrease in A) hydroxyprostaglandin dehydrogenase 15 (NAD) (* $p < 0.05$), a increase in B) glycerophosphodiester phosphodiesterase domain containing 3 (* $p < 0.05$), and a decrease in C) adiponectin expression levels in BM of myeloma-permissive C57Bl/KaLwRij mouse strain, as compared to C57Bl/6 mouse bone marrow (** $p < 0.005$). ($n = 3$ per group for expression studies) D) C57Bl/KaLwRij mice have decreased concentrations of total adiponectin (** $p < 0.005$) (C57 $n = 8$, KaLwRij $n = 7$) and decreased HMW adiponectin in the serum determined by ELISA, in comparison to C57Bl/6 mice. ($n = 4$ per group) F) BMSCs from myeloma-permissive C57Bl/KaLwRij mice do not express adiponectin compared to normal BMSCs (ST2 and primary C57Bl/6 BMSCs). Data are shown as mean \pm S.E.M. (Mann-Whitney U test)

concentrations of adiponectin are decreased in myeloma-permissive mice; however the function of decreased adiponectin in myeloma pathogenesis would need to be further investigated.

Adiponectin induces myeloma cell apoptosis

The function of adiponectin in multiple myeloma is unknown. Since our studies demonstrated a significant reduction in host-derived adiponectin in myeloma-permissive C57Bl/KaLwRij mice, we next investigated receptor expression in cells of the bone marrow microenvironment. Primary BMSCs from both C57Bl6 and C57Bl/KaLwRij mice express both adiponectin receptor 1 (AdipoR1) and 2 (AdipoR2) (Figure 20A).

The detection of adiponectin receptor expression on 5TGM1 MM cells suggests that bone marrow-derived adiponectin may have direct effects on myeloma cells. Adiponectin signaling has been previously reported to be dependent upon phosphorylation-dependent activation of AMP kinase and MAP kinase. Treatment with recombinant adiponectin for 48 hours resulted in activation of the downstream signaling kinase, AMPK (Figure 20B). The level of AMPK activation was comparable to the activation seen after treatment with the known AMPK activator, aminoimidazole carboxamide ribonucleotide (AICAR) (Figure 20B). Additionally, adiponectin treatment also resulted in the activation of p38 (Figure 20C). Adiponectin is known to induce apoptosis in a number of solid tumors, including breast cancer, however its role in myeloma is unknown. Furthermore, the activation of AMPK has been shown to inhibit growth of

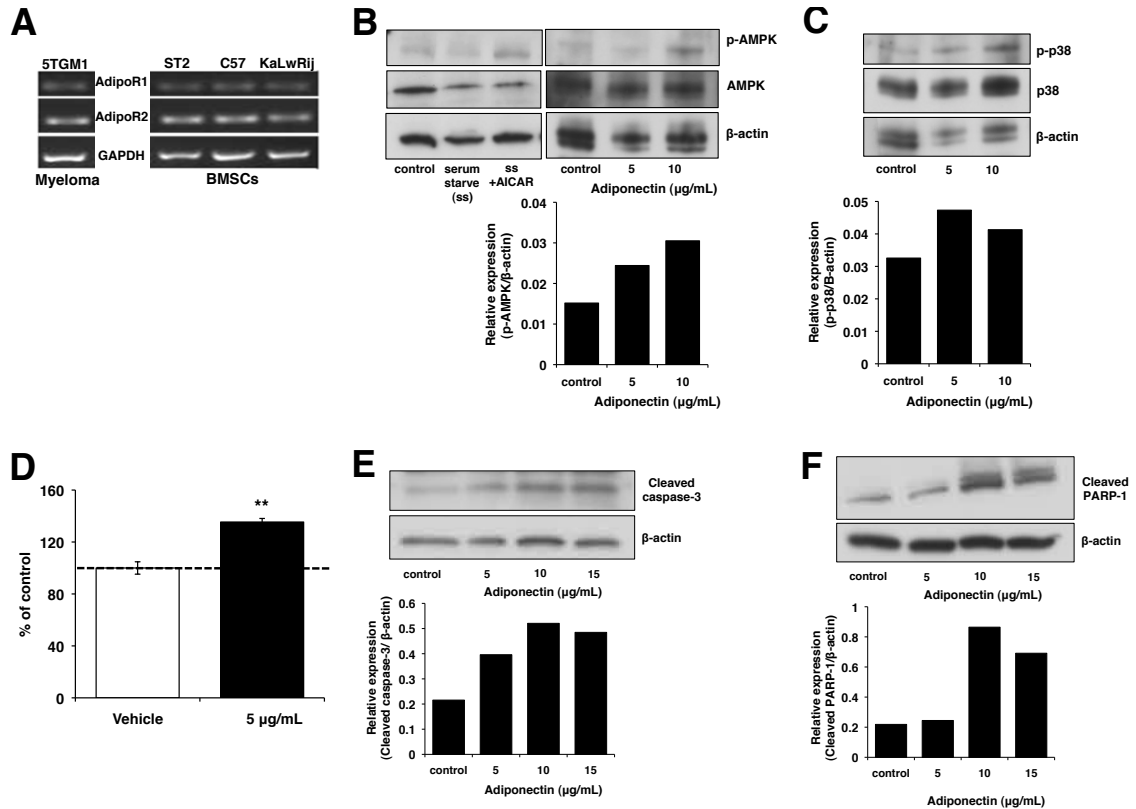


Figure 20. Adiponectin treatment induces myeloma cell apoptosis. A) Adiponectin receptor expression in BMSCs (ST2 and primary C57 and KaLwRij BMSCs) and 5TGM1 MM cells. B) Western blot showing activation of AMPK and C) p38 kinase in 5TGM1 MM cells upon treatment with 5 and 10 μg/mL adiponectin. D) 5TGM1 MM cells treated with 5 μg/mL of adiponectin for 48 hours showed a significant increase in apoptotic cells from vehicle-treated controls (** $p < 0.01$) as measured by annexinV and SYTOX staining and flow cytometric analysis. Data are shown as mean \pm S.E.M. (Mann-Whitney U test) E) Western blot showing cleavage of apoptotic caspase-3 and F) cleaved PARP-1 induced upon treatment with 5, 10, and 15 μg/mL recombinant adiponectin.

myeloma cells and other types of cancer cells (Gonzalez-Angulo and Meric-Bernstam 2010(Gonzalez-Angulo and Meric-Bernstam ; Woodard and Plataniias ; Baumann, Mandl-Weber et al. 2007). Given this inhibition, we wanted to determine the downstream effects of adiponectin on myeloma cells. 5TGM1 MM cells were treated with recombinant adiponectin and then assessed for apoptosis with annexin V and SYTOX AADvanced cell staining for flow cytometry. After 48 hours, 5TGM1 MM cells treated with adiponectin showed a significant increase in the percent of apoptotic cells, in comparison to vehicle treated control cells (Figure 20D). Finally, we investigated whether downstream apoptotic effectors were induced in response to adiponectin treatment. Following treatment with adiponectin, there was an increase in cleaved caspase-3 expression (Figure 20E) and increased PARP-1 cleavage (Figure 20F) present in 5TGM1 MM cells, indicative of apoptotic signaling activation.

Adiponectin is decreased in the serum of patients with MGUS and multiple myeloma

Given the similarities between the Radl model of myeloma in C57Bl/KaLwRij mice and human myeloma, we next wanted to determine whether our observations of decreased adiponectin in myeloma-permissive C57Bl/KaLwRij mice translated to the clinical setting. In collaboration with the Mayo Clinic serum, we obtained serum samples from patients with MGUS that subsequently progressed to MM, and patients with MGUS that had not progressed to myeloma over an average period of 19 years. Patient samples

were age-, sex-, and BMI-matched to normal controls. Measurement of total adiponectin concentrations demonstrated a significant difference between MGUS patients that or did not progress to myeloma, with a trend towards a reduction in serum adiponectin in those MGUS patients who did progress to myeloma, as compared to control (Figure 21A). Previous studies in the field of metabolism and diabetes suggest that high molecular weight (HMW) adiponectin is a better predictor of metabolic parameters, insulin sensitivity, and is thought to be more biologically active (Salani, Briatore et al. 2006; Bluher, Brennan et al. 2007). Accordingly, measurement of HMW adiponectin demonstrated not only a significant difference between MGUS patients that did or did not progress to myeloma, but also a significant decrease in those patients that did progress to myeloma, as compared to control (Figure 21B and C). Furthermore, analysis of serum paraprotein concentrations in those patients with MGUS that subsequently progressed to myeloma, revealed a clear trend towards a negative correlation between concentrations of serum paraprotein and adiponectin (Figure 21D). These data support our observations from a murine system of model that decreased adiponectin is associated with myeloma-permissive microenvironments, and so validate the further use of the 5T Radl model to investigate the role of adiponectin in myeloma pathogenesis *in vivo*. Furthermore, the significant difference between adiponectin concentrations in MGUS patients that do or do not progress to myeloma suggests that adiponectin may play a tumor suppressive role in myeloma development.

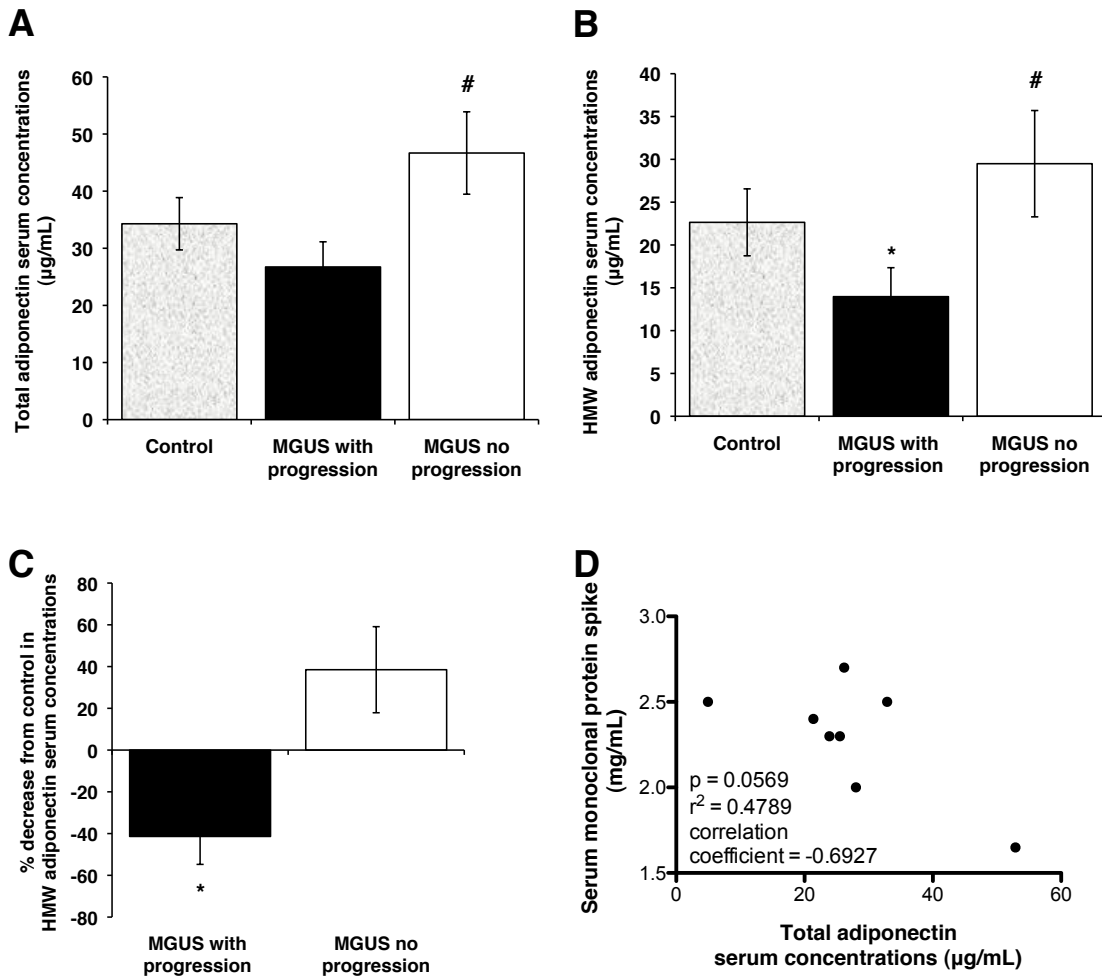


Figure 21. Decreased serum adiponectin in MGUS patients is associated with myeloma progression. Serum concentrations of both total and HMW adiponectin were measured by ELISA. A) Percent decrease from normal patient serum in total adiponectin in MGUS patients that either progress or do not progress to MM. B) HMW adiponectin concentrations are lower in MGUS patients that progress to MM, compared to control and MGUS patients that do not progress to MM. C) Percent decrease in HMW adiponectin is significant from normal patients compared to MGUS patients that progress to myeloma. D) A trend towards a negative correlation between plasma cell derived-serum paraprotein and total adiponectin concentrations present in the serum of MGUS patients. Data are shown as mean \pm S.E.M. * $p < 0.05$ compared to normal controls and # $p < 0.05$ compared to MGUS patient with progression (One-way ANOVA). (control $n = 16$, MGUS with progression $n = 9$, and MGUS with no progression $n = 7$)

Lack of host-derived adiponectin is important in myeloma pathogenesis

To specifically address the contribution of host-derived adiponectin to myeloma development *in vivo*, we investigated myeloma development in adiponectin deficient mice. Since we have previously shown that mice deficient in the recombinase activating gene-2 (RAG-2) are permissive to 5TGM1 MM cell growth and provide a tool for examining contributions of host-derived factors (Fowler, Mundy et al. 2009), we utilized these mice to determine how lack of host-derived adiponectin contributes to myeloma cell growth *in vivo*. RAG-2 deficient mice were bred with adiponectin deficient mice to obtain littermate adiponectin wildtype (RAG-2^{-/-}Adipo^{+/+}) and homozygous deficient (RAG-2^{-/-}Adipo^{-/-}) genotypes. Western blot analysis confirmed that the double knockout (RAG-2^{-/-}Adipo^{-/-}) mice do not express total adiponectin, or any of the other circulating isotypes (Figure 22A).

To determine whether myeloma development was more severe in mice that lack adiponectin, RAG-2^{-/-}Adipo^{-/-} and RAG-2^{-/-}Adipo^{+/+} mice were inoculated with 5TGM1 MM cells. Both wildtype and adiponectin-deficient mice developed myeloma in a period of approximately four weeks, similar to what is seen in the C57Bl/KaLwRij mice of the Radl model. RAG-2^{-/-}Adipo^{-/-} mice had a significant increase in tumor burden, as measured by myeloma-specific IgG2b serum concentrations following myeloma cell inoculation, in comparison to RAG-2^{-/-}Adipo^{+/+} mice (Figure 22B). Of note, this increase in tumor burden was seen as early as one-week post-tumor cell inoculation and persisted through the experimental endpoint. The osteolytic bone disease associated with myeloma

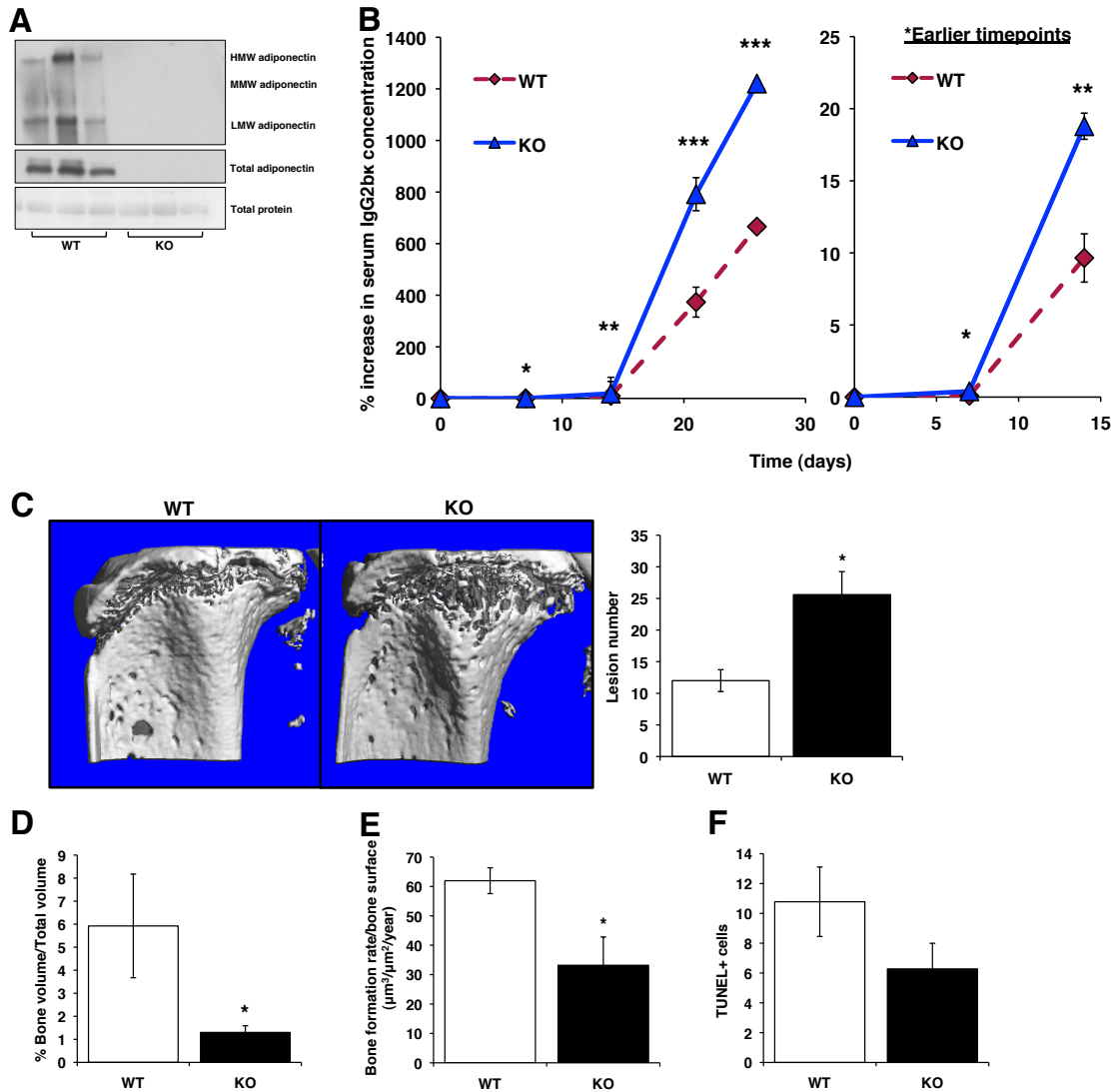


Figure 22. Lack of host-derived adiponectin exacerbates myeloma pathogenesis. Adiponectin deficient mice bred on a RAG-2 deficient background inoculated with 5TGM1 MM cells. A) Western blot showing adiponectin expression (total and various isoforms) in all three genotypes used in myeloma comparison study. B) A significant increase in tumor burden over time in KO RAG-2^{-/-}Adipo^{-/-}, represented by IgG2b serum concentrations. Data are shown as mean \pm S.E.M. *p<0.05, **p<0.001, and ***p<0.005 as compared to WT RAG-2^{-/-}Adipo^{+/+} (One-way ANOVA and Tukey-Kramer tests). Myeloma-associated bone disease was more severe in KO RAG-2^{-/-}Adipo^{-/-} with C) a significant increase in lesions through the cortical bone, D) a significant decrease in trabecular bone, and E) a significant decrease in bone formation rates, compared to WT RAG-2^{-/-}Adipo^{+/+} mice. F) TUNEL staining showing that myeloma-bearing RAG-2^{-/-}Adipo^{-/-} mice show a decrease in apoptotic myeloma cells present in the bone marrow cavity, in comparison to RAG-2^{-/-}Adipo^{+/+} mice. Data are shown as mean \pm S.E.M. *p<0.05, **p<0.001, and ***p<0.005 as compared to WT RAG-2^{-/-}Adipo^{+/+} (Mann-Whitney *U* test). (WT RAG-2^{-/-}Adipo^{+/+} n=3, KO RAG-2^{-/-}Adipo^{-/-} n=6)

was more severe in the RAG-2^{-/-}Adipo^{-/-} mice, which displayed increased lesions through the cortical bone (Figure 22C), decreased trabecular bone volume (Figure 22D), and decreased osteoblast activity (Figure 22E). There was also a decrease in osteoblasts and osteoclasts present on the trabecular bone surfaces (data shown as mean \pm S.E.M.; WT 8.06 \pm 0.423 versus KO 3.72 \pm 1.31 osteoblast surface/bone surface; WT 22.4 \pm 3.05 versus KO 13.4 \pm 3.08 osteoclast surface/bone surface); however this is likely due to the overall decrease in trabecular bone. Histological assessment of the bone marrow demonstrated a decrease in myeloma cells undergoing apoptosis in RAG-2^{-/-}Adipo^{-/-} mice compared to RAG-2^{-/-}Adipo^{+/+} mice (Figure 22F), providing further evidence of a myeloma-suppressive effect of adiponectin. There was no difference in myeloma cell proliferation in the RAG-2^{-/-}Adipo^{-/-} mice, compared to RAG-2^{-/-}Adipo^{+/+} mice (WT 0.0029 \pm 0.0007 versus KO 0.0010 \pm 0.0002 p-histone H3 positive myeloma cells/total cells). Given there was a significant increase in both tumor burden and the associated osteolytic bone disease in the RAG-2^{-/-}Adipo^{-/-} mice; this provides strong evidence that lack of circulating adiponectin has a role in myeloma pathogenesis.

To examine whether adiponectin deficient mice had a significant bone phenotype at time of tumor cell inoculation that may impact myeloma growth and progression, we evaluated the bone phenotype of adiponectin deficient mice at the time of tumor cell inoculation. Assessment of bone parameters in RAG-2^{-/-}Adipo^{-/-} mice by microCT and histomorphometry show a slight decrease in trabecular bone volume in RAG-2^{-/-}Adipo^{-/-} mice in comparison to RAG-2^{-/-}

	RAG-2 ^{-/-} Adipo ^{+/+}	RAG-2 ^{-/-} Adipo ^{-/-}
% BV/TV	5.48 ± 0.67	3.82 ± 0.75
OB.S./BS. (mm ² /mm ³)	15.6 ± 1.23	16.4 ± 1.96
OC.S./BS.	15.3 ± 1.27	17.5 ± 1.90
Tb.N (1/mm)	4.61 ± 0.35	3.95 ± 0.50
Tb.Th. (mm)	0.04 ± 0.003	0.04 ± 0.001
Tb.Sp. (mm)	0.22 ± 0.02	0.27 ± 0.05

Table 2. Bone parameters of RAG-2^{-/-}Adipo^{-/-} and RAG-2^{-/-}Adipo^{+/+} mice. MicroCT analysis of bone parameters in WT RAG-2^{-/-}Adipo^{+/+} and KO RAG-2^{-/-}Adipo^{-/-} mice, including trabecular bone volume (%BV/TV), osteoblast surface (OB.S./BS) and osteoclast surface to bone surface (OC.S./BS), trabecular number (Tb.N.), trabecular thickness (Tb.Th.), and trabecular spacing (Tb.Sp.). Data are shown as mean ± S.E.M. (WT n=9, KO n=5).

Adipo^{+/+} mice (Table 2), however this decrease was not significant. There were no significant differences between the RAG-2^{-/-}Adipo^{+/+} and RAG-2^{-/-}Adipo^{-/-} mice in any of the bone parameters measured.

L-4F increases adiponectin production *in vitro* and *in vivo*

Since our data strongly suggest that a decrease in host-derived adiponectin promotes myeloma, we hypothesized that pharmacological enhancement of adiponectin may represent a novel therapeutic approach. In order to increase adiponectin production we utilized L-4F, an apolipoprotein mimetic peptide, which has been previously shown to increase serum concentrations of the HMW form of adiponectin in obese mice when administered daily (Peterson, Drummond et al. 2008). Apolipoprotein mimetics have a strong lipid-associating ability because of their class A amphipathic helical structural motif (Sparrow, Gotto et al. 1973; Mishra, Palgunachari et al. 1995; Palgunachari, Mishra et al. 1996). The 4F refers to the number of phenylalanine residues present on the non-polar face of the helical structure (Figure 23A) (Mishra, Palgunachari et al. 2008). We undertook initial studies to confirm that treatment of C57Bl/KaLwRij mice, which we know to have decreased adiponectin, with L-4F also resulting in an increase in circulating adiponectin (Figure 23B). We next examined whether L-4F could induce adiponectin expression in BMSCs resident in the bone marrow microenvironment, particularly from the C57Bl/KaLwRij microenvironment that we previously demonstrated has decreased adiponectin. Both ST2 and primary C57Bl/KaLwRij BMSCs showed

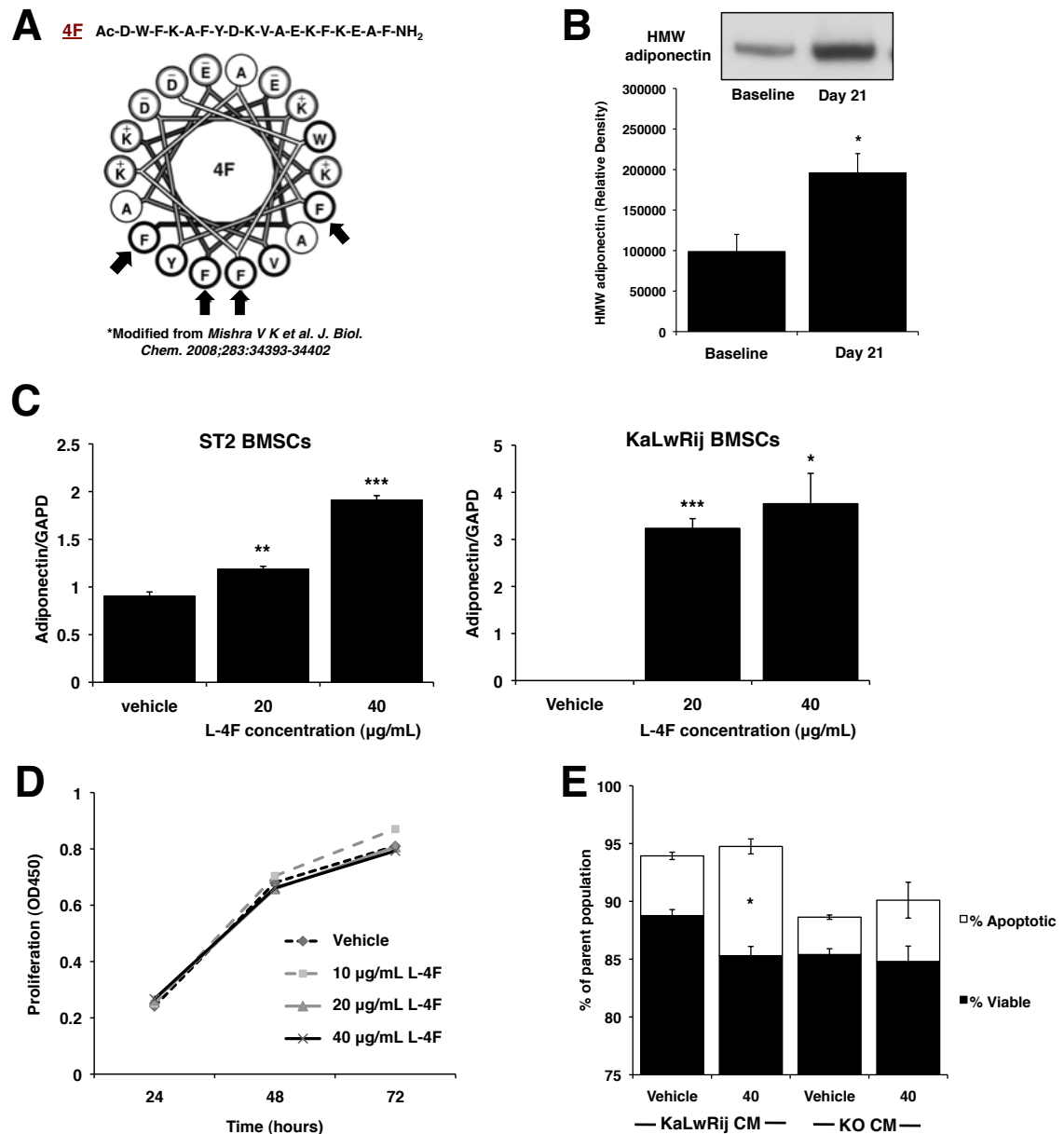


Figure 23. L-4F treatment increases adiponectin expression by BMSCs and increases adiponectin expression in vivo. A) The amino acid sequence of the L-4F peptide and the helical structure. The black arrows indicate the four-phenylalanine residues. B) Mice treated L-4F show increased serum concentrations of high molecular weight (HMW) adiponectin (* $p < 0.05$, Mann-Whitney U test). C) L-4F induced adiponectin expression in dose-dependent manner with 20 and 40 $\mu\text{g/mL}$ in BMSCs, determined by quantitative RT-PCR (** $p < 0.01$, *** $p < 0.005$ as compared to vehicle, one-way ANOVA). D) MTS assay over 72 hours of 5TGM1 MM cells treated with vehicle, 10, 20, and 40 $\mu\text{g/mL}$ of L-4F showed no direct effect on viability. E) 5TGM1 MM cell apoptosis measured by annexin V positive cells, after treatment with conditioned media from C57Bl/KaLwRij and RAG-2^{-/-}Adipo^{-/-} BMSCs treated with 40 $\mu\text{g/mL}$ of L-4F (* $p < 0.05$ as compared to vehicle conditioned media, Mann-Whitney U test. Data are shown as mean \pm S.E.M.

a significant induction of adiponectin expression following 48 hours of L-4F treatment (Figure 23C). This increase in expression was demonstrated at both 20 and 40 $\mu\text{g}/\text{mL}$ (Figure 23C). Interestingly, the C57Bl/KaLwRij BMSCs showed adiponectin expression following L-4F treatment despite basal expression being undetectable. In contrast, 5TGM1 myeloma cells treated with L-4F failed to induce adiponectin expression and adiponectin remained undetected in these cells.

Increasing concentrations of L-4F had a direct effect on myeloma cell viability (Figure 23D). However, treatment of 5TGM1 MM cells with the conditioned media from the C57Bl/KaLwRij stroma that were treated with L-4F showed a significant 82% increase in the percentage of apoptotic cells (Figure 23E). In contrast, treatment of 5TGM1 MM cells with conditioned media from L-4F-treated RAG-2^{-/-}Adipo^{-/-} BMSCs had no significant effect on apoptosis, indicating that L-4F can indirectly induce myeloma cell apoptosis via induction of adiponectin in BMSCs (Figure 23E). These results suggest that the BMSCs of the myeloma-permissive C57Bl/KaLwRij mice is not irreversibly altered since adiponectin expression can be restored and supports therapeutic potential of using L-4F *in vivo*.

L-4F treatment has potential as an anti-myeloma therapy

Given that L-4F treatment in mice increases adiponectin expression and production *in vivo*, we next wanted to address whether L-4F treatment of MM-bearing C57Bl/KaLwRij mice had an anti-tumor effect. Since our data

demonstrates that decreased adiponectin is present in MM-permissive microenvironments, prior to myeloma and suggests a role for adiponectin in the early stages of myeloma development, we first investigated the effect of L-4F, administered as a pre-treatment in order to increase host adiponectin prior to inoculation of myeloma cells. Following 28 days of daily L-4F treatment, C57Bl/KaLwRij mice showed a significant 28% increase in both high molecular weight (HMW) and total adiponectin concentrations present in serum (Figure 24A and B). This level of increase is equivalent to the reduction in adiponectin in MM-permissive C57Bl/KaLwRij mice, as compared to non-permissive C57Bl6 mice. At this time point, mice were inoculated with 5TGM1 MM cells. Treatment with L-4F or vehicle was continued throughout the experiment, in order to maintain the change in the host microenvironment. Measurement of tumor burden in vehicle and L-4F-treated mice demonstrated that mice treated with L-4F had a significant reduction in tumor burden throughout myeloma development and at experimental endpoint (Figure 24C and D). Interestingly, the decrease in tumor burden was seen early and throughout myeloma development, suggesting that increases in adiponectin are critical for tumor establishment (Figure 24D). The difference in tumor burden at early stages of disease is similar to the tumor burden in myeloma-bearing RAG-2^{-/-}Adipo^{+/+} versus RAG-2^{-/-}Adipo^{-/-} mice (Figure 22B). Immunohistochemical analysis of the bone marrow demonstrated a significant increase in the number of apoptotic myeloma cells present in the bone marrow of mice treated with L-4F in comparison to vehicle treated mice, determined by TUNEL staining (Figure 25A). There was also a significant decrease in Ki67

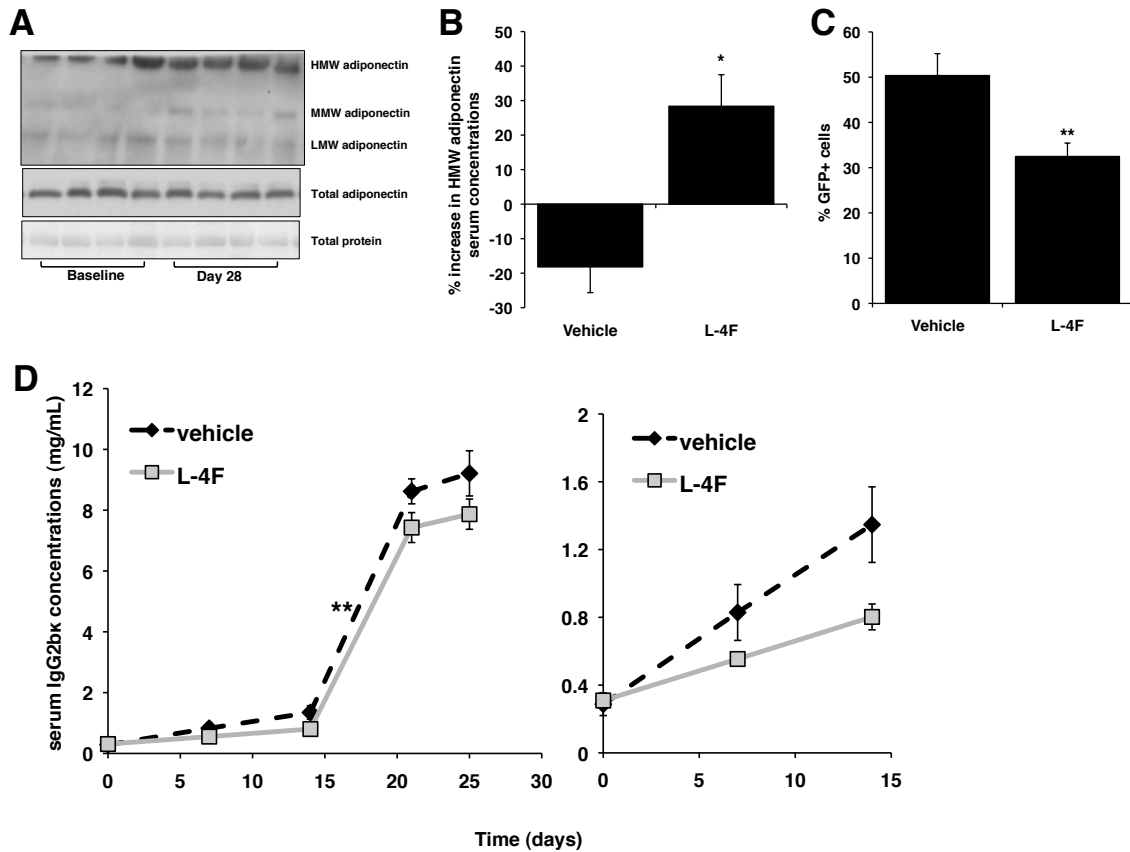


Figure 24. L-4F treatment results in decreased tumor burden in the myeloma model. Myeloma-bearing C57Bl/KaLwRij mice of the Rad1 myeloma model have decreased tumor burden with L-4F. A) Adiponectin western blot showing elevated HMW adiponectin serum levels after treatment with L-4F for 28 days. B) HMW adiponectin ELISA showed a significant increase in HMW adiponectin serum concentrations with L-4F treatment. C) Tumor burden represented by GFP positive 5TGM1 myeloma cells is significantly reduced within the bone marrow of L-4F treated mice, measured by flow cytometry. * $p < 0.05$, ** $p < 0.01$ as compared to vehicle (Mann-Whitney U test). D) L-4F treatment results in a significant decrease in the rate of tumor development, indicated by IgG2b serum concentrations over time. Data are shown as mean \pm S.E.M. * $p < 0.05$, ** $p < 0.01$ (One-way ANOVA). (Vehicle $n = 9$, L-4F-treated $n = 10$).

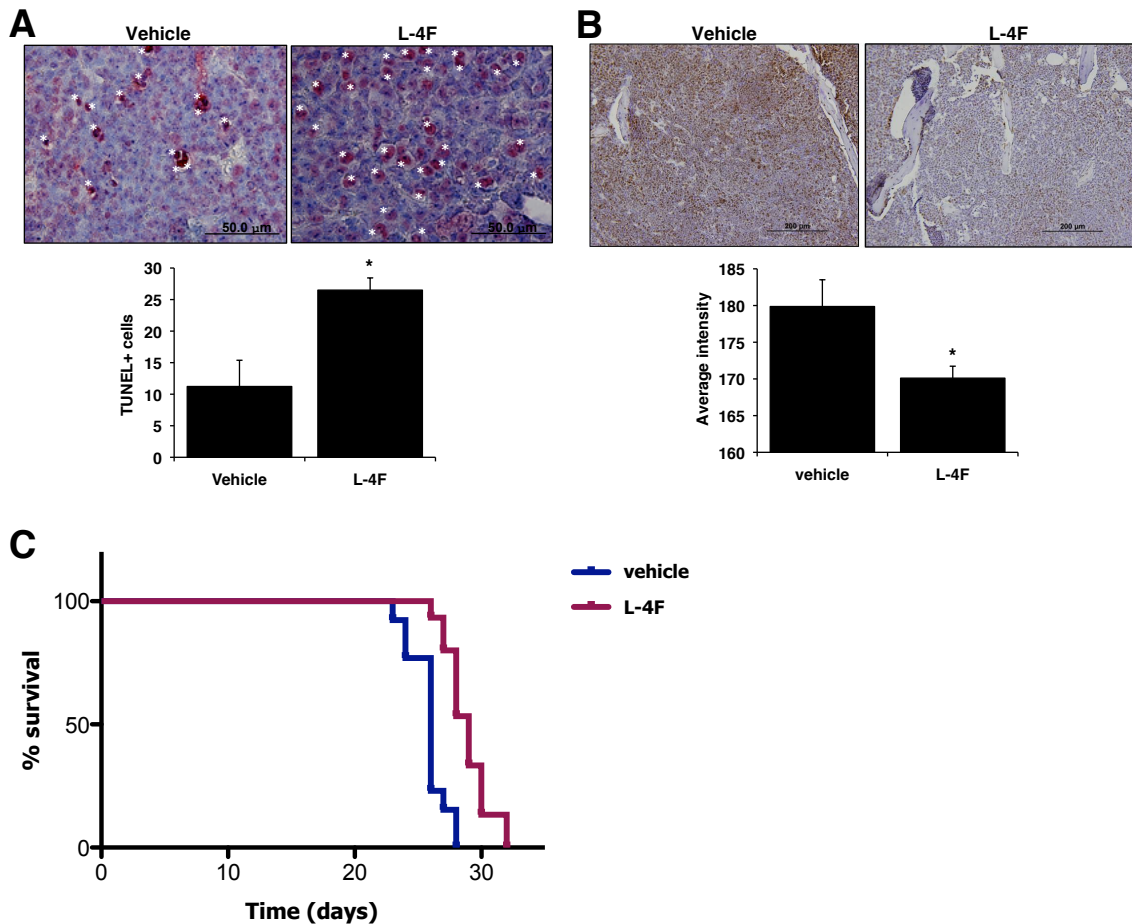


Figure 25. L-4F treatment increases myeloma cell apoptosis *in vivo* and increases survival. A) Mice treated with L-4F show a significant increase in TUNEL positive myeloma cells present in the BM, indicative of apoptosis. B) Mice treated with L-4F show a significant decrease in Ki67 staining present in the BM. Data are shown as mean \pm S.E.M. * $p < 0.05$ compared to vehicle (Mann-Whitney U test). (Vehicle $n = 9$, L-4F-treated $n = 10$). C) Kaplan-Meier plot showing L-4F treated mice had a significant increase in survival in comparison to vehicle treated mice. $p < 0.0001$, compared to vehicle treated mice (Log -rank Mantel-Cox test). ($n = 15$ per group)

staining and 55% decrease in phospho-histone H3 positive myeloma cells in the bone marrow of mice treated with L-4F, indicative of a decrease in overall myeloma cell proliferation (Figure 25B). In contrast, L-4F treatment of MM-bearing mice from time of tumor inoculation had no significant effect on tumor burden (38% \pm 13.5 reduction in IgG2b serum concentrations in L-4F treated mice), suggesting that the modification of the BM microenvironment prior to myeloma cell presence is critical for the anti-myeloma effect of L-4F and adiponectin. To further investigate the anti-myeloma effects of L-4F *in vivo*, we wanted to investigate the effects of L-4F on survival. Myeloma-bearing mice treated with L-4F showed an increase in survival in comparison to vehicle treated animals (Figure 25C).

Finally, since adiponectin is a secreted factor detected in the serum, we wanted to examine the effect of L-4F on myeloma growth independent of the BM microenvironment using a plasmacytoma model. C57Bl/KaLwRlj mice were treated with L-4F daily following subcutaneous injection of 5TGM1 MM cells. L-4F treatment resulted in a significant decrease in tumor growth, compared to vehicle treated mice (Figure 26A). Additionally, there was a significant increase in myeloma cells undergoing apoptosis in response to L-4F treatment (Figure 26B). These combined results demonstrate that increasing serum adiponectin levels via L-4F inhibits tumor cell growth and increases survival *in vivo*.

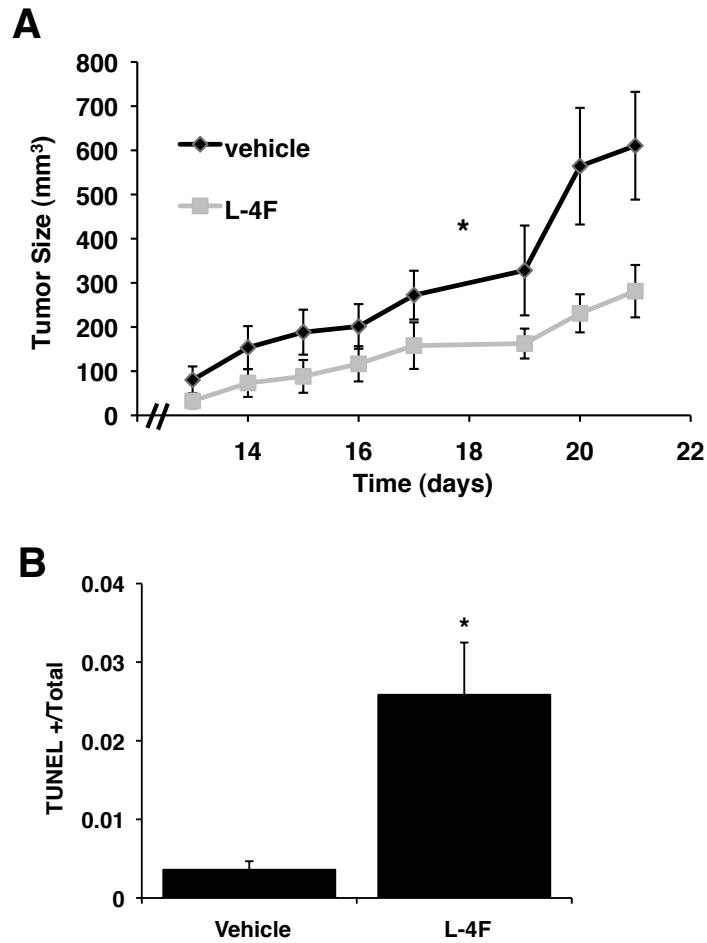


Figure 26. L-4F treatment decreases tumor growth in a plasmacytoma model. A) Mice treated with L-4F showed a significant decrease in tumor growth, compared to vehicle treated mice. B) L-4F treated mice had a significant increase in myeloma cells undergoing apoptosis in the subcutaneous tumor. Data are shown as mean \pm S.E.M. * $p < 0.05$ as compared to vehicle (One-way ANOVA for part A and Mann-Whitney U test for part B). (Vehicle $n = 7$, L-4F-treated $n = 8$)

L-4F treatment is beneficial to myeloma bone disease

Myeloma is associated with a destructive osteolytic disease; therefore when investigating new potential therapeutic options, it is important to consider the benefits to myeloma bone disease, in addition to anti-tumor effects. Myeloma bone disease is associated with a reduction in trabecular bone volume and an increase in osteolytic lesions and osteoclasts, plus a decrease in osteoblasts and bone formation. MicroCT analysis showed that myeloma-bearing mice treated with L-4F had significantly less osteolytic lesions present through the cortical bone, compared to vehicle-treated mice (Figure 27A). Histomorphometric analysis revealed although there was no significant difference in osteoclast number, L-4F treated mice showed a significant increase in bone formation rates compared to vehicle treated mice (Figure 27B).

Considering myeloma tumor burden and the associated bone are intrinsically linked, we wanted to determine whether L-4F had benefits to bone, independent of tumor cell presence; therefore we assessed the bones of non-tumor mice treated with either vehicle or L-4F. Following treatment with L-4F, mice showed a significant increase in trabecular bone volume measured by microCT analysis, in comparison to vehicle treated animals (Figure 28A). Additionally, histomorphometry showed that L-4F-treated mice had a significant increase in osteoblasts present on the bone surface (Figure 28B) and in bone formation rates (Figure 28C). Contrary to the increase in osteoblasts in response to L-4F, there was no significant change in osteoclasts present in the bone marrow (Figure 28B). These data provide strong evidence that L-4F has positive

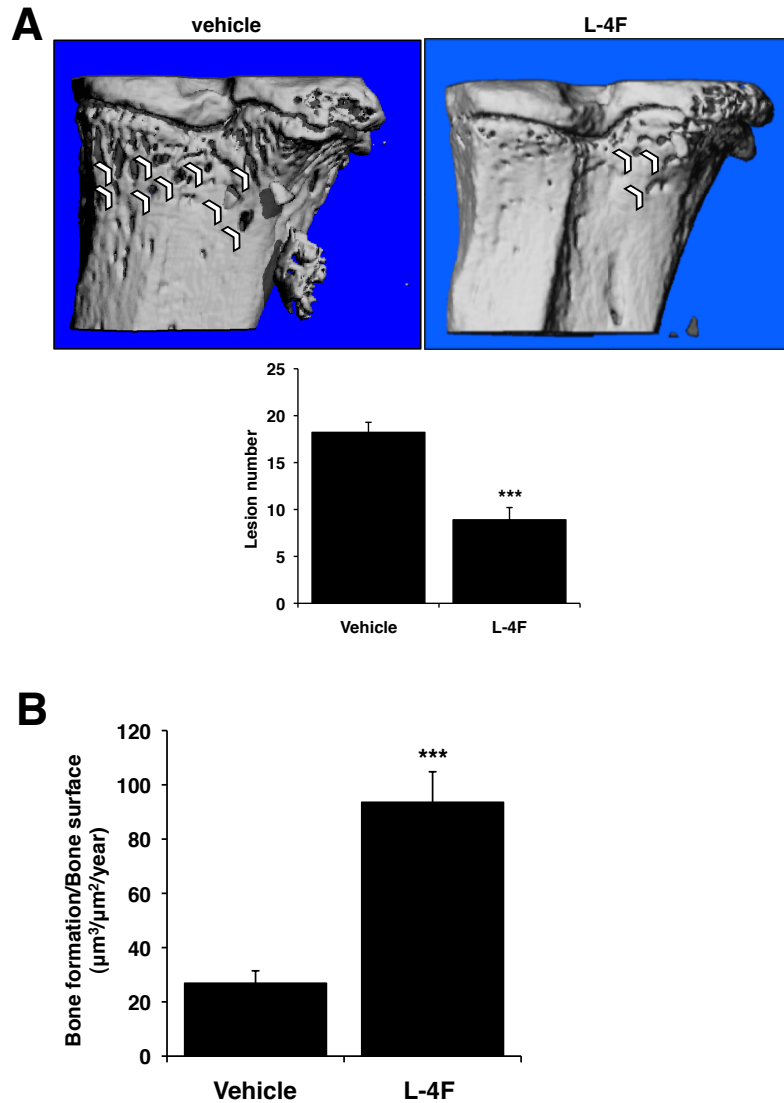


Figure 27. L-4F treatment reduces myeloma associated bone disease. A) Myeloma-bearing mice treated with L-4F had significantly less lesions (white arrowheads) through the cortical bone as compared to vehicle treated control mice. B) Myeloma-bearing mice treated with L-4F showed a significant increase in bone formation rates (per year), in comparison to vehicle-treated control mice. Data are shown as mean \pm S.E.M. *** $p < 0.001$ as compared to vehicle treated mice (Mann-Whitney U test). (Vehicle $n=9$, L-4F-treated $n=10$)

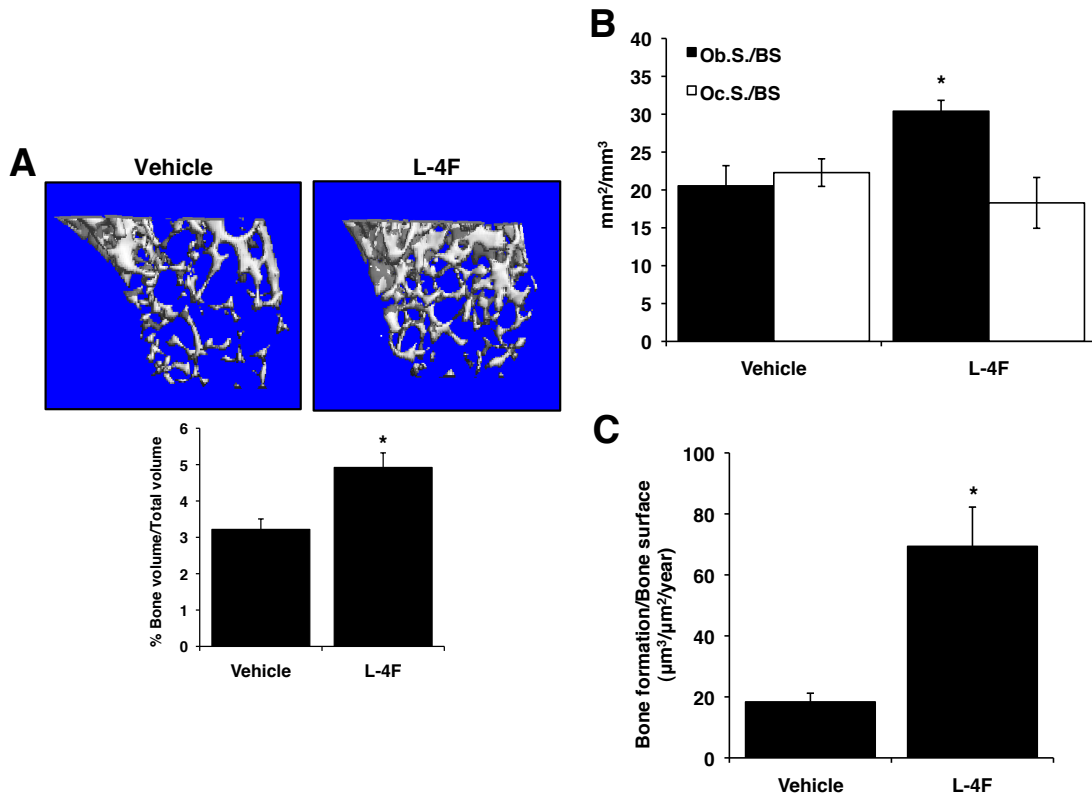


Figure 28. L-4F treatment has beneficial effects on bone. A) Non-tumor mice treated with L-4F showed a significant increase in trabecular bone volume, in comparison to vehicle-treated mice. B) Non-tumor mice treated with L-4F had a significant increase in osteoblasts per bone surface (Ob.S./BS) compared to vehicle-treated mice; however there was no difference in osteoclasts per bone surface (Oc.S./BS). C) L-4F treated mice also had a significant increase in bone formation rates, compared to vehicle-treated mice. Data are shown as mean \pm S.E.M. * $p < 0.05$ as compared to vehicle treated mice (Mann-Whitney U test). (n=5 per group)

effects on both myeloma bone disease and normal bone, likely mediated through increasing osteoblastic bone formation.

The anti-myeloma and bone effects of L-4F are mediated through adiponectin

Finally, to determine whether the beneficial effects of L-4F are mediated specifically through the promotion of adiponectin production, we performed a proof of principle experiment. RAG-2^{-/-}Adipo^{+/+} and RAG-2^{-/-}Adipo^{-/-} mice were treated with either vehicle or L-4F prior to tumor cell inoculation in order to elevate circulating concentrations of HMW adiponectin to the same level as the previous myeloma study. Following pre-treatment, these mice were inoculated with 5TGM1 MM cells while L-4F treatment continued throughout myeloma development. At experimental endpoint, we found that only WT RAG-2^{-/-}Adipo^{+/+} mice responded to L-4F treatment resulting in a decrease of tumor burden, compared to mice deficient in adiponectin (Figure 29A). Additionally, adiponectin deficient mice did not receive any beneficial bone effects from L-4F treatment. The WT RAG-2^{-/-}Adipo^{+/+} mice treated with L-4F had an increase in trabecular bone volume and bone formation rate compared to vehicle treated mice while KO RAG-2^{-/-}Adipo^{-/-} mice did not show these increases (Figure 29B and C). These data together provide evidence that L-4F mediates its anti-myeloma effect via stimulation of adiponectin expression and secretion.

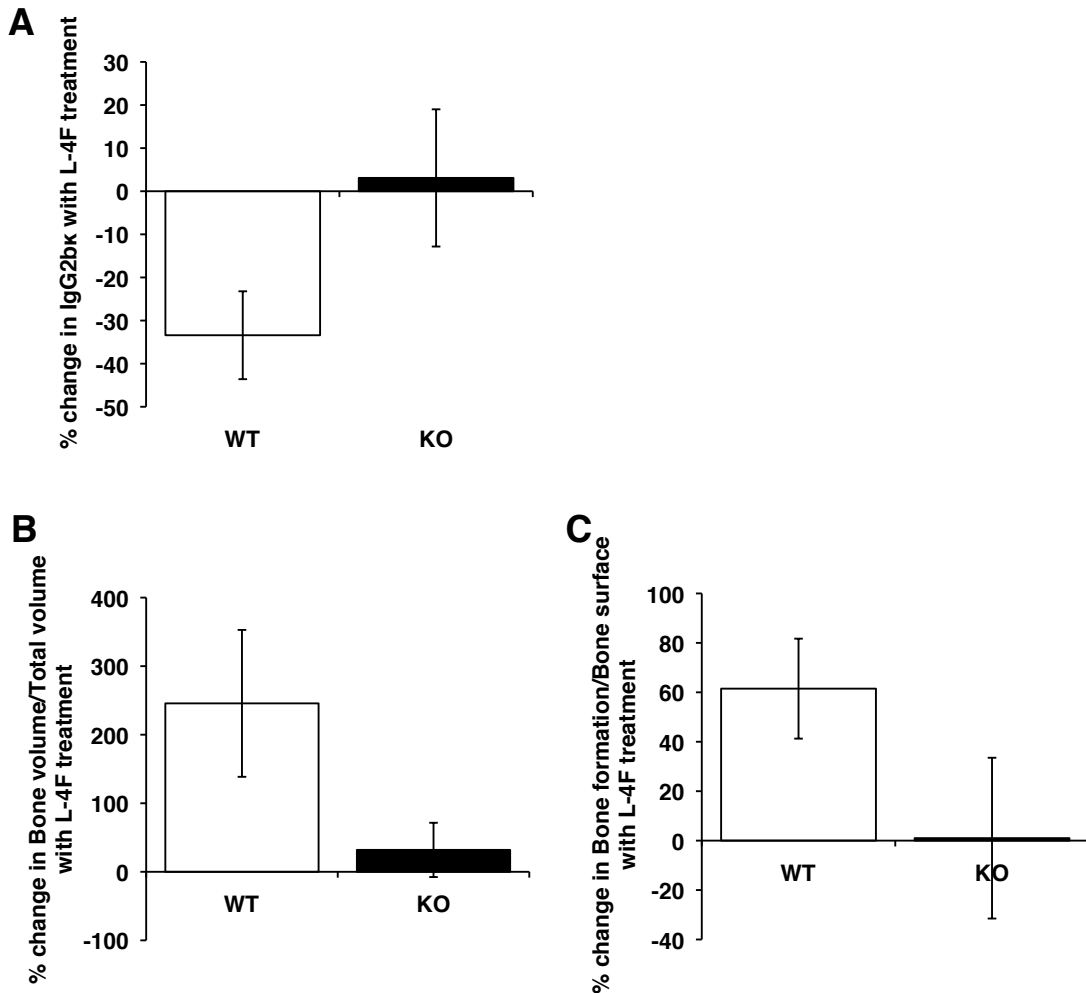


Figure 29. The anti-myeloma and bone effects of L-4F are mediated via adiponectin. A) Myeloma-bearing WT RAG-2^{-/-}Adipo^{+/+} mice treated with L-4F showed a decrease in tumor burden, compared to vehicle-treated mice. In contrast to KO RAG-2^{-/-}Adipo^{-/-} mice that did not show this decrease (p=0.887). Myeloma-bearing WT RAG-2^{-/-}Adipo^{+/+} mice treated with L-4F had an increase in B) trabecular bone volume and C) bone formation rate compared to vehicle treated WT mice; however KO RAG-2^{-/-}Adipo^{-/-} mice did not show an increase in bone volume in response to L-4F treatment (p=0.119 and p=0.058, respectively). Data are shown as mean ± S.E.M. (Mann-Whitney U test). (WT n=5, KO n=6)

Conclusions

Following the identification of decreased adiponectin in a myeloma-permissive microenvironment, we investigated the role of adiponectin in myeloma pathogenesis and its potential as a therapeutic target. Adiponectin was decreased in MM-permissive C57Bl/KaLwRij mice, as compared to non-permissive C57Bl6 mice. These observations in the murine myeloma model were supported by clinical evidence demonstrating that MGUS patients who progressed to myeloma had decreased adiponectin concentrations present in their serum. There are currently very few studies measuring circulating adipokines in MGUS patients. To date, there are only two studies demonstrating an association between abnormal adipokine production and risk of developing myeloma (Dalamaga, Karmaniolas et al. 2009; Reseland, Reppe et al. 2009). Our studies provide the first direct *in vitro* and *in vivo* evidence to link decreased adiponectin and myeloma pathogenesis, using both pre-clinical models and evidence from MGUS patients. Our study is the first time an adipokine has demonstrated such a potent anti-myeloma effect through the induction of apoptosis. Finally, adiponectin deficiency exacerbated myeloma pathogenesis while increasing circulating adiponectin in MM-bearing mice resulted in reduced tumor burden, the prevention of myeloma bone disease, and increased overall survival. These data demonstrate that decreased adiponectin contributes to myeloma pathogenesis, and establishes the potential therapeutic benefit of increasing adiponectin in the treatment of both MGUS and myeloma patients.

In this study, we demonstrate both pharmacologically and genetically, that reduced or absent adiponectin is important in myeloma progression and suggest that adiponectin may be tumor suppressive in myeloma. We demonstrated that adiponectin activates AMPK resulting in myeloma cell apoptosis. Previous studies by Baumann et al. demonstrated that activation of AMPK inhibited myeloma cell growth *in vitro*; however this was never demonstrated using physiologically relevant activators of AMPK, such as adiponectin (Baumann, Mandl-Weber et al. 2007). Adiponectin was shown to induce apoptosis through the activation of the caspase cascade in endothelial cells resulting in decreased angiogenesis (Brakenhielm, Veitonmaki et al. 2004). Additionally, there is recent evidence in the cancer field showing that AMPK may have an important role in controlling cancer because of its role in regulating cell growth and metabolism (Fogarty and Hardie ; Gonzalez-Angulo and Meric-Bernstam ; Woodard and Plataniias).

We have demonstrated that decreased adiponectin is important during early stages of myeloma development. Observations in the clinical setting have found that MGUS often precedes myeloma development (Landgren, Kyle et al. 2009; Weiss, Abadie et al. 2009). We found decreased adiponectin in MGUS patients that progress to myeloma compared to those that do not progress, which supports our hypothesis that adiponectin is important during the initial stages of disease. Supporting this observation, adiponectin deficient mice have higher tumor burden even at early time points in myeloma development compared to wildtype mice. Furthermore, L-4F only has a potent anti-myeloma effect with pre-

treatment to modify the microenvironment prior to tumor cells being present within the bone marrow. All of these results together suggest that adiponectin has a protective effect against myeloma establishment and progression, which manifests early in disease development. It is critical to identify risk factors in MGUS patients that are predictive of myeloma progression and our studies suggest that decreased adiponectin may predict such progression.

These studies suggest adiponectin and L-4F-mediated adiponectin production may be potential therapeutic agents for MGUS patients to prevent progression to MM, and that this approach may be more effective in MGUS than MM. Adiponectin is a secreted factor and can act through endocrine pathways. The strong anti-myeloma effect of L-4F in the plasmacytoma model supports the potent effects of L-4F-mediated adiponectin expression on myeloma cells and also the therapeutic potential for treatment of other cancers not localized to the BM.

A destructive osteolytic bone disease is often associated with MM. We demonstrate in MM-bearing mice that L-4F, through increased adiponectin production, can benefit bone disease by enhancing osteoblastic bone formation resulting in increased bone volume. Additionally, we show that L-4F has positive effects on bone without the presence of tumor cells, suggesting that L-4F may a beneficial treatment option for other conditions where bone health is compromised. Adiponectin not only acts on bone through endocrine pathways as a secreted hormone but also in an autocrine/paracrine fashion through local production within the bone marrow microenvironment that acts to promote bone

formation and osteogenesis (Shinoda, Yamaguchi et al. 2006). Adiponectin can have direct effects on both osteoblasts and osteoclasts, by stimulating proliferation and inhibiting RANKL-mediated osteoclastogenesis, respectively (Oshima, Nampei et al. 2005; Yamaguchi, Kukita et al. 2007; Williams, Wang et al. 2009). Some of the current data in the literature are conflicting in regards to the effects of adiponectin on bone; however our data suggest that its effects are mainly through stimulation of osteoblasts. Finally, the additional benefits of L-4F on bone suggest that increasing adiponectin would be beneficial for MGUS patients that experience bone loss. Drake, et al. recently demonstrated that MGUS patients experience a generalized bone loss despite the absence of lytic lesions.

The phenotype in AMPK β subunit knockout mice suggests that the beneficial effects of L-4F on bone may be through adiponectin-mediated AMPK activation. L-4F acts to stimulate adiponectin production, which then activates AMPK. Mice with a germline deletion of the AMPK β subunits have low AMPK activity in tissues and decreased trabecular bone, in both mass and density (Quinn, Tam et al.). Interestingly, the double deficient generated for these studies did not appear to have a dramatic bone phenotype as was observed in other studies using adiponectin deficient mice (Williams, Wang et al. 2009). The lack of a strong bone phenotype in the RAG-2^{-/-}Adipo^{-/-} mice is supportive that a decrease in overall bone is not responsible for increasing the severity of myeloma and associated bone disease; therefore is more likely due to the lack of protection against myeloma progression. Also of interest to better understanding

the effect of L-4F on bone is a recent study by Huang et al. showing that adiponectin stimulated BMP-2 expression in osteoblasts. Stimulation of BMP-2 may be a possible indirect downstream effect of L-4F on bone. The effects of L-4F on resident bone marrow cells is a future direction for this work and will be discussed further in the next chapter.

Obviously, of additional interest in the future is the effect of other adipokines and adiposity, in general, on myeloma progression. Other adipokines, including leptin and resistin are shown to be elevated in myeloma patients (Alexandrakis, Passam et al. 2004; Pamuk, Demir et al. 2006; Dalamaga, Karmaniolas et al. 2009; Reseland, Reppe et al. 2009). In contrast to examining some adipokines in myeloma patients, little to no investigation has been performed in patients with MGUS. The studies presented in this chapter are the first to provide evidence using *in vivo* models of disease and to provide some mechanism by which an adipokine contributes to myeloma progression. Given the close association of adiponectin and other adipokines with adiposity and obesity, it is difficult to separate the role of adipocytes in myeloma development. In the next chapter, the future directions of this work will be discussed involving diet-induced obesity in the context of the myeloma model.

The proposed mechanism by which circulating adiponectin concentrations have negative effects on myeloma progression is displayed in Figure 30. In healthy physiological conditions, adiponectin is at normal or even high circulating concentrations. These levels of adiponectin are protective against myeloma growth and progression, and adiponectin could be acting to directly inhibit

myeloma cell growth by inducing apoptosis. The protective effects could be acting on the local microenvironment to prevent a generalized bone loss often seen in older individuals and MGUS patients. The results presented in this chapter suggest that decreased adiponectin in early stages of disease results in the loss of tumor suppression and ultimately progression to myeloma. Our studies suggest that adiponectin acts on both osteoblasts within the microenvironment to stimulate bone formation and directly inducing apoptosis of myeloma cells. As the BMSC studies presented in chapter IV suggest, a generalized bone loss may be important in for myeloma progression; therefore benefits to overall bone health are critical. Future studies are needed to determine whether other *in vivo* models with bone loss are permissive to myeloma and whether adiponectin treatment could prevent myeloma growth in these mice.

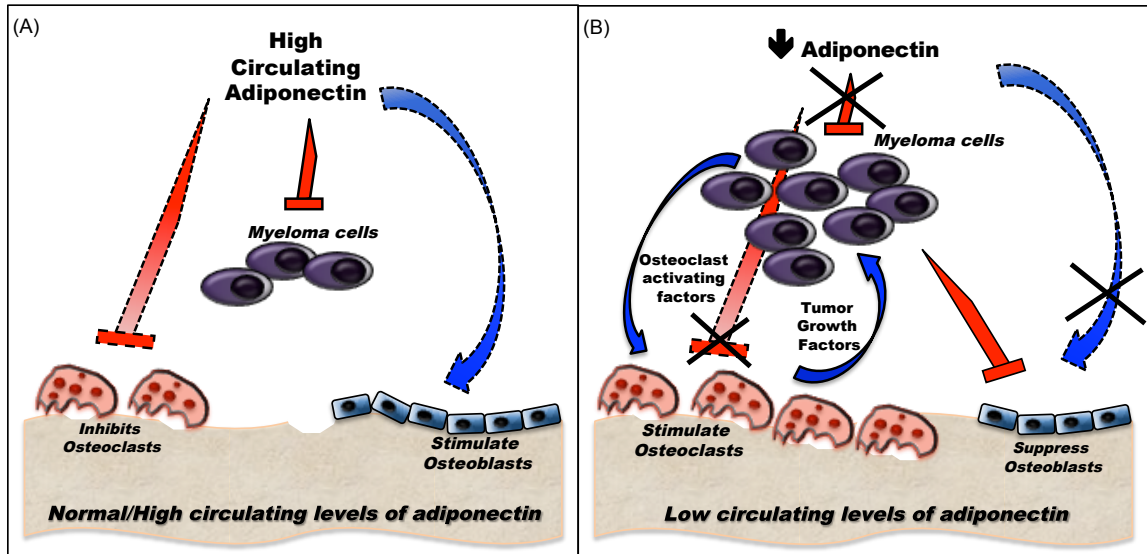


Figure 30. Circulating adiponectin concentrations contribute to myeloma pathogenesis. (A) When normal circulating levels of adiponectin are present in early stages of myeloma, adiponectin acts to suppress tumor growth, stimulate osteoblast activity, and inhibit osteoclast activity*. Additionally, L-4F treatment increases circulating adiponectin concentrations, which is shown to have a protective effect against myeloma progression. (B) When adiponectin concentrations are low, myeloma cells are no longer suppressed and can act to suppress osteoblasts and stimulate osteoclasts*.

**previously shown in the literature*

CHAPTER VI

CONCLUSIONS AND SIGNIFICANCE

The combined results from the studies described in this dissertation provide compelling evidence that the bone marrow microenvironment is critical for myeloma development. This evidence includes (i) differences between myeloma-permissive and non-permissive strains, (ii) contributions of altered BMSCs in myeloma establishment and progression, and (iii) decreased host-derived adiponectin playing a role in myeloma pathogenesis (Figure 31A and B). These studies provided novel concepts of investigation in the field of myeloma as demonstrated by this work receiving numerous forms of recognition either through grants, awards, or oral presentations at international conferences:

Awards

- 2010 ASBMR Young Investigator Travel Grant Award, Toronto, Canada.
- 2009 Lai Sulin Scholarship Award from Vanderbilt University Graduate School
- 2008 Young Investigator Award from ASBMR, Montreal, Canada.
- 2008 Cancer Induced Bone Disease Travel Award, Edinburgh, Scotland.

Presentations

J.A. Fowler, et al. Myeloma-associated fibroblasts suppress osteoblasts *in vivo* independent of tumor presence. 31th Annual Meeting of the American Society for Bone and Mineral Research, Denver, Colorado (2009)

J.A. Fowler, et al. Decrease in bone marrow-derived adiponectin contributes to myeloma pathogenesis *in vivo*. 31th Annual Meeting of the American Society for Bone and Mineral Research, Denver, Colorado (2009)

J.A. Fowler, et al. Host bone marrow-derived stromal cells promote myeloma initiation and development of osteolysis. 30th Annual Meeting of the American Society for Bone and Mineral Research, Montreal, Canada (2008)

J.A. Fowler, et al. Essential role of host bone marrow-derived stromal cells in the initial establishment and progression of multiple myeloma. VII International Meeting on Cancer Induced Bone Disease, Edinburgh, Scotland (2008).

First, we are able to demonstrate critical differences between 5TGM1 myeloma-permissive and non-permissive strains of mice. The concept of permissive versus non-permissive is a common theme throughout all the work presented in this dissertation. We found significant differences in myeloma establishment and progression in various strains of mice despite similar genetic backgrounds. The most interesting example is the difference of tumor establishment in the C57Bl/KaLwRij used in the Radl model and the lack of tumor take and growth in the C57Bl6 mice of the same genetic background. The differences between C57Bl/KaLwRij and C57Bl6 mice were examined further in chapters IV and V with the use of the BMSCs and performing the microarray analysis.

In chapter III, a role for the immune system in myeloma development was implicated. Of specific interest was the difference in tumor establishment between two immunocompromised strains of mice. Athymic nude mice do not develop characteristics of myeloma, whereas RAG-2 deficient mice develop pathology identical to the Radl C57Bl/KaLwRij mice. Although the use of RAG-2 deficient mice will not allow for the investigation of the immune system, specifically B and T cells, in myeloma, our results demonstrates that a lack of T cells is not sufficient to permit myeloma development *in vivo*. Since the major difference between RAG-2 deficient mice and nude mice is the absence of B cells, this raises the intriguing possibility that the development of 5T myeloma in RAG-2 deficient mice may not be simply due to immunodeficiency, but may in part be dependent on specific B cell regulation. Nude mice are also known to

have increased natural killer cell and macrophage activity, and it is possible that these differences may also contribute to their inability to permit development of myeloma (Budzynski and Radzikowski 1994). A generalized suppression of B lymphopoiesis is one clinical feature of patients with advanced multiple myeloma. The reduction of normal immunoglobulin levels found in these patients is attributed to defects in normal B cell differentiation (Pilarski, Mant et al. 1984; Pilarski, Ruether et al. 1985; Jacobson and Zolla-Pazner 1986; Pilarski, Andrews et al. 1986; Duperray, Bataille et al. 1991; Rawstron, Davies et al. 1998). Secreted factors from patient myeloma cell lines and marrow cells have a role in suppressing normal B cell proliferation (Farnen, Tyrkus et al. 1991; Quesada, Leo et al. 1995). The contribution of B cell deficiency in disease development and growth is difficult to study in human myeloma patients, as the pathology is already extensive upon diagnosis. Not only do our data demonstrate that RAG-2 deficient mice provide a useful tool for studying the host microenvironment in myeloma development, but these studies also suggest that lack of mature B cells may have a role on myeloma development.

Cancer-associated stroma and fibroblasts have received a reputation for promoting cancer progression mainly in solid or epithelial-derived tumors. As the studies presented in chapter IV have demonstrated, hematological malignancies can be influenced by the stromal microenvironment. In this chapter, 14M1 BMSCs were utilized that were originally isolated from myeloma-bearing C57Bl/KaLwRij mice. Our initial characterization of the 14M1 BMSCs showed that these cells express markers of both fibroblasts and cancer-associated

fibroblasts; therefore a thorough examination is necessary to properly distinguish the C57Bl/KaLwRij microenvironment effects from the effects produced by cancer-associated stroma. In the future, primary BMSCs from either non-tumor or myeloma-bearing C57Bl/KaLwRij mice will be used in co-inoculation experiments similar to the 14M1 BMSC studies.

The significance of the research described in this dissertation is the demonstration of the significant contributions that the bone marrow microenvironment has in myeloma establishment and progression. The studies focused on adiponectin are the first to demonstrate the role of an adipokine in inducing myeloma cell apoptosis. The therapeutic potential of both adiponectin and more specifically L-4F, could lead to improved survival of patients with multiple myeloma. Additionally, the evidence of decreased adiponectin serum concentrations in MGUS patients may provide a prognostic indicator for which MGUS patients might be at risk for progression to multiple myeloma. Screening MGUS patients for adiponectin levels may provide an opportunity for clinical intervention prior to progression.

Future directions for this work would include several areas of investigation. First, would be to explore the possible link between Dkk1 secretion and suppression of adiponectin production. Given the protective effect that osteoblasts appear to have against myeloma progression, as we saw in chapter IV with Dkk1 knockdown in BMSCs, it will be interesting to examine factors produced by the osteoblasts that might be responsible for this effect, as was described in the previous chapter. Osteoblasts are one of the major producers of

adiponectin within the bone marrow microenvironment. Data that was not included in this dissertation provides supporting evidence for Dkk1 being responsible for suppression of adiponectin production. We have found that Dkk1 transgenic mice have decreased circulating serum concentrations of adiponectin. Additionally, we have seen that primary BMSCs from C57Bl/KaLwRij mice have high expression of Dkk1 in comparison to BMSCs from the myeloma non-permissive C57Bl6 mice. These data show an inverse relationship between high Dkk1 and decreased adiponectin. We also have evidence suggesting that elevated Dkk1 is upstream of adiponectin suppression. In 14M1 BMSCs that secrete concentrations of Dkk1, upon overexpression of adiponectin in these cells there is no suppression of Dkk1 expression. Furthermore, in the studies described in chapter IV when 14M1 BMSCs are inoculated alone, we see decreased circulating adiponectin levels in these mice. The clinical evidence also supports this hypothesis. We demonstrated that MGUS patients who progress to myeloma have decreased concentrations of serum adiponectin. Drake, et al. have documented that elevated Dkk1 serum concentrations are not only present in myeloma patients but also in MGUS patients who progress to myeloma. These initial observations provide ample cause to investigate the links between Dkk1 and adiponectin in the context of MGUS and MM.

A second topic of examination in the future of this work will be to understand the role of diet-induced obesity as a risk factor for myeloma pathogenesis. The epidemic of obesity has become increasingly problematic worldwide in recent years. Obesity is associated with numerous ailments

including type II diabetes and cardiovascular disease, and is considered a state of low-grade systemic inflammation (Wang, Goalstone et al. 2004). This association with pathology has led to the examination of adipose tissue in both physiologic and pathologic processes. Adipose tissue was previously thought of as simply an inert tissue dedicated to energy storage. However in recent years, studies have demonstrated its role as an endocrine organ with active secretory functions. Adipose tissue releases various factors and cytokines including tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) (Fantuzzi 2005). Of particular interest would be to determine whether mice that are otherwise non-permissive to myeloma cell growth could develop myeloma while on a high-fat diet. C57Bl6 mice on a high fat diet for a period of 16 weeks showed decreased expression of adiponectin in the adipose tissue (Bonnard, Durand et al. 2008). These studies would be interesting in terms of further understanding the role of adiponectin in myeloma. Additionally, these studies would examine the role of increased adipose tissue in myeloma pathogenesis. In conjunction with high fat diet, mice under caloric restriction would also be a necessary part of these studies. Contrary to the expected result, young C57Bl6 mice under caloric restriction displayed high bone marrow adiposity in addition to lower bone mineral density and decreased trabecular bone volume, compared to mice on a normal diet (Devlin, Cloutier et al.). The results presented in chapter V in this dissertation and previous research in the cancer field has shown a role for adipokines, as well as obesity in cancer risk. There is already epidemiological evidence linking obesity with various types of cancer and most importantly with MGUS (Landgren,

Rajkumar et al. ; Miller, Lipsitz et al. ; Saxena, Fu et al. ; Wei, Wolin et al.), providing ample support for further investigation. Finally, bone marrow adipocytes are also a source of Dkk1, providing another link between Dkk1 and decreased adiponectin (Figure 30C).

In addition to our own initial observations linking Dkk1 and adiponectin, there are also links between metabolic diseases, such as type I and type II diabetes, and bone loss. In type I diabetes, bone loss is implicated as the cause of increased fracture risk (Bouillon 1991; Meyer, Tverdal et al. 1993; Forsen, Meyer et al. 1999; Schwartz, Sellmeyer et al. 2001) and subsequent delay in healing of these fractures (Herskind, Christensen et al. 1992; Hofbauer, Brueck et al. 2007). The bone phenotype seen in mice with type I diabetes induced by streptozotocin injection had osteoblast-specific suppression (Coe, Irwin et al.), similar to that seen in the in MM-bearing adiponectin deficient mice. In these diabetic studies, the authors demonstrated that osteoblast death was mediated by TNF- α production, which is stimulated by the inflammatory state of diabetes. Interestingly, initial examination of the myeloma permissive C57Bl/KaLwRij mice indicated that they have elevated serum concentrations of TNF- α , compared to the non-permissive C57Bl6 mice.

Another area of interest would be to investigate the anti-inflammatory capabilities of adiponectin and L-4F. Among the differentially expressed genes, the gene for hydroxyprostaglandin dehydrogenase-15 (15-PGDH or 15-HPGD) was identified as being significantly down regulated in the bone marrow of myeloma-permissive C57Bl/KaLwRij mice. However, measurement of C-reactive

protein, a marker indicative of inflammation, showed no elevation in C57Bl/KaLwRij mice. Prostaglandin E₂ (PGE₂) levels are regulated by this enzyme, as 15-HPGD is responsible for the degradation of this prostaglandin (Tai, Cho et al. 2006). 15-HPGD is often reduced in various types of cancer (Backlund, Mann et al. 2005; Ding, Tong et al. 2005) and associated with elevated COX-2 expression. Cyclooxygenase-2, or COX-2, is the rate-limiting enzyme for PGE₂ production (Wang and Dubois). The effect of prostaglandins on adipokine production has become evident in the literature. Peeraully et al have shown that PGD₂ reduced adiponectin mRNA expression and secretion in adipocytes (Peeraully, Sievert et al. 2006). In these studies, the authors showed that these prostaglandins stimulated the adipocyte production of IL-6 and MCP-1, which both play a role in myeloma pathogenesis (Cao, Luetkens et al. ; Pellegrino, Ria et al. 2005; Huston and Roodman 2006). Independent from the effect on adiponectin, prostaglandins also have dramatic effects on the bone. Two-month old C57Bl6 mice treated with PGE₂ demonstrated a significant reduction in trabecular bone volume and number (Gao, Xu et al. 2009). As discussed in chapter IV of this dissertation, we have data suggesting that decreased bone volume and altered stroma within the bone marrow microenvironment are critical for myeloma establishment and growth.

Osteolytic bone disease is one of the many devastating features associated with multiple myeloma. Despite advances in myeloma research, the contributions of various cell types found within the bone marrow microenvironment to myeloma bone disease are not fully elucidated. Our current

knowledge is limited due to the difficulty of studying the intact bone marrow microenvironment in its entire complexity; however some of the work within this dissertation will expand the available tools for studying the host microenvironment. It will be critical in the coming years to begin to understand what components of the bone marrow are important for progression and some of these have been discussed within this chapter and outlined in Figure 30C. The relationship between myeloma cells and these cells within the microenvironment contribute to the destructive bone disease, which is one of the defining features of multiple myeloma. Emerging evidence in cancer research of recent years not only demonstrates genetic alterations to the cancer cells but also to the surrounding microenvironment. The future of effective cancer therapeutics will have a dual focus; treating both the tumor cells and the altered microenvironment.

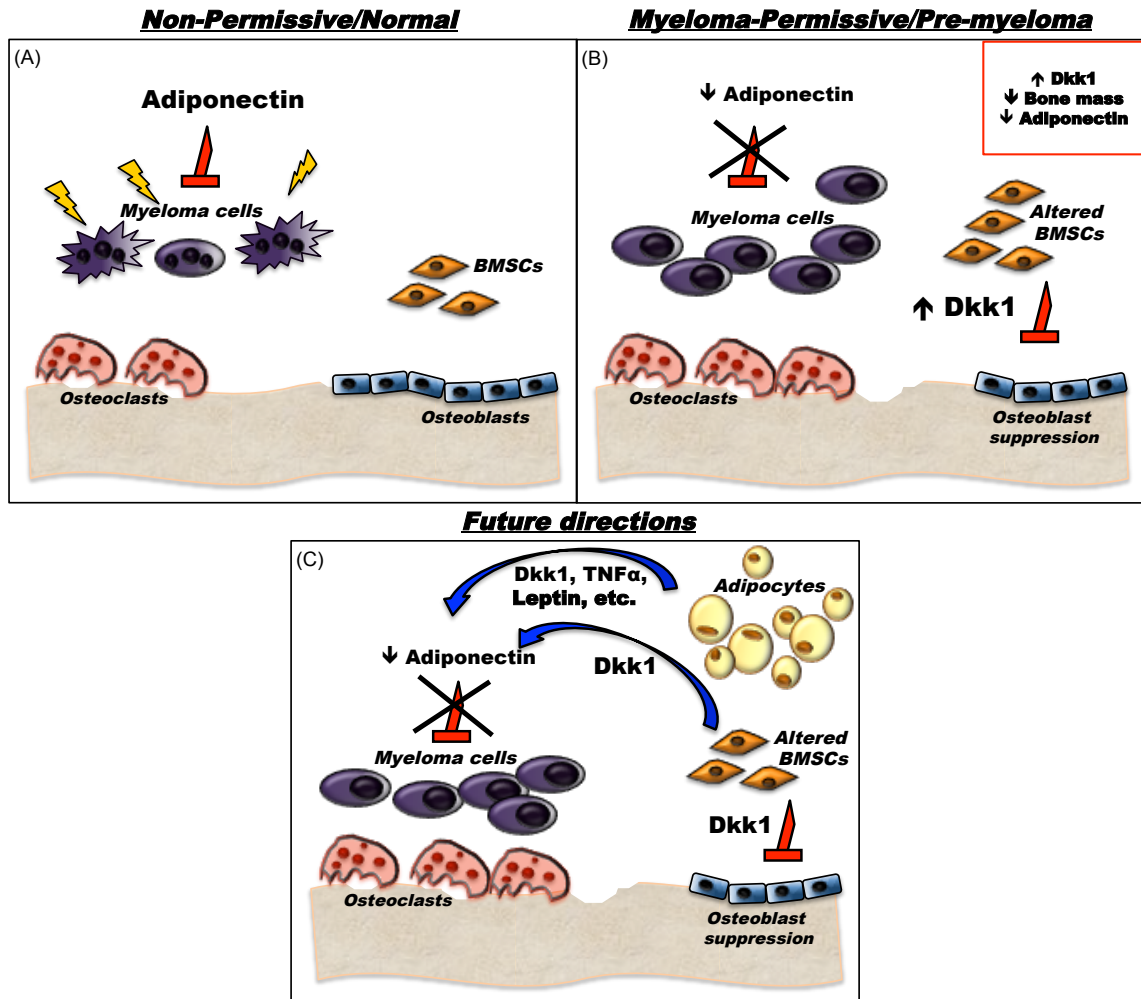


Figure 30. Bone marrow microenvironmental changes that contribute to myeloma pathogenesis. A) In a normal non-permissive bone marrow microenvironment, circulating adiponectin concentrations act to prevent myeloma cell growth. Additionally, the BMSCs within this microenvironment have not become altered and therefore provide the necessary factors for normal bone remodeling and hematopoiesis. B) In a permissive or pre-myeloma bone marrow microenvironment, circulating adiponectin levels are decreased resulting in lack of protection against myeloma progression. Additionally, BMSCs have altered expression of secreted factors such as Dkk1. The increased Dkk1 secretion from the BMSCs can modify the bone marrow microenvironment also making it permissive for myeloma progression. C) Future directions of this work will include investigating the link between Dkk1 and adiponectin. Dkk1 is produced not only by altered BMSCs but also by adipocytes. Preliminary data suggest that elevated Dkk1 can cause a reduction in circulating adiponectin concentrations. Also of interest is examining the role of adipocytes and fat-induced obesity in myeloma

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