# IL-3-MEDIATED OSTEOBLAST INHIBITION IN MULTIPLE MYELOMA

by

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Multiple myeloma is a plasma cell malignancy that localizes to the bone. It is diagnosed in 15,000 new patients per year, making it the second most common hematologic malignancy. The major source of morbidity in these patients is due to bone destruction induced by the myeloma cells leading to severe bone pain and pathologic fractures. Bone destruction in myeloma is mediated by an increase in osteoclast activity, the cells that normally resorb bone, with a concomitant decrease in osteoblast number and function, the cells that normally rebuild bone. The cause of the decrease in osteoblasts is not well understood. Interleukin-3 (IL-3) is upregulated in myeloma compared to normal controls and can mediate osteoclast activation in myeloma. This dissertation investigates the potential role of IL-3 as an osteoblast inhibitor in myeloma. First, IL-3 blocked osteoblast differentiation in a primary murine osteoblast culture system in a dose-dependant manner. Importantly, IL-3-mediated osteoblast inhibition occurred at IL-3 levels present in bone marrow samples from patients with myeloma. IL-3 did not inhibit osteoblast differentiation in cell lines, indicating that the IL-3 effects were not direct. Conversely, IL-3 caused proliferation in $CD45^+$ hematopoietic cells present in the primary murine cultures, and depletion of $CD45^+$ cells from these cultures resulted in a loss of IL-3 inhibition of osteoblast differentiation. Reconstitution of cultures with $CD45^+$ cells resulted in restoration of the ability of IL-3 to inhibit osteoblasts. These $CD45^+$ cells were shown to be $CD11b^+$ and in the monocyte/macrophage lineage. Further studies were conducted into the mechanism of IL-3-mediated osteoblast inhibition. Cell-to-cell contact was required between

osteoblasts and $CD45^+$ hematopoietic cells, and separation of the cell population by transwell cultures abolished IL-3 inhibition of osteoblasts. Transcript levels of several integrins expressed on osteoblasts were not increased by treatment with IL-3, indicating that increased binding of $CD45^+$ cells to osteoblasts is not the mechanism required for osteoblast inhibition. Contact between $CD45^+$ cells and osteoblasts could result in increased expression of a juxtacrine factor that mediates IL-3 inhibition of osteoblasts. In myeloma, IL-3 can mediate proliferation of malignant cells, stimulation of osteoclast activity, and inhibition of osteoblast activity, which ultimately leads to exacerbation of lytic lesions in these patients. Thus, IL-3 is a potential target for myeloma therapy.

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## PREFACE

This work would not have been possible without the help of many people over the years. First, I would like to thank Dr. David Roodman for allowing me to be a part of his lab and for encouraging me in many aspects of my training. He has challenged and supported me to become a successful researcher, and has encouraged my increasing independence in planning and executing all aspects of research including developing ideas and hypotheses to planning and executing experiments to interpreting and presenting results. His ability to wear different hats as a clinician, researcher, educator, and a leader in his field is an inspiration. I hope to have him as a mentor and a colleague as I continue to develop my own career.

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Lastly, I would like to thank my family for being endlessly supportive through a process that seems to never have an end. My father, Ken Ehrlich, has always been encouraging in whatever path I choose and always insists that there is a light at the end of the tunnel. Finally, I believe him. My mother, Joyce, has been endlessly supportive and a source of joy for the whole family. My sisters and brother, Wendy, Cindy, Amy, and Bryan, have been an inspiration to me, and have always tolerated my gross stories at the dinner table (and I think my brother actually enjoys them). Last, but certainly not least, I would like to thank my husband, Sean Fulton, for his support in every aspect of my life. Not only has he always lent an ear, but also he has challenged, encouraged, and helped me to be a better person. And on top of everything, he is a computer expert and has very patiently helped me through many technical problems along the way.

# 1. INTRODUCTION

## 1.1. Thesis Goals and Objectives

Multiple Myeloma (MM) is a plasma cell malignancy which accounts for 10% of all hematologic malignancies. Malignant plasma cells localize to the bone marrow and cause extensive bone destruction in a majority of patients. Bone destruction is mediated by a vast increase in the number and activity of osteoclasts, the normal bone resorbing cells. This increase in osteoclast activity is not followed by an increase bone formation by osteoblasts, the cells that produce bone, to repair the lesions. In fact, osteoblasts are functionally inhibited in early stages of MM disease, and at later stages, both osteoblasts number and function are inhibited. This results in severe bone destruction, which leads to lytic lesions visible by x-ray, pathologic fractures, severe bone pain, and hypercalcemia. Interestingly, when these patients are treated and there is no longer evidence of myeloma in their bone marrow, they still do not repair their lesions. These results suggest either a permanent inhibition of osteoblast activity or a total loss of osteoblast precursors capable of differentiating to bone-forming cells.

The identity of any osteoblast inhibitors in MM has been elusive. Recent studies have suggested Dickkopf1 (DKK1) as a potential inhibitor in MM (Tian et al., 2003). DKK1 inhibits the WNT signaling pathway, which is known to be important in osteoblast differentiation during development (Westendorf et al., 2004). Other candidates in MM are IL-7 (Giuliani et al., 2005)

and IGFBP-4 (Feliers et al., 1999). The role of these potential osteoblast inhibitors is described below.

Recently, our laboratory showed that abnormal transcriptional regulation in myeloma leads to an increase in several cytokines including interleukin-3 (IL-3). We examined the role of interleukin-3 (IL-3) as a mediator of bone destruction in MM. These studies showed that IL-3 was increased in the marrow plasma in 75% of patients with MM. Importantly, IL-3 stimulated both the growth of MM cells and osteoclast activity. The role of IL-3 as an osteoblast inhibitor in MM had not been previously investigated. Therefore, the goal of this dissertation was to establish an in vitro assay for osteoblast function and examine IL-3 as a potential osteoblast inhibitor in MM. I hypothesized that myeloma cells, directly or indirectly, suppress osteoblast differentiation and function through overexpression of cytokines such as IL-3.

This chapter describes normal bone biology, the bone microenvironment in MM, and highlights cytokine mediators of bone destruction in MM, and in particular IL-3 as an osteoclast activator in MM. In chapter 2, I studied the inhibitory effects of IL-3 on osteoblasts and showed these effects were indirect through a second hematopoietic cell. Chapter 3 further examines the mechanism of osteoblast inhibition mediated by IL-3, and I will show evidence that cell-cell contact is required between the hematopoietic cell and the osteoblasts to mediate inhibition. Chapter 4 describes general conclusions from these studies as well as future directions to investigate the actions of IL-3 in MM in vitro and in vivo.

## 1.2. Normal Bone Biology

### 1.2.1. Normal Osteoclast Biology

In the normal adult, bone is continuously destroyed and rebuilt to maintain calcium/phosphate homeostasis, control bone volume in response to mechanical load, and in fracture healing. On a cellular level, bone homeostasis is regulated by the function of osteoclasts (OCL) and osteoblasts (OBL), which mediate bone resorption and formation, respectively. Osteoclasts are derived from hematopoietic cells in the monocyte/macrophage lineage and are the major bone-resorbing cell. OCL precursors fuse to form multinucleated cells, which then differentiate to have bone-resorbing capacity. The major mediator of osteoclastogenesis is receptor activator of NF-kappaB ligand (RANKL), which is expressed on stromal cells/osteoblasts and binds to its receptor RANK on osteoclasts. RANK/RANKL interaction is involved in fusion and activation of OCL (Katagiri and Takahashi, 2002). This interaction can be blocked by osteoprotegerin (OPG), the soluble decoy receptor for RANKL, which acts as a negative regulator of osteoclast formation. Many tumors can metastasize to the bone, and secrete local or systemic osteoclast activators that can either increase fusion of precursors to form more osteoclasts or increase activity of already formed osteoclasts (Roodman, 2004).

### 1.2.2. Normal Osteoblast Biology

Osteoblasts are derived from an undifferentiated, pluripotent mesenchymal cell, which can also give rise to chondrocytes, myocytes, or adipocytes. The major factors involved in driving these pluripotent cells into OBL differentiation are Bone Morphogenetic Proteins (BMPs). BMPs are members of the TGF superfamily (except BMP-1) (Katagiri and Takahashi, 2002). In vivo, BMPs induce ectopic bone formation when de-mineralized bone is implanted into muscle. In vitro, BMPs induce expression of important markers of OBL differentiation including alkaline phosphatase (ALP), type I collagen, and osteocalcin (OC), and stimulate

mineralization. BMPs signal through Smad proteins which are important regulators of BMP function. Signaling through the regulatory Smad proteins (R-Smads) causes BMPs to increase OBL formation, while signaling through the inhibitory Smads (I-Smads) blocks BMP-induced osteoblastogenesis (Katagiri and Takahashi, 2002).

Differentiation of mesenchymal stem cells into osteoblasts and finally to osteocytes is transcriptionally regulated mainly by Runx2/cbfa1. Runx2 target genes include several markers of osteoblast differentiation such as alkaline phosphostase (ALP), type I collagen, and osteocalcin (OC) (Kobayashi and Kronenberg, 2005). Runx2 can interact with several of the Smad proteins and therefore regulates BMP-stimulated osteoblastogenesis. Runx2 deficient mice as well as mice overexpressing Runx2 have defective bone formation, indicating that Runx2 plays a complex role in the regulation of OBL formation. In fact, mice lacking Runx2 are completely deficient in bone formation (Fujita et al., 2004).

### 1.2.3. Osteoclast and Osteoblast Coupling

Bone formation occurs at sites of previous bone resorption, and OBL are recruited to sites of bone resorption in a tightly coupled manner. OCL resorb bone and release factors which cause reactive bone formation; possible candidate coupling factors include TGFβ/BMP family members. Bone coupling results from the following sequence of events: stimulus (e.g. mechanical load), stimulation of stromal cells, osteoclastogenesis, bone resorption, osteoblastogenesis, and bone formation (Manolagas et al., 1996) (see Figure 1.1). Cessation of OCL activity by OBL is likely mediated by TGFβ which then recruits OBL to sites of OCL resorption. Other factors that can cause OBL recruitment in vitro are platelet-derived growth factor (PDGF) or bone matrix fragments released by OCL. PDGF and TGFβ along with other

factors in the bone microenvironment cause proliferation of OBL in vitro. Finally, BMPs and 1,25-dihydroxy vitamin D (1,25-$(OH)_2D_3$) mediate differentiation and mineralization of new bone matrix (Mundy et al., 1995).

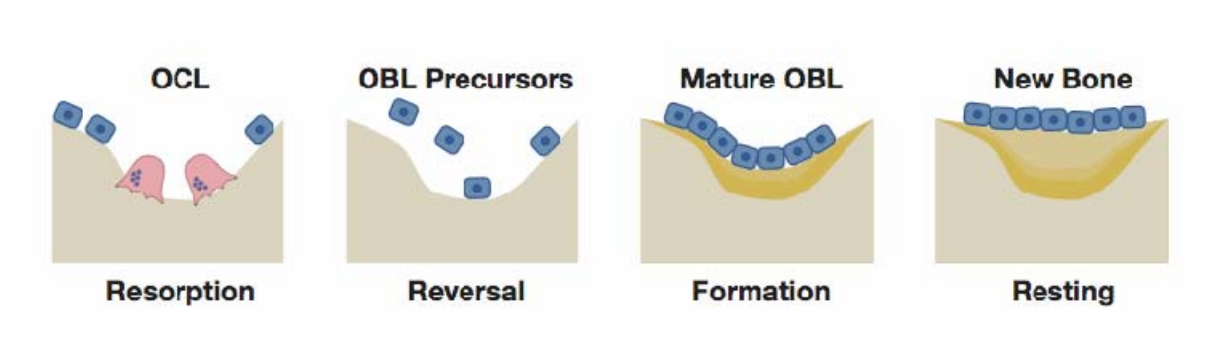



**Figure 1.1: Coupling of osteoclasts and osteoblasts in normal bone remodeling.** Normal bone remodeling occurs in a series of steps. First, osteoclasts are activated and resorb bone matrix. Immature osteoblasts are then recruited to the area where they differentiate into mature and active osteoblasts. Finally, new bone is formed and cells return to their resting state.

### 1.3. Bone Destruction in Multiple Myeloma

Multiple myeloma is diagnosed in approximately 15,000 people per year with a total incidence of 50,000 (Anderson et al., 2002). It accounts for 1% of all malignancies and 10% of hematologic malignancies. The average age at diagnosis is 65. Even with advances in diagnosis and treatment, the survival rates have been relatively unchanged. The median survival is only 4-5 years, and myeloma is uniformly fatal. Current treatment for MM includes high dose chemotherapy and, if possible, allogenic stem cell transplant (Hus et al., 2004). The primary source of morbidity in patients with myeloma is bone pain which is mediated by an increase in

osteoclast activity and a decrease in osteoblast activity leading to lytic bone lesions. Bone lesions are detected in 70-80% of all patients with MM. Treatment with bisphosphonates, which are potent inhibitors OCL activity, can result in slowing progression of bone destruction and can reduce tumor burden mediated by growth factors released from OCL. Treatment with bisphosphonates will not reverse bone lesions in MM as these patients have a defect in osteoblast function as well (Roodman, 2004). Genetics of MM

### 1.3.1. Cytokines Involved in OCL Stimulation in MM

Several malignancies metastasize to the bone, and cause bone destruction via osteoclast activation including breast cancer, lung cancer, and multiple myeloma. Osteoclast activation can result from an increase in systemic mediators of OCL activity such as parathyroid hormone (PTH) and PTH-related protein (PTHrP) or local factors in the bone marrow microenvironment (Roodman, 2004). Several of these factors result in upregulation of RANKL expression or downregulation of OPG, causing an increase in osteoclast formation and activity. The major cytokines that have been identified that directly or indirectly stimulate OCL activity in myeloma are IL-6, tumor necrosis factor-α (TNFα), macrophage inflammatory protein-1α (MIP-1α), and IL-7 and are reviewed below.

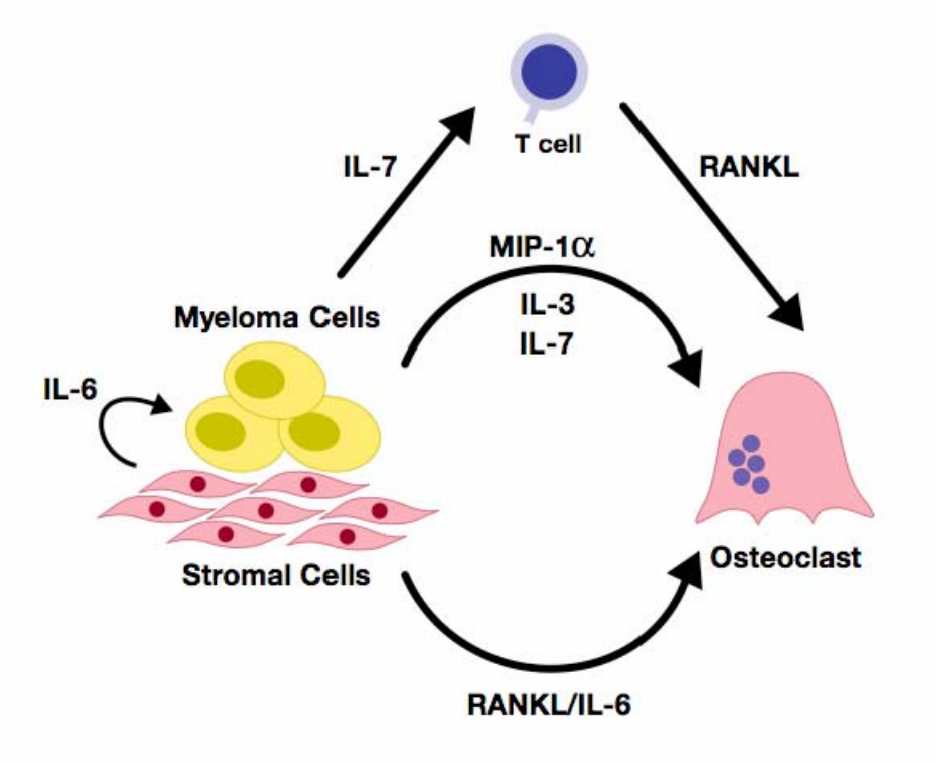



**Figure 1.2: Osteoclast Stimulators in MM.** Factors secreted from myeloma cells that increase osteoclast activation include MIP-1α, IL-3, and IL-7. IL-6 from myeloma cells and myeloma cell binding to stromal cells causes stromal cell upregulation of RANKL and IL-6. RANKL binds to RANK on osteoclasts resulting in an increase in precursor fusion and differentiation. IL-7 released from myeloma cells also causes an increase in RANKL expression in T cells.

#### 1.3.1.1. IL-6

The role of IL-6 in MM bone disease has been controversial. Although several studies have shown a correlation between IL-6 levels with tumor burden in MM (DuVillard et al., 1995; Solary et al., 1992), others have not been able to do so (Choi et al., 2000; Sati et al., 1998). DuVillard et al reported that IL-6 levels in myeloma correlated with tumor burden and Durie & Salmon stage of disease, while the increase in IL-6 in monoclonal gammopathy of unknown

significance (MGUS), a benign increase in immunoglobulin levels that is sometimes a precursor to MM, was related to inflammation (DuVillard et al., 1995). A similar study showed an increase in IL-6 levels in patients with severe osteolytic lesions, whereas myeloma patients without osteolytic lesions had IL-6 levels that were similar to patients with MGUS (Silvestris et al., 2004). Further, Abildgaard et al, reported a correlation between IL-6 levels and markers of bone turnover. This study used serum and urine peptides of collagen cleavage as markers of bone turnover as well as histomorphometric characterization of osteoclast activation. They showed a strong correlation between IL-6 levels in MM marrow samples and serum and urine markers of bone turnover. In contrast, serum levels of IL-6 did not correlate with bone turnover (Abildgaard et al., 2000). This latter point illustrates that bone destruction in MM is local rather than systemic and demonstrates the importance of measuring cytokines in the bone marrow microenvironment.

The source of IL-6 production in myeloma is also controversial. Though a few studies report that IL-6 is expressed by myeloma cells (Sati et al., 1998), most agree that IL-6 is produced by cells in the bone marrow microenvironment in response to contact with MM cells. Marrow stromal cells, osteoblasts, and osteoclasts all can express IL-6 in vitro. Karadag et al showed that primary human osteoblasts increase IL-6 production when cocultured with myeloma cell lines. The increase in IL-6 was greatest when MM cells were in contact with osteoblasts, but could also occur when conditioned medium from myeloma cultures were added to osteoblasts, indicating a soluble factor may be responsible for the effect. They also reported that IL-6 expression is increased in cocultures of MM cells with bone marrow stromal cells, but to a lesser extent than in osteoblast cultures. In this system, normal stromal cells represent a mixture of osteoblasts, adipocytes, and fibroblasts (Karadag et al., 2000). Merico et al cultured stromal

cells from patients with myeloma and were able to detect IL-6 expression in vitro (Merico et al., 1993).

Abe et al demonstrated that OCL also expressed IL-6 when cocultured with MM cells. They used peripheral blood mononuclear cell derived osteoclast cultures that lacked stromal cells and found that IL-6 was also expressed by osteoclasts when in contact with myeloma cells. Since IL-6 is a growth and anti-apoptotic cytokine for MM cells (Anderson et al., 1989), this study suggested that osteoclasts promote the growth and survival of myeloma cells in vitro. This effect requires cell-cell contact between OCL and MM cells, since separation of OCL and MM in transwell cultures abolished the growth stimulatory effects on MM cells. Importantly, the addition of a neutralizing antibody to IL-6 partially inhibited the growth stimulatory effects of OCL on MM cells indicating that IL-6 was acting in a juxtacrine fashion. In this study, OCL supported myeloma cell growth better than stromal cell cultures. It is unclear if IL-6 is enhancing OCL formation in MM by acting on OCL precursors or simply increasing MM tumor burden which in turn increases OCL formation. IL-6 can induce OCL formation in vitro (Kurihara et al., 1990), but it is unclear if sufficient levels of IL-6 are produced to induce OCL formation in MM.

#### 1.3.1.2. TNFα

Several studies have shown an increase in TNFα in patients with MM (Sati et al., 1999), but the role of TNFα in bone disease is unclear. Silvestris et al showed a dramatic increase in TNFα levels in plasma from myeloma patients with severe osteolytic lesions compared to myeloma patients without skeletal involvement or patients with MGUS (Silvestris et al., 2004). However, Abildgaard et al also reported an increase in TNFα levels in 15 of 16 bone marrow

plasma samples from myeloma patients, but were not able to find any correlation with biochemical or histomorphometric indices of bone metabolism (Abildgaard et al., 2000). Thus, although TNFα can induce OCL formation (Kitaura et al., 2004; Nanes, 2003), its role in MM bone disease remains to be defined.

#### 1.3.1.3. T lymphocytes and IL-7

The role of immune cells in the bone destruction associated with multiple myeloma is just beginning to be identified. Recently, Giuliani et al have proposed a role for T lymphocytes in mediating RANKL-induced osteoclastogenesis (Giuliani et al., 2002). The receptor activator of NF-κB ligand (RANKL)/osteoprotegrin (OPG) pathway is critically important in mediating osteoclast (OCL) activation in multiple myeloma (MM) (Pearse et al., 2001). Classically, RANKL is upregulated in stromal cell/osteoblasts in response to MM cell contact. T cell-induced osteoclastogenesis also has been demonstrated in autoimmune arthritis and estrogen deficiency-induced osteoporosis. Takayanagi et al reported that T lymphocytes regulate osteoclastic bone destruction in rheumatoid arthritis by expressing both RANKL which upregulates osteoclast activity, and IFN-γ which is a potent inhibitor of osteoclast activity (Takayanagi et al., 2000). Several studies have shown that IL-7 can mediate bone destruction in post-menopausal osteoporosis by increasing the osteoclast stimulatory activity of T lymphocytes in estrogen deficiency. IL-7 treatment of mice for 20 days results in increased osteoclast activity and was associated with increased levels of B and T lymphocytes. IL-7 did not directly stimulate osteoclast formation in vitro, but required other cells in the bone marrow microenvironment to induce osteoclasts (Miyaura et al., 1997). Toraldo et al clearly showed that although IL-7 increases both B and T cells in the bone marrow, T cells were responsible for the increase in osteoclastogenesis, and B cells alone were not sufficient for increased osteoclast activity.

Athymic nude mice with normal levels of B220+ B cell precursors but lacking in T cells were not sensitive to IL-7 induced bone destruction, and reconstitution of athymic mice with T lymphocytes restored the effects of IL-7 on OCL formation (Toraldo et al., 2003). Weitzmann et al also reported an increase in RANKL expression on T lymphocytes in response to IL-7 and increased secretion of soluble RANKL. Further, the osteoclast stimulatory effects of IL-7-stimulated T cells could be blocked by the addition of OPG, a potent RANKL antagonist (Weitzmann et al., 2000).

In multiple myeloma, binding of MM cells to stromal cells/osteoblasts leads to RANKL overexpression by the stromal cells. This causes a direct upregulation of osteoclast activity by RANKL binding to the RANK receptor on osteoclasts precursors (Pearse et al., 2001). Giuliani et al proposed an alternative pathway for RANKL upregulation of osteoclastogenesis (Giuliani et al., 2002). They showed that myeloma cells also induced expression of RANKL on T lymphocytes by a soluble factor which may contribute to MM-induced bone destruction. MM cells cultured in transwell plates with activated T lymphocytes increased RANKL protein expression in T cell culture supernatants. MM cells also decreased IFN-γ production by T lymphocytes, a potent inhibitor of osteoclastogenesis leading to further stimulation of osteoclast activity. A neutralizing antibody to IL-7 inhibits T cell production of RANKL induced by myeloma cells, indicating that IL-7 is the soluble factor responsible for the T cell activity. Several MM cell lines and highly purified $CD138^{+}$ MM cells from patients express IL-7 in vitro, and IL-7 levels are increased in peripheral blood and bone marrow samples from patients with MM compared to healthy controls. OPG, the RANKL inhibitor, partially but not completely blocks the IL-7/T cell mediated OCL activity indicating RANKL-dependent and –independent pathways (Giuliani et al., 2002).

Colucci et al reported a similar role for T lymphocyte activation in OCL stimulation in MM (Colucci et al., 2004). They demonstrated that in vitro cultures of bone marrow-derived OCL from patients with MM showed decreased survival if T lymphocytes were depleted from these cultures. They also found that T lymphocytes from MM patients overexpressed RANKL. Conversely, they found an overexpression in the RANKL inhibitor, OPG. OPG can also act as a decoy receptor for TNF-related apoptosis-inducing ligand (TRAIL) which is a death signal primarily for tumor cells. This study reported an increase in TRAIL expression effectively sequestering OPG and increasing the RANKL/OPG ratio to favor OCL formation (Colucci et al., 2004).

Thus, T lymphocytes appear to play an important role in MM bone disease. They produce RANKL in response to IL-7 secreted by MM cells. In addition, IL-7 suppresses T-cell production of IFN-γ, which further increases OCL formation.

#### 1.3.1.4. MIP-1α

Using a human myeloma cDNA expression library derived from marrow samples from MM patients, our laboratory identified MIP-1α as a novel osteoclastogenic factor produced by myeloma cells (Choi et al., 2000). ELISA results of freshly isolated bone marrow plasma detected elevated concentrations of human MIP-1α (hMIP-1α) (range 75-7784 pg/ml) in 62% (8/13) of patients with active myeloma, in 3/18 (17%) patients with stable myeloma (range 75-190.3 pg/ml), as well as in conditioned media from 4/5 lymphoblastoid cell lines (LCLs) derived from MM patients. Mildly increased levels of MIP-1α were only detected in 3/14 (21%) patients with other hematologic diagnoses (range 80.2-118.3 pg/ml; median value of 96 pg/ml) and were not elevated in marrow samples from normals (0/7). Furthermore, recombinant hMIP-1α induced OCL formation in human bone marrow cultures, and importantly, addition of a

neutralizing antibody to MIP-1α to human bone marrow cultures treated with freshly isolated marrow plasma from MM patients, blocked the increased OCL formation induced by these marrow plasma samples in 3/5 patients. Anti-MIP-1α had no effect on control levels of OCL formation. Marrow plasma samples from normal controls did not induce OCL formation. Thus, high levels of MIP-1α are present in marrow samples from MM patients with active disease, but not in marrow from patients with other hematologic disorders or normal controls. These data support an important role for MIP-1α as an osteoclastogenic factor in patients with active myeloma. Consistent with our observations is the recent publication by Abe et al, who showed that elevated levels of MIP-1α were present in 15/20 MM patients, and that MIP-1α induced rabbit OCL formation (Abe et al., 2002) and Uneda et al who showed that the expression levels of MIP-1α produced by myeloma cells are correlated with bone lesions in 16 out of 18 MM patients expressing elevated MIP-1α levels (Uneda et al., 2003).

Most importantly, blocking MIP-1α expression *in vivo* decreases bone destruction in an animal model of MM. Alsina et al developed an *in vivo* model of human myeloma bone disease, using the human myeloma derived cell line ARH-77, which could engraft in SCID mice. ARH-77 transplanted SCID mice (ARH-77 mice), but not control mice, that survived irradiation developed hind limb paralysis 28-35 days after injection of ARH-77 cells and became hypercalcemic (1.35 - 1.46 mmol/L), a mean of 5 days after becoming paraplegic. Lytic bone lesions were detected radiographically in all the hypercalcemic mice examined. No lytic lesions or hypercalcemia developed in the control animals. Histologic examination of tissues from the ARH-77 mice showed infiltration of myeloma cells in the liver and spleen, and marked infiltration of myeloma cells in vertebrae and long bones, with loss of bony trabeculae and increased OCL numbers. Human MIP-1α levels were markedly increased in ARH-77 mouse

marrow plasma samples (range 20-5000 pg/ml), but were undetectable in control animals. Other bone-resorbing human and murine cytokines such as IL-6, IL-1α or IL-1β, transforming growth factor-alpha (TGFα), lymphotoxin, PTHrP, HGF, and TNFα were not significantly increased in ARH-77 mouse sera or marrow plasma, compared to control mice, although ARH-77 cells produce IL-6 and lymphotoxiņin vitro (Alsina et al., 1996).

To investigate the role of MIP-1α in myeloma bone disease in vivo, ARH-77 cells were stably transfected with an antisense construct to MIP-1α (AS-ARH) and tested for their capacity to induce MM bone disease in SCID mice. Mice treated with AS-ARH cells lived longer than controls and, unlike the controls, they showed no radiologically identifiable lytic lesions. Importantly, animals infused with empty vector-transduced cells (EV-ARH) developed lytic bone lesions and increased OCL formation. In contrast, animals infused with AS-ARH cells did not demonstrate increased OCL formation or bone resorption. In addition, tumor burden in the bones of animals treated with AS-ARH cells was markedly decreased compared with animals infused with EV-ARH. AS-ARH cells could be detected histologically in the bone marrow sections from animals transfected with the antisense construct to MIP-1α, but they were rare (Choi et al., 2001). Similarly, Oyajobi et al demonstrated that neutralizing antibodies to MIP-1α could block bone destruction by murine myeloma cells in vivo (Oyajobi et al., 2003). These data demonstrated that MIP-1α plays an important role in osteoclast stimulation in myeloma, and blocking MIP-1α has a profound effect on bone destruction and tumor growth.

### 1.3.2. Known Osteoblast Inhibitors in MM

When patients with MM are in remission from their disease and there is no longer evidence of malignant cells in the marrow, these patients do not repair their bone lesions (Anderson et al., 2002). Bisphosphonate treatment will stop bone resorption, but patients will not repair their lesions. Lytic lesions are visible by radiographic imaging or MRI, but bone scanning dose not reveal a defect or underestimate the extent of bone disease. Bone scans detect reactive bone formation by osteoblasts after osteoclastic bone resorption. In MM patients, OBL function is impaired, so bone scans do not readily reveal a defect (Leonard et al., 1981).

MM bone destruction is caused by increased osteoclast number and activity with a concomitant decrease in osteoblastic bone formation. In early stage disease (stages I to II), normal coupling remains intact where an increase in OCL number results in a proportional increase in OBL number. Even in early stages, the increase in OBL number is not associated with a relative increase in OBL function so that a three fold increase in OCL and OBL number is associated with only a one and a half fold increase in bone formation leading to a net bone loss due to loss of OBL functional capacity. In late stage disease (stage III), the amount of eroded bone surface is increased from stage II, but the number of OCLs is not significantly increased. This is caused by an uncoupling of bone remodeling such that OBL number and functional capacity are both decreased relative to the increased number of OCLs (Taube et al., 1992).

Hjorth-Hansen et al also show marked osteoblastopenia in a mouse model of MM. The MM cell line, JJN-3 was injected into irradiated SCID mice which resulted in a 99% reduction in OBL per millimeter bone perimeter and a 70% reduction in bone volume (Hjorth-Hansen et al., 1999) which confirms the uncoupling of OBL activity from OCL action in MM. Silvestris et al showed that OBL derived from patients with MM are more susceptible to apoptosis induced by TNFα (Silvestris et al., 2004). Previous studies showed that myeloma cells secrete a soluble

OBL inhibiting factor (Evans et al., 1989), but very little is known about the identity of these agents that can suppress osteoblast funtion in MM. Possible candidates are DKK1 and other factors in the WNT pathway, IL-7, and other soluble factors. A summary of known osteoblast inhibitors in myeloma is shown in Figure 1.3.

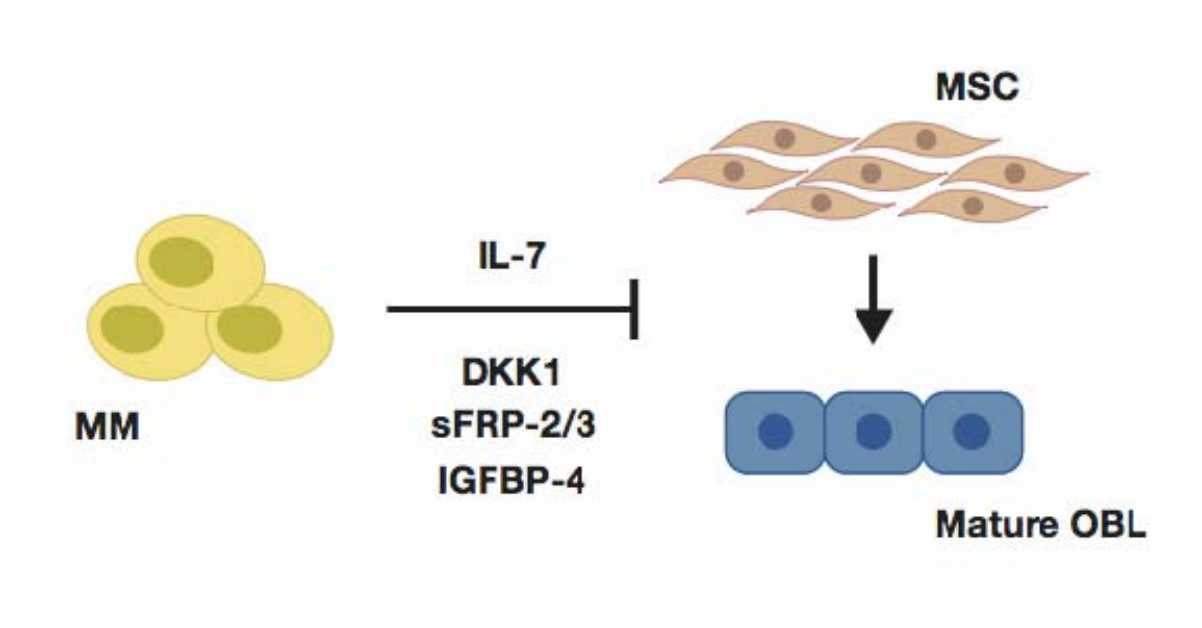



**Figure 1.3: Possible osteoblast inhibitors in MM.** Several soluble factors have been proposed to be osteoblast inhibitors in myeloma. DKK1, sFRP-3/4, and IL-7 are all thought to be secreted from myeloma cells. Differentiation of mesenchymal stem cells (MSC) into mature osteoblasts may be inhibited by these factors.

#### 1.3.2.1. DKK1 and the WNT Signaling Pathway

The WNT signaling pathway is important in the growth and development of osteoblasts as well as functioning in early lymphopoiesis (Westendorf et al., 2004). Classically, WNT binds to LRP5 or 6, another soluble mediator which then binds to the frizzled receptor. Signal transduction from the frizzled receptor results in dephosphorylation and stabilization of β-

catenin. β-catenin localizes to the nucleus and increases expression of target genes. Activation of the β-catenin pathway can lead to activation of OBL differentiation in cell lines in vitro (Bain et al., 2003). There are several soluble inhibitors of the WNT pathway including Dikkopf1 (DKK1) and secreted frizzled receptor-like proteins (sFRP). DKK1 binds to LRP5 and 6, sequestering them from binding to WNT thereby inhibiting proper signaling.

Tian et al investigated the role of DKK1 in osteoblast inhibition in myeloma (Tian et al., 2003). They discovered DKK1 to be upregulated in myeloma plasma cells by microarray analysis, and confirmed its expression by ELISA and immunostaining. DKK1 expression was greater in patients with one or more lytic lesions by MRI compared to MM patient with no lytic lesions indicating a possible role for DKK1 in bone disease. However, they were not able to show a correlation between DKK1 levels and the severity of myeloma. In fact, more aggressive myeloma patients did not express DKK1. As an in vitro model of OBL development, they used the murine mesenchymal cell line, C2C12, which can be committed to the OBL pathway with the addition of BMP-2. OBL differentiation was measured by alkaline phosphatase expression. Recombinant human DKK1 added to BMP-2-treated C2C12 cultures inhibited ALP expression by the C2C12 cells. Importantly, plasma from patients expressing high levels of DKK1 also blocked ALP expression, and this inhibition could be reversed by the addition of a neutralizing antibody to DKK1 (Tian et al., 2003). Further studies have revealed that DKK1 is likely not expressed directly from plasma cells, but rather induced in stromal cells by the presence of malignant plasma cells (Oyajobi et al., 2004).

Contrary to the above findings, Giuliani et al used primary OBL cultures from normal patients, and showed that DKK1 did not inhibit OBL formation in early (14 day, colony forming unit-fibroblast, CFU-F) or late (21 day, colony forming unit-osteoblast, CFU-OB) cultures. In

fact, DKK1 had a slight stimulatory effect on CFU-OB cultures. Similarly, DKK1 did not inhibit Runx2 activity, the primary OBL-specific transcription factor except for a slight inhibitory effect at high levels of DKK1 (Giuliani et al., 2005).

The sFRP family proteins are also soluble inhibitors of WNT signaling. These decoy receptors blocks binding of WNT to the membrane bound receptor, frizzled. sFRP-2 and –3 have also been investigated as possible mediators of OBL inhbition in MM. In preliminary studies, Oshima et al showed that sFRP-2 was secreted by primary myeloma cells and cell lines, and recombinant sFRP-2 inhibited differentiation of the OBL-like cell line, MC3T3-E1. Importantly, conditioned media from MM cell lines inhibited mineralization by in MC3T3-E1 cells, and this was partially reversed by immunodepletion of sFRP-2 (Oshima et al., 2004). FRZB, also known as sFRP-3, has been shown in two studies to be upregulated in malignant plasma cells of patients with MM (Davies et al., 2003; De Vos et al., 2001). Davies et al also showed a downregulation of the downstream effector gene CTNNB1 or β-catenin. The role of sFRP-3 has not been investigated in OBL inhibition in MM. The function of WNT signaling mediators in inhibiting OBL differentiation in MM, including DKK1 and sFRP, remain to be clarified.

#### 1.3.2.2. IL-7

Estrogen deficiency due to ovariectomy resulted in loss of bone mass in a model of osteoporosis. Weitzmann et al reported increased levels of IL-7 in these mice. In this report, they showed that IL-7 also blocked new bone formation after ovariectomy. In mouse calvarial osteoblast cultures, osteoblast activity was suppressed with both basal and BMP2-stimulated treatment (Weitzmann et al., 2002).

Guiliani et al has proposed a role for IL-7 in OBL inhibition in MM. In a recent study, they showed that IL-7 inhibits OBL differentiation in early and late OBL cultures (14 and 21 days, respectively) in a dose-dependant manner. IL-7 also inhibited Runx2 activity in OBL cultures. Importantly, treatment of bone marrow stromal cells with a myeloma cell line or plasma from a patient with MM blocked CFU-F formation at 14 days, and this inhibition was partially reversed by the addition of a neutralizing antibody to IL-7 (Giuliani et al., 2005).

#### 1.3.2.3. Others

A study by Feliers et al investigated the role of insulin-like growth factor (IGF) modulation in MM. IGFs increase bone formation in vitro and in vivo, and their function is modified by IGF binding proteins (IGFBPs) of which IGFBP-2 and –4 are negative regulators. They showed that ARH-77, a myeloma cell line, and plasma cells from four patients tested expressed IGFBP-4. Since IGFs are important in bone formation, they hypothesized that IGFBP-4 overexpression may inhibit bone formation, but they did not test the effects of IGFBP-4 on OBL cells in vitro (Feliers et al., 1999).

TNFα has been shown to inhibit OBL formation in vitro (Abbas et al., 2003; Bu et al., 2003), but since TNFα levels in MM do not correlate with bone disease (see section 1.3.1. above), it is unlikely that TNFα plays a role in OBL inhibition in MM.

## 1.4. IL-3 in MM

Interleukin-3 (IL-3) is a hematopoietic growth factor that causes proliferation of most blood cell lineages, though IL-3 knockout animals have normal hematopoiesis likely due to

redundancy in growth factor functions (Sugita et al., 1999). IL-3 is normally expressed from CD4+ T lymphocytes. The receptor for IL-3 consists of a heterodimer of the IL-3-specific alpha chain and a common beta chain that is also shared with GM-CSF and IL-5 (de Groot et al., 1998). The role of IL-3 in myeloma is only beginning to be understood and is reviewed below.

### 1.4.1. IL-3 Is a Myeloma Growth Factor

Recent studies have demonstrated that IL-3 plays a role in MM. IL-3 levels are increased in marrow and blood of patients with MM. Merico et al demonstrate an increase in protein levels in peripheral blood serum samples in 40% of patients with MM. Serum from patients from MM increased the growth of an IL-3-dependent cell line which could be reversed by the addition of a neutralizing antibody to IL-3 (Merico et al., 1993). Lee et al showed increased IL-3 mRNA expression in 75% patient bone marrow samples from MM patients and an increase in protein levels in bone marrow plasma samples. The mean level of IL-3 in bone marrow was 66.4 +/- 12 pg/mL in myeloma patients vs. 22.1 +/- 8.2 pg/mL in healthy controls (Lee et al., 2004).

In multiple myeloma, IL-3 may act as a growth factor for myeloma cells. Two studies have shown that malignant plasma cell precursors from the peripheral blood of patients with myeloma can form plasma cells in vitro with the addition of IL-3 and IL-6 (Bergui et al., 1989; Goto et al., 1992), implicating IL-3 and IL-6 as a differentiation factors for plasma cell precursors. Conversely, growth of mature myeloma cells is stimulated by IL-3 or IL-6 and the combination does not further increase MM cell growth compared to either cytokine alone (Anderson et al., 1989; Lee et al., 2004). Thus, IL-3 and IL-6 are independent growth factors for myeloma cells.

### 1.4.2. IL-3 Is an Osteoclast Activator in MM

Although some reports using murine marrow disagree (Khapli et al., 2003; Lorenzo et al., 1987), IL-3 stimulates human osteoclast formation in vitro. Fujikama et al reported that IL-3 stimulated formation of multinucleated OCL derived from peripheral blood mononuclear cells. In their system, the effects of IL-3 were mediated by macrophage-colony stimulating factor (M-CSF), since an antibody to M-CSF blocked the OCL stimulatory effects (Fujikawa et al., 2001). Myint et al showed that IL-3 stimulated OCL activation in vivo. IL-3 treatment of osteopetrotic op/op mice corrected the osteopetrosis with an increase in OCL in the femurs of these mice. Further, osteopetrosis reverses with age in op/op mice, and aged mice had increased IL-3 levels in their serum (Myint et al., 1999).

Choi et al showed that overexpression of MIP-1$\alpha$, one of the major OCL stimulators in MM, is caused by abnormal transcriptional regulation by the Acute Myeloid Leukemia-1A and -1B (AML-1A and –1B) transcription factors (Choi et al., 2003). Normally, AML-1A is a truncated form and negative regulator of AML-1B. AML-1A is normally expressed at low levels. In MM, overexpression of AML-1A and suppression of AML-1B leads to abnormally high levels of MIP-1$\alpha$ (Choi et al., 2003). The AML-1 class of genes is also known to regulate expression of several other genes including IL-3 (Nimer et al., 1996; Uchida et al., 1997). Lee et al clearly show that stable transfection of two myeloma cell lines with AML-1A resulted in overexpression of IL-3 as compared to empty vector or AML-1B transfection. Consistent with this, patients with MM who had an increased AML-1A:AML-1B ratio also had increased IL-3 by RT-PCR (Lee et al., 2004).

Lee et al also examined the role of IL-3 in OCL stimulation in myeloma patients. They found that IL-3 induced OCL formation in vitro at levels of IL-3 that were found in marrow

plasma from patients with myeloma. Further, bone marrow plasma samples from patients stimulated OCL formation in vitro, and a neutralizing antibody to IL-3 inhibited OCL formation in cultures treated with marrow plasma that contained high IL-3 concentrations. They found that IL-3 acted early in OCL differentiation (Lee et al., 2004). Barton and Mayer also report that IL-3 increased the number of OCL precursor cells and increased fusion into mature OCL (Barton and Mayer, 1989). These studies confirm the importance of IL-3 as an OCL stimulatory factor in myeloma.

### 1.4.3. IL-3 and Osteoblast Inhibition in MM

Because IL-3 was upregulated in MM, I sought to investigate its potential role in OBL inhibition in MM. In the following chapters, I will provide evidence that IL-3 inhibits the development of OBL in vitro. First I will show that IL-3 inhibits osteoblast differentiation at concentrations found in patients with myeloma using an in vitro model of OBL differentiation. Futher, I will show that IL-3 does not act directly on OBL in vitro, but rather through a secondary hematopoietic cell in the monocyte/macrophage lineage. Lastly, I will provide preliminary data into the mechanism of OBL inhibition by IL-3.

# 2. IL-3 IS A POTENTIAL INHIBITOR OF OSTEOBLAST DIFFERENTIATION IN MULTIPLE MYELOMA

## 2.1. Summary

Bone destruction in multiple myeloma is characterized both by markedly increased osteoclastic bone destruction and severely impaired osteoblast activity. We reported that IL-3 levels are increased in bone marrow plasma of myeloma patients compared to normals and that IL-3 stimulates osteoclast formation. However, the effects of IL-3 on osteoblasts are unknown. Therefore, to determine if IL-3 inhibits osteoblast growth and differentiation, we treated primary mouse and human marrow stromal cells with IL-3 and assessed osteoblast differentiation. IL-3 inhibited basal and BMP-2 stimulated osteoblast formation in a dose-dependent manner, without affecting cell growth. Importantly, marrow plasma from patients with high IL-3 levels inhibited osteoblast differentiation which could be blocked by anti-IL-3. However, IL-3 did not inhibit osteoblast differentiation of osteoblast-like cell lines. In contrast, IL-3 increased the number of $CD45^+$ hematopoietic cells in stromal cell cultures. Depletion of the $CD45^+$ cells abolished the inhibitory effects of IL-3 on osteoblasts, and reconstitution of the cultures with $CD45^+$ cells restored the capacity of IL-3 to inhibit osteoblast differentiation. These data suggest that IL-3 plays a dual role in the bone destructive process in myeloma by both stimulating osteoclast and indirectly inhibiting osteoblast formation.

## 2.2. Introduction

As reviewed in Chapter 1, in advanced multiple myeloma, normal bone remodeling is uncoupled with bone destruction no longer linked to new bone formation (Taube et al., 1992). Thus, MM bone destruction is caused by both increased osteoclast (OCL) numbers and activity with a concomitant decrease in osteoblastic (OBL) bone formation.

The factors involved in mediating OCL activation in MM including RANKL, MIP-1α, IL-6, and IL-7 have been a subject of intense investigation and are reviewed in Chapter 1. In contrast to OCL stimulators, the basis for the decreased OBL activity in MM bone disease has not been clearly defined, and only one inhibitor prior to the studies in this dissertation has been published. Tian et al recently reported that DKK1, an inhibitor of the WNT-signaling pathway, which is involved in osteoblastogenesis, is produced by myeloma cells (Tian et al., 2003). DKK1 gene expression levels correlated with the extent of bone disease in MM. Surprisingly, DKK1 expression was not increased in patients with a more aggressive type of myeloma (Tian et al., 2003). Other potential OBL inhibitors that have been suggested are IL-11, IGF binding protein 4 (IGF-BP4), and soluble Frizzled related protein-2 (sFRP-2). IL-11, which is increased in a subset of MM patients and may block new bone formation, is thought to be secreted from the OBL themselves (Hjertner et al., 1999). IGF-BP4 is also produced by MM cells and may inhibit IGF-I-stimulated osteoblast growth (Feliers et al., 1999). However, neither of these factors are produced by the majority of MM patients. sFRP-2 and sFRP-3, other inhibitors of the *wnt* signaling pathway, have also been reported in preliminary studies to be produced by myeloma cells and partially block OBL differentiation in the murine OBL-like cell line, MC3T3-E1 (Oshima et al., 2004).

We recently reported that IL-3 levels in bone marrow plasma from patients with MM are increased in approximately 70% of patients compared to normal controls. We demonstrated that IL-3 increased osteoclast formation and MM cell growth in vitro (Lee et al., 2004). In the current study, I tested the hypothesis that cytokines overexpressed in the MM marrow microenvironment and are implicated in osteoclastic bone destruction (e.g. RANKL, MIP-1α, and IL-3) may also inhibit osteoblast differentiation in MM. In this chapter, I show that IL-3 inhibited OBL differentiation in vitro in both mouse and human primary osteoblast cultures, and that IL-3 blocked differentiation of pre-osteoblasts to mature osteoblasts in vitro at concentrations comparable to those seen in marrow plasma from patients with MM. Importantly, bone marrow plasma from patients with MM with high levels of IL-3 blocked OBL formation in human cultures, and this inhibition was partially reversed with the addition of a neutralizing antibody to human IL-3. The inhibitory effects of IL-3 were increased in the presence of TNFα, a cytokine induced in the MM marrow microenvironment (Callander and Roodman, 2001), and that the effects of IL-3 were not through increased expression of TNFα. Interestingly, the effects of IL-3 were indirect and were mediated by $CD45^+/CD11b^+$ monocyte/macrophages in both human and mouse primary culture systems. Taken together, these data support a dual role for IL-3 in MM bone disease, acting as both a stimulator of osteoclast formation and an inhibitor of osteoblast differentiation.

## 2.3. Materials and Methods

*Materials*

α-Minimum Essential Medium (αMEM), trypsin/EDTA, Phosphate Buffered Saline (PBS), reverse transcriptase, *Taq* DNA polymerase, and 10x PCR Enhancer were purchased from Invitrogen (Carlsbad, CA). Ascorbic acid, β-glycerophosphate, Triton X-100, Alkaline Phosphatase Yellow (pNPP) Liquid Substrate for ELISA, Bradford reagent, silver nitrate, and sodium thiosulfate were purchased from Sigma (St. Louis, MO). Fetal calf serum was purchased from JRH Biosciences (Lenexa, KS). RNA-Bee was purchased from Tel-Test, Inc. (Friendswood, TX). Anti-mouse β-catenin (C-18) and anti-mouse CD45 (M-20) were from Santa Cruz (Santa Cruz, CA). MTT assay was from the American Type Culture Collection (Manassas, VA). Recombinant mouse IL-3, recombinant human TNFα, recombinant human bone morphogenic protein-2, goat anti-mouse neutralizing antibody, and mouse TNFα ELISA kit were from R&D Systems (Minneapolis, MN). Anti-PE and anti-FITC MicroBeads and LD columns were from Miltenyi Biotec (Gladbach, Germany). PE-conjugated anti-CD45 and FITC-conjugated anti-CD3, anti-CD11b, and anti-CD19 were purchased from BD Biosciences (San Jose, CA). B6D2F1 mice were purchased from Charles River Laboratories (Wilmington, MA).

*Primary mouse stromal cell cultures:*

Primary stromal cells were obtained from 4-6 week old B6D2F1 mice. Whole marrow was flushed from hind-limb long bones and plated in αMEM with 10% heat-inactivated FCS. Marrow from 2 mice was plated per 10-cm dish. After 4 days, non-adherent cells were discarded and adherent cells were washed with media and removed with trypsin/EDTA. Cells were split

1:2 and replated in αMEM with 10% FCS. This cell population, which has been reported to be stromal cell-enriched and monocyte-depleted (Abbas et al., 2003), was used for further experimentation.

Stromal cells were plated at $10^4$ cells/well in a 48-well plate with 0.5 mL osteogenic media with or without additional cytokines. The osteogenic media was αMEM with 10% heat-inactivated FCS, 50 mg/L ascorbic acid and 10 mM β-glycerophosphate. Media was replaced every 3 days with fresh osteogenic media with or without additional cytokines. After 10 days, of culture the cells were assayed for alkaline phosphatase activity as described below. After 3 weeks in culture, the mineral deposition was determined by the von Kossa method as described below.

*Alkaline phosphatase assay:*

Murine stromal cells cultured for 10 days in osteogenic media were washed two times with phosphate buffered saline (PBS) and lysed with 200 μL 0.1% Triton X-100 using one freeze-thaw cycle (-80$^o$C) to ensure complete cell lysis. The cell lysates were transferred to Eppendorf tubes and centrifuged at 12,000 rpm for 3 minutes at 4$^o$C to clear cellular debris. 50μL of cell lysate was transferred to a 96-well plate, and 150μL room-temperature pNPP was added to each well. Optical density at 405 nm was read at time 0 and 30 minutes afterward, and the zero value subtracted from the 30-minute value to asses activity. Alkaline phosphatase (ALP) activity levels were reported as μmol pNPP hydrolyzed per minute per well corrected as percent control. ALP in cell lines was corrected for protein and was reported as μmol pNPP hydrolyzed per min per microgram protein and was corrected as percent control.

*von Kossa staining (for mineral deposition):*

Murine stromal cells cultured for 3 weeks in osteogenic media were washed with cold PBS and fixed in 10% formalin for 15 minutes. Cells were then washed three times with deionized water, leaving the final wash on for 15 minutes. 200 μL of 5% silver nitrate were added to each well. The plates were kept in the dark for 15 minutes then exposed to room light for 1-2 hours until the stained areas turned dark brown/black. Cells were then extensively washed with deionized water. 250 μL of 5% sodium thiosulfate was added for 2 minutes to remove non-specific staining. Cells were again washed 3 times with deionized water and allowed to air dry. Wells with von Kossa positive staining were scanned and imported into Adobe Photoshop (Adobe Systems, Inc., San Jose, CA). Histogram values were obtained from each well and background levels were subtracted.

*Semi-quantitative RT-PCR:*

Total RNA was extracted from the cells with the use of RNA-Bee according to the manufacturer's protocol. RNAs were precipitated with isopropanol, and the pellets washed with 70% ethanol, briefly air dried, dissolved in water and stored at –80$^{o}$C. RT-PCR analyses were performed using the PerkinElmer (Branchberg, NJ) PCR apparatus. After reverse transcription, the PCR was carried out under the following conditions: 94$^{o}$C for 30 seconds, 60$^{o}$C for 30 seconds, and 72$^{o}$C for 1 minute for 20 to 35 cycles depending on relative amount of PCR products for glyeraldehyde-3-phosphate dehydrogenase (GAPDH) detected, which was used as an internal control with the same PCR conditions. PCR for IL-3Rα required the addition of 2x PCR Enhancer. The PCR primers for IL-3Rα, alkaline phosphatase (ALP), osteocalcin (OC), and GAPDH are listed in Table 1.1. The PCR products were sequence analyzed to confirm their

identity. Bands were quantified using ImageJ software (National Institutes of Health, Bethesda, MD) and corrected for GAPDH levels.

| gene | | sequence | size (bp) | annealing temp |
|---|---|---|---|---|
| IL-3Rα | sense | 5'-TGC ACT ACC GGA TGT TCT GG-3' | 636 | 60 |
| | antisense | 5'-ACT TCC TCC ACC ACA GCA GG-3' | | |
| ALP | sense | 5'-AAC CCA GAC ACA AGC ATT CC-3' | 517 | 60 |
| | antisense | 5'-GAG ACA TTT TCC CGT TCA CC-3' | | |
| OC | sense | 5'-GCA GCT TGG TGC ACA CCT AG-3' | 430 | 60 |
| | antisense | 5'-GGA GCT GCT GTG ACA TCC ATA C-3' | | |
| Runx2 | sense | 5'-CCC AGC CAC CTT TAC CTA CA-3' | 308 | 60 |
| | antisense | 5'-CAG CGT CAA CAC CAT CAT TC-3' | | |
| GAPDH | sense | 5'-ACC ACA GTC CAT GCC ATC AC-3' | 451 | 60 |
| | antisense | 5'-TCC ACC ACC CTG TTG CTG TA-3' | | |

Table 1.1. PCR primers

*Western blot*

Whole cell extracts were prepared by adding 200 μL of SDS gel loading buffer to each well of a confluent 6-well plate and separated by SDS-PAGE on 8% polyacrylamide gels and transferred to nitrocellulose membranes. After blocking with 5% skim milk, blots were incubated with anti-β-catenin (1:100) (C-18, Santa Cruz) overnight at 4$^{o}$C, followed by anti-goat IgG conjugated to horseradish peroxidase (1:1000) (Santa Cruz) and visualized by chemiluminescence on x-ray film. Anti-actin-HRP (1:2000) (C-11, Santa Cruz) was used as an internal control on the same membrane after stripping the anti-β-catenin Ab.

*$CD45^{+}$ cell depletion*

Primary mouse or human BM cells were washed two times with PBS. $10^{6}$ cells were resuspended in 45 μL PBS and 5 μL PE-conjugated anti-CD45 antibody was added. The

suspensions were incubated for 15 minutes at 4°C, then washed with bead buffer (PBS with 2 mM EDTA and 0.5% BSA). Cells were resuspended in 80 μL and 20 μL of anti-CD45 bound magnetic beads were added. Cells were incubated with the beads for 30 minutes at 4°C. Columns were wetted with buffer and placed under a magnetic field (MACS, Miltenyi). Cells bound to beads were added to the columns and two column volumes of buffer were added to obtain a flow-through containing CD45 depleted cell population. Non-specific primary antibody bound beads were used as a control. CD45 depleted cells were counted and plated at $10^4$ cells/well in a 48-well plate as usual with or without cytokines. After 10 days culture, ALP activity was determined as above. Efficiency of depletion was determined by flow cytometry as described below.

*Flow cytometry*

Flow cytometry analysis was carried out as previously described (Hentunen et al., 1999). Preparations of $10^6$ or fewer cells were incubated with fluorescein isothiscyanate- or phycoerythrin-conjugated monoclonal antibodies or isotype controls for 20 minutes at 4°C then washed with PBS. Labeled cells were resuspended in PBS for flow analysis. Samples were collected using FACSort (Becton Dickinson) and analyzed with FlowJo software (Tree Star, Inc., Ashland, OR). Four parameters were analyzed: forward scatter, side scatter, and two-color fluorescence intensity. Regions were set using forward vs. side scatter. Ten thousand events were collected for each sample.

*Statistical analysis*

Results are reported as the mean +/- standard deviation for typical experiments done in three or four replicate samples and were compared by the Student *t* test. Results were considered significantly different for p values less than 0.05. All experiments were preformed at least three times to insure reproducibility of the results.

## 2.4. Results

### 2.4.1. IL-3 inhibits OBL differentiation in vitro

Since the effects of IL-3 are species specific, the effect of murine IL-3 on murine OBL differentiation was determined. To determine if osteoblast precursors could respond to IL-3, IL-3Rα expression was measured by RT-PCR and flow cytometry. Primary murine stromal cell/osteoblasts obtained after eight days of culture and induced to differentiate along the osteoblast lineage with the addition of osteogenic media for 10 days expressed IL-3Rα mRNA (data not shown), the receptor subunit that is specific to IL-3 activity.

Since osteoblastic cells expressed IL-3R, we determined the effects of IL-3 on the capacity of these cultures to differentiate into OBLs. Murine stromal cell/osteoblast cultures treated for 10 days with osteogenic media expressed alkaline phosphatase (ALP), a marker of osteoblast differentiation (Aubin, 2001). ALP activity was dose-dependently decreased in murine stromal cell cultures by the addition of murine IL-3 (Figure 2.1A). Maximum inhibition of ALP activity was reached in cultures treated with 1 ng/mL IL-3, which inhibited ALP expression by approximately 80%. Importantly, the dose required to significantly inhibit ALP, 100 pg/mL, was the mean level present in bone marrow plasma of patients with multiple

myeloma (Lee et al., 2004). Another cytokine implicated in myeloma, IL-6 (Callander and Roodman, 2001), had no effect on OBL differentiation, while the known OBL inhibitor, TNFα (Abbas et al., 2003; Bu et al., 2003), also inhibited OBL differentiation in these cultures.

It was then determined if IL-3 could inhibit OBL differentiation in the presence of a potent OBL differentiating agent. The addition of bone morphogenic protein 2 (BMP2) to the osteogenic media in primary murine osteoblast cultures induced OBL differentiation with an increase in ALP expression at 10 days that was seven-fold greater than osteogenic media alone. IL-3 (0.01-10 ng/mL) was still capable of inhibiting OBL differentiation even in the presence of BMP-2 (Figure 2.1B). After 3 weeks in culture, primary osteoblast cultures were stained for mineral deposition by the von Kossa method. Mineral deposition by differentiated osteoblasts was also blocked by IL-3 (Figure 2.1C). Mineralization was also decreased in a dose-dependent manner at IL-3 concentrations detectable in marrow plasma from patients with MM.

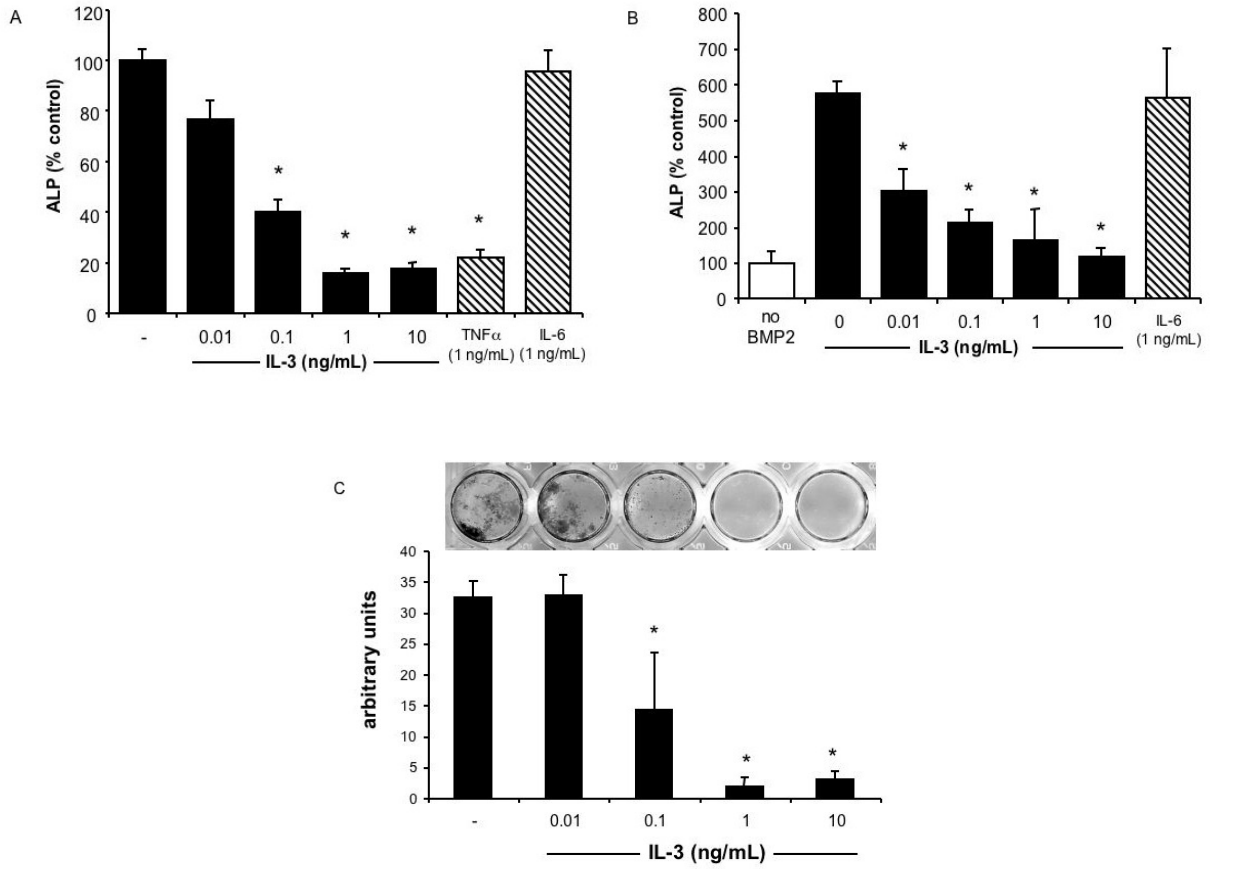



**Figure 2.1: OBL differentiation is inhibited by IL-3.** (A) Primary mouse OBL cells were cultured for 10 days in osteogenic media with or without the addition of IL-3, TNFα, or IL-6. ALP activity was measured as described in Methods. ALP activity was decreased in a dose-dependant manner by the presence of IL-3. TNFα and IL-6 are positive and negative controls, respectively. ALP activity in the untreated control ranged from 40 to 70 milli-units per $10^4$ cells plated. Inhibition by IL-3 ranged from 50 to 80% of control. Results represent the mean +/- standard deviation for 4 wells each concentration, and are representative of three independent experiments. * $p < 0.05$. (B) Cells were treated for 10 days with osteogenic media supplemented with BMP2 (50 ng/mL) with or without IL-3 and ALP activity was measured as described in Methods. BMP2 alone stimulated ALP expression, and was blocked by IL-3. IL-6 is a negative control. (C) Primary murine OBL cells were cultured for three weeks in osteogenic media with or without the addition of IL-3 then stained for mineral deposition by the von Kossa

method as described in the Methods section. Mineral deposition was inhibited in response to IL-3. Shown is a representative well from each concentration. The culture plate stained by the von Kossa method was scanned and quantified for relative amount stained using Adobe Photoshop. Quantification of von Kossa staining shows a dose dependent decrease in amount stained in response to IL-3. In all panels, data are represented as average +/- standard deviation for 4 wells each concentration, and is representative of three independent experiments. * $p < 0.05$.

### 2.4.2. IL-3 treatment of OBL blocks ALP and OC expression, but not β-catenin levels

Differentiation of osteoblasts from immature precursors to mature osteoblasts and finally to osteocytes is characterized by a distinct gene expression profile. OBL initially express high levels of ALP early and low levels of osteocalcin (OC). In contrast, OC is expressed at high levels in mature OBLs and osteocytes (Aubin, 2001). Semi-quantitative RT-PCR analysis demonstrated that IL-3 inhibited both ALP and OC mRNA expression in murine stromal cell cultures (Figure 2.2), consistent with our cell culture results. Interestingly, IL-3 did not inhibit expression of Runx2 mRNA, a transcription factor required for OBL differentiation (Komori, 2002), indicating that IL-3 does not directly inhibit osteoblast differentiation through Runx2 induced transcription, though this does not rule out regulation of Runx2 at the protein level. DKK1, a mediator of the β-catenin pathway, has been implicated in MM bone disease (Tian et al., 2003). Protein levels of β-catenin, the downstream effector of DKK1 (Westendorf et al., 2004), were unchanged by IL-3 treatment mouse OBL cultures (Figure 2.3), indicating that IL-3 does not block the β-catenin pathway.

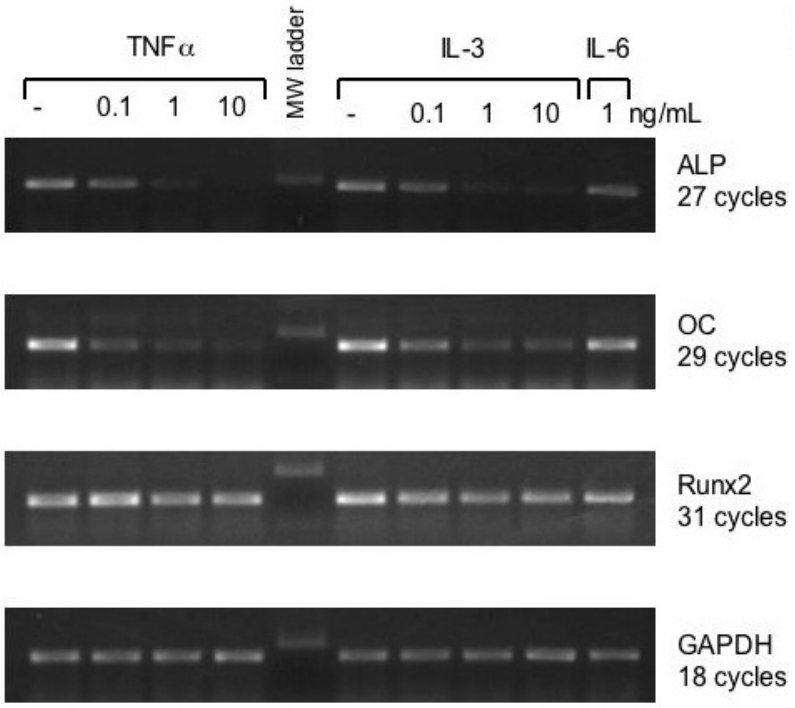



**Figure 2.2: IL-3 inhibits ALP and OC mRNA expression, early and late markers of OBL differentiation into osteocytes, but the inhibitory effects of IL-3 were not mediated by Runx2.** RNA was isolated from primary murine stromal cells cultured for 10 days with increasing concentrations of IL-3. RT-PCR was performed with primers specific for murine ALP, OC, and Runx2. IL-6 was a negative control for ALP, OC, and Runx2 response. GAPDH was used as an internal control.

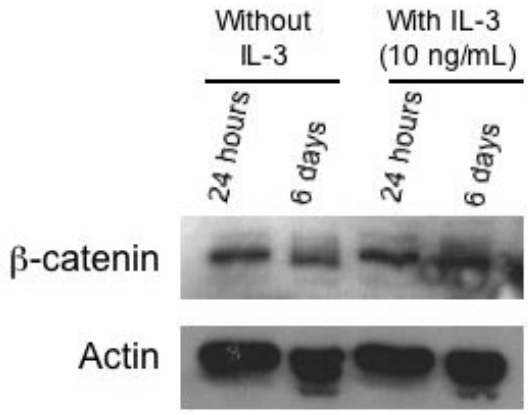



**Figure 2.3: IL-3 treatment does not affect the β-catenin pathway.** Whole cell lysates from murine stromal cell cultures treated with IL-3 for 24 hours or 6 days were prepared and subjected to Western blot analysis for β-catenin expression. β-actin served as an internal control.

### 2.4.3. IL-3 enhances the effects of TNFα on OBL inhibition

To determine if the inhibition of osteoblast differentiation by IL-3 could be enhanced by other cytokines present at low levels in the myeloma microenvironment, we tested combination of TNFα (0.01-10 ng/mL) and IL-3 on stromal cell cultures. As shown in Figure 2.4, low concentrations of TNFα increased the inhibitory effect of IL-3 on OBL differentiation compared to TNFα alone. We added 100 pg/mL IL-3 to cultures treated with increasing concentrations of TNFα (0.01-10 ng/mL). At low levels of TNFα (0.01-0.1 ng/mL), IL-3 and TNFα had an additive effect on lowering ALP expression. At higher levels of TNFα, ALP was fully inhibited, and no additional effects of IL-3 were seen.

### 2.4.4. IL-3 does not inhibit OBL-like cell lines

Since primary marrow stromal cell cultures are a mixed cell population, we then determined if IL-3 was directly inhibiting OBL differentiation by testing the effects of IL-3 on pre-osteoblast cell lines. When MC3T3-E1 cells which are derived from mouse calvarial osteoblasts (Wang et al., 1999) or the C2C12 myoblastic cells (Katagiri et al., 1994) were induced to express ALP with BMP2, the addition of IL-3 did not block ALP expression (Figure 2.5A). In contrast, TNFα inhibits ALP levels in MC3T3-E1 and C2C12 cells (data not shown).

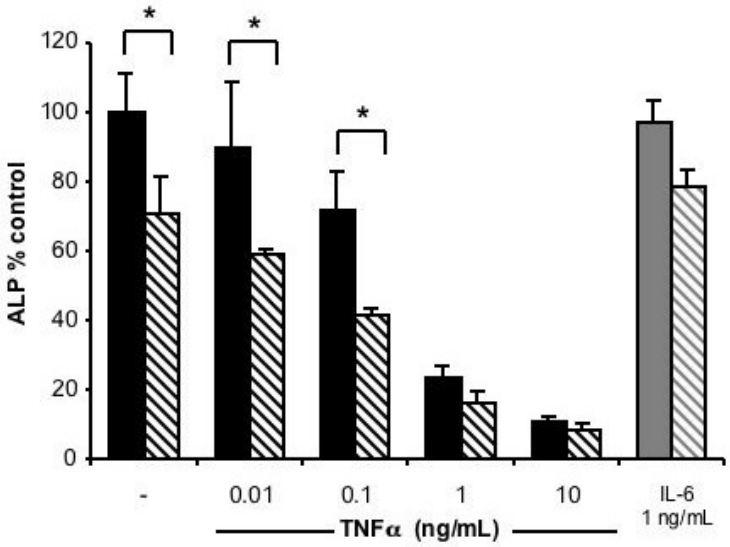



**Figure 2.4: IL-3 increases the capacity of TNFα to inhibit osteoblast differentiation.** Primary murine osteoblast cultures were treated with osteogenic media and increasing amounts of TNFα (0.01-10 ng/mL) without (solid bars) or with (striped bars) IL-3 (100 pg/mL). IL-6 treatment +/- IL-3 (gray and gray striped bars) is a negative control. ALP activity was determined as described in Methods. Data are represented as average +/- standard deviation for 4 wells for each concentration. * $p < 0.05$

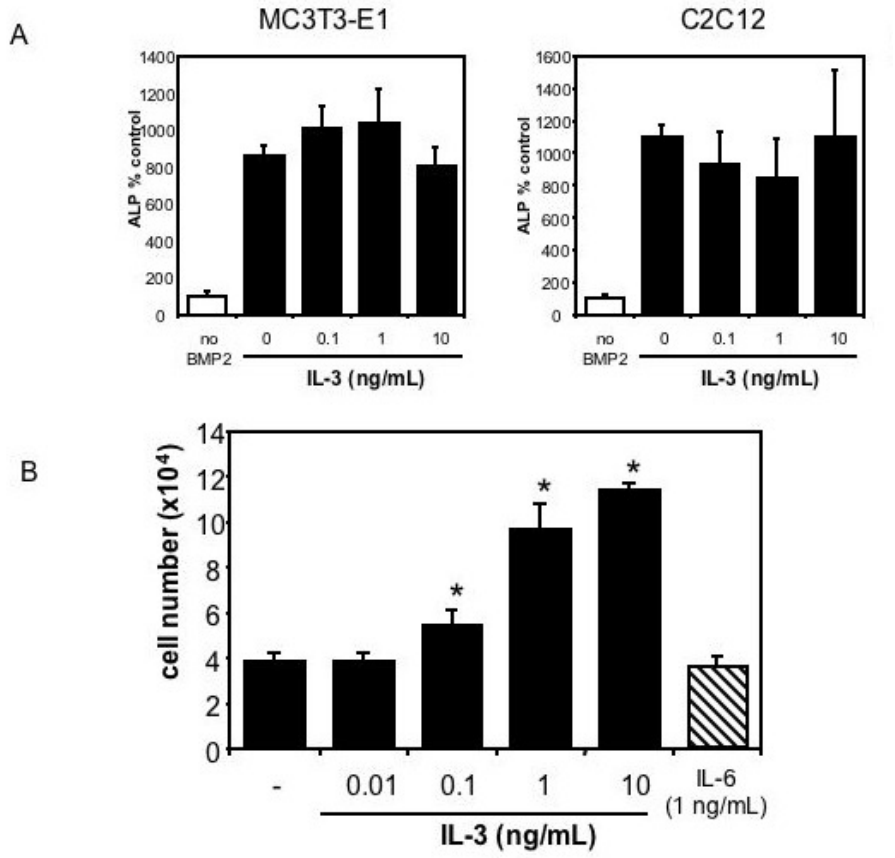



**Figure 2.5: IL-3 does not inhibit ALP levels in osteoblast-like cell lines MC3T3-E1 and C2C12, and increases cell number in primary murine cultures.** (A) MC3T3-E1 or C2C12 were cells treated with 50 ng/mL BMP2 to stimulate OBL differentiation and increasing concentrations of IL-3 (0.1-10 ng/mL). ALP levels measured at 10 days were corrected for protein content and represented as percent control cultures (without BMP2 stimulation). (B) Cell number in primary murine cultures was determined by the MTT assay after a 10-day culture of primary osteoblast cells with or without the addition of IL-3. Low concentrations of IL-3 (0.01-0.1 ng/mL) did not decrease cell number. High concentrations of IL-3 (>1 pg/mL) increased cell number. Data are represented as average +/- standard deviation for 4 wells for each concentration. * $p < 0.05$.

### 2.4.5. $CD45^+$ cells mediate the effects of IL-3

To exclude that IL-3 was simply toxic to the cultures and thus inhibiting osteoblast differentiation, cell growth was measured by the MTT assay. IL-3 did not induce cell death or block cell growth at low concentrations (Figure 2.5B) in murine stromal cell cultures, and at high concentrations (1-10 ng/mL), cell number was increased. I then determined if IL-3 was affecting the cell type distribution in the primary cultures, in particular the percentage of hematopoietic cells since IL-3 stimulates growth of hematopoietic cells (Gianella-Borradori, 1994). Therefore, flow cytometry studies were performed to determine the percentage of $CD45^+$ cells, a marker for hematopoietic cells (Sasaki et al., 2001) that is not expressed on osteoblastic cells in the cultures (Sun et al., 2003; Suva et al., 2004). As shown in Figure 2.6A, thirty percent of the cells in murine stromal cell/OBL cultures were $CD45^+$ cells.

To determine if IL-3 acted through hematopoietic cells to inhibit osteoblast differentiation, $CD45^+$ cells were depleted from both human BM and murine stromal cell cultures, and their response to IL-3 was determined. $CD45^+$ cells were removed from the stromal cells cultures by binding the $CD45^+$ cells to a PE-conjugated anti-CD45 antibody and then, using magnetic beads linked to an anti-PE antibody, depleted on cell separation columns. A non-specific PE-conjugated primary antibody was used as a control. The efficiency of depletion was confirmed by flow cytometry, and was essentially 100% efficient (Figure 2.6A). The $CD45^+$ population was then obtained by washing the remaining cells from the depletion column. This population was approximately 25% $CD45^-$ (Figure 2.6A) and was added back to the CD45 depleted population. The $CD45^-$ cell population and the "add back CD45" population were cultured as before in osteogenic media for 10 days with or without the addition of IL-3. As

shown in Figure 2.6B, $CD45^+$ cell depletion from primary murine OBL cultures abrogated the inhibitory effects of IL-3 on OBL differentiation. Adding back the $CD45^+$ population reconstituted the inhibitory effects of IL-3. The effect of the known OBL inhibitor, TNFα, was not changed by the depletion of murine $CD45^+$ cells (Figure 2.6B).

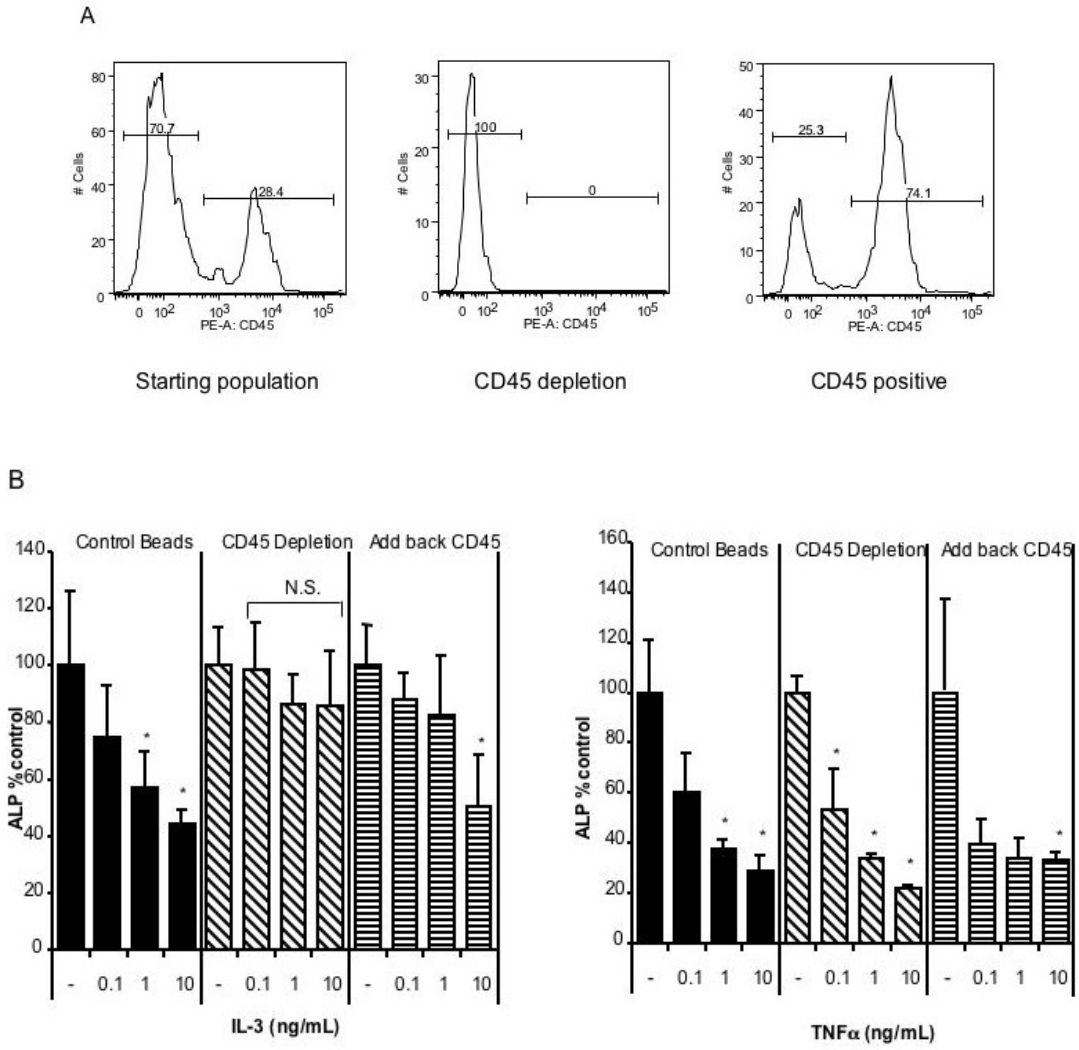



**Figure 2.6: CD45$^+$ cell depletion from primary OBL cultures blocks the inhibitory effects of IL-3.** (A) Murine bone marrow was cultured in αMEM + 10% FCS media for 8 days as described in Methods. These cells were the starting population for further osteoblastic differentiation studies. Flow cytometry analysis shows a PE-CD45$^-$ population of approximately 70% and a PE-CD45$^+$ population of 30%. CD45$^+$ cells were then depleted from the total population by magnetic bead depletion as described in Methods. The resulting population was essentially 100% CD45$^-$ by flow cytometry. The CD45$^+$-enriched population was recovered after depletion and was approximately 25% CD45$^-$ and 75% CD45$^+$. (B) The CD45-depleted or reconstituted cell populations or control cells were plated as usual in osteogenic media with

varying concentrations of IL-3 or TNFα (0.1-10 ng/mL), and ALP activity was determined as usual after 10 days in culture. Solid bars are normal cells, striped bars are cells exposed to control beads, and checked bars are CD45 depleted cells. Depleting $CD45^+$ cells results in a cleaner stromal cell/osteoblast population, so the ALP level per well was approximately two times control antibody depletion. Data is represented as percent of media alone (without IL-3 treatment) for each group and is mean +/- standard deviation for 3 wells each concentration for a typical experiment. Similar results were seen in two independent experiments. * $p < 0.05$, NS = not significant.

### 2.4.6. $CD45^+$ cells are $CD11b^+$

Finally, flow cytometry was used to determine which hematopoietic subpopulations were represented in the $CD45^+$ population. Markers used were CD3, CD11b, and CD19 for T cells, monocyte/macrophages, and B cells, respectively. Figures 2.7A-C show that the $CD45^+$ cells were $CD3^-/CD11b^+/CD19^-$ and belong to the monocyte/macrophage lineage.

### 2.4.7. IL-3 inhibition of OBL is not mediated by TNFα

We next examined if IL-3 inhibited osteoblastogenesis by inducing the secretion of a known osteoblast inhibitory factor such as TNFα (Abbas et al., 2003) by $CD45^+$ cells. ELISA analysis of conditioned media from murine stromal cell cultures did not detect elevated levels of TNFα at days 3, 6, and 9 of culture. TNFα levels were 0-20 pg/mL at all time points. These TNFα levels are lower than required to inhibit osteoblastogenesis in these cultures and were not changed by treatment with IL-3. In addition, neutralization of TNFα in the cultures by the addition of an anti-mouse TNFα antibody did not block the inhibitory effects of IL-3 (data not shown).

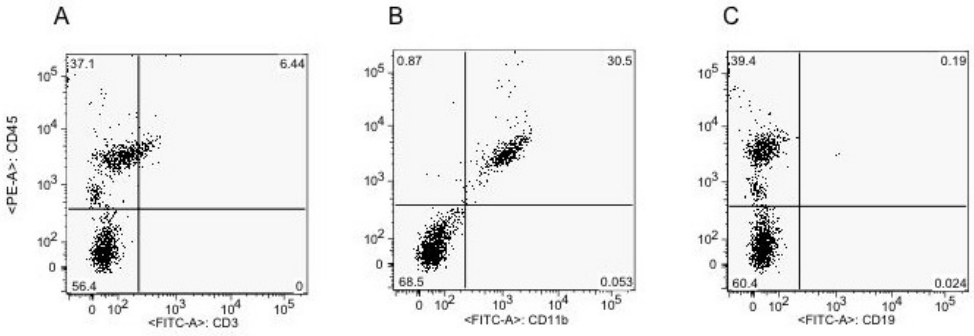



**Figure 2.7: CD45+ cells in murine stromal cell cultures are CD11b$^+$ monocyte/macrophages by two-color flow cytometry analysis.** Mouse bone marrow adherent cells were labeled with PE-conjugated anti-CD45 antibody and FITC-conjugated anti-CD3 (A), anti-CD11b (B), or anti-CD19 (C). CD45$^+$ cells were predominately CD11b$^+$ monocyte/macrophages and negative for T and B cell markers (CD3 and CD19, respectively). Number represents the percentage of cells in each quadrant.

### 2.4.8. Confirming data from our collaborator Nicola Giuliani

(Cattedra di Ematologia, Universita Degli Studi di Parma, Parma, Italy)

Our collaborator was able to perform two important experiments to confirm the effects of IL-3 in myeloma-induced OBL differentiation. Firstly, he tested the effects of IL-3 on human osteoblast differentiation to confirm that the effects were not species specific. Colony-forming unit-fibroblast (CFU-F) and –osteoblast (CFU-OB) were obtained by culture of human bone marrow aspirates maintained for 14 days or 21 days in OBL differentiation media, respectively. Both CFU-F and CFU-OB were significantly inhibited by IL-3 with a maximal effect at 20 ng/ml (CFU-F vs. control: -70%, CFU-OB vs. control: -40%; $p<0.05$) (data not shown). Similar to our experiments in mouse, $CD45^+$ cell depletion of primary human BM cultures significantly decreased the inhibitory effect of IL-3 on CFU-F formation indicating that IL-3 is working by the same indirect mechanism in human as well as mouse cultures.

Secondly, to test the potential involvement of IL-3 in the inhibition of osteoblast bone formation in MM, he determined the effects of BM plasma obtained from MM patients on osteoblast differentiation. BM plasma with low and higher IL-3 levels (range: less than 5 pg/ml or greater than 50 pg/ml) were tested. BM plasma significantly inhibited CFU-F formation in BM cultures. The addition of a neutralizing monoclonal antibody to IL-3 significantly decreased the inhibitory effects of BM plasma from patients with high IL-3 (>50 pg/ml) on CFU-F formation, but had no effect on BM plasma with low IL-3 levels. A similar effect was observed on CFU-OB formation (data not shown).

## 2.5. Discussion

MM bone disease results from both osteoclastic bone destruction and decreased or absent OBL activity. Both histologic and isotopic scanning studies have demonstrated that OBL activity is decreased or absent at the sites of bone destruction and that bone formation is suppressed in MM patients (Leonard et al., 1981). However the basis for decreased OBL activity is unknown. In the current study, I tested the hypothesis that cytokines/chemokines produced by MM that increase OCL formation also decrease OBL differentiation. The major mediators of OCL activity in MM that are known include RANKL, MIP-1α (Callander and Roodman, 2001), and IL-3 (Lee et al., 2004). Others have demonstrated that RANKL does not inhibit OBL differentiation but in fact may increase OBL growth and differentiation (Lam et al., 2002). In preliminary studies, I previously found that MIP-1α or RANKL by itself did not block OBL differentiation (unpublished results) even though stromal cell/OBLs express the CCR5 receptor for MIP-1α (Abe et al., 2002), and $CD45^+$ cells present in the stromal cell population express RANK (Kang et al., 2004). Therefore I focused my studies on IL-3. Lee et al (Lee et al., 2004) reported that 75% of patients with MM have increased IL-3 levels with a median value of 66.4 pg/mL compared to 22.1 pg/mL in healthy controls.

To determine the effects of IL-3 on OBL activity, we utilized both primary human and murine stromal cell systems. Since the IL-3 protein is not homologous between human and murine systems, we used both to determine if the effects of IL-3 on OBL differentiation were generalizable between species and if future studies in murine models of myeloma were feasible. In these studies, OBL differentiation in both human and murine cultures was significantly inhibited in vitro by 100 pg/mL of IL-3, comparable to the levels seen in patients with myeloma.

Further, bone marrow plasma from patients with MM with high IL-3 inhibited OBL differentiation in human CFU-F cultures. The inhibitory effects of plasma from patients with high IL-3 were partially reversed by the addition of a neutralizing antibody to human IL-3. In contrast, the inhibitory effects of marrow plasma from patients with normal IL-3 levels were unchanged by the addition of anti-IL-3.

These results show that, unlike other inhibitors of OBL differentiation that act directly on OBL precursors, IL-3 acts indirectly via monocyte/macrophages. As shown in Figure 6A, $CD45^+/CD11b^+$ hematopoietic cells were present in these primary stromal cell cultures. Importantly, depletion of the $CD45^+$ cells in primary OBL cultures blocked the capacity of IL-3 to inhibit OBL differentiation. Importantly, reconstitution of the $CD45^-$ cultures with $CD45^+$ cells restored the capacity of IL-3 to inhibit OBL differentiation. Consistent with IL-3 acting indirectly to block OBL differentiation, IL-3 did not inhibit differentiation of the pre-osteoblast cell lines, MC3T3-E1 or C2C12 (Figure 5A), even though they express IL-3Rα by RT-PCR (data not shown).

Other factors present in the MM marrow microenvironment also can enhance the inhibitory effects of IL-3 on OBL differentiation. Low concentrations of TNFα increased the inhibitory effects of IL-3 on OBL differentiation (Figure 4). TNFα levels in myeloma have been reported to be increased, but there is no correlation between TNFα levels and severity of bone disease in MM (Hideshima et al., 2001; Sati et al., 1999). These results suggest that the low levels of TNFα found in patients with myeloma that would normally not be sufficient to inhibit OBL differentiation may inhibit OBL differentiation when IL-3 is present. The effects of IL-3 on OBL differentiation are not simply a result of increased TNFα production by monocyte/macrophages responding to IL-3 since TNFα levels were not increased in vitro by the

addition of IL-3, and the inhibitory effects of IL-3 were not blocked by the addition of anti-TNFα neutralizing antibodies (data not shown).

IL-3 by itself does not fully account for the inhibition in OBL differentiation in MM. As shown by the studies with marrow plasma from MM patients with high IL-3 levels, anti-IL-3 only partially reversed the inhibitory effects of IL-3 on OBL differentiation. Recently Giuliani et al have shown a potential role for IL-7 as an OBL inhibitor in MM (Giuliani et al., 2005). These data suggest other OBL inhibitors, such as DKK1, sFRP-2 or -3, or IL-7 may act in conjunction with IL-3 to suppress OBL activity in MM.

Thus in MM there appears to be at least two classes of OBL inhibitors: one that acts directly on OBL such as DKK1 or sFRP-2 or -3, and one that appears to act indirectly, IL-3. IL-3 may act as a bifunctional mediator of MM bone disease, both increasing OCL formation and suppressing OBL differentiation. The identity of the factor(s) induced by IL-3 or its mechanism of action is, as yet, unknown. However, IL-3 does not affect Runx2 expression or DNA binding activity, suggesting that it may affect other signaling pathways involved in OBL differentiation. In preliminary experiments, IL-3 treatment, in contrast to DKK1, did not directly affect the WNT signaling pathway in stromal cell cultures, suggesting that the IL-3 induced inhibitor has a different mechanism of action.

# 3. STUDIES OF THE MECHANISM OF IL-3 SUPPRESIVE EFFECTS ON OSTEOBLASTS

## 3.1. Summary

IL-3 is involved in several aspects of bone destruction in multiple myeloma. IL-3 stimulates osteoclastic bone resorption, and is a growth factor for myeloma cells. IL-3 also inhibits osteoblastic bone formation at levels present in the bone marrow of patients with myeloma. This study sought to investigate the mechanisms underlying IL-3-mediated osteoblast inhibition. I previously showed that osteoblast inhibition by IL-3 was indirect through $CD45^+/CD11b^+$ monocyte/macrophages present in primary mouse osteoblast cultures. Here I show that a minimum of 9% $CD45^+$ cells is required to mediate these effects and it is cell dose dependent. I also show that cell-to-cell contact is required between $CD45^+$ hematopoietic cells and $CD45^-$ osteoblast precursors. Finally, I show that IL-3 is not the only hematopoietic stimulator that can cause osteoblast inhibition, as GM-CSF also can inhibit osteoblastogenesis.

## 3.2. Introduction

Osteoblast inhibition in multiple myeloma is a poorly understood phenomenon. The few candidates that have been implicated in this inhibition are IL-3, DKK1, and IL-7. I have shown

in Chapter 2 that IL-3 inhibits osteoblast differentiation in myeloma. This study focuses on the potential mechanisms responsible for IL-3-mediated inhibition of osteoblasts.

$CD45^+$ hematopoietic cells are required for IL-3-mediated inhibition. CD45 is a glycoprotein that is expressed on all nucleated hematopoietic cells (Donovan and Koretzky, 1993). Hematopoietic cells can be further divided by the presence of surface markers specific to each lineage. Major sub-classes of non-granulocytic hematopoietic cells include CD3, CD11b, and CD19 which are markers for T lymphocytes, monocyte/macrophages, and B lymphocytes, respectively. We previously showed that the $CD45^+$ cells present in primary murine osteoblast cultures are responsible for the actions of IL-3 and are $CD11b^+$. Cells in this lineage include monocytes, which are present in peripheral blood, tissue macrophages, osteoclasts, dendritic cells, and natural killer cells.

Potentially, adhesive molecules are important in mediating the interaction between $CD45^+$ hematopoietic cells and $CD45^-$ osteoblasts. Integrins are responsible for cell-to-cell communication as well as cell-to-matrix binding. Integrin $\alpha$ and $\beta$ subunits form heterodimers to become functional adhesion molecules (Elangbam et al., 1997). Several integrins are expressed on osteoblasts including $\alpha5\beta1$ which binds to the extracellular matrix (Luthen et al., 2005; Yang et al., 2005). The nature of the $CD45^+/CD11b^+$ cells is unknown, so it is difficult to predict which integrins may be important. The alpha class includes 15 integrins, and the beta class has 8. Here we screened several integrin subunits including $\alpha5\beta1$ for regulation by IL-3 in murine osteoblast cultures. In this study, I hypothesized that interactions between $CD45^+$ hematopoietic cells and $CD45^-$ osteoblasts either via cell-to-cell contact or a soluble factor is responsible for osteoblast inhibition mediated by IL-3.

## 3.3. Methods

*Semi-quantitative RT-PCR*

Primary mouse bone marrow cultures were plated at $5x10^5$ cells per well in a 6-well plate. For time course experiments, RNA was isolated using RNA-Bee (Tel-Test, Inc, Friendswood, TX) as previously described (Section 2.3.) every day for days 1-10. For integrin expression, RNA was isolated after 10 days in culture. RNA was reverse transcribed using Super Script II Reverse Transcriptase (Invitrogen, Carlsbad, CA) following the manufacturers instruction. Polymerase chain reaction (PCR) was carried out as previously described (See section 2.3.) using primer sets for integrins in Table 1. Primer sets for alkaline phosphatase (ALP) and osteocalcin (OC) are listed in Chapter 2, Table 2.1. Primer sets for integrins are listed below in Table 3.1. Sources for integrin primer sets are as follows: α3 and α5, (Sutherland et al., 1993); α4 and β1, (Bowen and Hunt, 1999); β5, (Zhang et al., 1998).

| gene | | sequence | size (bp) | anneal temp |
|---|---|---|---|---|
| α3 | sense | 5'-ATT GAC TCA GAG CTG GTG GAG GAG-3' | 516 | 58 |
| | antisense | 5'-TAC TTG GGC ATA ATC CGG TAG TAG-3' | | |
| α4 | sense | 5'-TCC AAA AAT CCC CTA TCC TCT C-3' | 440 | 58 |
| | antisense | 5'-AAG CCA TCC TGC TGC AAA C-3' | | |
| α5 | sense | 5'-CTG CAG CTG CAT TTC CGA GTC TGG-5' | 275 | 58 |
| | antisense | 5'-GAA GCC GAG CTT GTA GAG GAC GTA-3' | | |
| β1 | sense | 5'-TGT TCA GTG CAG AGC CTT CA-3' | 452 | 58 |
| | antisense | 5'-CCT CAT ACT TCG GAT TGA CC-3' | | |
| β5 | sense | 5'-GCG AAA AGA TGC TCT GCA-3' | 600 | 58 |
| | antisense | 5'-GCC GCA TGT GCA ATT GTA-3' | | |
| GAPDH | sense | 5'-ACC ACA GTC CAT GCC ATC AC-3' | 451 | 60 |
| | antisense | 5'-TCC ACC ACC CTG TTG CTG TA-3' | | |

Table 2.1. Integrin PCR primers

*CD45 depletion experiments*

Total bone marrow was isolated from normal B6D2F1 mice, and cultured for 8 days in αMEM and 10% fetal calf serum as described in Chapter 2. Adherent cells from these cultures were then subjected to CD45-depletion or control-depletion using columns as described above. The $CD45^-$ population was plated in 48-well plates at a concentration of $1x10^4$ per well. The $CD45^+$ population was added to the wells in varying ratios ranging from 1:10 (CD45 neg:pos) to 1:1. All cell subsets were subjected to single-color flow cytometry as described in Chapter 2 to determine the percentage of $CD45^+$ cells in each group. From this percentage, the relative percentage of CD45 positive and negative cells in each well was calculated. Cells were cultured for 10 days in osteogenic media (αMEM, 50 mg/mL ascorbic acid, 10 mM β-glcerophosphate, and 10% FCS) with or without the addition of IL-3 (0.1-10 ng/mL). Alkaline phosphatase activity was measured as previously described (Chapter 2). Alkaline phosphatase (ALP) activity levels are reported as μmol pNPP hydrolyzed per minute per well corrected as percent control.

Transwell experiments used a 12-well plate with 0.4 micron pores. Cells were plated at $2x10^4$ cells per well. $CD45^-$ cells were plated in the lower chamber of a transwell plate and $CD45^+$ cells were plated in the upper chamber at a 1:1 ratio. IL-3 was added to the top wells, but could diffuse through the pores. Cells were cultured as usual in osteogenic media for 10 days, and ALP activity was determined for the $CD45^-$ population in the lower well.

In "CD45 primed" experiments, the $CD45^-$ and $CD45^+$ populations were plated in separate 48-well plates. $CD45^+$ cells were then treated with or without IL-3 (10 ng/mL) for 6 days. After 6 days, $CD45^+$ cells were trypsinized and removed from the plate using a cell scraper. $CD45^+$ cells were counted and the IL-3 "primed" population or "unprimed" population was added to the initial $CD45^-$ cells and cultured for an additional 6 days, or to a plate of freshly

prepared adherent bone marrow cells and cultured for 10 days in osteogenic media without additional cytokines. After this time period, ALP levels were assessed as previously described.

*Conditioned media*

Adherent bone marrow cells were cultured as usual in a 48-well plate in osteogenic media with the addition of IL-3 (10 ng/mL). At days 3, 6, and 9, conditioned media from these cultures were collected. Conditioned media were filtered through a 0.22 micron filter to ensure sterility. Day 6 conditioned media was added to freshly isolated adherent cell cultures at concentrations of 10%, 20%, and 30% in osteogenic media. A neutralizing antibody to IL-3 (R&D Systems, Minneapolis, MN) was added to ensure that residual IL-3 in the conditioned media was not inhibiting osteoblast formation. Media was changed with the addition of new conditioned media every three days as usual. After 9 days in culture, ALP activity was assessed as described above.

*Cytokine treatment of OBL cultures*

Adherent bone marrow cells were plated in 48-well plates at a concentration of $1x10^4$ cells per well in osteogenic media. GM-CSF, MIP-1$\alpha$, or IL-6 was added as indicated at 0.01-10 ng/mL. Soluble IL-6R was added at a fixed concentration of 10 ng/mL to cultures treated with IL-6. RANKL was added at 5-100 ng/mL. All cytokines were obtained from R&D Systems (Minneapolis, MN). Media was changed every three days with fresh cytokines added to the media. After 10 days of culture, ALP activity was assessed as described above.

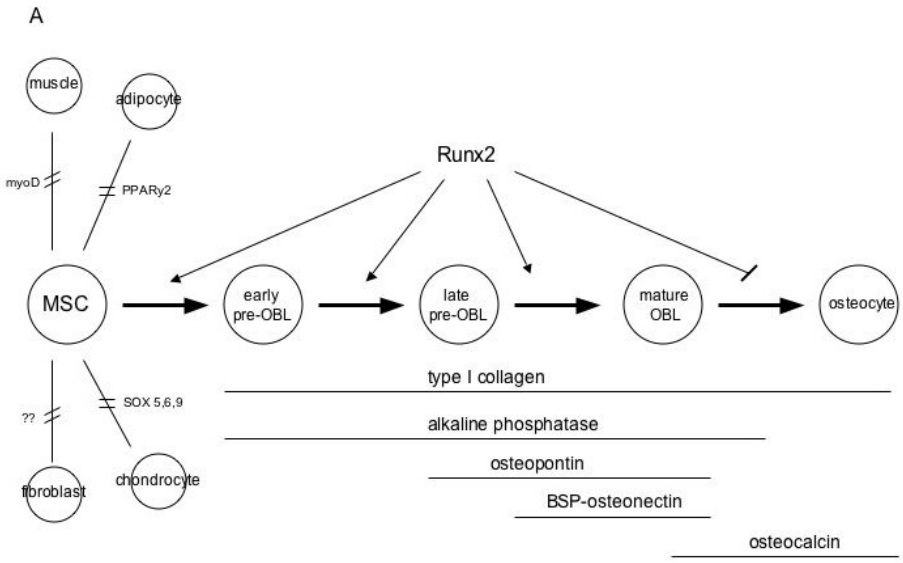
*Statistical analysis*

Results are reported as the mean +/- standard deviation for typical experiments done in three or four replicate samples and were compared by the Student *t* test. Results were considered significantly different for p values less than 0.05. All experiments were preformed at least three times to insure reproducibility of the results.

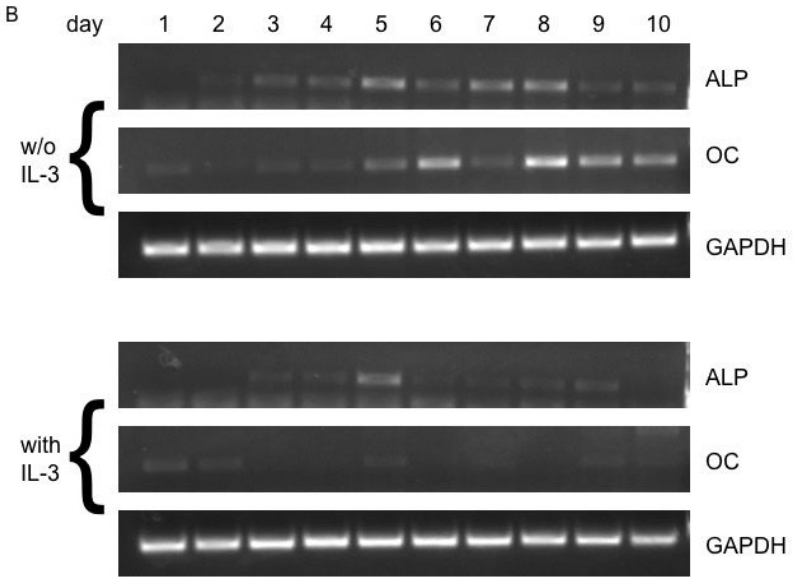
## 3.4. Results

### 3.4.1. IL-3 blocks early OBL differentiation

Osteoblasts progress through several stages of differentiation from mesenchymal stem cells to mature osteoblasts and finally to osteocytes. Early osteoblasts are identified by an increase in alkaline phosphatase (ALP) while late osteoblasts are identified by osteocalcin (OC) activity (Aubin, 2001) (Figure 3.1A). ALP and OC levels were assessed by semi-quantitative RT-PCR. In the 10-day primary mouse OBL culture system, both ALP and OC were undetectable in the early stages of the cultures. ALP peaked at approximately 5 days and then decreased, but was still detectable at day 10. OC peaked at 8 days of culture and remained elevated to the end of the culture period (Figures 3.1B and C). IL-3 blocked OBL differentiation at an early stage, so that ALP expression peaked at day 5 at a low level and then plummeted. OC levels never increased in the cultures treated with IL-3 (Figure 3.1B and C).




Adapted from: Aubin, 2001



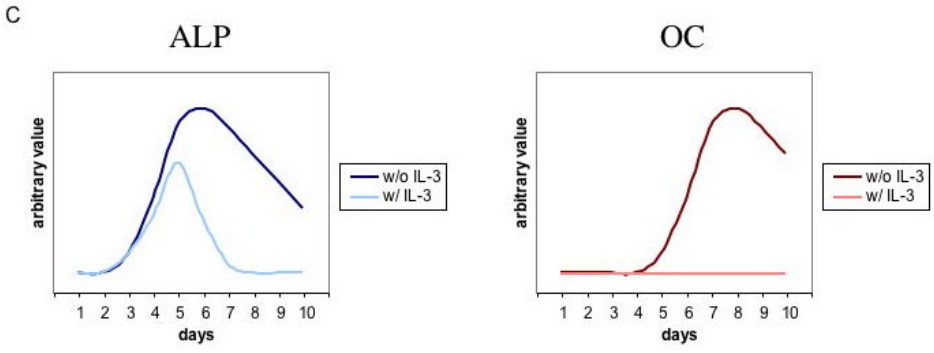



**Figure 3.1: IL-3 inhibits OBL differentiation by day 5 in primary mouse bone marrow cultures.** (A) Osteoblasts are derived from mesenchymal stem cells (MSC) and go through several stages of differentiation. Pre-OBL are identified by high ALP levels and low OC while mature OBL have high OC and decreasing ALP. (B) RNA was isolated every day in a 10 day OBL culture without (top) or with (bottom) the addition of IL-3. Semi-quantitative RT-PCR was carried out using primers specific for ALP or OC. GAPDH levels were measured to ensure that each sample had the same amount of RNA. (C) Relative levels of ALP and OC were approximated from the intensity of the bands in B. Dark blue represents ALP levels without IL-3, and light blue is with IL-3. Dark red and light red represent OC levels without and with IL-3, respectively. Graphs are not exact values, but rather an approximation based on the trends in PCR intensity.

### 3.4.2. IL-3 inhibition of OBL is $CD45^+$ cell dose dependent

We previously determined that the inhibitory effects of IL-3 are indirect in the mouse and human in vitro OBL culture systems and mediated through $CD45^+$ hematopoietic cells (Chapter 2). Figure 3.2A shows flow cytometry analysis of the percentage of $CD45^-$ and $CD45^+$ cultures in each population when CD45 cells were depleted using magnet-associated antibody depletion columns. The starting population was 30% $CD45^+$. Control depletion experiments without the primary antibody revealed that approximately 70% $CD45^+$ cells adhered to the columns indicating some non-specific binding to the column leaving a final CD45+ concentration of 9%. $CD45^+$ cell depletion with the anti-CD45 primary antibody was complete, with 100% $CD45^-$ cells in the cell population that passed through the column. The $CD45^+$ cell population that was recovered from the column was less pure and was comprised of 30% $CD45^-$ and 70% $CD45^+$ cells. The $CD45^+$ cell population was added back to the $CD45^-$ population at a ratio of 10:1 (CD45 neg:pos) and increased stepwise to a 1:1 ratio. The final concentration of $CD45^+$ cells was calculated and is reported in Figure 3.2B. When $CD45^+$ cells were depleted from OBL cultures, the inhibitory effects of IL-3 were lost. Interestingly, when $CD45^+$ cells were depleted and 100% of the cells cultured were $CD45^-$, ALP levels dropped by approximately 50% compared to the starting population indicating that hematopoietic cells may have the ability to support OBL growth. Adding back the enriched $CD45^+$ cell population that was contaminated with 30% $CD45^-$ cells resulted in an increase in total OBL cells in the cultures, and an increase in the ALP levels observed. With the addition of IL-3, ALP levels were decreased significantly when $CD45^+$ cells comprised greater than 9% of the total cell population. Figure 3.2C shows results with IL-3 treatment as a percentage of ALP present in cultures without IL-3. Clearly,

when $CD45^+$ cells were added back to the $CD45^-$ cultures in increasing concentrations, ALP levels were inhibited by IL-3 treatment in a cell dose dependent manner.

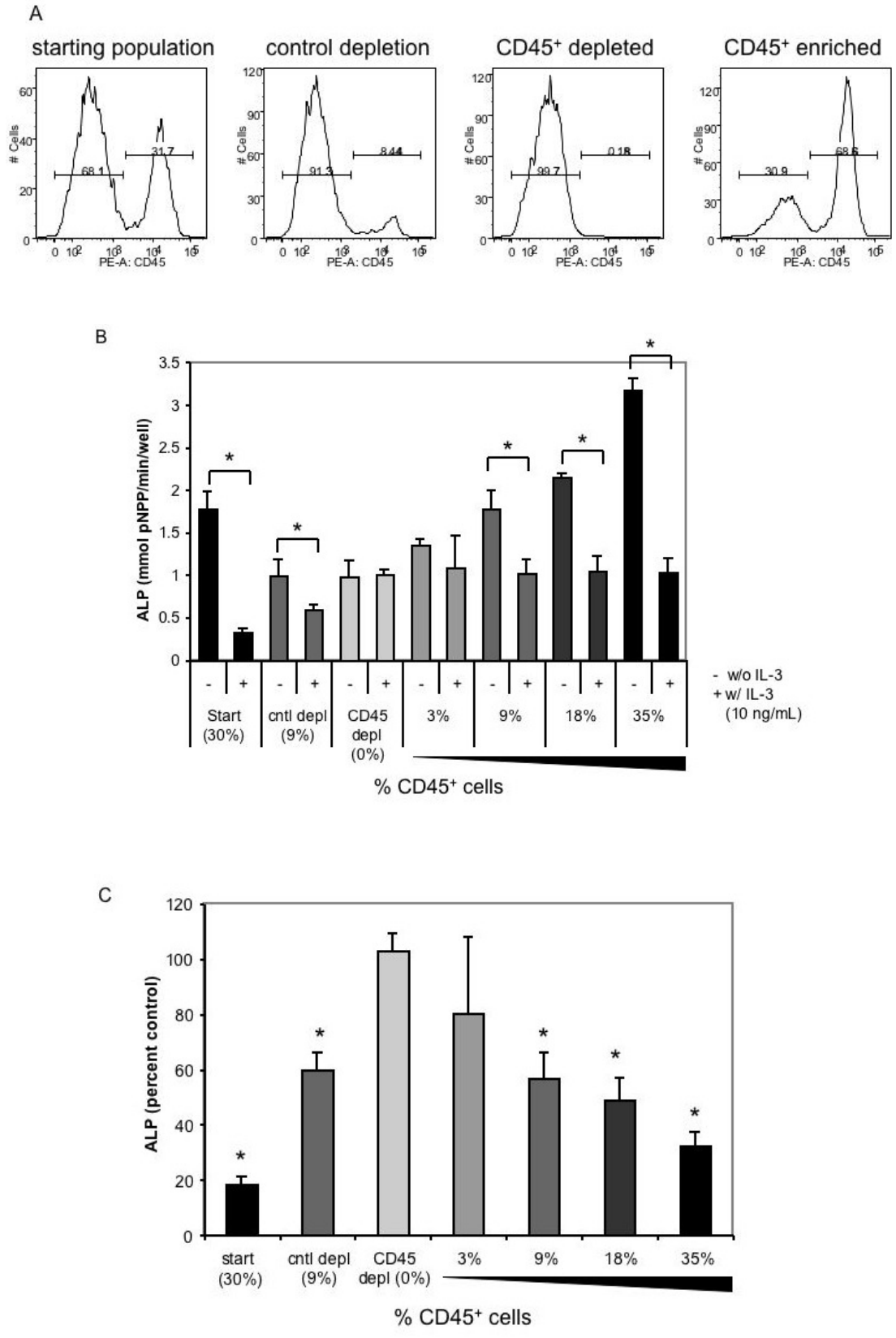

A
starting population
control depletion
CD45+ depleted
CD45+ enriched
# Cells
PE-A: CD45
68.1
31.7
91.3
8.44
99.7
0.18
30.9
68.6
B
ALP (mmol pNPP/min/well)
- w/o IL-3
+ w/ IL-3
(10 ng/mL)
Start (30%)
cntl depl (9%)
CD45 depl (0%)
3%
9%
18%
35%
% CD45+ cells
C
ALP (percent control)
start (30%)
cntl depl (9%)
CD45 depl (0%)
3%
9%
18%
35%
% CD45+ cells

**Figure 3.2: $CD45^+$ cells mediate IL-3 inhibition of OBL in a dose-dependent manner.** (A) $CD45^-$ and $CD45^+$ cell populations were separated using antibody-bound magnetic columns. The starting population was before column depletion, and control depletion was without the primary antibody on depletion columns. All populations were analyzed for percentage of $CD45^-$ and $CD45^+$ cells by single color flow cytometry. (B) $CD45^-$ cells were plated at $1x10^4$ per well in a 48 well plated. $CD45^+$ cells were added where appropriate at increasing concentrations starting from 1:10 to 1:1 (pos:neg). Final percentage of $CD45^+$ cells was calculated based on the flow cytometry data for each population. Cells were cultured with or without the addition of IL-3 (10 ng/mL), and ALP activity was measured after 10 days in culture as usual. Results represent the mean +/- standard deviation for 3 wells each concentration, and are representative of two independent experiments. * $p < 0.05$. (C) ALP activity with IL-3 was represented as a percent inhibition compared to without IL-3 for each population.

### 3.4.3. IL-3 inhibition of OBL requires cell-to-cell contact with $CD45^+$ cells

To determine the mechanism of action of IL-3-mediated inhibition of OBL differentiation, we first sought to determine if IL-3's inhibitory actions required cell-to-cell contact between $CD45^+$ cells and $CD45^-$ OBL precursors. $CD45^-$ and $CD45^+$ populations were separated as before. $CD45^-$ osteoblastic cells were plated in the bottom well of a transwell system, and $CD45^+$ cells were added to the top. IL-3 had no effect on $CD45^-$ cells alone. In the transwell system when cells were not in contact, IL-3 did not suppress OBL differentiation (Figure 3.3).

To confirm that IL-3 effects were not mediated by a soluble factor, OBL cultures were treated with IL-3, and conditioned media were collected every three days. This conditioned media was added to a new OBL culture to determine if there were soluble factors secreted by $CD45^+$ cells that mediate IL-3 actions. A neutralizing antibody to IL-3 was added to ensure that residual IL-3 in the culture media did not affect OBL differentiation. As a control, cells were also treated directly with IL-3 and IL-3 plus a neutralizing antibody. The inhibitory IL-3 effects were reversed by the anti-IL-3, so any inhibitory effects on ALP detected could not have been due to IL-3. Increasing concentrations of conditioned media from control or IL-3 treated cultures both had a slight inhibitory effect on ALP activity likely due to cellular debris from the initial cultures. Importantly, conditioned media from cells treated with IL-3 did not show greater inhibition than control cultures without IL-3 (Figure 3.4). In fact, there was a slight stimulatory effect on ALP levels by the IL-3 conditioned media, likely due to non-specific stimulators in these cultures.

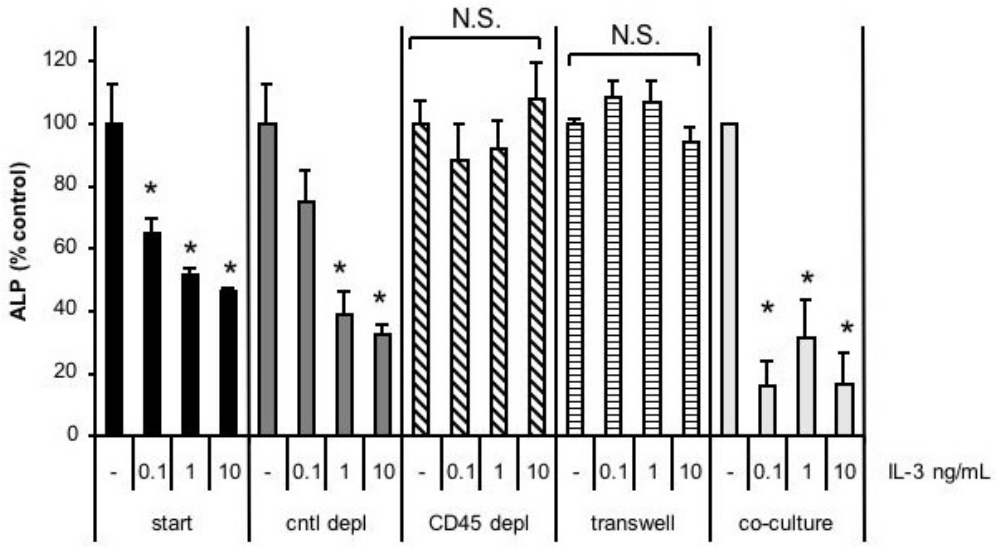



**Figure 3.3: IL-3 mediated OBL inhibition requires cell-to-cell contact between $CD45^-$ OBL and $CD45^+$ hematopoietic cells.** $CD45^-$ and $CD45^+$ cell populations were separated as in Methods. $CD45^-$ cells were plated in the bottom chamber of a transwell plate. $CD45^+$ cells were plated in the top chamber. In the co-culture control wells, $CD45^+$ cells were added directly to the $CD45^-$ cells without the transwell insert. ALP activity was measured in the bottom well of the transwell plates after 10 days in culture. Results represent the mean +/- standard deviation for 3 wells each concentration, and are representative of three independent experiments. * $p < 0.05$, N.S.=not significant.

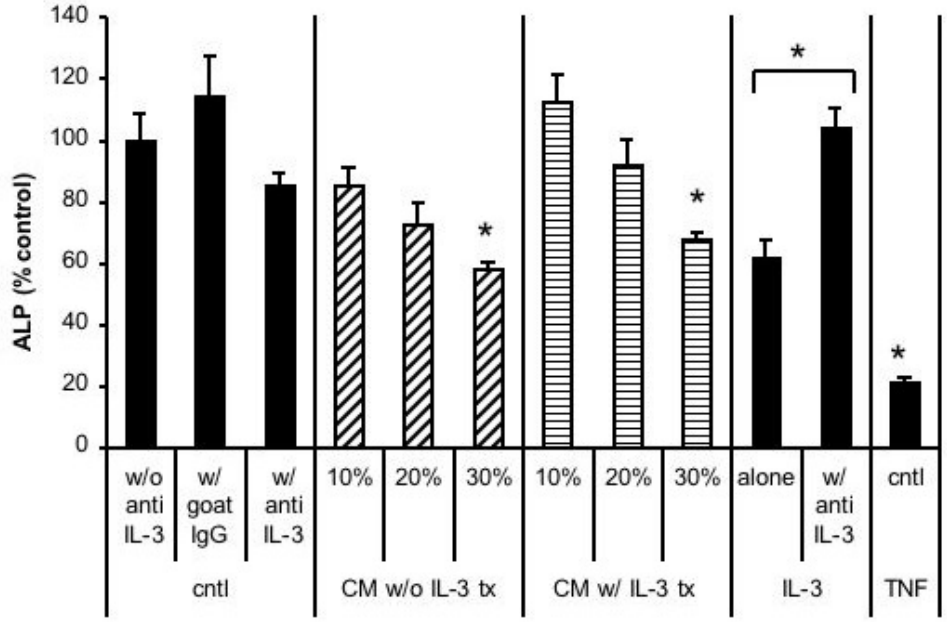



**Figure 3.4: Conditioned media (CM) from IL-3 treated OBL cultures did not inhibit OBL formation greater than CM from OBL cultures without IL-3.** Total mouse adherent cells were cultured in osteogenic media as usual with or without IL-3 (10 ng/mL). Conditioned media was collected every 3 days. A second 10-day culture was treated with media conditioned from day 3-6 of the original culture at increasing concentrations. A neutralizing antibody to IL-3 was added to each well to ensure that residual IL-3 from the original culture was blocked. CM from both control and IL-3 treatment showed slight inhibitory effects, but this was likely from non-specific inhibitory peptides. IL-3 treated CM did not inhibit ALP levels more than control CM. Results represent the mean +/- standard deviation for 3 wells each concentration, and are representative of two independent experiments. * $p < 0.05$.

### 3.4.4. Pretreatment of $CD45^+$ cells with IL-3 does not inhibit OBL

To determine if $CD45^+$ cells could be activated with IL-3 treatment or if they required IL-3 to be present during culture with OBL, $CD45^+$ cells were "primed" with IL-3 as described above. The $CD45^-$ OBL and $CD45^+$ hematopoietic cells were cultured separately. $CD45^+$ cells were then cultured in the presence or absence of IL-3. After 6 days of IL-3 treatment, the $CD45^+$ cells were trypsinized and added onto the $CD45^-$ cultures or a new culture of total adherent cells without additional IL-3 treatment. At the end of the culture period, ALP was measured. Figure 3.5 shows that pre-treatment of $CD45^+$ cells with IL-3 had no effect on OBL differentiation.

### 3.4.5. IL-3 does not increase expression of selected integrins in OBL cultures

Because cell-to-cell contact is required for the inhibitory effects of IL-3 on OBL, relative levels of selected integrins were measured by semi-quantitative RT-PCR. Importantly, $\alpha5\beta1$ is the integrin highly expressed on OBL (Luthen et al., 2005; Yang et al., 2005). $\alpha3$ mRNA was expressed at very low levels, and was undetectable in OBL cultures even at high cycle number. The other integrin mRNAs were expressed at high levels in the mixed OBL culture system, but none were increased by IL-3 treatment of OBL cultures (Figure 3.6).

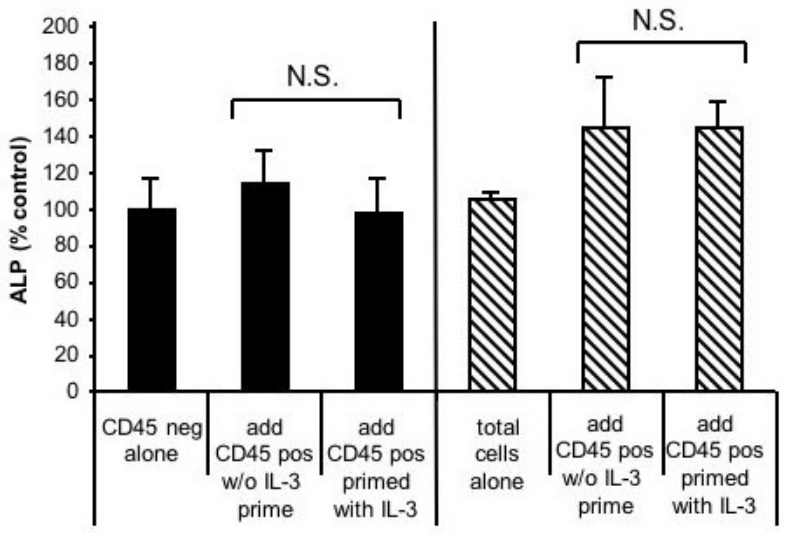



**Figure 3.5: CD45$^+$ cells must be in the presence of IL-3 to inhibit OBL differentiation, and cannot be "primed" with IL-3.** CD45$^-$ and CD45$^+$ cell populations were separated as before, and each plated separately. CD45$^+$ cells were cultured with or without IL-3. After 6 days, CD45$^+$ cells were trypsinized and added to the CD45$^-$ cells already in culture (black bars) or to a new plate of adherent bone marrow cells (striped bars). ALP activity was measured at 10 days. Priming CD45$^+$ cells with IL-3 did not result in ALP inhibition in CD45$^-$ OBL cultures or total adherent cell cultures. Results represent the mean +/- standard deviation for 3 wells each concentration, and are representative of two independent experiments. N.S.=not significant.

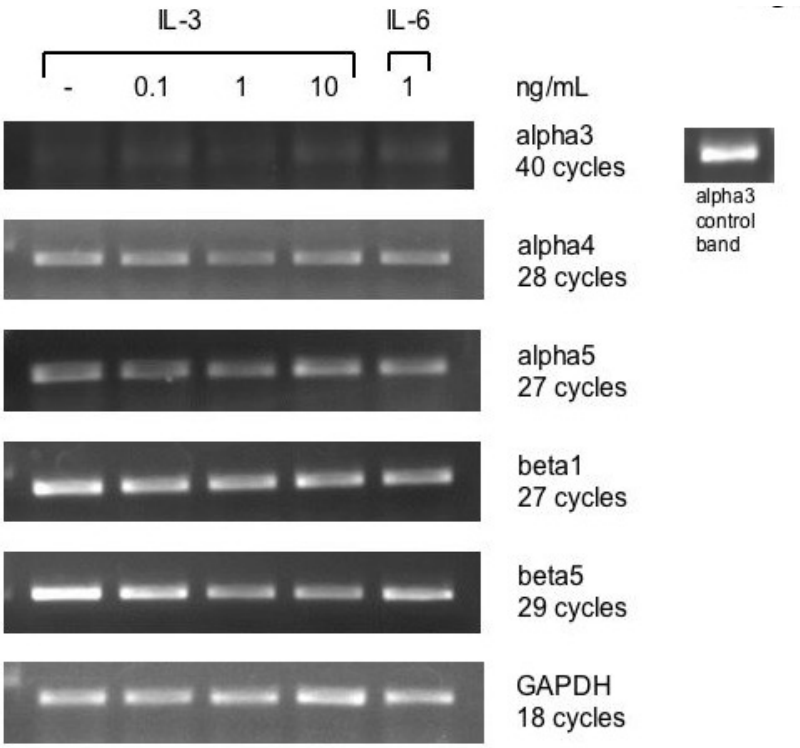



**Figure 3.6: IL-3 treatment does not result in upregulation of integrins α3, α4, α5, β1, or β5.** RNA from 10 day OBL cultures treated with IL-3 was analyzed for integrin expression by RT-PCR with primers that were specific for each transcript. IL-6 was a negative control for OBL inhibition. GAPDH was used as an internal control. An RNA sample with high levels of α3 was used as a control.

### 3.4.6. GM-CSF, a hematopoietic growth factor, can inhibit OBL differentiation

We previously showed that the $CD45^+$ cells were also $CD11b^+$ and in the monocyte/macrophage lineage, which is also the lineage from which osteoclasts are derived. Because IL-3 can stimulate osteoclast formation in vitro, we determined if other osteoclastogenic factors could inhibit bone osteoblast function in this system. RANKL and MIP-1α are both factors that are known to be important in osteoclast function in myeloma. Mouse adherent cell cultures were plated with or without these factors. RANKL was added at 5-100 ng/mL, which is the level of RANKL required for osteoclast differentiation. MIP-1α was added at 0.01-10 ng/mL, which is also the level required to stimulate osteoclast differentiation in vitro (Han et al., 2001). Neither of these factors had any effect on OBL differentiation in vitro (Figures 3.7A and B, respectively). IL-6, which has also been shown to be important in MM as a myeloma growth factor, was also tested for its inhibitory function. In mouse systems, the action of IL-6 requires the addition of soluble IL-6 receptor (sIL-6R). The combination of IL-6 and sIL-6R had no inhibitory effects on ALP activity (Figure 3.7C).

Since IL-3 is a growth factor for hematopoietic cells, we determined if other hematopoietic growth factors such as GM-CSF, that also utilize the common β subunit of the receptor complex, could inhibit OBL differentiation. GM-CSF did inhibit ALP activity in the same concentration range as IL-3. ALP was significantly inhibited at 0.01 ng/mL, and maximum inhibition was achieved at a concentration of 10 ng/mL of GM-CSF, which resulted in 90% inhibition of ALP activity at 10 days (Figure 3.7D).

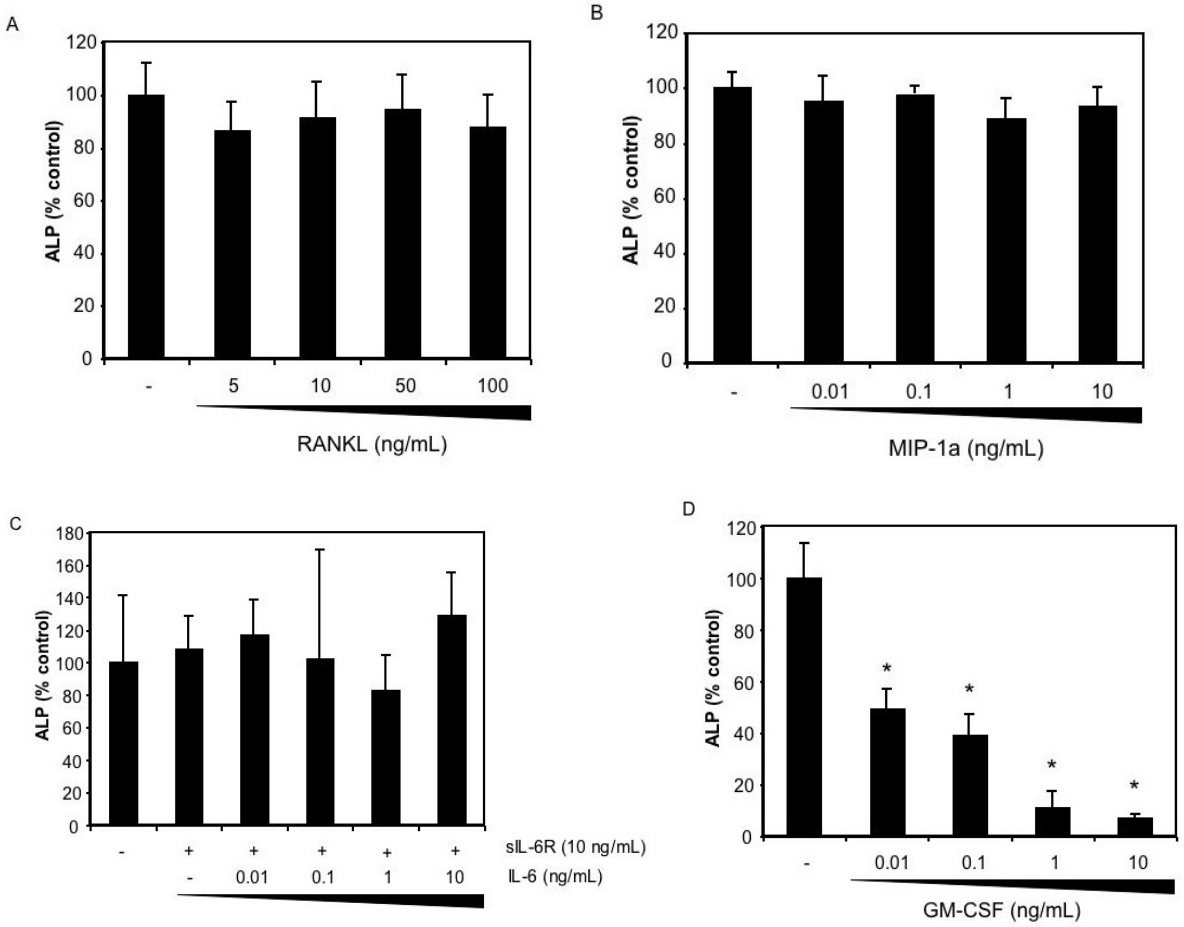



**Figure 3.7: OBL differentiation is not inhibited by other factors important in MM OCL activation or MM cell growth, but is inhibited by GM-CSF, a hematopoietic growth factor.** Adherent mouse bone marrow cells were cultured in the presence of RANKL (A), MIP-1α (B), IL-6 + sIL-6R (C), or GM-CSF (D). RANKL levels were 5-100 ng/mL, which is required for OCL activition in vitro. Cytokine levels were 0.01-10 ng/mL, which encompases the level present in MM bone marrow samples. ALP activity was assessed after 10 days in culture. ALP was not inhibited by RANKL, MIP-1α, or IL-6 + sIL-6R, but was inhibited by GM-CSF. Results represent the mean +/- standard deviation for 3 wells each concentration, and are representative of three independent experiments. * $p < 0.05$.

## 3.5. Conclusions

The inhibitory effect of IL-3 on OBL has been clearly shown in Chapter 2. I also showed that IL-3 inhibition of OBL is indirect and is mediated through $CD45^+/CD11b^+$ hematopoietic cells in mouse and human primary cultures. The nature of the interactions between $CD45^+$ cells and OBL precursors are unknown and several potential mechanisms responsible for IL-3-mediated OBL inhibition are explored in this chaper.

As mesenchymal stem cells (MSC) differentiate from pre-OBL to mature OBL, the cells express different markers of differentiation. Studies delineated in Chapter 2 have shown that IL-3 inhibited OBL differentiation and this inhibition was not through a block in Runx2 expression, the major OBL-specific transcription factor. Runx2 activation is important in several stages of OBL differentiation (Figure 3.1A), so lack of an effect on Runx2 does not reveal which differentiation stage IL-3 was blocking. Alkaline phosphatase is an early marker of OBL differentiation, and it peaks in mouse primary OBL cultures at day 5. Osteocalcin is a late marker, and it peaks at 8 days in culture. This indicates that OBL differentiation in mouse bone marrow cultures begins at the MSC or early pre-OBL, which does not express either ALP or OC, and culminates in mature OBL, which has high OC and decreasing ALP expression. The addition of IL-3 blocks OBL differentiation by day 5 of culture with a sharp but blunted rise and then precipitous fall in ALP levels. The results indicate that IL-3 blocks differentiation of late pre-OBL into mature OBL. This is not simply a result of IL-3 killing OBL precursors since OBL proliferation continues in these cultures (Chapter 2, Figure 2.5B).

Since the effects of IL-3 were indirect through $CD45^+$ hematopoietic cells, we sought to discover if the effects were through a secondary soluble factor or if cell-to-cell contact was required. Transwell plates were used to separate the two cell populations. When $CD45^+$ cells

were not in contact with OBL, ALP activity was not inhibited. Similarly, conditioned media from total adherent cell cultures treated with IL-3 did not inhibit ALP levels compared to treatment with conditioned media from adherent bone marrow cells cultured without IL-3. These experiments showed that $CD45^+$ cells must be in contact with OBL to mediate inhibitory effects. This did not rule out the possibility of a soluble factor that acts in a juxtacrine fashion requiring contact between $CD45^+$ cells and osteoblasts. Because $CD45^+$ cells could not be "primed" with IL-3, the cytokine has to be present during the interaction with OBL to mediate its inhibitory effects. A minimum of 9% $CD45^+$ cells are required in OBL cultures to block OBL differentiation.

Because cell-to-cell contact was required we sought to determine if integrins important in OBL function were upregulated possibly mediating an increase in binding and the inhibition of OBL. The expression level of several integrins was examined including $\alpha5\beta1$ that is important for OBL differentiation. None of the integrins studied was upregulated by the addition of IL-3 to primary OBL cultures. Therefore, increased transcription of $\alpha3$, $\alpha4$, $\alpha5$, $\beta1$, or $\beta5$ is not responsible for the inhibitory effects of $CD45^+$ when they bind to OBL. Other adhesion molecules may be involved, but their delineation is beyond the scope of this study.

Lastly, other soluble factors were assayed to determine if they could cause OBL inhibition in this in vitro assay of OBL differentiation. RANKL and MIP-1$\alpha$ are major mediators of OCL activation. Since OCL are in the $CD45^+/CD11b^+$ monocyte/macrophage lineage, we sought to determine if they could cause OBL inhibition as well. RANKL, MIP-1$\alpha$, and IL-3 all act at different stages of OCL precursor differentiation. Although RANKL and MIP-1$\alpha$ do not inhibit OBL, this does not rule out the possibility that the $CD45^+/CD11b^+$ cells causing OBL inhibition are osteoclasts. GM-CSF, which causes proliferation of several

hematopoietic cells, also blocked OBL differentiation. Therefore, IL-3-mediated inhibition was not unique. This study did not determine if GM-CSF and IL-3 inhibit OBL differentiation through the same mechanism, and other growth factors in this hematopoietic lineage such as M-CSF, G-CSF, and SCF were not examined for their capacity to inhibit OBL differentiation.

Thus, this study investigated the mechanism of IL-3 inhibition of OBL mediated by $CD45^+$ cells. IL-3 inhibited OBL differentiation in the pre-OBL stage. Further, cell-to-cell contact was required between $CD45^+$ hematopoietic cells and $CD45^-$ osteoblast precursors, and a minimum of 9% $CD45^+$ cells were required. Lastly, IL-3 is not a unique hematopoietic growth factor that can inhibit OBL because GM-CSF also inhibited OBL differentiation.

# 4. GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

Bone destruction is a major source of morbidity in patients with multiple myeloma. Mediators of bone destruction have been under intense investigation, and significant progress has been made on their identification. Osteoclast activators that have been identified in recent years include RANKL, MIP-1α, and IL-3. Several other cytokines have been implicated as tumor growth factors, which also contribute to causing bone destruction. It has long been known that osteoblasts are suppressed in myeloma (Taube et al., 1992), but the cause of this inhibition is unknown. A few potential osteoblast inhibitors have been identified including DKK1 and IL-7. However, it is likely that other inhibitors of OBL differentiation are present in the myeloma microenvironment, just as there are several potent stimulators of osteoclast formation produced or induced by myeloma cells.

## 4.1. IL-3 Is an Inhibitor of Osteoblast Differentiation

In the current study, the hypothesis that cytokines/chemokines produced by MM that increase OCL formation also decrease OBL differentiation was tested. These studies focused on IL-3. Chapter 2 showed the importance of IL-3 as an OBL inhibitor in MM. In an in vitro model of osteoblastogenesis, IL-3 inhibited OBL formation. Importantly, IL-3 inhibition of OBL in vitro occurred at IL-3 levels that are found in myeloma patient bone marrow plasma samples. Data from our collaborator confirmed that the inhibitory effects of bone marrow

plasma from patients with myeloma could be partially reversed by blocking IL-3. Importantly, IL-3 did not directly inhibit osteoblast differentiation because IL-3 did not inhibit osteoblastogenesis in OBL-like cell lines. Primary osteoblast culture systems were not comprised of a single cell population and IL-3 normally acts as a hematopoietic factor, therefore the presense of a hematopoietic cell population that was $CD45^+/CD11b^+$ was confirmed in these cultures that mediated the effects of IL-3. Without the presence of these $CD45^+$ cells, IL-3 actions were abrogated. Importantly, these $CD45^+$ cells were found to be in the monocyte/macrophage lineage.

Chapter 3 investigated the mechanism of IL-3-mediated inhibition of OBL differentiation. Clearly, $CD45^+$ cells were necessary for IL-3-mediated OBL inhibition, and cell-to-cell contact was required for this action. Figure 4.1. summarizes the mechanism of IL-3 inhibition on osteoblasts in myeloma. IL-3 is likely released from the myeloma cells themselves as we previously reported. Though the nature of the inhibition of OBL by $CD45^+$ cells is not understood and cell-to-cell contact is required between $CD45^+$ cells and osteoblasts, it does not appear to be mediated by increased expression of $\alpha 3$, $\alpha 4$, $\alpha 5$, $\beta 1$, or $\beta 5$ integrins. The possibility of a secondary, soluble factor were not ruled out by these studies, but this factor is not TNF$\alpha$, which inhibits OBL in vitro and is possibly increased in the myeloma marrow microenvironment (Sati et al., 1999). Another hematopoietic growth factor, GM-CSF, can also inhibit OBL differentiation suggesting that activation of cells in the monocyte/macrophage lineage by hematopoietic growth factors that induce OBL inhibition may be responsible for the effects of IL-3. Products of activated macrophages include GM-CSF (Cavaillon, 1994), but GM-CSF has not been reported to be increased in myeloma. These data suggest a previously unidentified macrophage-derived mediator appears responsible for the effects of IL-3 on OBL differentiation.

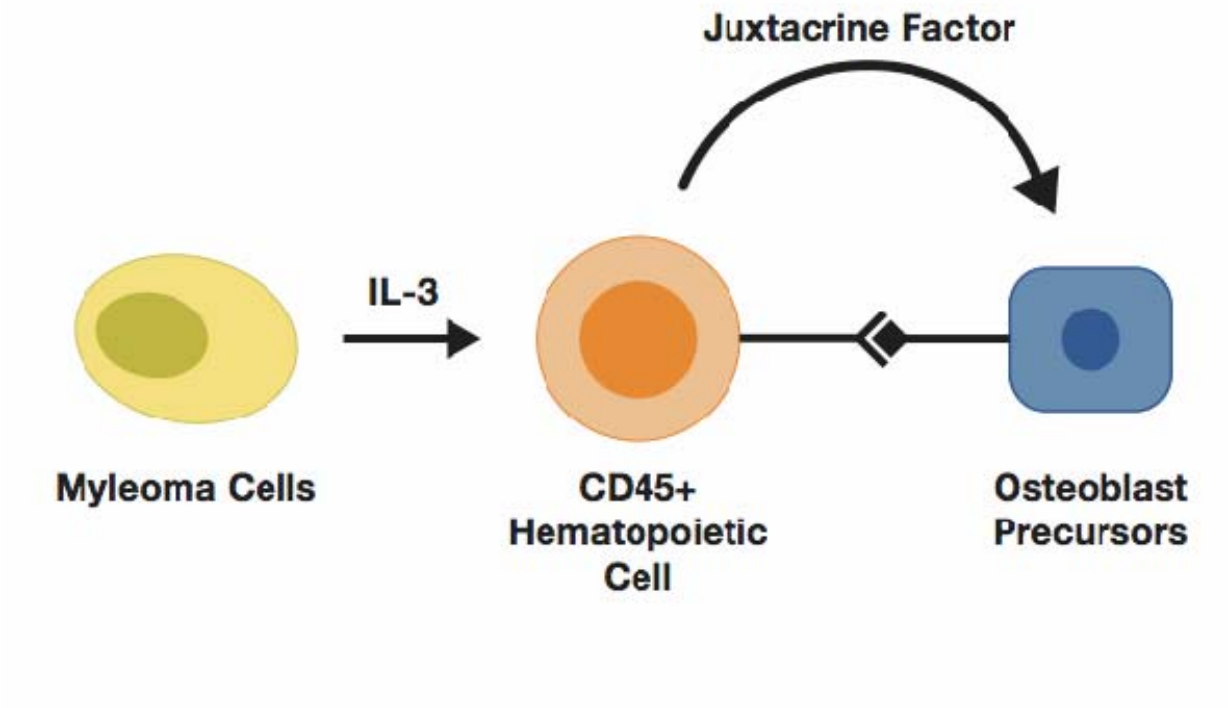



**Figure 4.1: IL-3 from myeloma is an indirect inhibitor of osteoblast differentiation.** Myeloma cells secrete IL-3, which acts on $CD45^+$ hematopoietic cells in this culture system. This, in turn, increases adhesion of $CD45^+$ cells to osteoblast precursors. $CD45^+$ cell contact with osteoblasts blocks their differentiation. Other than cell binding, a secondary factor may be involved in a juxtacrine manner.

## 4.2. Future Directions

Based on the data presented, the mechanism of IL-3 inhibition in myeloma is not fully understood, and several issues need to be explored in future studies before it can be clarified. First, the nature of the $CD45^+$ cell has not been clearly defined. The $CD45^+$ cells responsible for OBL inhibition in vitro are $CD11b^+$ in the monocyte/macrophage lineage, but cells with this

marker can differentiate into several hematopoietic lineages including osteoclast, macrophages, neutrophils, and natural killer cells. Interestingly, IL-3 can affect lineage commitment of cells in the macrophage lineage skewing their differentiation into the dendritic cell lineage (Suzuki et al., 2004). It is interesting to speculate that dendritic cells may be involved in IL-3-mediated osteoblast inhibition in myeloma. GM-CSF can also induce dendritic cell (DC) lineage commitment (Lutz, 2004), therefore the mechanism of inhibition by IL-3 and GM-CSF could be through this mechanism. The role of dendritic cells in myeloma is a topic of active investigation. Brown et al. determined that the level of immature dendritic cells in the peripheral blood of patients with MM was normal, but their ability to express costimulatory molecules was blunted (Brown et al., 2001). Recently, Hashimoto et al. have suggested that DC differentiation was also decreased in MM and that this block in DC differentiation resulted from MIP-1α inducing OCL differentiation and blocking DC differentiation of a common precursor in the monocyte lineage (Hashimoto et al., 2005). Dendritic cells also express the CD45 and CD11b markers; CD11c expression on these $CD45^+$ cells in primary OBL cultures could indicate that they are in the dendritic cell lineage, which should be investigated further.

The lack of IL-3 effect in transwell cultures or via conditioned media from IL-3-treated OBL cultures does not rule out the possibility of a secondary soluble inhibitory factor. This secondary factor could be secreted at low levels and require close association of $CD45^+$ cells and OBL (juxtacrine effects), or cell-to-cell contact between the two cell populations may be necessary to cause secretion of the secondary factor. The $CD45^+$ hematopoietic cells in primary OBL cultures are in the monocyte/macrophage lineage. Macrophages are known to secrete many factors including IL-1, IL-6, IL-8, IL-10, IL-12, TNFα, Interferon-α (IFN-α), IFN-γ, M-CSF, G-CSF, GM-CSF, MIP-1, MIP-2, TGFβ (Cavaillon, 1994), and prostaglandins (Villamon et al.,

2005). Based on the studies reported here, several of these factors can be ruled out as the juxtacrine mediator of IL-3-mediated inhibition of OBL. IL-6 and MIP-1 do not have inhibitory effects in primary OBL cultures (Chapter 3). TNFα is not increased in OBL cultures with IL-3 treatment, and anti-TNFα does not block the inhibitory effects of IL-3 on OBL differentiation (Chapter 2). The colony-stimulating factors (M-CSF, G-CSF, and GM-CSF) do not have direct effects on OBL (Hofstetter et al., 1996). CSFs could affect the hematopoietic cells in primary OBL cultures, but likely are not the juxtacrine factor that is released by IL-3 to inhibit OBL. TGFβ and prostaglandins stimulate OBL differentiation in vitro and increase mineralization in vivo (Bonewald, 1996; Zhang et al., 2002), and therefore are not likely candidates. Many of the other factors secreted by macrophages have not been investigated for their effects on OBL differentiation. Interestingly, Tanabe et al show that IL-1α can inhibit OBL differentiation in rat cultures (Tanabe et al., 2004). Cell-surface associated growth factors such as TGFβ or SCF should also be investigated as cell-to-cell contact between the $CD45^+$ cells and the OBL is important for OBL inhibition. We are currently analyzing conditioned media from OBL cultures for the presence of cytokines using "cytokine arrays," which can screen for the levels of twenty cytokines. If any cytokines or chemokines are upregulated in IL-3 treated OBL cultures, their importance in IL-3-mediated OBL inhibition can be rigorously tested in vitro and in vivo.

If no known adhesion molecules or cytokines are found to be important in these screening tools, gene array analysis can identify a large number of genes that are up- or down-regulated in IL-3 treated stromal cell cultures. Gene arrays and virtual subtraction of gene levels in control and IL-3 treated cultures could identify genes altered by IL-3 treatment of osteoblast cultures. The subpopulations of primary stromal cell cultures also could be separated into $CD45^+$ hematopoietic cells and $CD45^-$ osteoblasts as well as the interaction of both populations together

to determine genes that are altered by the interaction of the two subsets. One obvious drawback of gene array analysis is that many genes are up or down regulated with the possibility of false positives or negatives. Gene expression levels as well as protein levels have to be confirmed by alternative analyses such as Northern or Western blots, respectively. Another major drawback to gene array analysis is the discovery of genes that are positively or negatively regulated but are not physiologically relevant. Narrowing candidate genes to adhesive molecules or other known osteoblast inhibitors could help limit the target gene population. Only extensive analysis of the importance of these candidate genes in the established model of IL-3-mediated osteoblast inhibition can confirm their importance.

The role of IL-3 in OBL inhibition in vitro has been clearly shown in this dissertation, and IL-3 present in bone marrow samples of patients with myeloma can inhibit OBL differentiation. To confirm the biologic significance of these results, in vivo studies must be undertaken to show the role of IL-3 in the intact bone microenvironment. Several in vivo models have been established for studying myeloma bone destruction. Our lab established the ARH-77 model of myeloma bone destruction in vivo (Alsina et al., 1996). In this model, ARH-77 cells, which are a human myeloma cell line, are injected into immunocompromised mice. The ARH-77 cells home to the bone marrow cavity and cause lytic lesions with an increase in osteoclast activity. Because IL-3 is species-specific, human IL-3 from the myeloma cell line would not affect mouse bone marrow cells in this model. The model would have to be adapted to use either a mouse myeloma cell line of which there are few or a human marrow microenvironment. Only one mouse myeloma cell line grows easily in vitro, 5TGM1, but this cell line does not secrete IL-3 (unpublished data). Alternatively, a human microenvironment could be utilized as in the scid-hu model developed by Yaccoby et al (Yaccoby et al., 1998). Here, a small piece of human fetal

bone is implanted under the skin of immunocompromised mice. The major drawback of this model is that although the initial bone marrow milieu is human (donor), likely mouse (host) hematopoietic cells are recruited to the bone fragment resulting in murine-derived osteoclasts. Staining for human- or murine-specific proteins could easily identify the donor or host origin of the osteoclasts and other hematopoietic cells.

As an alternative to these models, we are also developing the "ossicle model" which uses primary mouse adherent cells cultured in the same manner as the in vitro OBL cultures above. These marrow stromal cells were implanted subcutaneously in immunocompromised mice within a synthetic matrix. After 4-6 weeks, the stromal cells calcified and developed a marrow-like cavity that recapitulates the marrow microenvironment in ectopic bone. The benefit of this model is the ease of manipulation of the stromal cell/osteoblast population. Although hematopoietic cells in this model are derived from the host mouse, the osteoblasts are from the donor mouse cultures. IL-3 actions on osteoblasts are through hematopoietic cells, which are from the wildtype host mouse. Once the identity of the adhesive interactions responsible for hematopoietic cell-mediated osteoblast inhibition, mice lacking this adhesive factor could be used to have an in vivo model with the key factor only "knocked out" of the osteoblasts. Again, IL-3 is species specific, so the requirement for mouse myeloma cells is apparent. Alternatively, because we know that exogenous IL-3 inhibits osteoblasts in vitro, normal cells or myeloma cells transfected to constitutively express murine IL-3 could be injected into these mice to demonstrate the importance of IL-3 in vivo.

The obvious goal of discovering potential mediators of bone destruction in any disease is to take this discovery "bench to bedside." Targeting and inhibiting IL-3 in myeloma would have the potential to decrease tumor burden and block bone destruction. Figure 4.2. reviews the

effects of IL-3 in myeloma. The effects of IL-3 are three-fold as a myeloma growth factor, an osteoclast stimulator, and an osteoblast inhibitor. IL-3 is potentially partially responsible for uncoupling of bone mediators in myeloma. IL-3 stimulates osteoclasts increasing bone destruction. Osteoclast stimulation normally causes an increase in new bone formation. In myeloma, IL-3 blocks osteoblast differentiation inhibiting new bone from being laid down. Because IL-3 is an important hematopoietic factor, the potential for interfering with immune regulation is great, though other cytokines, such as GM-CSF, that share some functions with IL-3, may compensate for this loss. Alternatively, once the secondary mediator of IL-3 OBL inhibition is identified, either adhesive interactions or a juxtacrine factor, it can be targeted directly, isolating the effects of IL-3 on OBL and not blocking normal hematopoiesis. Developing therapies targeting the production of IL-3 by myeloma cells or the actions of IL-3 in bone destruction could reduce tumor burden and bone sequelae in patients with MM.

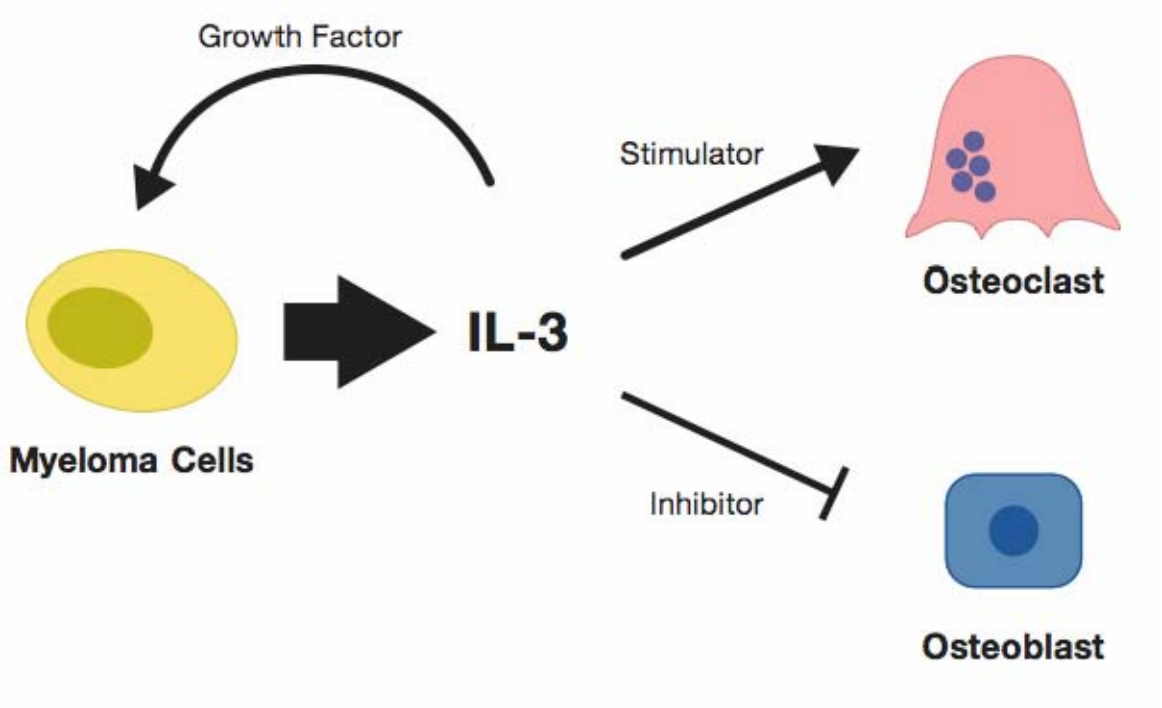



**Figure 4.2: IL-3 in myeloma affects osteoblasts, osteoclasts, and malignant cells themselves to cause bone destruction.** IL-3 stimulates osteoclast formation, which destroys bone. Osteoblasts are inhibited so that new bone formation is blocked. Finally, IL-3 stimulates myeloma cell growth, which increases secretion of other osteoclast stimulators and osteoblast inhibitors in a vicious cycle.

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