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Chemotherapy-Induced Damage of the Bone Marrow Microenvironment

Stephanie Lynne Rellick

Dissertation submitted to the School of Medicine at West Virginia University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

In

Cancer Cell Biology

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ABSTRACT

Chemotherapy-Induced Damage of the Bone Marrow Microenvironment

Stephanie Lynne Rellick

The bone marrow microenvironment is characterized as the anatomical site including specialized niches that support stem cells. In addition, these niches also provide both soluble and physical cues leading to the differentiation of stem cells into all the cells of the blood. The studies in this dissertation focus on two supportive niches in the bone marrow microenvironment, osteoblasts (HOB) and bone marrow stromal cells (BMSC), in the setting of high dose chemotherapy and the potential damage that chemotherapy treatment causes to the cells of the bone marrow microenvironment.

In the first study, we investigated the effects of melphalan and Etoposide (VP-16) treatment on osteoblasts. Previous studies from our lab had shown that chemotherapy treatment increased the amount of active TGF-β secreted from BMSC, leading to decreases in the ability to support pro-B cells. Here we describe the novel observation that osteoblasts pre-treated with chemotherapy have increased active TGF-β and a decreased capacity to support human embryonic stem cells (hESC), CD34+ bone marrow derived cells and pro-B cells. We also evaluated the effects of adding recombinant TGF-β (rTGF-β) to osteoblasts to mimic the autocrine and paracrine TGF-β in the microenvironment during chemotherapy treatment. rTGF-β treatment of osteoblasts increased TGF-β secretion and also led to a decreased ability to support hESC, CD34+ bone marrow derived cells and pro-B cells. Microarray analysis of the cells treated directly with chemotherapy or rTGF-β or conditioned media from BMSC that were treated with chemotherapy suggested that many genes are changing in response to all of these treatment groups, indicating that osteoblasts are a vulnerable cell population that can be affected by high dose chemotherapy, potentially resulting in decreased hematopoietic support.

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We also investigated Interleukin-6 (IL-6), a known hematopoietic factor important in both myeloid and lymphoid differentiation, acute and inflammatory immune responses and bone metabolism. Neuroendocrine modulation of the bone marrow microenvironment is thought to be important in both hematopoiesis and immune regulation. We investigated the roles of neurotrophins in the bone marrow and their effects on BMSC. We show that BMSC express functional neurotrophin receptors and that treatment of BMSC with two neurotrophins, NGF or BDNF, led to an increase in IL-6 expression. Increased IL-6 is associated with a number of inflammatory diseases and our data support the idea that increased neurotrophins in the bone marrow microenvironment could lead to dysregulated hematopoiesis.

Additionally, we also evaluated the effects of chemotherapy treatment of BMSC and HOB, focusing on IL-6. Previous data has suggested IL-6 to be involved in graft versus host disease and we investigated the effects of melphalan on IL-6 expression in BMSC and HOB. Interestingly, we determined that melphalan treatment led to a decrease in IL-6 mRNA and protein, and compared to other chemotherapeutic agents used in our studies, melphalan had the most pronounced effect. We also evaluated the effects of recombinant IL-6 (rIL-6) in combination with melphalan and determined that the addition of rIL-6 restored both IL-6 mRNA and protein expression, suggesting that pathways associated with IL-6 expression may be disrupted. The decrease in IL-6 could potentially affect hematopoiesis and further studies in vivo need to be completed. Additionally, melphalan is used as first-line therapy in the treatment of multiple myeloma (MM). IL-6 is a proliferative factor in MM, allowing for disease progression. The melphalan-induced decrease in IL-6 observed in our studies may, in part, contribute eradicating the tumor population by decreasing the potent proliferative factor, IL-6.

Collectively, these data contribute to our understanding of alterations to the bone marrow microenvironment that occur during high dose chemotherapy and emphasize the importance of understanding the mechanisms that underlie the potential damage leading to altered ability to support normal hematopoiesis.

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LIST OF ABBREVIATIONS

5-FU	5-fluorouracil
Ang-1	angiopoietin-1
AP-1	activator protein-1
BDNF	brain-derived neurotrophic factor
BFU-E	blast forming units- erythrocyte
BM	bone marrow
BMEC	bone marrow endothelial cell
BMSC	bone marrow stromal cell
CFU	colony forming unit
CFU-F	colony-forming unit-fibroblast
CNS	central nervous system
CXCL12	chemokine (CXC motif) ligand 12 also known as SDF-1
EPO	erythropoietin
FGF	fibroblast growth factor
Flt-3	FMS-like tyrosine kinase 3
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony stimulating factor
HA	hyaluronan
hESC	human embryonic stem cell
HOB	human osteoblast
HPC	hematopoietic progenitor cells
HSC	hematopoietic stem cell
ICAM-1	Intercellular adhesion molecule
IFN-γ	Interferon-γ
IL	Interleukin
IL-6	Interleukin-6
JAK	Janus kinase
LTBMC	long term bone marrow culture
MM	multiple myeloma

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Mpl	thrombopoietin receptor
MSC	mesencymal stem cells
NF-IL6	nuclear factor-Interleukin-6
NGF	nerve growth factor
NT	neurotrophin
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Sca-1	stem cell antigen-1
SCF	stem cell factor
SLAM	signaling lymphocyte activation molecule
SNO	Shaped N-cadherin positive
SOCS	suppressor of cytokine signaling
STAT	signal transducers and activators of transcription
TGF-β	transforming growth factor-β
TNF-α	tumor necrosis factor-α
TPO	thrombopoietin
VCAM-1	vascular cell adhesion molecule-1
VLA-4	very late antigen-4
VLA-5	very late antigen-5
VP-16	Etoposide

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Chapter I

The Bone Marrow Microenvironment as the Site of Hematopoiesis and a Potential Target of Chemotherapy-Induced Damage

Literature Review

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I. Physical components of the bone marrow

Hematopoiesis is the process by which all the cells of the blood are formed. This process is dependent on multipotent hematopoietic stem cells (HSCs) which are found in the bone marrow of adults. The bone marrow microenvironment is characterized as the anatomical site that includes specialized niches where stem cells are associated with other cells that influence their behavior[1]. The function of these niches is to support hematopoietic progenitor cell proliferation and differentiation, self-renewal and provide a sanctuary for HSCs to remain quiescent. Spangrude et al described different types of HSCs including both long-term and short-term repopulating HSCs which were capable of self-renewal and multipotent progenitors which have very low or no self-renewal capacity[2]. Human HSCs are characterized by their surface markers with a phenotype of $CD34^+$, $lineage^-$, $CD38^-$, $CD90^+$ and $CD45RA^-$[3]. In addition to self-renewal, HSCs must also be able to differentiate into all the cells of the blood in both the lymphoid and myeloid lineages.

HSCs have a number of receptors and adhesion molecules that are necessary for homing, which is the process by which HSCs move through the blood, cross the endothelial cell layers and take up residence in a variety tissues or the bone marrow, and adhesion in the bone marrow, which is the physical tethering of HSCs to the adherent, supportive cells of the bone marrow microenvironment[4]. Some examples of these interactions are the presence of the CXCR4 receptor on the surface of HSCs, which responds to CXCL12, a soluble factor produced by the bone marrow and osteoblasts to attract HSCs[4]. Once in the bone marrow, VLA-4 and VLA-5 on HSCs, interact with VCAM-1 and fibronectin respectively on the surface of bone marrow stromal cells (BMSC), human osteoblasts (HOB) and bone marrow endothelial cells (BMEC), mediating cell adhesion and retention in the marrow[5]. Some additional signaling pathways important for maintenance of HSCs in the BM are thrombopoietin (TPO)/Mpl and Tie2/Angiopoietin-1 (Ang-1)[6-8]. The Notch/Jagged and TGF-β signaling pathways will be discussed later. TPO is a factor that regulates platelet production by binding to its receptor,

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Mpl, and stimulating the production of megakaryocytes[6;9]. Recent studies have shown that TPO signaling may also have important roles in maintenance of quiescence. Buza-Vidas et al showed that long-term repopulating HSCs had higher expression of Mpl[9], and Qian et al, using a TPO knock-out mouse, showed that these mice gradually lost HSCs, having a 150 fold decrease in HSCs, decreased BM cellularity and multilineage defects by one year[6]. Additionally, Arai et al showed that TPO was produced by the bone cells in the endosteal niche, administration of Mpl neutralizing antibody to mice suppressed the quiescence of long-term repopulating cells and exogenous treatment with TPO restored the quiescent cell population[7]. Tie2/Angiopoietin signaling has also been associated with maintenance of quiescence. Arai et al demonstrated that Tie2 receptors were found on quiescent HSCs and furthermore, these cells formed a "side population" of cells that were adherent to osteoblasts[8]. The same group also showed that osteoblasts produced Ang-1, and that the interaction of Tie2/Ang-1 allowed for maintenance of long term repopulating HSCs[8;10]. Some receptors on the surface of HSCs have important roles in the maintenance of quiescence such as Notch1 and TGF-β receptors[11;12]. In order for normal hematopoiesis to occur, HSCs are dependent on the niches in the bone marrow for support and maintenance of signaling pathways to promote self-renewal as well as differentiation. These niches are discussed in more detail in the following sections.

The bone marrow has been described as having two hematopoietic support niches that are anatomically and functionally distinct[13;14]. These include the endosteal niche consisting of mesenchymal stem cells, osteoblasts and bone marrow stromal cells (BMSCs)[15-17] and the vascular niche consisting of bone marrow endothelial cells[18-20]. Each of the cell types within these niches contribute to hematopoiesis and stem cell regulation through the expression of cellular adhesion molecules such as VCAM-1 and ICAM-1 and integrins like VLA-4 and VLA-5, extracellular matrix proteins including collagens, proteoglycans and glycoproteins, and production of soluble factors including TGF-β and Interleukin-6, which will be discussed in further detail as a focus of the current study.

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Early studies by Lord and Gong showed that there are higher numbers of colony forming units (CFU) cells closer to the bone surface than to the center of the marrow cavity[21;22]. Nilsson et al and others showed that while most cells enter the bone marrow through blood vessels in the center of cavity, stem cells are distributed closer to the bone in the endosteal niche, while committed progenitors were located closer to the center of the bone cavity[23;24]. The spatial organization of the niche also correlates with the specific cell types found within it. A precursor stem cell of osteoblasts and bone marrow stromal cells are mesenchymal stem cells (MSCs) and they were first characterized by Friedenstein as non-hematopoietic cells that have colony forming unit-fibroblast (CFU-F) activity, adherence to plastic and the potential to differentiate into multiple lineages such as osteoblasts, adipocytes, BMSCs and other supportive cells of the microenvironment[25;26]. MSCs have a fibroblastic phenotype and express a variety of surface markers including VCAM-1, Stro-1 and ICAM-1[27;28]. MSCs are rare in the bone marrow, reported to represent 1 out of 10,000 nucleated cells[29]. There are a number of studies that are examining the idea of co-transplanting MSCs with HSC transplantation[30;31]. It is thought that this would help with hematopoietic recovery by providing a pool of supportive microenvironment cells that could help replace cells damaged by chemotherapy regimens.

One of the major components of the endosteal niche are BMSCs that, in concert with other components of the microenvironment, support reconstitution of hematopoiesis. BMSCs are described as fibroblastic reticular marrow cells that are located in the region between the bone and endothelial niche, also referred to as the subendosteal niche[32;33]. Dorshkind described these cells as “nonhemopoietic, fixed tissue cells in the medullary cavity”[34]. BMSC were defined by their ability to form colonies referred to as fibroblast colony forming units[25;35]. Another characteristic of BMSC is that they are able to maintain long-term bone marrow cultures[36-39]. These cells, in part, form the cellular network that helps in the regulation of hematopoiesis. These BMSCs have been characterized as expressing a variety of surface markers such as VCAM-1 that interacts with VLA-4 on the surface of B cell precursors, allowing

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for their retention in bone marrow[40;41]. VCAM-1 expression can be regulated through cytokines present in the marrow. Dittel et al showed that TGF-β had the ability to decrease VCAM-1 protein on the surface of BMSCs while Interleukin-1β and Interleukin-4 can increase VCAM-1[42]. They also noted a correlation between the amount of VCAM-1 on the surface of the BMSC and the ability to support B cell precursors. In addition to surface markers, BMSCs also express extracellular matrix proteins such as collagen I, III, IV, V, VI, fibronectin and laminin and a number of proteoglycans such as hyaluronic acid and heparin sulfate[34;43-45]. Another important component of hematopoietic support is the production and secretion of soluble factors including SDF-1 (CXCL12), Interleukin-6 (IL-6), IL-3, IL-7, IL-11, granulocyte-macrophage colony stimulating factor (GM-CSF), stem cell factor (SCF) and flt-3 that not only allow for the homing of stem cells to the bone marrow but also support the differentiation and maturation of HSCs for reconstitution of the immune system as well as all the cells of the blood[34;46;47]. BMSC also secrete factors that are negative regulators of hematopoiesis such as TGF-β and interferon-γ (IFN-γ)[48;49].

Another critical component of the endosteal niche are osteoblastic cells. There are different types of osteoblasts. There are spindle-shaped N-cadherin positive (SNO) osteoblasts as well as osteoblasts that are N-cadherin negative[50]. Literature has suggested that it is the SNO cells that line the endosteal surface and that this is the location of the most primitive hematopoietic cells[10;21;22;50;51]. One of the main roles of the osteoblastic niche is to maintain stem cell quiescence, which occurs through both soluble factors as well as cellular adhesion molecules[52;53]. Work by Calvi and Zhang showed that osteoblasts are a target of parathyroid hormone in the bone marrow[50;54]. In a mouse model where there was expression of osteoblast-specific parathyroid hormone receptor, it was shown that these mice had increased bone mass and increased numbers of osteoblasts[54;55]. Importantly, these transgenic mice also had increased Jagged1 expression, which is the ligand for the Notch signaling pathway[11]. This increased Jagged1 expression also correlated with an increase in HSCs, suggesting that

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osteoblasts have a critical role in the maintenance of Notch signaling and HSC expansion. Calvi et al also showed that the transgenic mice had increases in the N-cadherin positive population of osteoblasts[11]. Another factor secreted by osteoblasts is osteopontin (Opn)[56]. Opn has been reported to help HSCs home to the bone marrow and be retained in the endosteal niche. When HSCs are transplanted into a healthy microenvironment, they reside specifically in the endosteal regions[57]. In an Opn -/- mouse model, HSCs had a random distribution throughout the bone marrow[57]. Additionally, Opn-/- mice also had increased BrdU incorporation in HSCs, suggesting Opn is a negative regulator of HSCs and is important in maintaining quiescence[57]. Osteoblasts have also been characterized as having a number of different cellular adhesion molecules such as VCAM-1 and fibronectin[58]. VCAM-1 is important for interaction with Very Late Antigen-4 (VLA-4) and Very Late Antigen-5 (VLA-5) interacts with fibronectin, both of which are found on hematopoietic progenitor cells and can help with homing to the bone marrow and adhesion once they are in the marrow space[58;59].

There is some controversy in the field of osteoblasts as components of the stem cell niche, specifically related to the presence of N-cadherin and its role in maintenance of stem cells through homotypic interactions with osteoblasts. Kiel et al did not detect N-cadherin in purified HSCs using a variety of methods and suggested that it was the N-cadherin negative population of bone marrow cells that reconstituted irradiated mice[60]. In a mouse model in which N-cadherin was conditionally deleted, it was shown that there was no effect on HSC maintenance or hematopoiesis, as the cellular composition of the bone marrow and the number of progenitor cells was not affected[61]. The lack of N-cadherin also did not affect the ability of HSCs to reconstitute irradiated primary and secondary recipients[61]. These studies suggest that N-cadherin expression by HSCs is not essential for regulation of the niche.

Whether N-cadherin is necessary or not has yet to be determined, but osteoblasts are critical for HSCs to survive and maintain the niche. Work by Visnjic et al showed deficits in hematopoiesis in mice where osteoblast deficiency was induced. A transgenic mouse model

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with herpesvirus thymidine kinase gene under the control of a collagen alpha 1 type I promoter allowed for lineage specific expression of the gene in osteoblasts[62]. These mice lost lymphoid, myeloid and erythroid progenitors in the bone and had significantly decreased HSCs. When osteoblasts were allowed to recover, hematopoiesis started to recover in the bone marrow[62]. Chitteti et al showed that CFU expansion was increased when HSCs were cultured with osteoblasts[63]. This suggests that osteoblasts have critical roles in the regulation of hematopoiesis, most likely through both physical and soluble factors.

An additional niche of the bone marrow relevant to stem cell support is the endothelial niche. The endothelial niche serves as the barrier separating the bone marrow from the blood, making it a critical component in a healthy bone marrow microenvironment[18;64]. The main cells found in this niche are endothelial cells and hematopoietic cells. Both of these cells come from a common precursor in the embryonic stage called the hemangioblast[65]. The bone marrow endothelial cells (BMEC) make a network of thin-walled and fenestrated sinusoidal vessels that are unique to the bone marrow[19]. The endothelial niche is important in two different roles within the microenvironment. The first role is homing to the marrow and allowing HSCs to enter the bone marrow cavity where the cells then migrate to the endosteal niche[18-20;64]. The second role is mobilization and egress of mature cells into the blood[4;64]. Prior to entry into the blood, hematopoietic progenitor cells are supported by both BMSC and BMEC while they mature and differentiate[66;67]. The most well studied cell type is the support of megakaryocytes through the secretion of factors such as FGF-4, SDF-1 and thrombopoietin (TPO), and through adhesion molecules like VCAM-1 while they mature and differentiate[20;68;69]. Rafii et al showed the ability of BMEC to support multi-lineage differentiation[68]. When hematopoietic progenitors were either in direct contact with BMEC or in transwells, the progenitors expanded 5-7 fold in one week and by two weeks in co-culture, 70-80% of the cells were myeloid and 14-19% were megakaryocytes. They suggested this ability of BMEC to support progenitor cell differentiation was through the production of cytokines such as IL-6, GM-CSF, G-CSF and Kit-ligand[20].

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The physical structure of the bone marrow vasculature has also been studied both in its support of progenitor cells as well as how progenitor cells can influence the recovery of the endothelial cells following chemotherapy treatment[70]. Studies have shown that the integrity of the vessels is maintained by the surrounding hematopoietic cells, specifically megakaryocytes, which secrete vascular endothelial growth factor-A (VEGF-A)[70]. Following treatment with cytotoxic agents or radiation, the vessels of the bone marrow are destroyed. Post-treatment, there is a regeneration of the sinusoidal vessels and this is thought to occur through vascular progenitors and HSCs that are not affected by the therapy[16]. One critical protein involved in this process is MMP-9. Heissig et al showed that MMP-9 led to the release of Kit ligand allowing for progenitor cells to translocate to the vascular region[71]. When mice that were MMP-9 deficient were treated with 5-FU, there was impaired "hemangiogenic" recovery in the bone marrow[66]. Additional studies by Avecilla et al showed that using neutralizing antibodies against VE-cadherin following 5-FU treatment led to similar effects, including disruption of VCAM-1 and subsequently the lack of recovery of megakaryocytes[66]. Other experimental results showing the close interaction between endothelial cells and HSCs was the identification of stimulated lymphocyte activating molecules (SLAM) markers on the surface of HSCs[72]. Kiel et al showed that more than 50% of HSCs that expressed SLAM markers were found in the vascular region of the bone marrow, indicating that this niche is a supportive niche of HSCs. Finally, work by Butler et al demonstrated that HSCs in contact with endothelial cells cultured under serum free conditions were still able to expand long-term repopulating HSCs both *in vitro* and *in vivo*[73], providing further evidence that the endothelial niche is able to support HSCs and hematopoiesis.

Within all of these niches, there are a vast number of adhesion molecules and cytokines that must be present and tightly regulated to ensure proper support of hematopoietic stem cells. We have chosen to focus on the effects of chemotherapy on TGF-β and Interleukin-6 as two important cytokines in the bone marrow necessary for maintenance of quiescence and

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differentiaion of hematopoietic stem cells.

II. Factors influencing the niche

All of the supportive cells of the BM microenvironment, including BMSC, osteoblasts and BMEC secrete soluble factors that are critical for the maintenance of quiescence and self-renewal as well as to promote differentiation of hematopoietic progenitor cells into all the cells of the blood including lymphoid cells (B cells, T cells and NK cells), myeloid cells (monocytes, megakaryocytes, macrophages, neutrophils, etc)[74]. Chemotherapy exposure of the microenvironment during pre-transplant regimens has the potential to damage the supportive cells, and subsequently, can disrupt the secretion of soluble factors necessary for hematopoiesis. We have chosen to examine the effects of chemotherapy treatment on two of these soluble factors, TGF-β as a mediator of HSC quiescence, and Interleukin-6 as factor important in the differentiation of both myeloid and lymphoid progenitors, and both of these factors and their role in the microenvironment will be described in detail.

A. TGF-β

One soluble factor that is critical in the BM microenvironment is TGF-β, which is secreted by BMSCs and HOB. TGF-β is a factor that controls proliferation, cellular differentiation, apoptosis and hematopoiesis[12;75]. There are three different isoforms, TGF-β1, TGF-β2 and TGF-β3, and all the isoforms have distinct functions[75]. TGF-β1 has been shown to inhibit proliferation of hematopoietic progenitors and stem cells[76;77]. TGF-β2 has been reported to positively regulate HSCs[77] and TGF-β3 has been shown to have inhibitory effects on progenitors[75]. TGF-β is a member of a superfamily of growth factors that are divided into two families which are characterized both by sequence similarity among members as well as the signaling pathways that each family activates[75]. The first family is the TGF-β/ activin/ nodal family which primarily use Smad2 and Smad3 for signaling[75]. The second family is the bone morphogenetic protein/ growth and differentiation factor/ Muellerian inhibiting substance and these factors mainly signal

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through Smad1, Smad5 and Smad8[75]. TGF-β signaling occurs through TGF-β receptors on the surface of cells through a heteromeric receptor complex[78]. TGF-β binds to TGF-β Type II receptors and the Type II receptors then recruit TGF-β Type I receptors and subsequently phosphorylates serine residues on the cytoplasmic domain. This then allows for the phosphorylation of receptor Smads (R-Smads), which are Smads 1, 2, 3, 5 and 8[79]. The R-Smads form dimers and then complex with Smad4. This complex then translocates into the nucleus where it can regulate transcription of target genes. In addition, inhibitory Smads (I-Smads) also exist as a way to negatively regulate TGF-β signaling[79]. I-Smads will compete with R-Smads for binding to Smad4. I-Smads also tag the receptors for degradation by recruiting Smurf, an E3 ubiquitin ligase[80].

Several studies have shown the role of TGF-β1 in maintaining quiescence of HSCs. Massague et al showed that TGF-β1 is able to down regulate c-myc, which is a known growth promoting factor[81]. Studies by Cheng and Scandura demonstrated the ability of TGF-β1 to induce cyclin dependent kinase inhibitors, which inhibit cell cycle progression[82;83]. In addition to these factors, TGF-β has also been reported to affect the proliferation of HSCs by downregulating the receptors for granulocyte macrophage colony stimulating factor (GMCSF), Interleukin-3 (IL-3) stem cell factor (SCF) and IL-6[84-86]. The levels of TGF-β1 in the microenvironment have been shown to be extremely important. TGF-β at low, physiological levels is thought to induce CD34 antigen expression on stem cells, and this was sufficient to maintain hematopoietic immaturity[86;87]. Pierelli et al supported this finding by demonstrating that TGF-β1 upregulated both CD34 mRNA and protein[88]. They showed this led to Smad activation and decreased phosphorylation of p38, which may be another method of regulation of quiescence. In addition to maintaining quiescence, Basu et al showed that TGF-β1, at low levels, modulates SDF-1 responsiveness of CD34+ cells to help with homing to the bone marrow[89]. *In vivo* studies to determine the exact mechanisms by which TGF-β1 regulates quiescence have been difficult as the TGF-β1 knock-out mouse is 50% embryonic lethal at

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embryonic day (E)10.5 caused by defective hematopoiesis and yolk sac vasculogenesis[90;91]. For the mice that do survive, they die after 3 weeks due to inflammatory disease and wasting[92]. Yaswen et al were able to show that this defect was something inherent to the HSCs as this disease is transplantable[93]. The TGF-βRII knock-out mouse has a phenotype identical to the TGF-β1 knock-out mouse, suggesting an important role for TGF-β signaling in early embryonic development[94]. Capron et al used a conditional TGF-β1 -/- murine model[95]. These mice had no evidence of inflammatory disease at 8-10 days and their studies demonstrated that these animals had impaired short and long term repopulating activity that correlated with decreased homing, suggesting TGF-β1 has an important role in homeostasis of HSCs, including homing to the marrow.

While TGF-β may play important roles in maintenance of quiescence, there are also studies that suggest TGF-β is not necessary in this capacity. Larsson et al used a conditional knock-out of the TGF-βRI[96]. In their first study, they showed that the TGF-βRI null mice had normal hematopoiesis with respect to cell numbers as well as the ability of the progenitor cells to differentiate. The HSCs from the TGF-βRI null mice were also able to repopulate both primary and secondary recipients following bone marrow transplantation. In additional studies, they treated both knock-out mice and controls with 5-fluorouracil (5-FU) and completed competitive transplantation assays[97]. They found no difference in susceptibility of HSCs to damage and with serial transplants, recipients developed hematopoietic failure at the same time, regardless of the bone marrow from which they were derived. They suggested this shows that TGF-β does not maintain the stem cell pool *in vivo*.

TGF-β is not only involved in regulation of hematopoiesis as it has been implicated in a variety of other disease settings. TGF-β has been associated with osteoporosis, atherosclerosis and tissue and organ fibrosis[98]. TGF-β has also been implicated in cancer. It has been shown to lead to increases in extracellular matrix production and increases in angiogenesis[98;99]. Additionally, TGF-β has been shown to have the ability to be immunosuppressive by

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suppressing infiltrating immune cells[100].

B. Interleukin-6

IL-6 as a classic inflammatory cytokine

Another factor of interest to our lab is interleukin-6 (IL-6), as a factor that has important roles in the hematopoietic system. IL-6 is secreted from both BMSCs and osteoblasts and has both proliferative and anti-proliferative effects[101;102]. It is a pro-inflammatory cytokine that is secreted by T-cells and macrophages in response to tissue injury or pathogens, leading to acute phase responses and fever[101;102]. IL-6 also has important roles in bone metabolism by stimulating osteoclast formation. In the bone marrow microenvironment, IL-6 is important for inducing B-cell differentiation, stimulation of T-cells[103], enhancement of hematopoietic colony formation and the maturation of megakaryocytes[101]. IL-6 belongs to a family of cytokines that have redundant and overlapping signaling pathways due to the use of a common signal transducer, gp130[102]. IL-6 signaling occurs through a hexameric complex at the cell membrane[104]. An IL-6 molecule interacts with a membrane-bound IL-6 receptor. This interaction leads to an association with gp130, which is a glycoprotein also found in the membrane that allows for transduction of the signal[105]. Upon stimulation with IL-6, gp130 becomes phosphorylated on tyrosine residues[106] leading to the activation of signal transducers and activators of transcription (STATs), including, STAT3. The STAT proteins then interact with members of the Janus Kinase family (JAKs),[107] and IL-6 primarily activates JAK1 and STAT3. IL-6 signaling can also lead to the activation of Ras signaling[108]. The signaling cascades that begin in the cytoplasm can then signal to transcription factors which can enter the nucleus and regulate IL-6 expression. The promoter region of the IL-6 gene contains response elements that include binding sites for activator protein-1 (AP-1), Nuclear Factor-IL-6 (NF-IL6) and NF-κB[109] that modulate IL-6 transcription. There are also factors that have been shown to be important in the negative regulation of IL-6 signaling, including Suppressor of cytokine signaling (SOCS) proteins.

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SOCS proteins are negative regulators of cytokine-mediated homeostasis[110;111]. There are a number of SOCS proteins but SOCS3 has been shown to be a critical regulator of IL-6. A SOCS3 knock-out is embryonic lethal and conditional knock-out leads to neutrophilia and inflammatory diseases, suggesting important roles for the protein in IL-6 associated inflammatory diseases.

In addition to the membrane-bound IL-6 receptor, a soluble form of the receptor also exists. There are two possible mechanisms that have been described that lead to the production of the soluble IL-6 receptor. The first mechanism is through differential mRNA splicing, with the soluble IL-6 receptor lacking the trans-membrane domain[112]. The second mechanism suggests that the soluble receptor is produced by cleavage of the membrane-bound receptor by an endogenous protease, although no endogenous protease has been identified[113]. The soluble IL-6 receptor binds to IL-6 molecules with a similar affinity to that of the membrane-bound receptor and can interact with gp130 on the membrane, leading to IL-6 signaling in the same manner. Gp130 is ubiquitously expressed throughout the body on a variety of cell types and tissues. Hibi et al. suggested that the presence of the soluble IL-6 receptor/ IL-6 complex, and the ubiquitous expression of gp130, may allow cells that would not normally respond to IL-6 to become responsive[114]. This could potentially lead to a situation of systemic inflammation similar to graft-versus-host disease.

Interleukin-6 and neurotrophins

In addition to the regulation of hematopoiesis by the supportive cells of the bone marrow microenvironment, the neuroendocrine system is also thought to play important roles in the regulation of hematopoiesis and the immune system[115]. While the most well studied effects of neurotrophins have been on cells of the nervous system, studies have also shown that cells of the bone marrow express neurotrophin receptors as well as secrete neurotrophins[116]. Members of the neurotrophin family include nerve growth factor (NGF), brain-derived neurotrophic factor

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(BDNF), neurotrophin-3 (NT-3), neurotrophin-4 (NT-4) and neurotrophin-5 (NT-5)[116]. The members of the neurotrophin family signal through Trk receptors, which are receptor tyrosine kinases, on the surface of cells. The Trk receptors include TrkA, which binds NGF, TrkB which binds BDNF and NT4/5, and TrkC which binds NT-3. There is also a low affinity neurotrophin receptor p75NTR that has no tyrosine kinase activity but can bind the neurotrophins and interact with the Trk receptors to enhance or, in some instances, inhibit signaling[116].

Studies have shown that NGF has roles in colony formation as well as synergistic effects with GM-CSF allowing for the differentiation of myeloid cells[117]. It was also shown that leukemic cell lines expressed both neurotrophic factors as well as the receptors, suggesting potential roles for these receptors in normal hematopoietic cells[118]. NGF has the ability to interact with other hematopoietic cytokines, such as interleukin-2 (IL-2), where Brodie et al, using B lymphocytes as a model, showed NGF and IL-2 could increase each other's receptor expression[119]. In a model of stromal cells isolated from the thymus, it was shown that NGF increased IL-6 expression[120]. The role of BDNF in hematopoiesis was also studied using a BDNF knockout mouse[121]. These mice had reductions in the number of B lymphocytes both in the spleen and the bone marrow, suggesting this neurotrophin factor has roles in B lymphocyte development. These data provide strong evidence that factors commonly thought to regulate cells of the nervous system also have paracrine effects on cells of the hematopoietic system.

Work by our lab has also shown the presence on neurotrophin receptors present on the surface of BMSC[122]. BMSC respond to NGF and BDNF by increasing IL-6, and increases in IL-6 are associated with a number of inflammatory diseases such as systemic lupus erythematosus and rheumatoid arthritis[118]. The presence of both neurotrophins and their receptors on BMSC provide a possible mechanism of disruption of normal hematopoiesis if not tightly regulated (Chapter 2).

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Interleukin-6 deficiency

While most literature on IL-6 focuses on increased levels of IL-6, the interest of our lab is the impact of an IL-6 deficit in the BM microenvironment. In order to study the different aspects of IL-6 signaling, a number of transgenic models have been used. Gp130 knock-out is embryonic lethal so experiments to study this common signaling receptor use conditional deletions post-natally[123-125]. This mutation still led to impaired acute phase immune response, thrombocytopenia, demyelination of nerves and the development of emphysema. Efforts to study individual members of the IL-6 family have been more challenging as many of these family members are redundant *in vivo*[102]. IL-6 knock-out mice are not embryonic lethals, indicating IL-6 is not critical for embryonic development. However, IL-6 knock-out mice have hematopoietic deficiencies, decreased acute phase response, defective bone maintenance and an increase in susceptibility to infections with a variety of different pathogens[126;127]. It has been documented that an IL-6 deficient environment decreases DNA synthesis in progenitor cells and excessive TGF-β production by BMSCs inhibits IL-6 production[128;129]. Exogenous IL-6 has also been combined with some radiation therapies because the therapy causes a decrease in IL-6, and the addition of IL-6 as part of the therapy accelerates hematopoietic recovery[130]. Preliminary data from our lab suggests that chemotherapy treatment decreases IL-6 protein *in vitro* (Chapter 3). Based on association of IL-6 deficits with sub-optimal hematopoietic recovery, further investigation of the mechanisms by which chemotherapy alters IL-6 expression is warranted.

III. Damage of the microenvironment by cancer therapies

Myeloablative chemotherapy and total body irradiation are commonly used to prepare individuals for bone marrow or stem cell transplantation. While the goal of these regimens is to eliminate tumor cells, the residual damage to the supportive cells of the microenvironment can affect the hematopoietic recovery of these patients long-term. The earliest studies by

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Chamberlin and Fried showed the effects of radiation and chemotherapy on hematopoietic stromal cells[131;132]. In their studies, they observed that the hematopoietic cells (CFU) were able to recover, while the hematopoietic stroma did not recover in models where damaged femurs were transplanted into isogeneic hosts. It is well documented that chemotherapy induces stromal cell damage. Patients receiving standard chemotherapy regimens had a reduced capacity to form confluent monolayers when BMSCs were isolated from aspirates[133]. Stromal cells damaged by chemotherapy and radiotherapy can affect the ability of the BMSCs to self-repair and can also lead to chronic states of osteoporosis by altered bone metabolism as well as a decreased number of functional mature cells in the blood[134;135]. Galotto et al demonstrated that patients receiving allogeneic bone marrow transplants have serious and irreversible stromal damage as measured by CFU-F frequencies that did not recover to the levels of normal control patients even after 12 years[136]. They reported that CFU-F frequencies were reduced by 60-90% in bone marrow transplant patients. In another study, damage of the bone marrow was evaluated using cell culture assays[137]. One year post-treatment, bone marrow samples were isolated from normal donors or patients that had received peripheral blood stem cell transplantation for a variety of different cancers including breast cancer, non-Hodgkin lymphoma and Hodgkin lymphoma. The samples were analyzed using colony forming assays and long term bone marrow cultures (LTBMCs). They determined that the numbers of committed progenitors were significantly reduced in all the groups that had received prior treatment. A healthy stromal cell compartment should have cobblestone areas, adipocytes and support colony formation. The LTBMCs also showed that the adherent layer of cells from patients treated with chemotherapy did not have these properties and could not support progenitor cells[137].

Damage to MSCs, which have the ability to differentiate into multiple cell types such as osteoblasts, adipocytes or BMSC, have also been studied to understand how damage to the cells that can "reconstitute" the supportive cells of the bone marrow are affected by

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chemotherapy and how hematopoietic recovery might be affected by this damage. Kemp et al evaluated MSCs and how this population of cells is affected by high dose chemotherapy[31]. MSCs from patients with hematological malignancies were characterized and compared to untreated patients. It was shown that the MSCs isolated from patients with hematological malignancies were not able to expand in culture, making them unable to be stimulated to determine if the cells had the capacity to differentiate into different cell types. The cells also had decreased CD44 staining, which is a cellular adhesion molecule important for MSC interaction with HSCs. These data suggest that co-transplantation with MSCs may be beneficial.

Many efforts have been made to determine ways to help patients receiving transplants have more rapid hematopoietic recovery. Some of the first factors investigated were recombinant human GM-CSF and G-CSF[138]. The use of these factors showed increases in neutrophils and in a trial by Klumpp et al, it was demonstrated that administering G-CSF led to significant decreases in time to neutrophil engraftment, and the patients had decreased hospital stays, decreased treatment with antibiotics and a more rapid myeloid recovery[139]. Erythropoietin (EPO), another hematopoietic factor that controls red blood cell production and is commonly administered to patients to help with recovery from anemia[138]. IL-6 was investigated to determine if giving it to patients post-transplant would help promote immune system recovery[140]. This cytokine did provide some moderate effects by increasing megakaryopoiesis and thrombocytopoiesis, the cytotoxic effects, including fevers, chills, fatigue, anemia and erythema have made clinicians cautious about use of the cytokine post-transplant. Some groups have also evaluated administering factors during chemotherapy to aide in hematopoietic recovery. Matrosova et al demonstrated that 5-fluorouracil (5-FU) decreases hyaluronic acid (HA) in the bone marrow and HA has been shown to be important for hematopoiesis *in vitro*[141]. Mice given HA while being treated with 5-FU displayed a more rapid recovery of hematopoiesis. They showed increased hematopoiesis in the bone marrow of these mice, suggesting not only that HA had important roles in hematopoiesis but also that administering factors may help with

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hematopoietic recovery. While a variety of hematopoietic factors have been investigated to help with hematopoietic recovery, no individual factor or combination of factors has been identified that allows complete hematopoietic recovery.

TGF-β, as an important factor in the microenvironment for maintenance of quiescence, has been previously described. Although there are many studies that address the effects of chemotherapy on TGF-β, most of them describe increases in TGF-β as a negative prognostic factor. In several reports, it has been documented that chemotherapy can lead to an increase in TGF-β levels. In a study by Butta et al, it was demonstrated that Tamoxifen led to an induction of TGF-β1 as seen in biopsy samples from patients with breast cancer[142]. Furthermore, TGF-β inhibitors are currently being used in both pre-clinical and clinical trials as well as neutralizing antibodies as a method to reduce cancer progression and to aid in hematopoietic recovery[143]. TGF-β is also being used as a prognostic factor in radiotherapy for non-small cell lung cancer to determine which patients can receive escalated therapy. The higher the TGF-β levels were in the patient, the greater the toxicity[144]. In a different cancer setting, it was shown that chemotherapy treatment for Acute Lymphoblastic Leukemia led to increased TGF-β levels, decreased ability of stromal cells to support hematopoiesis, and post-treatment anemia[145]. Based on the data describing increases in TGF-β in response to chemotherapy, one could deduce that this increase in TGF-β detrimentally affects the stem cell population in the bone marrow.

Collectively, these observations emphasize the microenvironment as a site that is vulnerable to change imposed by diverse treatments. The dynamic nature of the microenvironment is reflected, in part, by fluctuation in soluble factors produced by microenvironment cellular components. Our model of chemotherapy-induced stromal damage, uses the chemotherapeutic agent melphalan, with VP-16 included in some experiments as an agent we have characterized to some degree previously. VP-16 (Etoposide) is a topoisomerase II inhibitor, which leads to strand breaks in the DNA making it a popular anti-cancer agent. It

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has been used in Ewing's sarcoma, lung cancer, lymphoma, leukemia and in pre-transplant regimens before bone marrow or stem cell transplants. Melphalan belongs to the nitrogen mustard DNA alkylating class of drugs. Its mechanism of action is to add alkyl groups to the guanine residue in DNA. Melphalan is currently used as front-line therapy for the treatment of multiple myeloma, ovarian cancer, breast cancer as well as being used as part of a pre-transplant chemotherapy regimen for autologous stem cell and allogeneic stem cell transplantations, making it a clinically relevant agent for our experiments[146;147]. Studies by Down et al showed that melphalan was more toxic to early developing cobblestone area-forming cells. In this study, mice were treated with melphalan or a number of other chemotherapeutic agents for 24 hours and the marrow compartment was isolated from femurs. Early and late cobblestone areas were then established in culture[148].

Based on all these data, more studies are necessary to understand the mechanisms by which high dose chemotherapy regimens lead to damage of the supportive components of the bone marrow microenvironment and how this damage leads to a decreased capacity to support both HSCs and hematopoietic progenitor cells.

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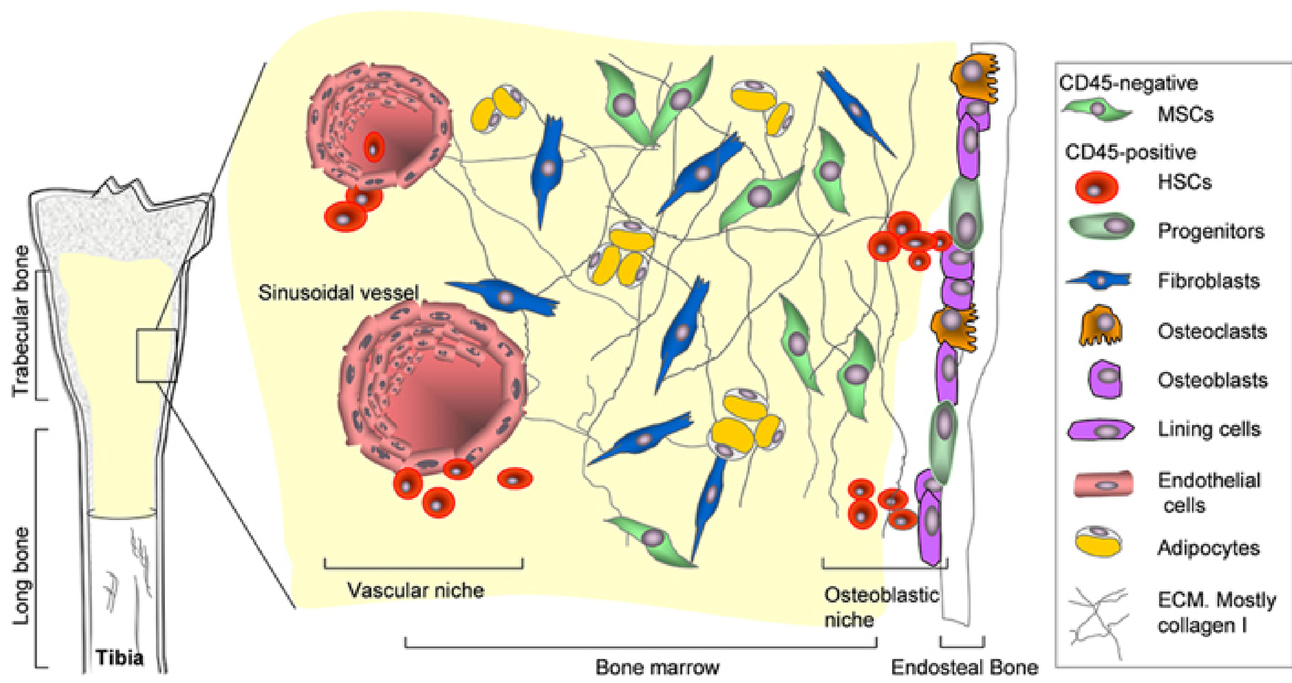

Figure 1. Architecture of the bone marrow microenvironment.

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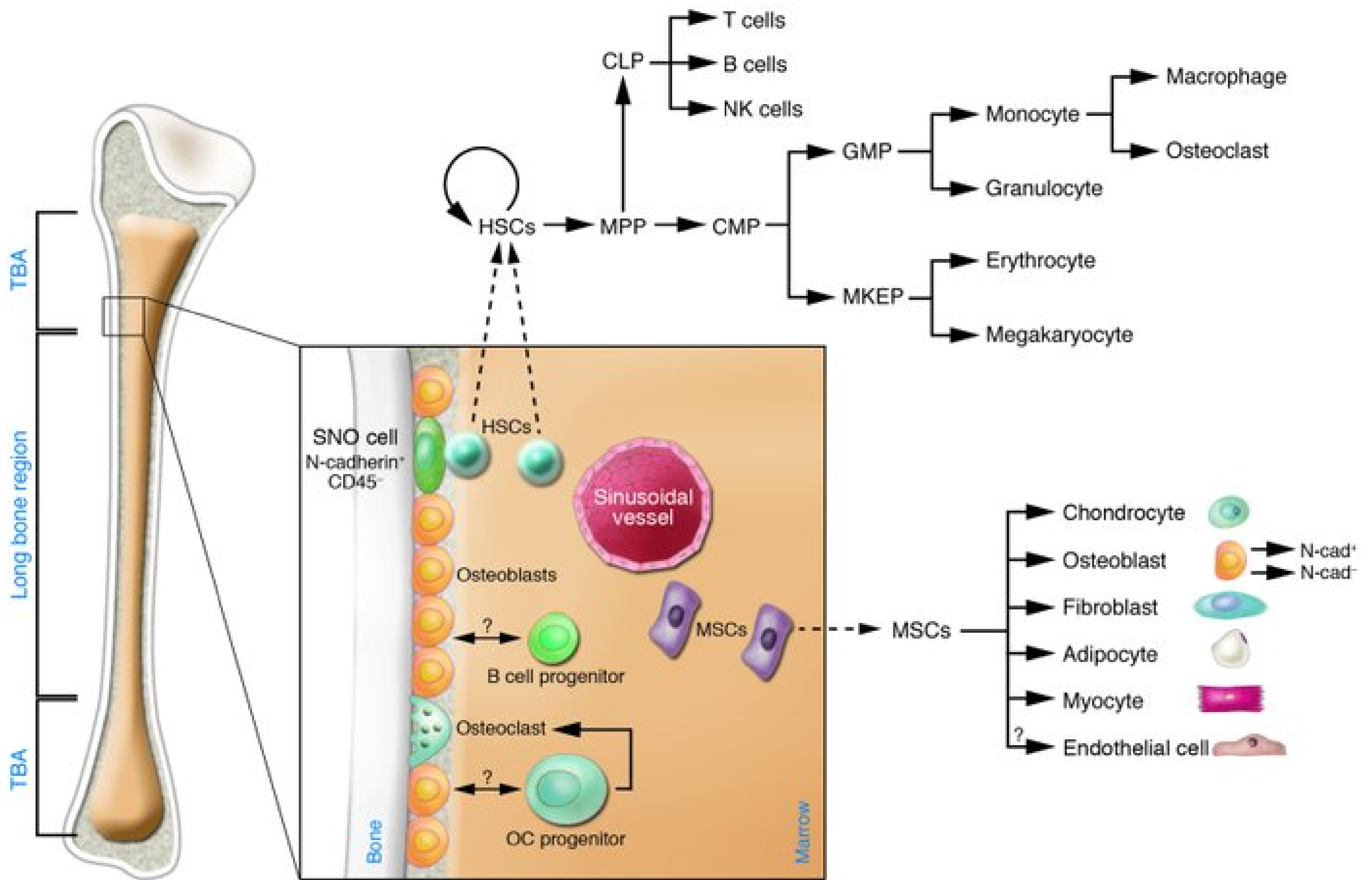

Figure 2: Bone marrow microenvironment and cell differentiation

Yin T and Li L. 2006. The stem cell niches in bone. J. Clin. Invest.

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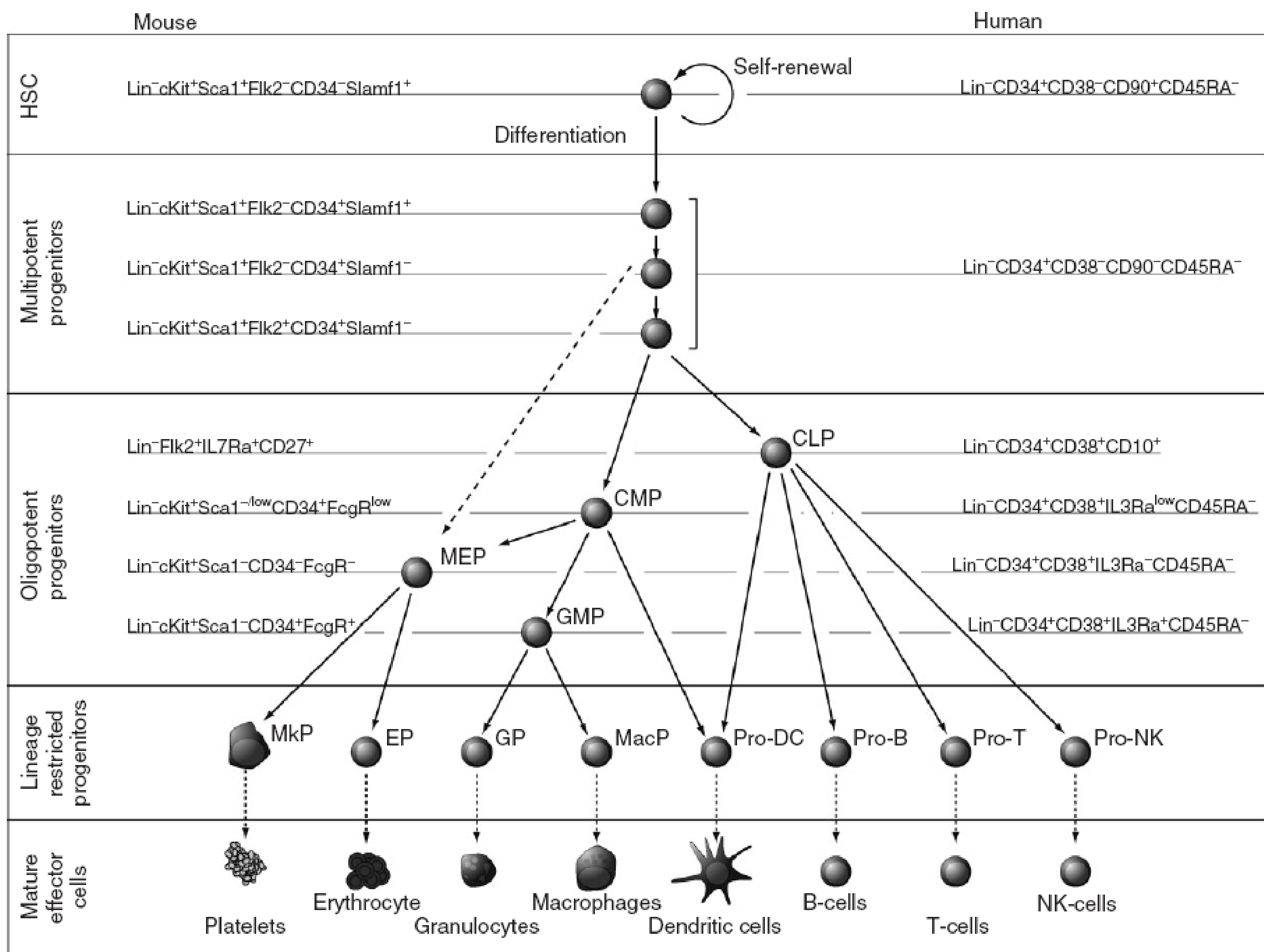

Figure 3: Hematopoietic hierarchy and characterization of stem cell markers

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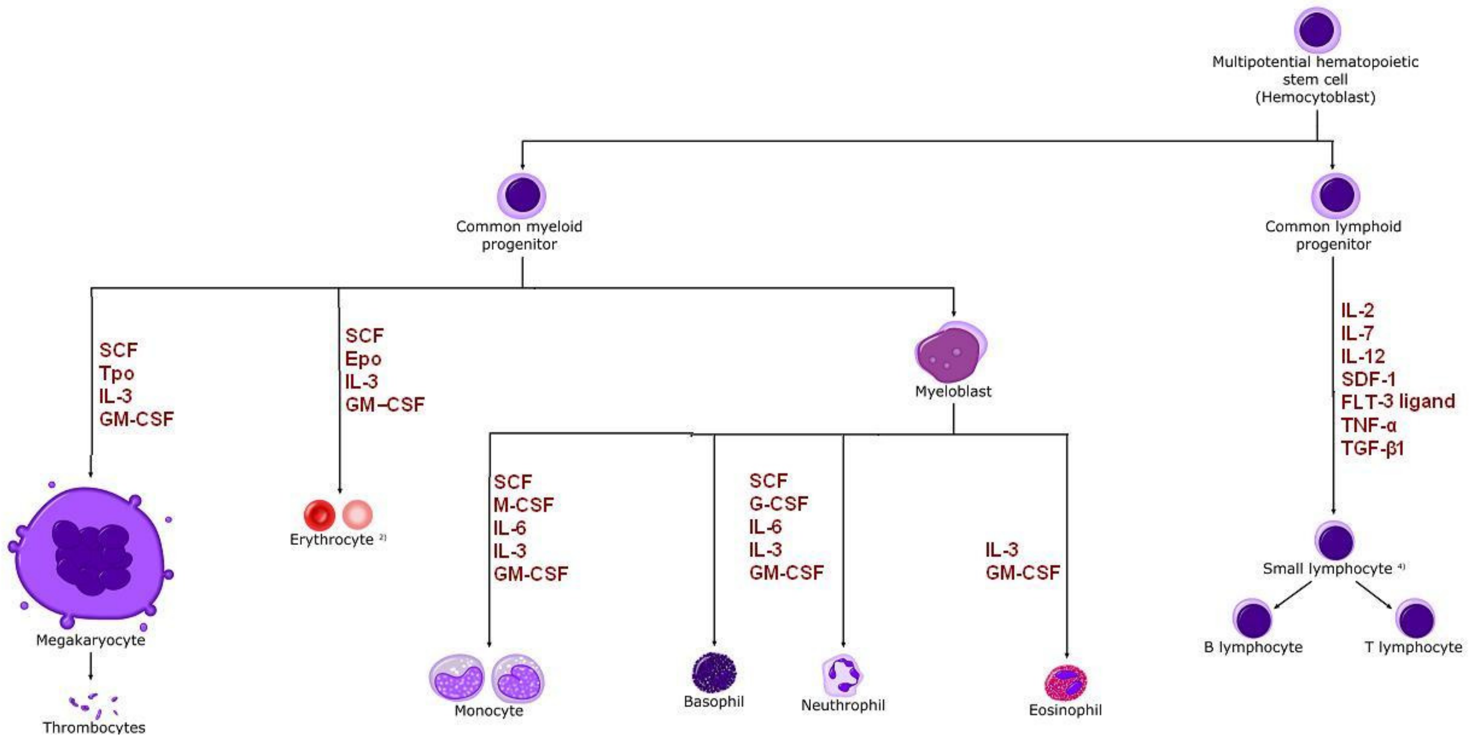

Figure 4: Soluble factors involved in cell differentiation

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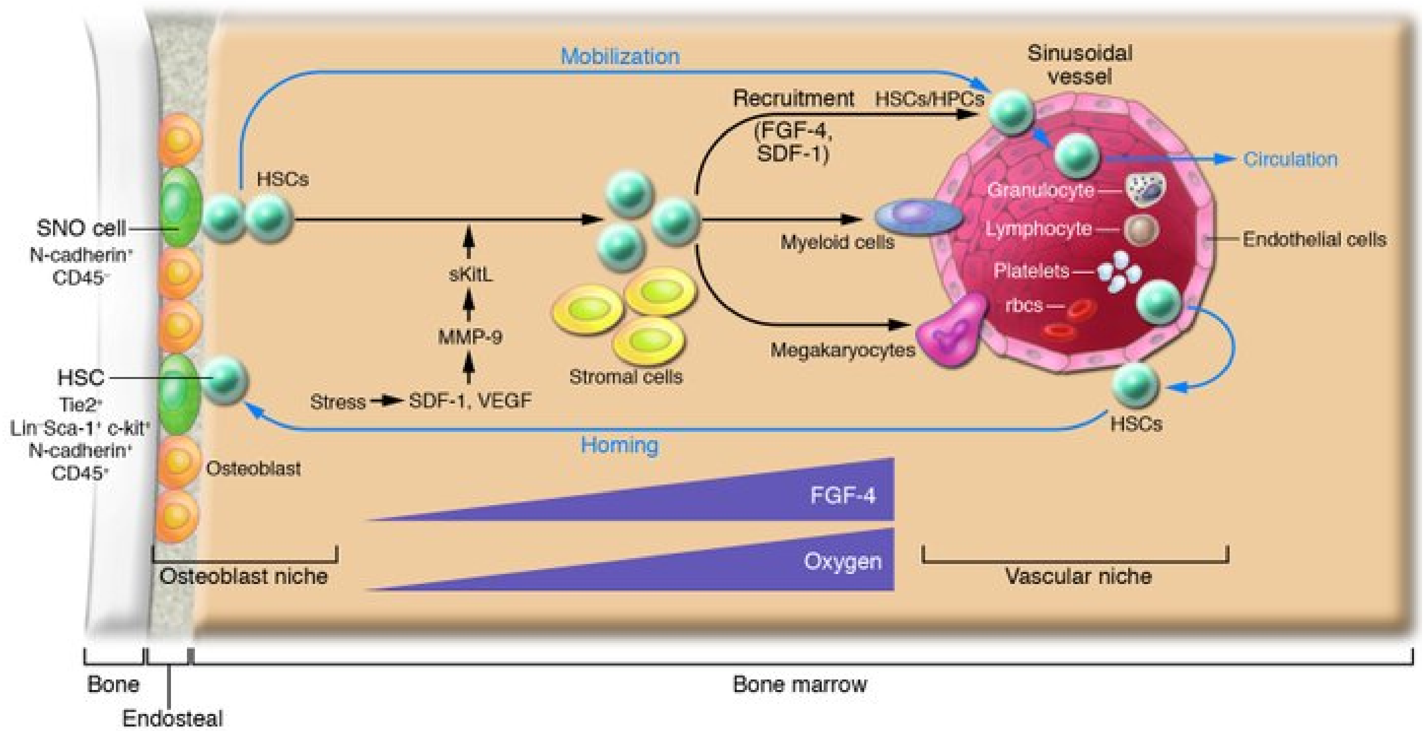

Figure 5: HSC interaction with endosteal and vascular niches

Yin T and Li L. 2006. The stem cell niches in bone. J. Clin. Invest.

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Chapter II

Bone Marrow Osteoblast Damage by Chemotherapy

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Abstract :

Hematopoietic reconstitution, following bone marrow or stem cell transplantation, requires a microenvironment niche capable of supporting both immature progenitors and stem cells with the capacity to differentiate and expand. Osteoblasts comprise one important component of this niche. We determined that treatment of human primary osteoblasts (HOB) with chemotherapy resulted in increased phospho-Smad2, consistent with increased TGF-β activity. This increase was coincident with reduced HOB capacity to support immature B lineage cell chemotaxis and adherence. The supportive deficit was not limited to committed progenitor cells, as human embryonic stem cells (hESC) or human CD34+ bone marrow cells co-cultured with chemotherapy or rTGF-β pre-treated HOB had profiles distinct from the same populations co-cultured with untreated HOB. Functional support deficits were downstream of changes in HOB gene expression profiles following chemotherapy. Chemotherapy induced damage of HOB suggests vulnerability of this critical niche to therapeutic agents frequently utilized in pre-transplant regimens and suggests that dose escalated chemotherapy may contribute to post-transplantation hematopoietic deficits by damaging structural components of this supportive niche.

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Introduction:

The stem cell niche hypothesis was first presented in 1978 by Schofield who suggested that stem cells were associated with accessory cells that influence their behavior [1]. Studies from several labs have expanded our appreciation of the unique anatomical niches within the marrow microenvironment and have characterized areas of optimal stem cell support [2]. The niche's cellular components consist of osteoblasts (HOB), bone marrow stromal or mesenchymal stem cells (BMSC, MSC), and endothelial cells [3;4]. Recent work has demonstrated the importance of the interaction of osteoblasts and stem cells in the niche, suggesting that hematopoietic stem cells (HSC) can regulate MSC differentiation into osteoblasts and that they, in turn, play an important role in the support of B lymphocytes and differentiation of HSC [5;6]. Additionally, it has been shown that resting HSC are maintained in a quiescent state as a result of their close proximity to osteoblasts and that the number of HSCs change as a result of the number and type of osteoblasts present [7;8]. Studies describing BMSC have shown that damage by chemotherapy and radiotherapy can affect the ability of the BMSC to self-repair and leads to decreased numbers of functional immune system cells in the blood, with deficits persisting years after transplant [9;10]. The effects of chemotherapy on osteoblasts, and subsequently HSC and progenitor cell support, have not been as well characterized as those on BMSC, and warrant further investigation.

The stem cell niche is characterized, in part, by expression of specific cytokines, including TGF-β and CXCL12, to facilitate signaling between the niche components and HSC. Studies have demonstrated that chemotherapy increases the levels of active TGF-β resulting in decreased ability of BMSC to support HSC [11;12]. It has also been shown that TGF-β has crosstalk with CXCL12 and can stimulate the differentiation of progenitor cells to erythroid and myeloid cells resulting in a deficit of the primitive stem cell pool[13]. The importance of CXCL12 is demonstrated by its requirement for homing of progenitor cells to the bone marrow following transplantation [14;15]. We have previously demonstrated that diminished levels of CXCL12 in the

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supernatants of VP-16 treated BMSC results in loss of an optimal chemokine gradient to which CXCR4+ pro-B cells respond with CXCL12 subsequently shown to also be important in regulation of stem cell phenotype by Guo *et al.* [16;17]. Sugiyama *et al* showed that mice deficient in the CXCL12 receptor, CXCR4, had a reduction in HSC, in both vascular and endosteal niches, and increased sensitivity to myelotoxic stress compared to their wild-type counterparts [18]. Other studies of CXCR4 in the HSC niche have shown that CXCR4 is essential to maintain quiescence and retention of stem cells [19]. In the current study, we investigate chemotherapy-mediated damage of osteoblasts with emphasis on CXCL12 and TGF-β levels following chemotherapy. Global changes in HOB gene expression in response to melphalan were investigated to determine the vulnerability of osteoblasts to genotoxic stress. In addition, TGF-β, CXCL12 and VCAM-1 were investigated as representative osteoblast proteins involved in three critical functions of the endosteal niche; support of pluripotency, homing and stem cell retention [20;21]. Our results indicate diverse changes in gene expression profiles following HOB exposure to melphalan, conditioned media from BMSC pre-treated with melphalan, and following exposure to rTGF-β as one of the factors elaborated by chemotherapy damaged stroma [11]. Coincident with altered gene expression profiles treated HOB had increased levels of active TGF-β, reduced ability to support Oct-4 positive embryonic stem cell colonies, deficits in support of differentiation of CD34+ bone marrow cells, and reduced chemotactic support and adhesion of CXCR4+ pro-B cells. These data suggest that the niche in which hematopoietic recovery occurs may be more vulnerable to damage than previously appreciated.

Materials and Methods:

Cell Lines and Reagents

Human osteoblasts (HOB) were purchased from Promocell (Heidelberg, Germany) and maintained in osteoblast growth media. The CXCR4+/VLA-4+ pre-pro-B leukemic cell line JM-1

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was purchased from the ATCC (CRL-10423, Manassas, VA). The BMSC and IL-7 dependent murine pro-B cell line C1.92 was kindly provided by Dr. Kenneth Landreth and has been described in detail [22]. Human embryonic stem cells (H9, WiCell, Madison WI) were maintained on irradiated mouse embryo fibroblasts (MEF) and grown in DMEM-F12 media (Mediatech, Manassas, VA) supplemented with Knockout Serum Replacement (Gibco/Invitrogen, Carlsbad, CA), 2 mM L-glutamine (Mediatech), 0.05 μM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO), non-essential amino acids and B-FGF solution. H9 cells were moved to HOB feeder layers where indicated. CD34+ bone marrow cells (ALLCELLS, Emeryville, CA) were grown in RPMI-1640 supplemented with 10% fetal bovine serum. Melphalan (Sigma-Aldrich) was reconstituted at a stock concentration of 2.5mg/ml or 50mg/ml immediately prior to use. VP-16 (Etoposide, Bristol Myers Squibb, New York, NY) was stored at a concentration of 33.98mM and diluted immediately prior to use. Human rTGF-β (R&D, Minneapolis, MN) was used at a concentration of 10ng/ml. In all experiments that include rTGF-β, it was added to the culture every 6 hours based on its short half-life. Human rIL-3 (R&D) was used at a concentration of 100 ng/ml.

Adhesion Assay

HOB were pre-treated with 50μg/ml melphalan, 50μM VP-16, or 10ng/ml rTGF-β for 24 hours. C1.92 pro-B cells were stained with CellTracker Green (Invitrogen) according to the manufacturer's instructions. The HOB adherent layer was thoroughly rinsed following treatment and $1x10^5$ fluorescently labeled C1.92 pro-B cells were added in co-culture for 24 hours. Subsequently, the media containing non-adherent C1.92 was aspirated and the cultures were gently rinsed. Remaining HOB and adherent C1.92 were trypsinized and C1.92 cells were enumerated using a FACSCalibur flow cytometer (BD, Franklin Lakes, NJ) with events counted

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for 30 seconds on high flow rate. Data were analyzed using WinMDI software.

Chemotaxis Assays

HOB were plated in the bottom chamber of a transwell at 100% confluence and were left either untreated or treated with 50 µg/ml melphalan, 50 µM VP-16, or 10 ng/ml rTGF-β for 24 hours. After 24 hours, 350 µL of supernatant was placed in the bottom of a transwell and JM-1 cells ($1x10^6$ cells/mL) were added to the top chamber, and incubated at 37°C for 4 hours. JM-1 cells migrated through the 5µm pores to the bottom chamber towards media supplemented with 100 ng/mL CXCL12 (positive control), towards media alone (negative control), or towards media conditioned by the chemotherapy treated or non-treated HOB. Cells were collected using a FACSCalibur flow cytometer (BD) with events counted for 30 seconds on high flow rate. Data were analyzed by WinMDI software.

ELISA

To complete the CXCL12 ELISA (R&D), HOB were plated at 100% confluence in a 96 well plate and left untreated or treated with 10 ng/ml rTGF-β, 50 µg/ml melphalan or 50 µM VP-16 for 24 hours. The media was then removed, cells were rinsed and fresh serum-free media was added to each well. Following 24 and 48 hours of incubation supernatants were collected and analyzed for CXCL12 following the manufacturer's instructions. The TGF-β ELISA (R&D) was completed using HOB plated at 100% confluence in serum free media and left untreated or treated with 10 ng/ml rTGF-β, 50 µg/ml melphalan or 50 µM VP-16 for 24 hours. The media was removed, cells were rinsed, and fresh serum-free media was added to each well. After 24 and 48 hours supernatants were collected and analyzed for TGF-β following the manufacturer's instructions.

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Fluorescent Microscopy

HOB cells were cultured on coverslips and left untreated or treated with 50 µg/ml melphalan, 50 µM VP-16, or 10 ng/ml rTGF-β for 24 hours, washed thoroughly and H9 stem cells added. Stem cell colonies were monitored for 2 days and counts based on colony morphology were completed. Cells were subsequently stained for Oct-4. To complete intracellular staining, cells were fixed in 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. After blocking for 30 minutes in 5% BSA/1X PBS, cells were incubated with mouse primary antibody (1µg) specific for human Oct-4 or the matched isotype control, in 5% BSA/1X PBS for 1 hour at RT. Coverslips were washed with 1X PBS, incubated with Alexa Fluor 488 labeled secondary α-mouse antibody (1 µg) at RT for 1 hour and mounted on glass microscope slides with ProLong Gold plus DAPI (Invitrogen). Confocal images were acquired using a Zeiss LSM510 confocal system connected to a Zeiss AxioImager microscope (Thornwood, NY). Photographs of human embryonic stem cells were taken using a Nikon Coolpix 990 camera. To complete phospho-Smad2 staining, HOB cells were plated on coverslips and left untreated or treated for 4 hours with 100 µg/ml melphalan, 100 µM VP-16, or with conditioned media from BMSC that were untreated or treated with 100 µg/ml melphalan or 100 µM VP-16 for 24 hours. Staining and imaging was completed as described above using a murine primary antibody (1.5 µg/coverslip), specific for human phospho-Smad2 (Cell Signaling Technology Inc., Danvers, MA) or matched isotype control.

Microarray

HOB cells were left untreated, or treated with 50µg/ml melphalan, 10ng/ml rTGF-β or conditioned media from BMSC (treated with 50µg/ml melphalan for 24 hours) for 6 hours. Total RNA was isolated from HOB using the RNeasy RNA isolation kit (Qiagen,Valencia, CA) with quality assessed by electrophoretic analysis on an Agilent Model 2100 Bioanalyzer. RNA

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samples had integrity numbers greater than 8.0 (8.4-10). RNA (250 ng) was used as the template for synthesis of internally labeled cRNAs using the Agilent QuickAmp Labeling kit and cyanine 3-CTP and cyanine 5-CTP (Perkin Elmer, Waltham, MA) and a modified QuickAmp protocol [23]. A total of 825 ng of cyanine 3- and cyanine5-labeled cRNAs was combined and hybridized onto Agilent Whole Human Genome 4 x 44 K microarrays at 65°C for 17 hours and washed according to the manufacturer's protocol. Slides were scanned on an Agilent DNA Microarray Scanner. HOB treated with rTGF-β and conditioned media were competitively hybridized against untreated HOB in a balanced block design with six replicates. Melphalan treated HOB and untreated HOB were hybridized against Stratagene Universal Reference RNA (Agilent Technologies, Santa Clara, CA) in a universal reference design with four replicates. Intersections of groups and corresponding statistically significant fold changes (details described in the supplemental section) for each experiment were imported into Ingenuity Pathway Analysis (IPA) software v 2.6 (Ingenuity Systems ®, Redwood City, CA, www.ingenuity.com). We performed a core analysis in IPA, using default settings, to search for networks associated with these lists of genes. Complete microarray data may be accessed at the NCBI Gene Expression Omnibus (GEO) database (GSE17860).

Real Time Reverse Transcriptase PCR

Total cellular RNA was isolated from HOB using the RNeasy RNA isolation kit (Qiagen). Real time RT-PCR was performed using 50ng RNA per reaction using the QuantiTech SYBR Green RT-PCR kit supplied by Qiagen. Primers specific for human CXCL12 were obtained from SuperArray (Frederick, MD) with 0.25μl used per reaction. Primers specific for TGF-β and the housekeeping gene (HPRT-1) were purchased from Real Time Primers, LLC (Elkins Park, PA). Samples were analyzed in triplicate using the Applied Biosystems 7500 Real-time PCR system (Foster City, CA). Amplification parameters included 50°C for 30 minutes, 95°C for 15 minutes,

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94°C for 15 seconds (x 45 cycles), 58°C for 30 seconds, and 72°C for 45 seconds. Changes in gene expression were determined using the Comparative Ct method and analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.

HOB and CD34+ cell co-culture

HOB were grown to confluence and were either untreated or treated with 25ug/ml melphalan or 10ng/ml rTGF-β1 for 24hrs. Following treatment, the cells were rinsed and human CD34+ bone marrow cells were added. Cultures were supplemented with recombinant IL-3 (100ng/ml) every 2 days. CD34+ bone marrow cells were collected 2 and 6 day post co-culture. Viability was determined and staining for the following cell surface makers was completed using the indicated antibodies: phycoerythrin (PE)–conjugated CD4 (clone SK3), CD19 (4G7), phycoerythrin-Cy7 (PE-Cy7)–conjugated CD56 (NCAM16.2), CD34 (8G12), peridinin-chlorophyll protein-Cy5.5 (PerCP-Cy5.5)–conjugated CD3 (SK7), CD33 (P67.6), fluorescein isothiocyanate (FITC)–conjugated CD8 (SK1), CD71 (L01.1), allophycocyanin (APC)–conjugated CD14 (MφP9), CD3 (SK7), and allophycocyanin-Cy7 (APC-Cy7)–conjugated CD45 (2D1) ([BD], San Jose, CA). The samples were stained in the following combinations: isotype controls and CD45, CD45, CD34, CD3, CD19, CD33, and CD71 and CD45, CD3, CD4, CD8, CD56, and CD14. Data were acquired with a FACSCanto II (BD) flow cytometer with a minimum of 30,000 events for each sample and analyzed with FACSDiva software (BD). Initial gating was based on forward (FSC)/ side scatter (SSC) to exclude debris and non-viable events. Thresholds for positivity were set such that greater than 99% of events within the gated region were negative for each isotype-matched control antibody. Positive events were back-gated to ensure that they constituted a discrete population by CD45/SSC, confirming specificity of antigen binding.

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Statistics

Data were analyzed using the Students–t test or ANOVA where appropriate with statistical significance of $p \leq .05$ denoted by an asterisk (*). Microarray data analysis is described in the supplemental section.

Results

Melphalan, or factors from chemotherapy damaged BMSC, affect TGF-β pathways in HOB.

Earlier observations have demonstrated that BMSC exposed to chemotherapy have higher levels of active TGF-β and diminished capacity to support pro-B cells and normal hematopoiesis [11-13;24]. Additionally, retrospective studies of patients that received allogeneic bone marrow transplants showed that they have serious and irreversible stromal damage as measured by CFU-F frequencies that did not recover to the levels of normal control patients even after 12 years, suggesting that the damage done to the supportive cells of the bone marrow is irreversible [25].

To determine if direct chemotherapy damage to HOB cells results in increased active TGF-β, HOB were treated with melphalan or VP-16 and the expression of total and active TGF-β was assessed. Direct exposure to chemotherapy (Figure 1A) does not alter the expression of TGF-β mRNA but does result in increases in active TGF-β capable of signaling, reflected by increased phosphorylation of Smad2 protein. However, direct treatment with rTGF-β (Figure 1 B) increases both TGF-β mRNA and protein expression. To mimic the indirect effects of soluble cues elaborated by damaged stroma on HOB, BMSC were treated with melphalan or VP-16, rinsed, and allowed to condition media that was then placed on HOB that had not been exposed to chemotherapy. HOBs exposed to conditioned media from damaged BMSC have higher levels

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of phosphorylated Smad2 than their counterparts exposed to conditioned media from untreated stroma (data not shown).

Chemotherapy or rTGF-β diminishes HOB support of human stem cells and adhesion of pro-B cells.

One of the critical functions of osteoblasts in the endosteal niche is to support stem cells, and post-transplantation, osteoblasts are crucial to efficient and sustained hematopoietic reconstitution [26]. Therefore, we investigated how chemotherapy or rTGF-β modulates the ability of osteoblasts to support human stem cells. Figure 2 shows that in the absence of treatment, osteoblasts support undifferentiated stem cell colonies characterized by morphology of dense round colonies with definitive, regular, cell borders. In contrast, after HOB pre-treatment with melphalan, VP-16 or rTGF-β, there is an increase in the number of differentiated stem cell colonies with irregular borders (Figure 2A). Oct-4 staining was completed on stem cell colonies as a measure of pluripotency potential, with a decrease in the ability to support Oct-4 positive colonies observed in HOB that had been pre-treated with chemotherapy or rTGF-β (Figure 2B).

Pre-treatment of HOB with rTGF-β or melphalan changes the differentiation pattern of CD34+ cells.

We next investigated the effects of these treatments on the ability of HOB to support CD34+ hematopoietic stem/ progenitor cell self-renewal and lineage differentiation. Figure 2C summarizes observations indicating that following exposure to rTGF-β or melphalan, distinct differentiation patterns of CD34+ bone marrow cells was supported. Deficits in total lymphocytes, with specific alteration of B cell and NK cell differentiation were seen subsequent to treatment. T-cells represented 0.1% of the lymphocyte cell population in the untreated group, while no T cells were detected in cultures that included HOB treated with rTGF-β or melphalan

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(data not shown). Additionally, melphalan treatment of HOB leads to a decreased granulocyte population, while exposure to rTGF-β enhances granulocyte differentiation and both rTGF-β and melphalan decreased erythrocyte progenitors. There was no change in monocytes with rTGF-β treatment and a slight decrease in monocytes with melphalan treatment (data not shown) and viability among all groups was similar (82-87%). Taken together, these data suggest that exposure of HOB to direct and indirect chemotherapeutic damage has functional consequences with respect to support of hematopoietic differentiation.

Alterations in osteoblast function after aggressive treatment could impact transplant engraftment and hematopoietic reconstitution [8;20]. For technical ease we utilized CXCR-4+/VLA-4+ C1.92 and JM-1 pro-B cells to investigate the effects of chemotherapy on the ability of HOB to support immature hematopoietic progenitor cell adhesion and chemotaxis. Experiments summarized in Figure 2D indicate that following HOB pre-treatment with chemotherapy or rTGF-β, C1.92 pro-B cells did not adhere as efficiently as they did to untreated controls. To determine if alterations in adhesion molecule expression were associated with decreased adhesion between C1.92 and HOB, VCAM-1, CD44 and Hyaluronan expression were evaluated on the HOB in the presence and absence of chemotherapy. No modulation of these proteins was detected on the HOB during chemotherapy exposure (data not shown).

Chemotherapy or rTGF-β diminishes HOB expression of CXCL12.

Inhibition of CXCL12 in the bone marrow has been shown to have a negative impact on chemotaxis leading to deficits in HSC homing and engraftment [14;27]. To further investigate the impact of chemotherapy and rTGF-β treatment on expression of osteoblast derived CXCL12, real time RT-PCR and ELISA were completed as described. Pre-treatment of HOB with chemotherapy or rTGF-β decreased the amount of CXCL12 mRNA and protein detected by real time RT-PCR and ELISA, respectively (Figure 3A and B). Jung *et al* have described how the

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regulation of SDF-1 by osteoblasts can affect homing and reported that treatment of osteoblasts with TGF-β decreased SDF-1 secretion which is consistent with our results [28]. Additionally, as a functional readout of a potential CXCL12 deficit in our model, chemotaxis of JM-1 cells toward HOB that were untreated or pre-treated with chemotherapy or rTGF-β was completed. Figure 3C summarizes data indicating that chemotaxis of JM-1 cells toward adherent layers of HOB was impaired by melphalan or rTGF-β treatment.

Direct and indirect chemotherapy induced damage results in global changes in HOB gene expression.

To elucidate the global changes that occur in osteoblasts with direct and indirect insult from chemotherapy, HOB were exposed to either rTGF-β or melphalan. In addition, HOB were exposed to conditioned media from BMSC that had been pre-treated with melphalan (drug removed prior to collection of conditioned media) to recapitulate signaling that may occur in response not only to active TGF-β elaborated by BMSC, but also in response to the collective soluble factors elaborated by BMSC in response to chemotherapy induced stress. Microarray analysis of gene expression was performed as described. The genes for which expression changed in each group individually, and common gene targets that overlap between treatment groups, are indicated in the Venn diagram (Figure 4A). HOB exposure to recombinant TGF-β resulted in the highest number of genes influenced across the treatment groups evaluated, with melphalan exposure also resulting in a robust effect. Twenty-five common genes significantly changed when the intersection of all treatment groups was considered. The Venn and network diagrams show the number of genes modulated due to treatment, and potential relationships between some of the responsive genes, as well as convergence on signaling molecules such as the NF-κB complex which emerged as a hub of signaling. The genes that were commonly up-regulated (red) or down-regulated (green) between treatment groups are shown and include: 4

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up-regulated, 2 down-regulated (Figure 4, intersection of all 3 treatments), 16 up-regulated, 3 down-regulated (Figure S1, CMM: melphalan), 26 up-regulated, 11 down-regulated (Figure S2, rTGF:CMM), and 97 up-regulated, 188 down-regulated (S3, rTGF: melphalan). This summary can be compared with those genes that were influenced, but in opposing directions, between groups (S4).

Discussion

Myelosuppressive and ablative therapies followed by stem cell transplantation is used to treat hematopoietic, breast, ovarian and brain tumors as well as childhood sarcomas, and immune deficiencies [29;30]. As the primary site of postnatal hematopoiesis, the functional integrity of the bone marrow microenvironment is critical for hematopoietic recovery. Earlier reports have suggested that BMSC are vulnerable to functional damage imposed by aggressive chemotherapeutic agents [12;13;31]. These studies have focused largely on the ability of stromal cells to generate fibroblastic colonies (CFU-F) or to support survival or expansion of committed progenitor cells when isolated from patients following treatment of subsequent to *in vivo* drug exposures [32].

Murine models of ablative treatment and stem cell transplant have shown long-term deficits in hematopoietic recovery and *in vitro* models have paralleled these documenting the inability of transplanted cells to migrate efficiently to the necessary anatomical niches for engraftment [33]. Observations of long-term hematopoietic deficits in bone marrow of transplantation patients suggest that the functionality of the developmental niches required for appropriate support of immature hematopoietic or stem cells may have been compromised by aggressive pre-transplant therapies. One study observed that at 1 year post transplant 61% of patients have subnormal values in one or more hematopoietic lineages [34]. Further, Nieboer *et al*. showed that at 5 years post transplant 15% of the patient population analyzed had low values in one or more hematopoietic cell lineages [35]. Investigation of the mechanisms that

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underlie damage of the hematopoietic and stem cell niche is further encouraged by retrospective studies of patients that received allogeneic bone marrow transplants in which patient HSC did not recover to the levels of control patients, even after 12 years, as measured by CFU-F frequencies, suggesting that the damage of the structural, hematopoietic supportive cells of the bone marrow can be sustained [36].

In the current study we characterized the impact of direct and indirect damage on osteoblasts and their subsequent ability to support progenitor and stem cells. Following transplantation and during development, HSC home to the endosteal niche which acts as a critical regulator of stem cell quiescence, proliferation, and conservation of the stem cell pool. Direct contact between osteoblasts and HSC is required for HSC survival [7;8;37] with a dynamic relationship demonstrated by the ability of HSC to regulate the cytokines expressed by osteoblasts in order to enhance their own survival. Studies by Calvi *et al*., and others, have shown that number of osteoblasts present in the niche directly modulates the numbers of HSC that can be supported by the niche [7;8;20].

Our data has shown that both direct exposure to chemotherapy as well as exposure to conditioned media from chemotherapy damaged BMSC increases the activity of osteoblast derived TGF-β, one of the known negative regulators of HSCs (Figure 1) [11-13]. Consistent with the literature suggesting that TGF-β activity leads to decreased expression of HSC surface cytokine receptors and a deficit in the stem cell pool,[13;38] Figures 2 and 3 summarize data that show the decreased ability of chemotherapy damaged osteoblasts to interact with, and support, both human embryonic stem cells as well as more differentiated pro-B cells. Direct treatment of HOB with active TGF-β results in a comparable reduction in pro-B cell adhesion and chemotaxis as well as diminished ability to support Oct-4 positive stem cells. In addition, HOB pre-treated with either rTGF-β or melphalan had a decreased ability to support differentiation of CD34+ bone marrow cells. Deficits occurred in the lymphocyte population, with decreased B cells, NK

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cells and T cells in addition to decreased erythroid and granulocyte progenitors. Collectively, these data suggest that chemotherapy induced increases in active TGF-β, from damaged BMSC and HOB, could contribute to deficits in the reconstitution of the hematopoietic niche.

Another consideration when evaluating the dynamic balance of the niche is the role of adhesion molecules physically tethering progenitor cells to supportive cells of bone marrow, which provides signals for their maturation and survival. Earlier reports have described the role of the VCAM-1/VLA-4 interaction in hematopoiesis. Ryan *et al* demonstrated that adhesion of B cell precursors to BMSC was dependent on this interaction and Dittel *et al* elucidated how cytokines could alter the surface expression of VCAM-1 [39;40].

In addition to the VCAM-1/ VLA-4 interaction, the CD44/ hyaluronan (HA) interaction has also been recognized for its role in hematopoiesis and homing of primitive cells to the bone marrow [41]. Avigdor *et al* demonstrated the important roles of CXCL12 with respect to the migration and anchorage of progenitors to the bone marrow through CD44/ HA [42]. It was based on these observations that we investigated the effects of chemotherapy on VCAM-1, CD44, and HA in our model of osteoblast damage. Figure 2D shows that pro-B cells co-cultured with chemotherapy or rTGF-β pre-treated osteoblasts are unable to adhere to the osteoblasts efficiently. However, investigation of the adhesion molecules VCAM-1, CD44 and Hyaluronan indicated no altered expression following either chemotherapy or rTGF-β exposure (data not shown). These observations suggest that the deficit in hematopoietic support, in our model, may be the result of changes in a soluble factor acting in either an autocrine or paracrine manner to diminish optimal cell:cell interaction or that an unidentifiable adhesion molecule is influenced by the treatment we tested. Paracrine effects could be modulated, in part, through alteration of integrin activity, which would not have been detected in our assay. These observations also suggest very specific effects of chemotherapy on stromal and osteoblast components of the niche, emphasizing the need to understand each population individually to understand the total response of the niche to therapy.

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We examined the effect of chemotherapy on osteoblast derived CXCL12. Data in Figure 3 shows that both chemotherapy and rTGF-β decrease CXCL12 mRNA and protein in osteoblasts [43]. The decrease in CXCL12 correlated with a decrease in chemotaxis of CXCR4+ JM-1 cells towards osteoblasts pre-treated with either melphalan or rTGF-β. However, in contrast to previous experiments in which we reported chemotactic deficits in VP-16 treated BMSC [16], no significant reduction in chemotactic support of CXCR4+ JM-1 cells was noted in the VP-16 treated HOB groups. These data suggest potential drug specific effects in which melphalan may target CXCL12 expression as well as other critical chemotactic factors in HOB, while VP-16 does not as globally affect chemotaxis regulators, allowing some maintenance of chemotactic support. One such factor for future investigation would include stem cell factor (SCF), which has been shown to synergize with CXCL12 in homing of stem cells and retention in their developmental niche [44].

Collectively, these data suggest that generation of active TGF-β in the endosteal niche can negatively affect production of CXCL12, thus impairing progenitor cells from homing to the bone marrow, engrafting, and reconstituting the patient's immune system. This observation of vulnerability of gene expression to genotoxic stress in HOB prompted us to investigate the magnitude of direct and indirect chemotherapy-induced damage to osteoblasts by microarray analysis. After 6 hours of treatment, the diverse changes observed in HOB gene expression alone allow for a better understanding of the significance of the potential damage to the niche and the subsequent impact on hematopoietic reconstitution that relies on balanced expression of several proteins. Interestingly, all 3 HOB treatments (rTGF-β, melphalan and conditioned media from melphalan pre-treated BMSC) evaluated in the microarray analysis identified NF-κB as a point of convergence (Figure 4). As with all gene expression pathway analysis, in the absence of targeted genetic manipulation or biochemical analysis, the interactions remain hypothetical. The most pronounced value of these data in the current study is to provide a sense of the responsiveness of osteoblasts to genotoxic stress, as well as to factors such as

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TGF-β, which may participate in both autocrine and paracrine signaling in the stem cell niche. Points of convergence, such as NF-κB, may then provide the focus for a more mechanistic investigation of cell signaling downstream of stress in the bone marrow microenvironment.

Central to our investigation was an interest in the influence of active TGF-β released from chemotherapy treated HOB as well as TGF-β that may be released from neighboring BMSC in a damaged microenvironment as just two potential sources of this growth factor. Studies by Batard *et al* have described the importance of low levels of TGF-β in the bone marrow microenvironment for maintenance of the stem cell pool through up-regulation of the CD34 antigen, a marker of primitive HSC [38]. Consistent with the need for rigorous control of total TGF-β levels, a number of studies have shown the benefit of TGF-β neutralization during therapy [45]. Lagneaux *et al* showed that stromal cells isolated from B-CLL patients had increased TGF-β production correlated with decreased colony-stimulating activity which was corrected by neutralizing TGF-β activity [13]. Using a murine model of breast cancer, Biswas *et al* showed that radiation or doxorubicin treatment increased levels of TGF-β which correlated with increased circulating tumor cells and increased metastasis [46]. These effects were abrogated by anti-TGF-β antibodies providing rationale for utilization of TGF-β inhibitors, such as GC1008 in clinical trials in the setting of renal cell carcinoma and malignant melanoma [47]. Based on our observations, application of TGF-β neutralizing antibodies may have utility through influence on both hematopoietic cells and the niche in which they develop.

Our observations indicate that osteoblasts are susceptible to genotoxic stress documented by alteration of gene expression profiles (Figure 4, S1-4) and functional deficits in hematopoietic cell support, including differentiation (Figure 2C). Further investigation will identify targets that may prove useful in augmenting hematopoietic recovery through "balancing" the stem cell niche following therapy-induced damage. Long-term hematopoietic deficits may, in fact, derive in part from the immediate changes in the niche that are imposed by aggressive therapeutic regimens. This aspect of marrow function may highlight an area in which better

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understanding could identify new therapeutic strategies to augment efficient patient recovery follow bone marrow transplantation.

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Figure legends:

Figure 1: ***Direct and indirect damage increase HOB levels of active TGF- β.*** **A)** HOB were treated with 50 μg/ml melphalan or 50 μM VP-16 for 24 hours and real time PCR completed (top) and HOB were treated directly for 4 hours with chemotherapy (100 μM VP-16 or 100 μg/ml melphalan), fixed and stained for detection of phospho-Smad2 (green), as a read-out of TGF-β activity (bottom) (DAPI stain for nuclei-blue). **B)** HOB were treated with 10 ng/ml rTGF-β for 24 hours and real time PCR completed (left). Additionally, HOB were treated with 10 ng/ml rTGF-β for 24 hours, the HOB layer was rinsed and new media was added and allowed to condition for 24 hours before being evaluated by ELISA to quantitate the amount of secreted TGF-β (right).

Figure 2: ***Chemotherapy or rTGF-β exposure diminished the ability of HOB to support human embryonic stem cells and CD34+ bone marrow cells and treatment diminishes HOB interaction with pro-B cells.*** HOB were pre-treated with 10 ng/ml rTGF-β, 50 μg/ml melphalan or 50 μM VP-16 for 24 hours. HOB were rinsed thoroughly and embryonic stem cells were co-cultured in complete media as recommended by WiCell. After 2 days of co-culture, stem cell colonies were counted, stained for Oct-4 as an indicator of potential for pluripotency **(A)** and designated as undifferentiated or differentiated based on classic morphology of well defined borders **(B).** To evaluate osteoblast support of CD34+ bone marrow cells, HOB cells were treated for 24 hours with 10 ng/ml rTGF-β, or with 25 μg/ml melphalan. After the 24 hour treatment, the HOB were thoroughly rinsed and 8.8x10^5 CD34+ cells were added in co-culture. Recombinant IL-3 (100 ng/ml) was added in all groups. CD34+ cells were collected at 2 and 6 days after co-culture and samples were given to pathology for analysis. Cellular populations were determined by the presence of surface markers **(C)**. To determine if treatment of HOB affected pro-B cell adherence, HOB were treated with 10 ng/ml rTGF-β, 50 μg/ml Melphalan or 50 μM VP-16 for 24 hours. Adherent layers of HOB were rinsed thoroughly and co-cultured with $1x10^5$ fluorescently labeled pro-B cells. After 24 hours the media was aspirated

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and non-adherent pro-B cells were gently rinsed. Remaining HOB and pro-B cells adherent to the HOB layer were then trypsinized and events within the easily distinguishable lymphoid gate, based on fluorescence and forward/side scatter, were counted on high flow rate for 30 seconds to enumerate number of pro-B cells attached to the HOB **(D).**

Figure 3: ***Chemotherapy or rTGF-β diminishes HOB expression of CXCL12 and support of pro-B cell chemotaxis.*** **A)** HOB were treated with 10 ng/ml rTGF-β, 50 μg/ml melphalan or 50 μM VP-16 for 24 hours. RNA was isolated and real time RT-PCR was performed for CXCL12. **B)** HOB were treated with 10 ng/ml rTGF-β, 50 μg/ml melphalan or 50 μM VP-16 for 24 hours. Growth factor or chemotherapy was removed and fresh media was added, allowed to condition for 24 or 48 hours, and then evaluated in a CXCL12 specific ELISA. **C)** HOB were treated with rTGF-β, 50 μg/ml melphalan or 50μM VP-16 for 24 hours. 350 μl of the supernatant was then removed and placed into the bottom chamber of a transwell. $1x10^5$ JM-1 progenitor cells were placed in the top chamber and allowed to migrate for 4 hours. After 4 hours the cells migrated were read on high flow rate for 30 seconds on a flow cytometer.

Figure 4: ***Intersection of global gene changes during direct and indirect chemotherapy.*** HOB cells were treated for 6 hours with 10ng/ml rTGF-β, 50 μg/ml melphalan, or conditioned media from BMSC (CMM) pre-treated with 50 μg/ml melphalan for 24 hours. BMSC exposed to melphalan were rinsed and fresh media was place on adherent layers to condition and to remove drug prior to stimulating HOB. After the 6 hour treatment, HOB RNA was isolated and microarray analysis was completed to evaluate global changes in gene expression. **A)** The Venn diagram summarizes the number of HOB genes that changed within each group as well as the changes between the groups. **B)** A gene summary was made of the genes that commonly were up-regulated (4, red) or down-regulated (2, green) for the intersection of all 3 treatments (rTGF-β:CMM:melphalan). **C)** A network diagram was

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generated for the intersection of all 3 treatment groups that highlights the convergence of potential pathways associated with those genes such as NF-κB. All genes listed were generated using a 2.5% FDR and 1.5 fold significant cut off.

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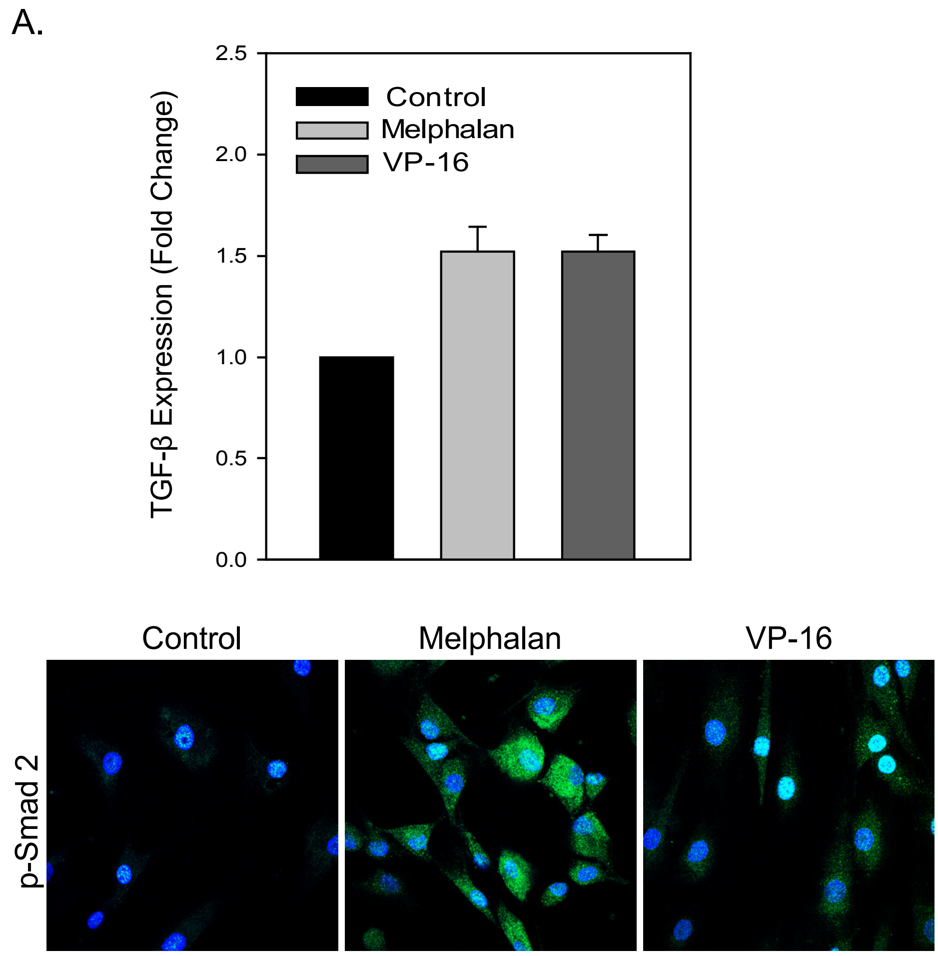

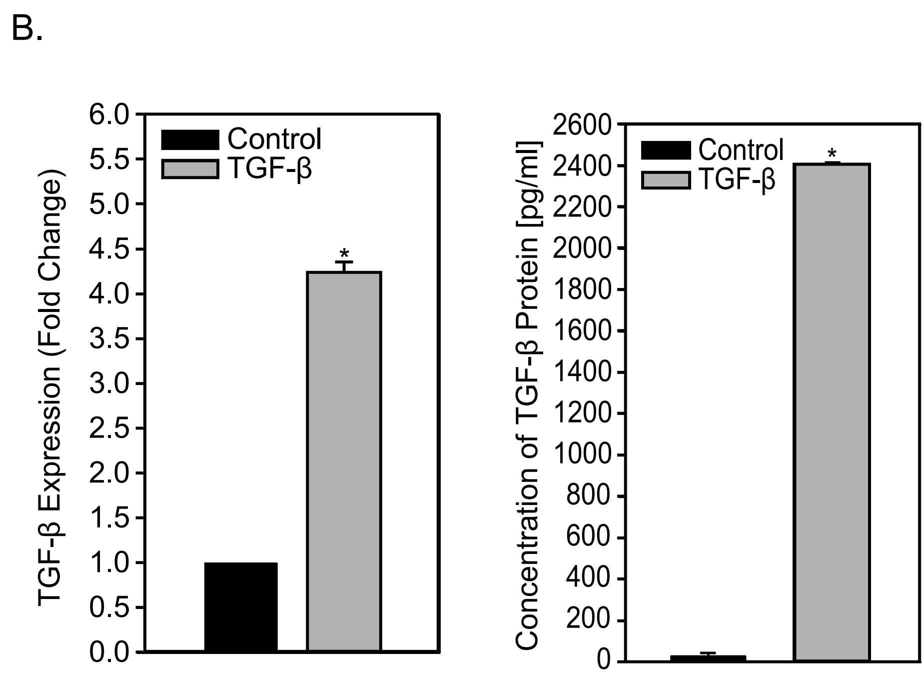

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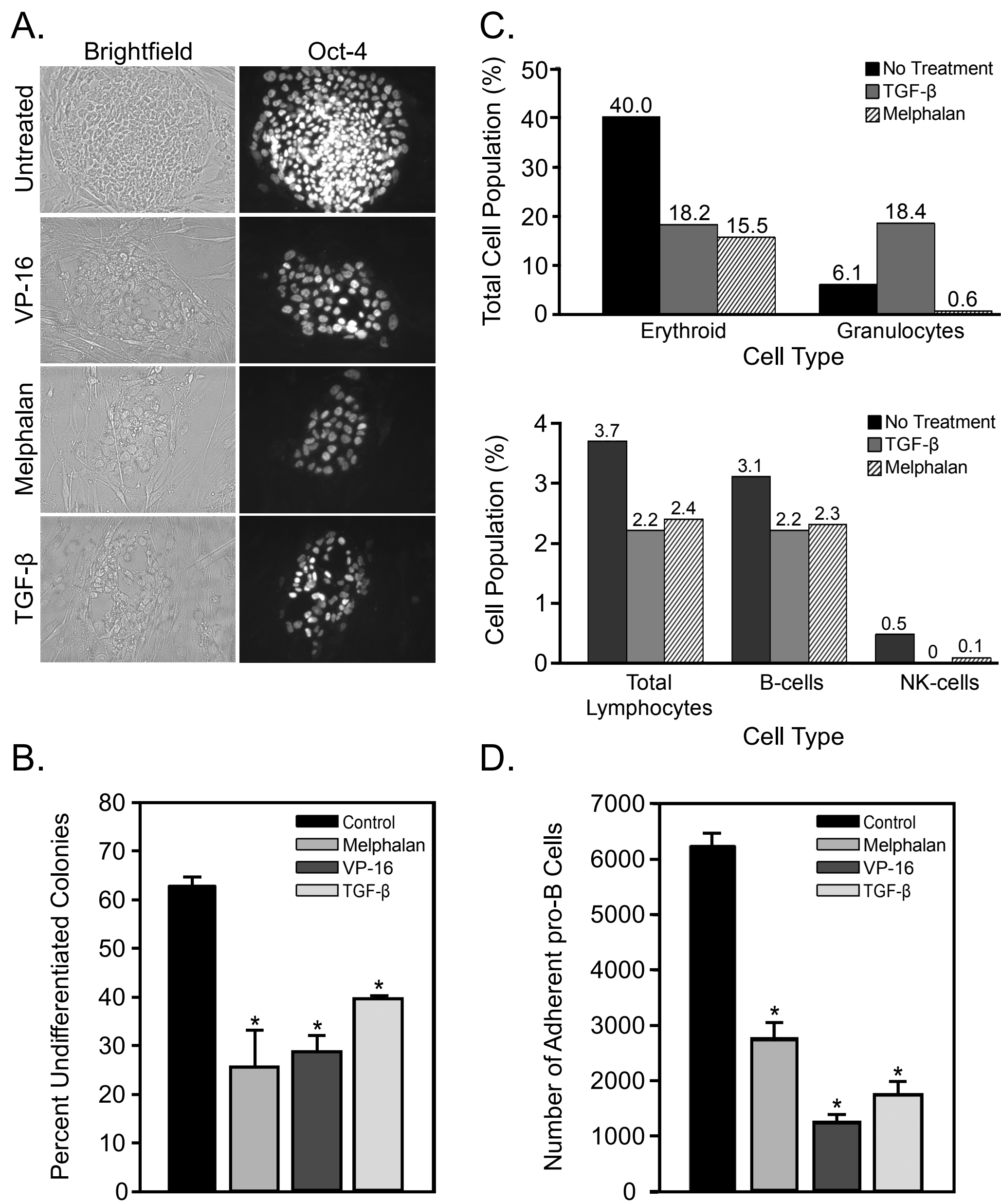

Figure 2: Chemotherapy or rTGF-β exposure diminished the ability of HOB to support human embryonic stem cells and CD34+ bone marrow cells and treatment diminishes HOB interaction with pro-B cells.

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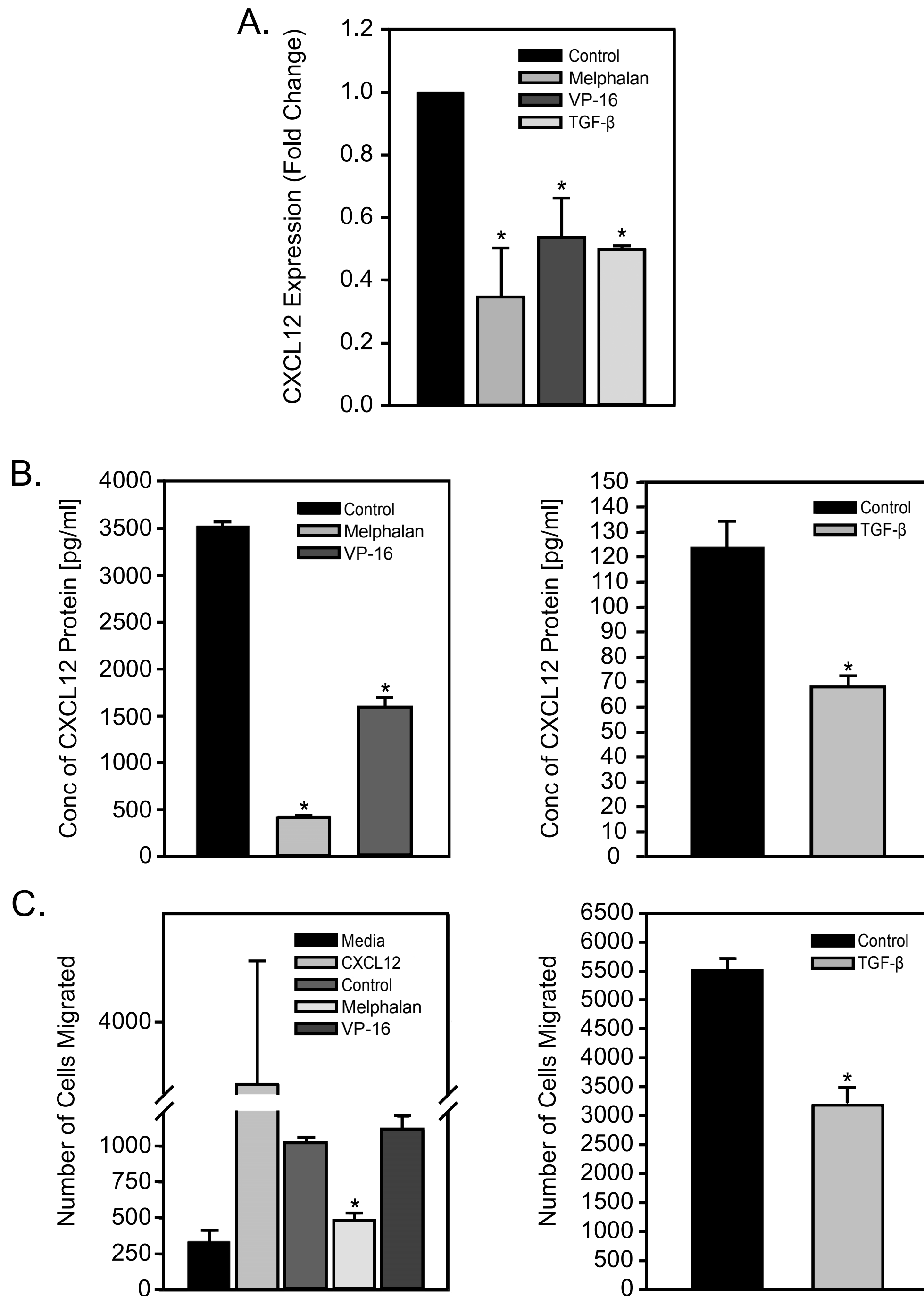

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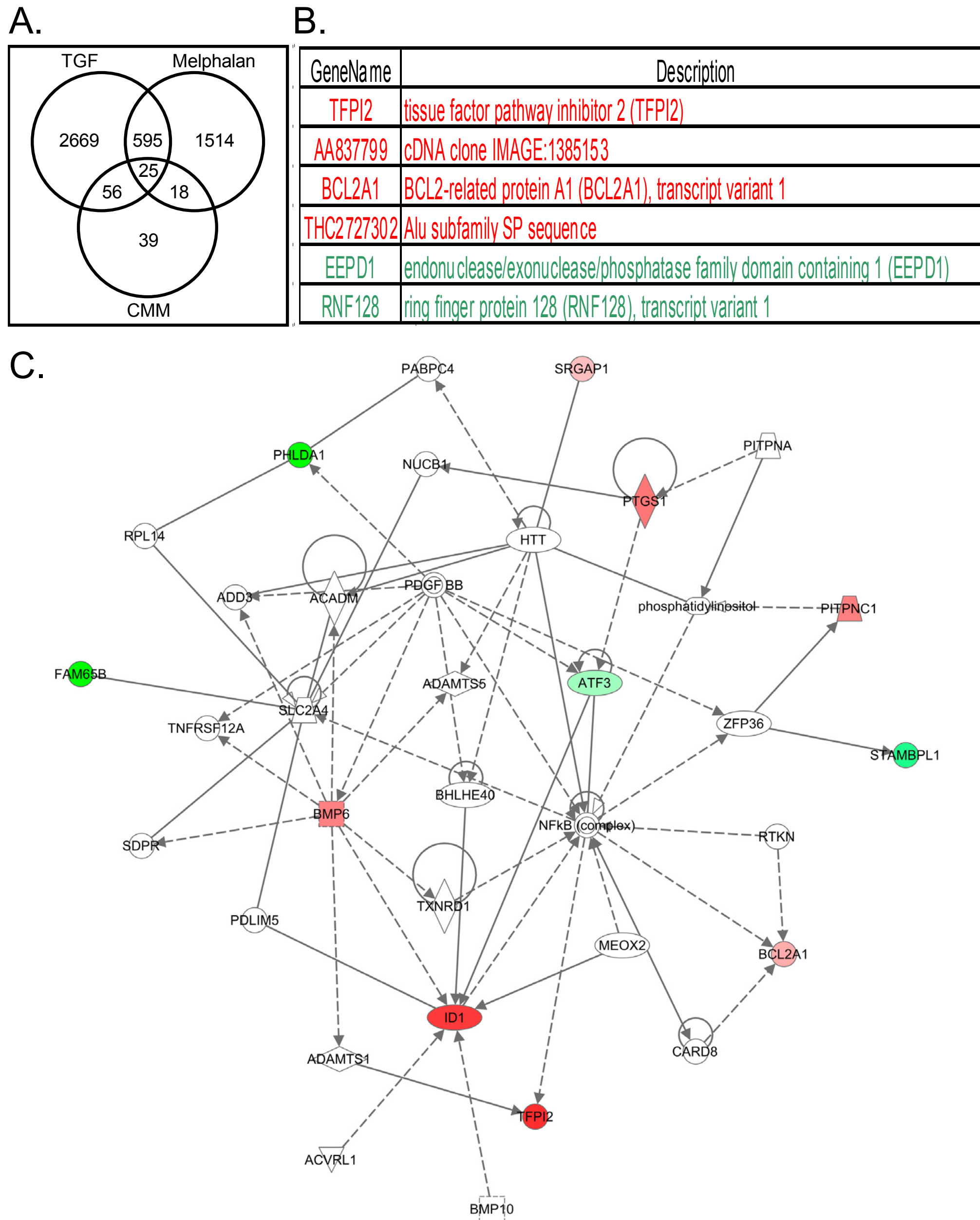

GeneName	Description
TFPI2	tissue factor pathway inhibitor 2 (TFPI2)
AA837799	cDNA clone IMAGE:1385153
BCL2A1	BCL2-related protein A1 (BCL2A1), transcript variant 1
THC2727302	Alu subfamily SP sequence
EEPD1	endonuclease/exonuclease/phosphatase family domain containing 1 (EEPD1)
RNF128	ring finger protein 128 (RNF128), transcript variant 1

Figure 4: Intersection of global gene changes during direct and indirect chemotherapy.

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Chapter III

Neurotrophins Regulate Bone Marrow Stromal Cell IL-6 Expression Through the MAPK Pathway

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Abstract

Background: The host's response to infection is characterized by altered levels of neurotrophins and an influx of inflammatory cells to sites of injured tissue. Progenitor cells that give rise to the differentiated cellular mediators of inflammation are derived from bone marrow progenitor cells where their development is regulated, in part, by cues from bone marrow stromal cells (BMSC). As such, alteration of BMSC function in response to elevated systemic mediators has the potential to alter their function in biologically relevant ways, including downstream alteration of cytokine production that influences hematopoietic development.

Methodology/Principle: In the current study we investigated BMSC neurotrophin receptor expression by flow cytometric analysis to determine differences in expression as well as potential to respond to NGF or BDNF. Intracellular signaling subsequent to neurotrophin stimulation of BMSC was analyzed by western blot, microarray analysis, confocal microscopy and real-time PCR. Analysis of BMSC Interleukin-6 (IL-6) expression was completed using ELISA and real-time PCR.

Conclusion: BMSC established from different individuals had distinct expression profiles of the neurotrophin receptors, TrkA, TrkB, TrkC, and $p75^{NTR}$. These receptors were functional, demonstrated by an increase in Akt-phosphorylation following BMSC exposure to recombinant NGF or BDNF. Neurotrophin stimulation of BMSC resulted in increased IL-6 gene and protein expression which required activation of ERK and p38 MAPK signaling, but was not mediated by the NFκB pathway. BMSC response to neurotrophins, including the up-regulation of IL-6, may alter their support of hematopoiesis and regulate the availability of inflammatory cells for migration to sites of injury or infection. As such, these studies are relevant to the growing appreciation of the interplay between neurotropic mediators and the regulation of hematopoiesis.

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Introduction

Neurotrophins are a family of proteins which are best characterized by their modulation of survival, differentiation and apoptosis of cells in the nervous system. This family includes NGF, BDNF, neurotrophin 3 (NT-3), and neurotrophins 4/5 (NT-4/5)[1]. Neurotrophins signal through the high-affinity tyrosine kinase (Trk) receptors, TrkA, TrkB, TrkC, and the low-affinity receptor, $p75^{NTR}$, a member of the tumor necrosis factor receptor family[1;2].

NGF is a survival factor essential for a large number of neuronal and non-neuronal cell types. The importance of neurotrophin signaling is highlighted by neurodegenerative conditions such as Alzheimer's disease, in which there is a dysregulation of pathways modulated by neurotrophic factors[3;4]. In addition to its role in neurological pathways, neurotrophin signaling has an impact on innate and adaptive immunity[5]. Alteration of NGF has been documented in autoimmune inflammatory diseases including multiple sclerosis[6], psoriasis[7], systemic lupus erythematosus[8] and rheumatoid arthritis[9]. Traumatic brain injury[10], neuroectodermal tumors[11] and endocrine disorders[12] are a few examples of many conditions also associated with increased neurotrophins. A positive correlation between NGF level and allergic asthma, airway hyperactivity, total IgE and the number of eosinophils in the serum has also been noted[13]. These observations suggest that neurotrophins may mediate hematopoietic responses to several clinically relevant conditions. Importantly, NGF has the potential to act systemically on distant organs, including the bone marrow which serves as the primary site of postnatal hematopoiesis[14;15].

BMSC provide the structural and physiological support for hematopoietic cell survival, proliferation and differentiation. Resident stem and immature hematopoietic progenitor cells mature under the influence of the bone marrow microenvironment to functional, mature cells of diverse lineages[14;15]. As such, exposure of this microenvironment to circulating neurotrophins, cytokines and growth factors has the potential to alter its function, resulting in the generation of hematopoietic populations that are markedly different than those in healthy individuals.

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In the current study, a cytokine that was consistently and significantly increased in BMSC exposed to NGF or BDNF was Interleukin-6 (IL-6). IL-6 is a multifunctional cytokine[16] modulated by other factors including IL-1, TNF-α, growth factors, hormones, and viral or microbial products[17-19]. Dysregulation of IL-6 production has been reported in the pathogenesis of several autoimmune diseases including rheumatoid arthritis (RA), systemic-onset juvenile chronic arthritis (JCA), autoimmune encephalomyelitis, psoriasis, antigen-induced arthritis (AIA), and Systemic Lupus Erythematosus[16;20-22]. IL-6 is a critical factor for hematopoiesis through regulation of the entry of hematopoietic stem cells into the cell cycle, proliferation of cells committed to the myeloid and lymphoid lineage, and maturation of B-cells into antibody producing cells[16;23-26]. Increased IL-6 expression in transgenic mice results in massive polyclonal plasmacytosis and malignant plasmacytoma[26]. In contrast, a reduction in hematopoietic progenitor cell support has been reported by IL-6 deficient bone marrow stromal cells[27]. These observations suggest that changes in IL-6 levels could impact on the development of hematopoietic populations available to participate in inflammatory responses with the novelty of our current study derived from consideration of the potential of systemic neurotrophic factors to modulate IL-6 in the marrow microenvironment through direct stimulation of BMSC.

Depending upon the cellular context, IL-6 transcription has been documented to be influenced by both NF-κB and MAPK (mitogen-activated protein kinase) cascades subsequent to NGF stimulation[28;29]. Studies have shown that NGF activates NF-κB in rat pheochromocytoma PC12 cells[30]. NF-κB is sequestered in the cytoplasm by the IκB family of proteins which become phosphorylated, and degraded by the proteasome with subsequent NF-κB translocation to the nucleus[31]. As a transcription factor involved in the control of inflammatory responses, cellular growth, and apoptosis[31], NF-κB is involved in the pathology of several diseases, including cancers, arthritis, chronic inflammatory bowel disesse, asthma and neurodegenerative diseases[32-36]. Neurotrophin stimulation of the MAPK pathway has been

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documented in the PC12 cell line[37], dorsal root ganglia, and transient receptor potential vanilloid 1 (TRPV1) (the capsaicin receptor) model[38]. Activation of the TrkA receptor by NGF in airway smooth muscle cells also leads to activation of the MAPK cascade including p38 MAPK, and extracellular-regulated protein kinase 1/2 (ERK1/2)[39]. Following stimulation, the MAPK family of proteins activate several downstream factors involved in regulating inflammation[37-39].

Based on the diverse set of pathologic conditions associated with dysregulated neurotrophic factors, many of which involve inflammation as a central feature, we investigated the effects of NGF and BDNF on BMSC function as a critical influence on regulation of hematopoietic cell development.

Materials and Methods

Ethics Statement

This study was conducted according to the principles expressed in the Declaration of Helsinki. The study was approved by the Institutional Review Board of West Virginia University. All patients provided written informed consent for the collection of samples and subsequent analysis.

Cell culture and reagents

P163, P164, PED299, PED604, PED62304 and GPBM 1-32 primary human bone marrow stromal cells (BMSC) were derived from consenting donors (written consent) with the approval of the West Virginia University Institutional Review Board. Establishment of BMSC and their characterization have been previously described in detail[40]. Because the characteristics of BMSC can be influenced by preparative regimens, all lines were established identically and evaluated at comparable passage number in the experiments presented. BMSC were maintained in Minimum Essential Medium, Alpha (α-MEM) (Mediatech, Manassa, VA)

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supplemented with 10% fetal bovine serum (FBS) (Hyclone, Pittsburgh, PA), 2mM L-glutamine (Mediatech) 100 mg/ml streptomycin, 100 IU/ml penicillin (Sigma, St. Louis, MO), and $5x10^{-5}$M 2-β mercapthanol (Sigma), at 37˚C in 6% CO_2. Of note, the concentration of 2- β mercapthanol used is less than that published as having the potential to stimulate cells to neural differentiation[41]. The adherent, fibroblastic BMSC utilized in all of our studies constitutively express VCAM-1, Fibronectin, SDF-1 (CXCL-12), VEGF, Thrombospondin and a variety of cytokines that influence both lymphoid and myeloid cell survival and expansion.

Flow cytometric analysis

Six different BMSC lines were grown to confluence (~10^6 cells), trypsinized, fixed in 10% formaldehyde for 30 minutes, and subsequently permeabilized in 70% EtOH for 30 minutes on ice. To reduce non-specific antibody binding, BMSC were blocked in 3% BSA in PBS for 15 minutes and subsequently incubated with 1µg per sample of rabbit polyclonal TrkA or TrKB, goat polyclonal $p75^{NTR}$ specific antibodies from Santa Cruz Biotechnology,Inc. (Santa Cruz, CA). For detection of TrkC, TrkC-PE (FAB373P) and isotype control goat IgG-PE were acquired from R&D systems (Minneapolis, MN). The additional isotype control antibody, rabbit IgG, was purchased from Southern Biotechnology (Birmingham, AL). Primary antibody binding was detected by incubation with 1µg per sample fluorescein isothiocyanate (FITC)–conjugated goat anti-rabbit (Santa Cruz Biotechnology) for Trk A and TrkB and FITC–conjugated rabbit anti-goat IgG(H+L) (Southern Biotechnology) for p75NTR. Data were acquired by counting 10000 events and analyzed using FACSCalibur (BD Biosciences, San Jose, CA). One representative cell line from the six lines examined was chosen for completion of the remaining experiments in the manuscript unless indicated.

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Western blot analysis

Confluent BMSC were treated with 100 ng/ml mouse NGF 2.5 S (Roche Applied Science) or recombinant human BDNF (Invitrogen, Carlsbad, CA) for 5 minutes, 30 minutes, 1 hour and 6 hours. Following treatment, BMSC were lysed in complete cell lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 0.25% Na-deoxycholate, 1 mM EDTA, and 1 mM NaF, 1 mM DTT, 1 mM PMSF, 1 mM activated Na_3VO_4, 1 µg/mL aprotinin, 1 µg/mL leupeptin, and 1 µg/mL pepstatin) on ice for 15 minutes. Following centrifugation at 20,000x g for 15 minutes, supernatants were collected and protein concentration was determined using the bicinchoninic acid protein assay (BCA). Proteins were resolved on SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell Bioscience, Keene, New Hampshire). Membranes were blocked in TBS/5% nonfat dry milk/0.1% Tween-20 at room temperature for 1 hour, and probed with the primary antibodies rabbit anti-phospho-Akt (Ser473) or rabbit anti-Akt, (Cell Signaling Technology, Inc, Danvers, MA). Additional antibodies included rabbit Erk 1/2 (Cell Signaling Technologies) and anti-phospho ERK1/2 purchased from Promega Corporation (Madison, WI). Mouse anti-GAPDH (Fitzgerald Industries International, Concord, MA) was used as a lane loading control. Washes were in TBS/0.1% Tween-20 following incubation with horseradish peroxidase-conjugated secondary antibodies. Luminol (Santa Cruz Biotechnology) generated signal was detected on x-ray film. Densitometric analysis was performed using the Fotodyne imaging system with Foto/Analyst version 5.00 software (FOTODYNE Inc., Hartland, WI) for image acquisition, and TotalLab version 2005 software for analysis.

Cellular Fractionation and Western Blot Analysis

BMSC were left untreated or treated with NGF (100ng/ml) for 30 min. Cells were trypsinized, pelleted and cellular fractionation was completed using the NE-PER cytoplasmic and nuclear extraction kit (Pierce Biotechnology, Rockford, IL). Protein concentration was

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determined using the bicinchoninic acid (BCA) protein assay (Pierce Biotechnology). Cytoplasmic and nuclear proteins were resolved on SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk/ 1X TBS/0.1% Tween-20 and probed with a mouse monoclonal p65 specific antibody. After incubation with anti-mouse HRP- conjugated secondary antibody, the signal was visualized using Immobilon chemiluminescence reagents (Millipore, Billerica, MA).

RNA Isolation

Total RNA was isolated from three BMSC lines treated with NGF or BDNF (100ng/ml) using the Qiagen RNeasy Mini kit following the recommendations of the manufacturer (Qiagen, Valencia, CA). Pelleted BMSC were lysed by centrifugation through QIA shredder spin columns and RNA was treated with 1U DNAse for 15 minutes at 24°C. Samples were quantified at 260nm (GENESYS-10UV, Spectronic, Unicom) and protein contamination determined by evaluation at 280 nm.

Microarray analysis

Gene expression profiles of BMSC were assessed using the Human Neurotrophin and Receptor Gene Array HS-018 (SuperArray, Frederick, MD) as a representative, but not exhaustive, approach for screening of NGF or BDNF induced changes in gene expression. BMSC RNA from untreated control and NGF or BDNF treated cells was converted to biotinylated cDNA using the Ampolabeling-LPR kit (SuperArray). Membranes were hybridized with cDNA probes overnight at 60°C with continuous agitation at 5–10 r.p.m and then washed as recommended by the manufacturer. Signal was detected on X-ray film with images scanned and analyzed with GEArray Expression analysis suite software (SuperArray). Signal intensities were normalized to *GAPDH* and *beta-actin* on each membrane. Only those genes having a 3 fold or higher change in expression were examined (GEO accession number GSE18537).

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Real-time PCR

To determine relative IL-6 expression, real-time PCR was used to validate data from microarray experiments. The one-step QuantiTect SYBR Green RT-PCR Kit (Qiagen) was used as recommended by the manufacturer. All reactions were performed in triplicate using 80 ng of RNA per reaction, IL-6 gene primers (#PPH08958A; SuperArray) or the housekeeping gene *GUSB* (beta glucuronidase) (Real Time Primers, Elkins Park, PA). Amplifications were completed using a 7500 real-time PCR system (Applied Biosystems, Foster City, CA). Amplification conditions included 50°C for 30 minutes, 95°C for 15 min, 45 cycles of 94°C for 15 seconds, 55°C for 30 seconds, and 72°C for 45 seconds. The relative quantitative method (DDCT) was used to evaluate gene expression in experimental and control cells for each gene examined[42].

ELISA

BMSC were cultured in a 96-well plate and serum deprived (1% FBS) for 24h before treatment with 100 ng/ml NGF or BDNF for 24 or 48 hours. Supernatants were collected and a human IL-6 ELISA completed (eBioscience, San Diego, CA) with 1/20 dilution of supernatants as recommended by the manufacturer. For experiments in which the effects of MEK and MAPK inhibition were studied, 20μM of the MEK inhibitor U0126 or 10μM of the p38 MAPK inhibitor SB 203580 (Promega) were added 2 hours before treatment with NGF or BDNF in select wells. Integrity of cell layers were confirmed prior to collection of supernatants.

Immunostaining and confocal microscopy

BMSC were grown to confluence on glass coverslips and treated with 100 ng/ml NGF, BDNF or rTNF-α (R&D Systems), for 5, 30 and 60 minutes. Following treatment, BMSC were rinsed in phosphate-buffered saline (PBS) and fixed in 4% formaldehyde at room temperature for 30 minutes, followed by rinsing and fixing in acetone for 10 minutes. Permeabilization of

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cells was completed with 0.5% Triton X-100 for 30 minutes. Nonspecific antibody binding was blocked by incubation of BMSC for 30 minutes in PBS/5% BSA. NF-κB localization was evaluated by incubation of BMSC with 2 μg/coverslip of mouse anti-human monoclonal NF-κB p65 (Santa Cruz Biotechnology) in PBS/5% BSA for 1 hour. Subsequently, Alexa Fluor 555 donkey anti-mouse IgG(H+L) (Invitrogen), was applied at a 1 μg/coverslip for one hour. Coverslips were then inverted on slides and mounted with Prolong Gold antifade reagent with 4, 6 diamidino-2-phenylindole (DAPI) (Invitrogen). Images were collected using a Zeiss LSM510 confocal on an AxioImager Z1 microscope (Carl Zeiss) with a 405-diode laser to excite DAPI and a 543 HeNe laser to excite the AlexaFluor 555-labeled secondary antibody. Cells were visualized using a 40x/1.30 oil objective. All confocal images were adjusted equally using Adobe Photoshop.

Statistical analysis

Data presented were expressed as mean ± SEM for triplicate samples. Statistical analysis was performed by ANOVA ($p \leq 0.01$ determined as significant) and Student t-test ($p \leq 0.01$ determined as significant).

<u>Results</u>

BMSC have distinct expression profiles for TrkA, TrkB, TrkC and p75^{NTR}.

To determine the expression pattern of neurotrophin receptors by BMSC, flow cytometry analysis of BMSC with antibodies specific for, TrkA, TrkB, TrkC and p75^{NTR} was performed. While the levels of expression for a given receptor varied between stromal lines derived from different individuals, all BMSC lines used in this study expressed both the high and low affinity receptors to respond to neurotrophins (Figure 1).

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BMSC demonstrate Akt phosphorylation following NGF or BDNF exposure.

Activation of Akt occurs in response to a variety of stimuli, and relevant to the current study, is a well documented consequence of neurotrophin stimulation[43]. Therefore, while not a investigative focus in this work, the phosphorylation of Akt was utilized simply as a read-out to determine if BMSC could respond to neurotrophin binding to the Trk receptors. Phosphorylation of Akt occurred rapidly following BMSC exposure to 100 ng/ml of NGF or BDNF. NGF treated BMSC Akt phosphorylation was detected as early as 5 minutes with the peak signal occurring at 30 minutes. Phosphorylated Akt began to diminish by 1 hour, and minimal signal was detected after 6 hours of exposure to NGF. In BDNF treated cells, peak phosphorylation of Akt occurred at 5 minutes and gradually diminished over 6 hours (Figure 2).

Exposure to NGF or BDNF increases IL-6 gene expression in the BMSC.

To investigate the cytokine pattern of BMSC exposed to neurotrophic factors, RNA isolated from BMSC exposed to NGF and BDNF was analyzed by microarray as described. While many genes were increased, and fewer decreased, following NGF or BDNF exposure, with a cut-off of 3 fold-change compared to untreated controls, Fibroblast Growth Factor-2 (FGF2) and IL-6 emerged as those genes markedly upregulated following NGF or BDNF treatment in this panel (Figure 3 A-B). In addition, the BDNF receptor (NTRK2) and CRH1 gene expression increased following NGF exposure, but did not meet the threshold cutoff in cells exposed to BDNF. IL-6 was chosen for further investigation to determine the signaling pathway that may underlie altered expression based on its well characterized role in hematopoiesis.

To validate our IL-6 microarray data, relative transcript levels were determined by quantitative real-time PCR. Consistent with the microarray data, IL-6 mRNA increased in BMSC after exposure to both NGF and BDNF (Figure 3 C-D).

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BMSC exposed to NGF or BDNF demonstrate increases in IL-6 protein consistent with gene expression changes

To evaluate effects of NGF or BDNF on IL-6 protein, BMSC were serum deprived (1% FBS) for 24 hours and subsequently exposed to 100 ng/ml of NGF or BDNF for 24 or 48 hours in triplicate. An IL-6 ELISA was performed on the supernatant collected from each time point to determine changes in the production of IL-6 protein. Consistent with the up-regulation of IL-6 message, exposure of BMSC to NGF and BDNF increased the level of IL-6 protein (Figure 4). To determine the optimal concentration of neurotrophins needed to induce NGF or BDNF signaling, resulting in IL-6 protein increase, a dose response curve was completed, using an IL-6 ELISA with doses of neurotrophins ranging from 0-100 ng/ml. A MTT assay was used to determine the concentrations of neurotrophins used were not toxic to the BMSCs (data not shown). To determine if there is a role for the sIL-6R in our model, BMSC were left untreated or stimulated with NGF or BDNF (10-100ng/ml) for 24 hours and a sIL-6R ELISA was completed. There was no detectable sIL-6R present in the untreated cells or in those cells treated with NGF or BDNF (data not shown).

Neurotrophins do not induce NF-κB signaling in BMSC

In neurons, it has been established that neurotrophin stimulation activates signaling through NF-κB[30]. Additionally, NF-κB is a known transcription factor for IL-6[28]. Using immunofluorescence, we determined whether exposure of BMSC to neurotrophins induced nuclear translocation of the p65 subunit of NF-κB. BMSC (one cell line) were exposed to either NGF or BDNF. Subsequent to NGF or BDNF exposure, no nuclear translocation of the NF-κB p65 subunit was noted. In contrast, rTNF-α, which was used as a positive control, induced rapid translocation of the p65 subunit from the cytoplasm to the nucleus in the BMSC (Figure 5A). To confirm the lack of p65 translocation to the nucleus with NGF stimulation, BMSC were left

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untreated or treated with NGF (100ng/ml) for 30 min. Following treatment, cellular fractionation was completed and the fractions analyzed by western blot with p65 specific antibodies. There was no detectable p65 in the nuclear fraction of untreated or NGF treated cells, while p65 was readily detected in the cytoplasmic fraction (Figure 5B). GAPDH was used as a fraction contamination control (data not shown).

NGF and BDNF induce MAPK ERK1/2 signaling pathway

Stimulation of neuronal TrkA with NGF has been shown to activate the MAPK components ERK1/2 and p38 MAPK[39]. To evaluate the role of neurotrophin induced MAPK signaling in BMSC, we analyzed phosphorylation of ERK1/2 as a read out of MAPK activation following BMSC exposure to NGF or BDNF. Phosphorylation of ERK occurred rapidly and transiently in both NGF and BDNF treated groups while no phosphorylation was detected in untreated control cells (Figure 6).

MAPK ERK1/2 pathway inhibitors blunted IL-6 production following NGF or BDNF exposure.

As shown previously (Figure 4), treatment with NGF or BDNF increased IL-6 protein in BMSC supernatants. The increase in IL-6 protein with NGF or BDNF stimulation without any inhibitors was statistically significant ($p=.001$ for NGF and $p=.002$ for BDNF) (Figure 7). To further investigate the role of ERK1/2 and p38 pathway in neurotrophin induction of IL-6, specific inhibitors targeting both pathways were utilized as discussed in methods. Pre-treatment of BMSC with U0126 and SB 203580 in concentrations held low enough to maintain specificity of inhibition resulted in approximately a 50% decrease of IL-6 upon exposure to NGF or BDNF (Figure 7). To investigate the role of the Akt pathway in neurotrophin induction of IL-6, BMSC were pre-treated as described above with Akt VIII, a specific Akt inhibitor, for 2 hrs prior to

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stimulation with NGF or BDNF. Akt inhibition did not decrease IL-6 protein detected in BMSC supernatants (data not shown).

Discussion

The purpose of this study was to determine if BMSC gene expression is responsive to NGF or BDNF as a means by which neurotrophic factors may indirectly influence hematopoiesis. Many *in vivo* and *in vitro* studies have investigated the role of neurotrophins in hematopoiesis. NGF has been demonstrated as an important factor for hematopoietic colony growth and differentiation[44]. Previous work documents its ability to directly influence proliferation, differentiation, and maturation of myeloid progenitors along with induction of migration, survival and activation of mature hematopoietic cells[45-47]. NGF is also a chemotactic stimulus for human leukocytes and macrophages[48;49]. Fewer studies have focused specifically on the response of BMSC to neurotrophic factors as the mechanism by which NGF or BDNF may indirectly influence hematopoiesis. However, there have been previous reports that document the expression of NGF receptors on BMSC[50] as well as the capacity of BMSC to produce and respond to NGF during normal hematopoiesis[51]. The role of BDNF on immune function and hematopoietic cell development is not as well defined as NGF, although impaired B cell development in bone marrow of BDNF deficient mice has been reported[52].

We have demonstrated expression of both high and low affinity receptors for neurotrophins by BMSC, with their level of expression varying between BMSC donors (Figure 1 A-D). While in some settings signaling through the low affinity receptor p75 following NGF exposure can result in apoptosis[53], in the presence of TrkA the signals are predominantly reported to be anti-apoptotic[54]. Co-expression of both high and low affinity receptors by human BMSC appear to have favored the anti-apoptotic effects of neurotrophin signaling. The neurotrophin receptors are functional as demonstrated by Akt-phosphorylation following stimulation of BMSC by NGF and BDNF (Figure 2 A-B). Phosphorylation of Akt, while it may

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influence survival of BMSC consistent with its role in several settings, was not being investigated in its survival context in our model. Rather, as a well characterized downstream target of neurotrophic signaling it was merely monitored as a measure of functionality of receptors in the absence of a clearly defined signal more immediately stimulated by NGF or BDNF in BMSC.

Through microarray analysis we demonstrated that cytokine gene expression by BMSC changes in response to NGF or BDNF exposure (Figure 3 A, B). Any focused panel of gene expression analysis is, by design, not an exhaustive evaluation of all the potential targets that respond to any stimulus. As such, we chose a focused evaluation, and subsequently targeted IL-6 for follow up based on its diverse involvement in innate immunity, hematopoiesis, and inflammatory responses[16]. Consistent with the microarray data, IL-6 gene and protein expression increased after exposure to both NGF and BDNF as determined by real-time PCR and ELISA respectively (Figure 3 C, D and Figure 4). In addition to IL-6, FGF2 was increased more than three-fold that of matched controls by both BDNF and NGF. Previous reports have indicated that elevated FGF2 decreased both stromal cell derived factor-1 (SDF-1;CXCL12) mRNA and protein *in vivo* and also diminished the capacity of BMSC to support the expansion of peripheral blood derived stem cells[55]. Uniquely, the Trk B receptor (NTRK2) was increased by NGF, but not BDNF, with the increase reaching our threshold cut-off of three times that of untreated cells. This increase suggests a potential synergistic relationship between these two neurotrophic factors in which an NGF increase may position BMSC cells to more robustly respond to BDNF through modulation of TrkB receptor expression. Consequently, subtle increases in circulating BDNF may have pronounced signaling potential when NGF increases have occurred previously.

The significant role of IL-6 in inflammation has been demonstrated by the diminished ability of IL-6 knockout mice to respond to environmental air pollutants exposure compared to wild-type control[56]. Of specific relevance to the current study, IL-6 overproduction in transgenic

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animals resulted in an increase in the number of megakaryocytes, plasmacytosis formation and extramedullary hematopoiesis[26;57], suggesting elevated IL-6 subsequent to circulating NGF or BDNF may contribute to dysregulated immune function. Of note, all reports are not consistent with a clear inflammatory response subsequent to increased NGF, with a recent study suggesting that NGF may, in fact, have anti-inflammatory actions via its regulation of calcitonin gene-related peptide (CGRP) in monocytes[58]. This recent work highlights the necessity of interpreting data within the confines of the specific model being investigated.

Our data do not rule out the possibility of NGF or BDNF acting on gene expression in BMSC through one, or several, intermediate factors. Increased expression or release of Substance-P following neurotrophic stimulation of cells has been described in diverse settings documenting its important role in the hematopoietic-neuro-immune axis in inflammation, as well as normal and malignant hematopoiesis[59-64]. Relevant to our model, Substance-P has been shown to participate in upregulation of inflammatory cytokines in fibroblastic cells[65;66] and more recently in a model of increased IL-6, IL-1β, and TNF-α in a model of burn-induced lung injury[67]. These reports raise the possibility that Substance-P may be an intermediate between neurotrophic factor exposure and altered gene expression in BMSC as well which has not been investigated. Studies that include targeted knock-out of Substance-P will be intriguing, and are required to determine if it is a required mediator of signaling in BMSC. However, regardless of outcome, the biological significance remains that functional changes in a critical component of the marrow microenvironment, BMSC, can be elicited by circulating NGF or BDNF.

Different reports suggest several pathways mediate IL-6 induction and the immunomodulatory effects of neurotrophins based on the stimulus and cell type. Our study demonstrated phosphorylation of ERK1/2 MAPK (Figure 6 A, B), with no nuclear translocation of NF-κB detected (Figure 5) following NGF or BDNF treatment of BMSC. Furthermore, we observe that ERK1/2 and p38 MAPK pharmacological inhibitors (U0126 and SB203580) significantly reduced IL-6 production by BMSC during exposure to NGF or BDNF (Figure 7).

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This observation is consistent with a recently reported model that included LPS stimulated BMSC in which SB-203580 inhibited IL-6 and IL-11 mRNA expression[68]. Our data suggest ERK and p38 MAPK pathways are required for optimal BMSC IL-6 induction by NGF and BDNF, with these pathways likely involved in baseline expression of IL-6 as a reduction in steady state expression was noted with both inhibitors in the absence of any neurotrophic stimulation. The inhibition noted following stimulation was approximately 50% suggesting that other pathways are also involved in NGF and BDNF stimulated IL-6 production. While a dose response of increased inhibitor concentration could be completed to determine if more pronounced inhibition can be achieved, the approach is not valid based on the loss of specificity that will occur at higher doses. Therefore, experiments were completed with the concentration limited to generate meaningful results.

Collectively these data suggest BMSC can be modulated by neurotrophins in a manner consistent with influence on hematopoietic cell proliferation and differentiation, reflected by a significant IL-6 increase in this study. As such, neurotrophins are positioned to regulate the availability of inflammatory cells derived from the marrow, through both direct[44-47] and indirect mechanisms. Taken together, our data suggest a central role for neurotrophins in the inflammatory process subsequent to infection and identify bone marrow stroma as a novel target for these factors. Further, this study broadens the context in which we should consider the consequences of dysregulated neurotrophin expression.

Acknowledgments

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Figure Legends

Figure 1. BMSC express neurotrophin receptors. BMSC established from different individuals were stained for high affinity receptors (A) TrkA, (B) TrKB, (C) TrkC and the low affinity receptor (D) p75NTR with specific antibodies followed by flow cytometric evaluation. BMSC expressed distinct profiles of neurotrophin receptors. Isotype matched controls are indicated by the solid histogram.

Figure 2. BMSC Akt phosphorylation increases following NGF or BDNF exposure. Following treatment with NGF or BDNF, BMSC were lysed and protein was resolved by SDS-PAGE and transferred to nitrocellulose membranes. (A) Membranes were probed with antibodies specific for phospho-Akt and total Akt; GAPDH was used as a lane loading control. (B) Densitometry demonstrates an increase in phospho-Akt:Total Akt following NGF or BDNF exposure.

Figure 3. NGF or BDNF changes the gene expression profile of BMSC and increases IL-6 mRNA. BMSC were treated with 100 ng/ml of (A) NGF or (B) BDNF for 18 hours. The cells were collected, RNA was isolated, and microarray analyses were performed. Graphs summarize the panel of gene expression changes in the BMSC treated with NGF or BDNF compared to untreated control cells. BMSC were treated with 100ng/ml of (C) NGF or (D) BDNF for 1, 2, 4 and 8 hours. The cells were collected, RNA was isolated and real time PCR was performed with the one-step QuantiTech SYBR Green kit as instructed by the manufacturer. BMSC IL-6 expression in response to NGF or BDNF exposure compared to untreated control cells is shown. Expression was normalized to the housekeeping gene *GUSB*. Statistical analysis was completed by ANOVA ($P \leq 0.0001$) with significance indicated by an asterisk.

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Figure 4. BMSC treatment with NGF or BDNF increases IL-6 protein. BMSC were exposed to 100 ng/ml of NGF or BDNF for 24 and 48 hours. The supernatant was then collected and an IL-6 ELISA was performed. An increase in BMSC IL-6 protein was noted in all treatment groups. Statistical analysis was completed by ANOVA ($P \leq 0.01$) with significance indicated by an asterisk.

Figure 5. Neurotrophins do not induce NF-κB signaling in BMSC. BMSC were treated with 100 ng/ml of NGF, BDNF, or TNFα for 5, 30 and 60 minutes. Following treatment, BMSC were fixed and probed with antibodies specific for NFκB p65 or its matched isotype control. Analyses of samples by confocal microscopy indicate cytoplasmic p65 in untreated control and translocation of p65 to the nucleus upon stimulation with a known positive stimulus, TNF-α (A). The subcellular localization of p65 did not change in response to NGF or BDNF. Representative images from 30 minutes exposure to stimuli are shown (original magnifications ×40). (B) BMSC were left untreated or treated with NGF (100ng/ml) for 30 min. and subcellular fractionation and western blot analysis completed with p65 specific antibodies. Treatment of BMSC with NGF did not change the subcellular localization of p65.

Figure 6. BMSC demonstrate activation of MAPK ERK following NGF or BDNF exposure. Following treatment with100 ng/ml of NGF or BDNF, BMSC were lysed and protein was resolved by SDS-PAGE and transferred to nitrocellulose membranes. (A) Membranes were probed with antibodies specific for phospho ERK1/2 and total ERK; GAPDH was used as a lane loading control. (B) Densitometric quantitation of increase in phosphorylated Erk:Total Erk following NGF or BDNF exposure.

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Figure 7. ERK and p38MAPK pathway inhibitors blunted IL-6 protein increase during NGF or BDNF exposure. A MEK1/2 inhibitor, U0126, and p38 MAPK inhibitor SB 203580 was introduced to BMSC cultures 2 hours prior to NGF or BDNF exposure. BMSC were then exposed to 100 ng/ml of either NGF or BDNF for 24 hours and an IL-6 ELISA was performed on collected supernatants. BMSC pre-treated with U0126 or SB 203580 demonstrated greater than 50% decrease in IL-6 protein upon exposure to NGF or BDNF. Statistical analysis was completed by ANOVA ($P \leq 0.01$) with significance indicated by an asterisk. Groups that included inhibitor were compared to matched group with no inhibitors. BMSC treated with NGF or BDNF without inhibitors had a significant increase in IL-6 protein ($p=.001$ and $p=.002$ respectively as determined by a Student's t-test).

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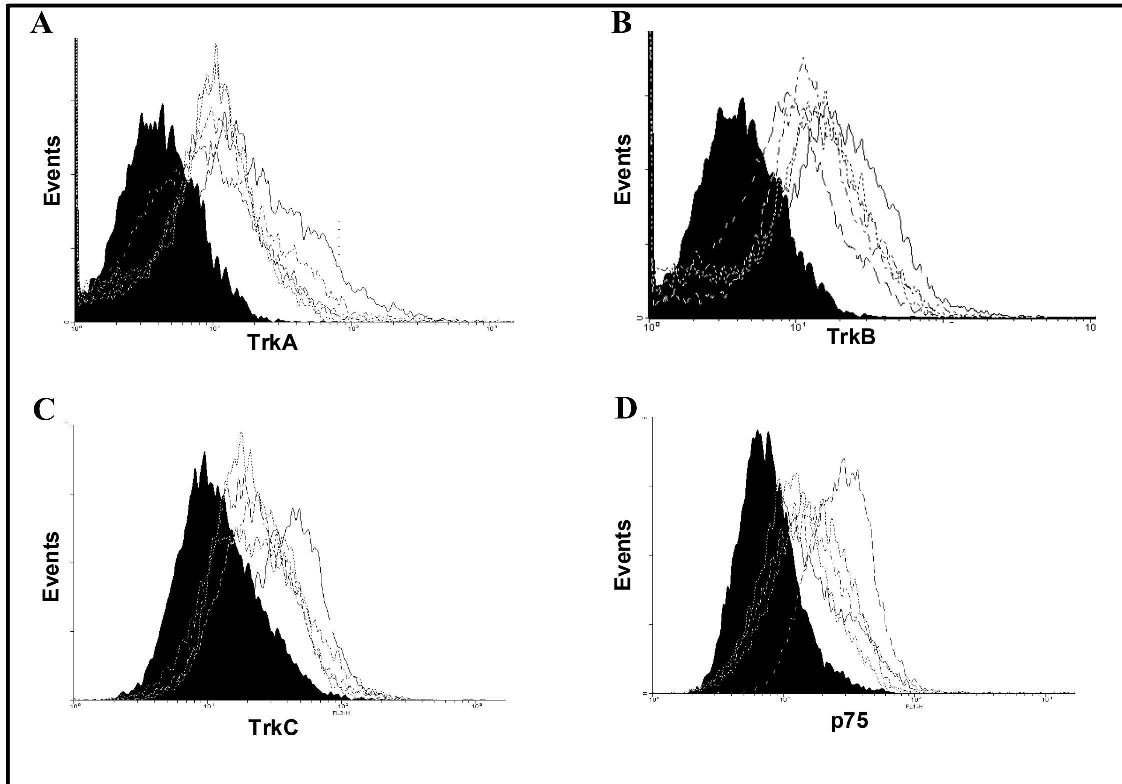

Figure 1. BMSC express neurotrophin receptors.

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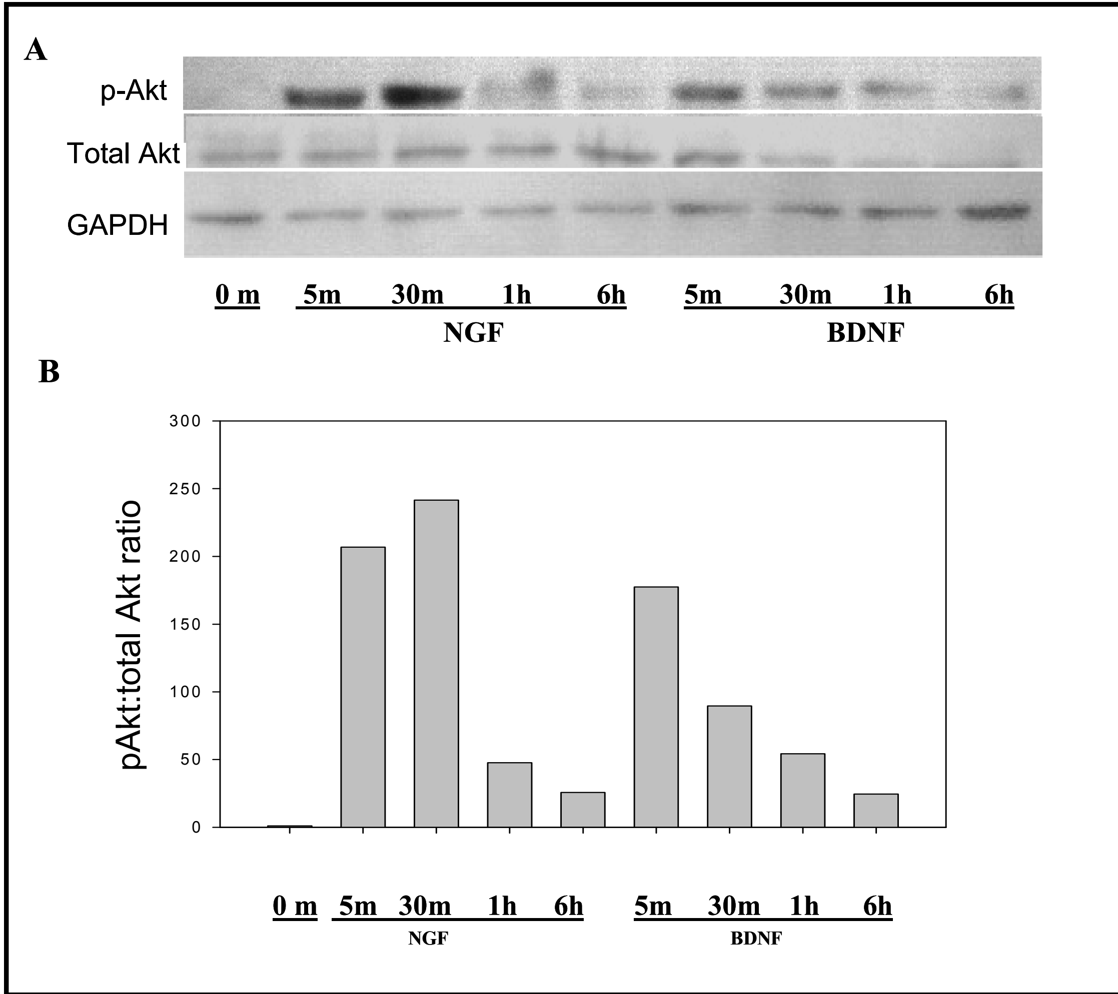

Figure 2. BMSC Akt phosphorylation increases following NGF or BDNF exposure.

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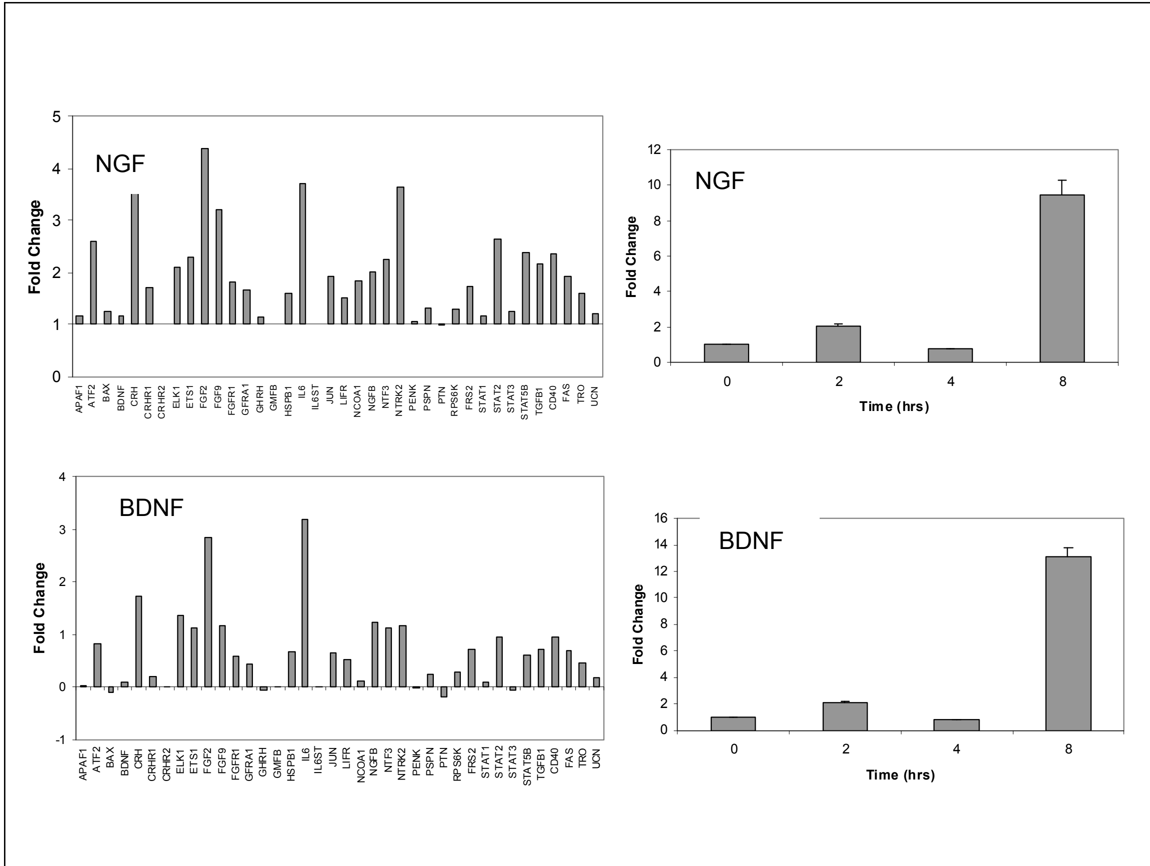

Figure 3. NGF or BDNF changes the gene expression profile of BMSC and increases IL-6 mRNA.

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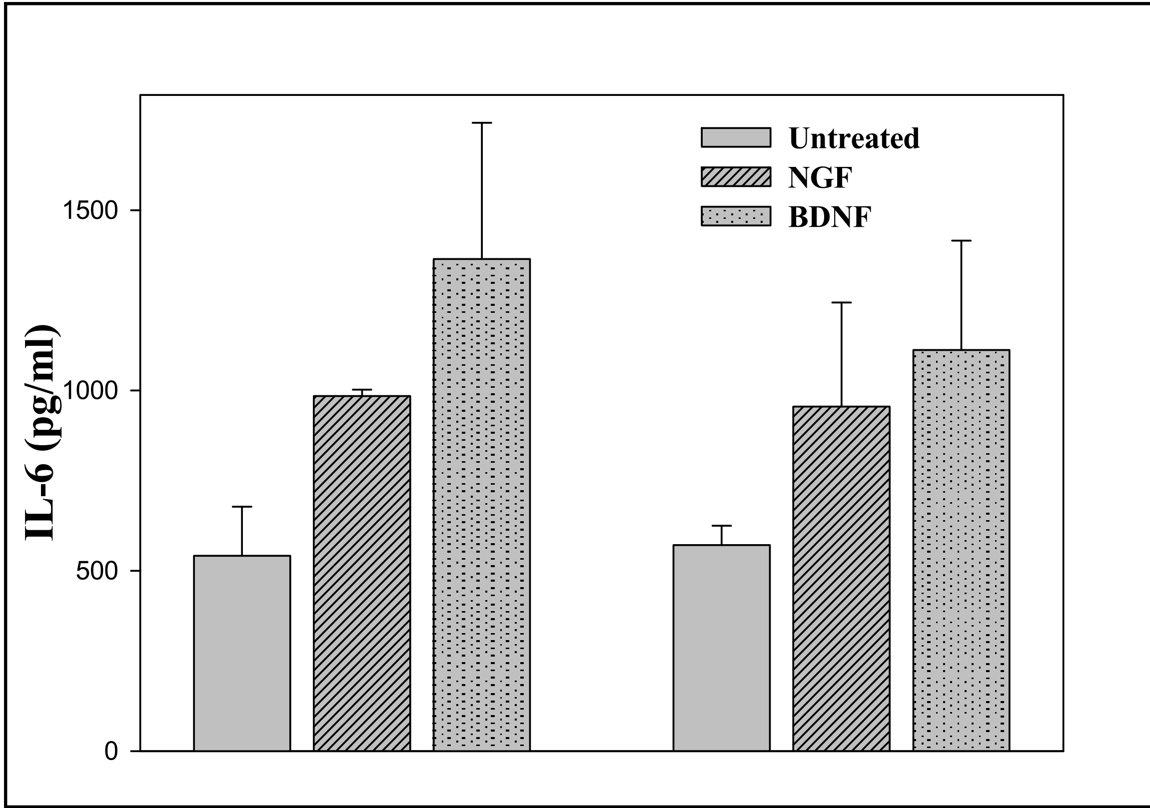

Figure 4. BMSC treatment with NGF or BDNF increases IL-6 protein.

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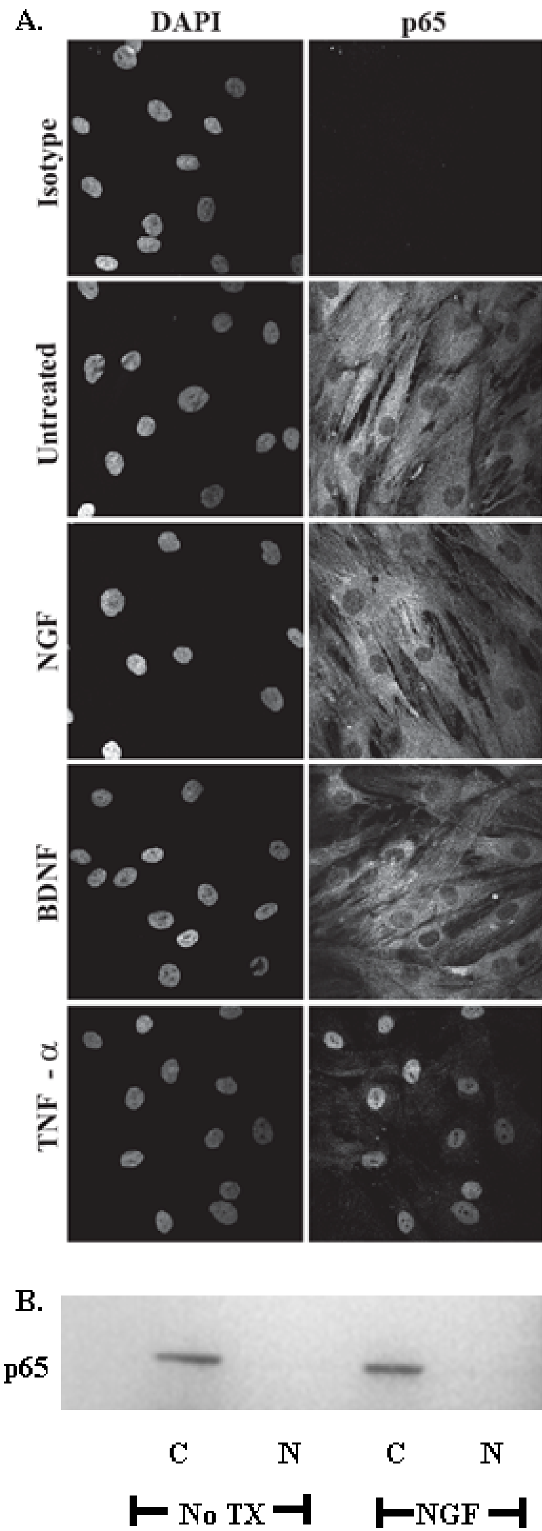

Figure 5. Neurotrophins do not induce NF-κB signaling in BMSC.

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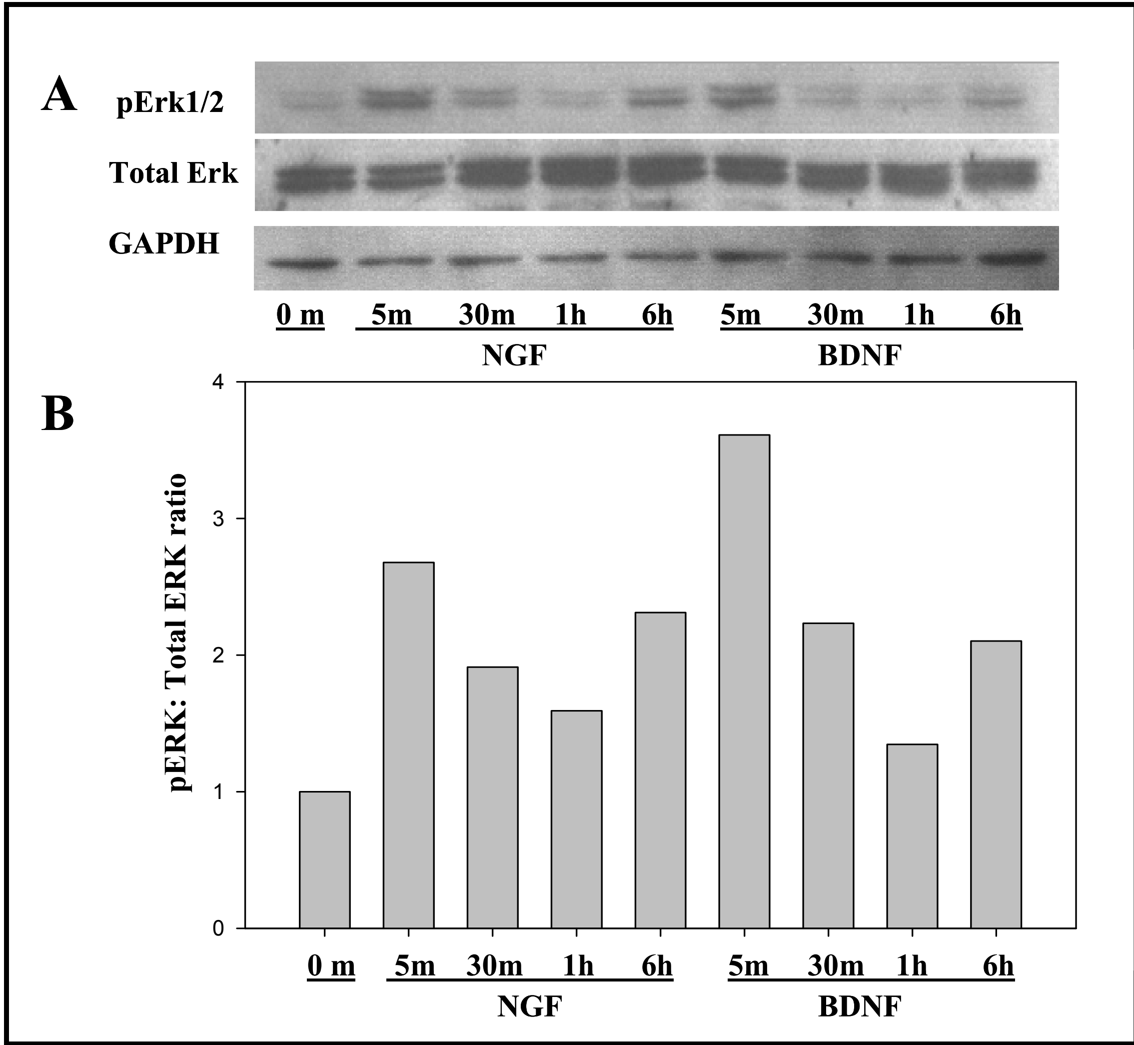

Figure 6. BMSC demonstrate activation of MAPK ERK following NGF or BDNF exposure.

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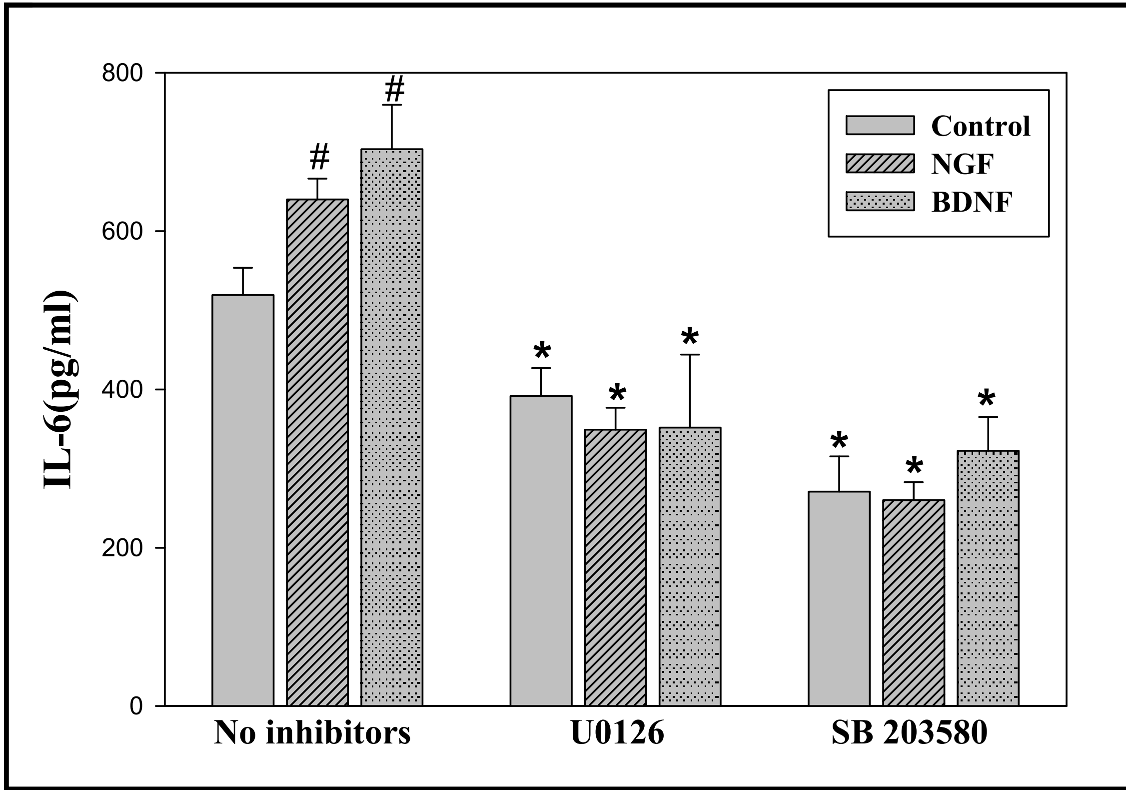

Figure 7. ERK and p38MAPK pathway inhibitors blunted IL-6 protein increase during NGF or BDNF exposure.

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Chapter IV

Melphalan Treatment Induces an Interleukin-6 Deficit in Bone Marrow Stromal Cells and Osteoblasts

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Abstract

Bone marrow stromal cells (BMSC) and osteoblasts are critical components of the microenvironment that support hematopoietic recovery following bone marrow transplantation. Aggressive chemotherapy not only affects tumor cells, but also influences structural and functional components of the microenvironment. Successful stem cell or bone marrow transplantation following myeloablative chemotherapy is dependent on the cellular components of the microenvironment to maintain their functionality through secretion of soluble factors and expression of cellular adhesion molecules. In the current study, we investigated the effects of chemotherapy treatment on BMSC and human osteoblast (HOB) expression of Interleukin-6 (IL-6).

IL-6 is a pleiotrophic cytokine which has diverse effects on hematopoietic cell development. The treatment of BMSC or HOB with melphalan leads to decreases in IL-6 protein expression. The decreased IL-6 protein is the most pronounced with melphalan treatment compared to several other chemotherapeutic agents tested. We also observed that melphalan decreased IL-6 mRNA and the addition of rIL-6 in combination with melphalan treatment restored both IL-6 mRNA and protein. Further investigation is necessary to determine the exact mechanism by which chemotherapy affects expression of IL-6, likely at a transcriptional level based on observations of decreased IL-6 mRNA with melphalan treatment. Collectively, these observations suggest that chemotherapy induced alteration of the bone marrow microenvironment, focusing on an IL-6 deficit, may result in decreased or defective hematopoietic support of early progenitor cells. In addition, the decrease in IL-6 protein may be important for the use of melphalan as a therapeutic agent for multiple myeloma, where IL-6 present in the bone marrow microenvironment is a proliferative factor and leads to disease progression.

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Introduction

The ability of the supportive cells of the bone marrow microenvironment, including bone marrow stromal cells (BMSC) and osteoblasts that comprise the endosteal niche, to maintain their functional integrity following chemotherapy or irradiation is vital for efficient reconstitution of hematopoiesis. The importance of specialized niches within the marrow environment that support stem cell self-renewal and a supply of mature blood cells has been described in detail[1-4].

Several groups have documented chemotherapy-induced stromal cell damage. In addition, BMSCs isolated from patients receiving standard chemotherapy regimens had a reduced capacity to form confluent monolayers[5]. Chemotherapy-induced damage diminishes the ability of the BMSCs to self-repair, ultimately leading to decreased numbers of functional mature blood cells[6]. Galotto et al demonstrated that the patients receiving allogenic bone marrow transplants have irreversible stromal damage measured using functional assays that showed CFU-F frequencies did not recover to normal levels even after 12 years post-transplant[7]. These investigations emphasize the vulnerability of the components of the endosteal niche.

Interleukin-6 (IL-6) is a pleiotropic cytokine that has important roles in expansion of hematopoietic progenitors, induction of acute-phase proteins for immune and inflammatory responses, and regulation of bone metabolism[8;9]. IL-6 is secreted from BMSCs and osteoblasts, and has proliferative and anti-proliferative effects. In the bone marrow microenvironment, IL-6 regulates B-cell differentiation and stimulation of T-cells, both necessary to maintain the immune system[10]. An IL-6 deficiency in the microenvironment decreases DNA synthesis in normal hematopoietic progenitor cells[11]. Long-term bone marrow cultures established from IL-6 knockout mice had delayed stromal cell layer development. Additionally, reduced hematopoietic support activity, measured by CFU-GM, BFU-E, and cobblestone areas,

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which are characteristic of active hematopoietic proliferation was noted in the absence of IL-6 as well [11]. Moreover, IL-6 deficient mice have impaired immune and acute-phase responses[12]. IL-6 deficient mice challenged with diverse viruses and pathogens demonstrated acute-phase inflammatory responses were compromised[12]. Relevant to the current study, Patchen et al observed that IL-6 administration following radiotherapy accelerated multilineage hematopoietic recovery in a murine model[13]. Based on the association of IL-6 deficits with sub-optimal hematopoietic recovery, we undertook investigation to determine whether chemotherapy dysregulates IL-6 expression in BMSC and osteoblasts as one factor involved in the dysregulated hematopoietic support capacity of the bone marrow microenvironment following dose-escalated chemotherapy.

In the current model of chemotherapy-induced damage we included the chemotherapeutic agents melphalan and VP-16. Melphalan is extensively used in pre-transplant chemotherapy regimens for autologous stem cell and allogeneic stem cell transplantations[14;15]. As such, damage imposed by chemotherapy on the microenvironment is of pronounced clinical relevance.

Our results indicated that BMSCs and osteoblasts express diminished IL-6 protein following chemotherapy treatment with melphalan and to a lesser extent, following VP-16 exposure. We also report chemotherapy-induced reductions of IL-6 mRNA that are not due to increased instability of mRNA in cells stressed by cytotoxic agents and are not associated with overt changes in three critical transcription factors. Collectively, our data suggest that melphalan treatment induces an IL-6 deficit in BMSCs and osteoblasts of the endosteal niche, potentially contributing to diminished ability of the bone marrow microenvironment to support reconstitution of hematopoiesis following myeloablative chemotherapy and subsequent bone marrow or stem cell transplantation. We also observed that recombinant IL-6, in combination with melphalan treatment, can help to restore IL-6 mRNA and protein levels suggesting that the

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addition of IL-6 is potentially able to restore disrupted IL-6 expression, leading to transcription and subsequently, translation of IL-6.

Materials and Methods

Cell culture and reagents

Primary human bone marrow stromal cells (BMSC) were derived from consenting donors with the approval of the West Virginia University Institutional Review Board. The cells were maintained in alpha-modification of Eagle's medium (α-MEM) supplemented with 2mM L-glutamine, 10% fetal bovine serum (Hyclone, Logan, UT), 100mg/ml streptomycin, 100 IU/ml penicillin and 5×10^{-5}M 2-β mercaptoethanol at 37°C in 6% CO_2. Primary human osteoblasts (HOB) were obtained from PromoCell (Heidelberg, Germany) and maintained in complete osteoblast growth media as recommended by the manufacturer. Phenotype of the osteoblasts is confirmed by both alkaline phosphatase staining and bone mineralization assays. In experiments that include chemotherapy treatment, melphalan (Sigma Aldrich, St. Louis, MO) was used at a concentration of [50 µg/ml]. Melphalan was dissolved in diluent at a concentration of 2.5 mg/ml immediately before use. Etoposide (VP-16) (Bristol-Myers Squibb, New York, NY) was stored at a concentration of 33.98 mM and a final concentration of 50 µM was used in all experiments. Methotrexate (Sigma) was used at [50 ug/ml], vincristine (Sigma) was used at [20 µg/ml], docetaxel (Sigma) was used at [50 µM] and carboplatin (Sigma) was used at [50 µM].

ELISA

BMSC and HOB were cultured in a flat bottom 96 well plate in α-MEM complete media or complete osteoblast growth media until confluent, and subsequently treated with melphalan [50µg/ml] for 24 hours. Following treatment, the media was replaced, supernatants collected at 2, 4, 6, 8, 24, and 48 hours post-treatment and the confluent layers of BMSC and HOB were

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lysed in RIPA buffer to allow quantitation of both supernatant and intracellular IL-6 protein levels. Following collection of samples at all timepoints, an IL-6 ELISA (eBioscience, San Diego, CA) was completed. BMSC and HOB were treated for 24hrs with melphalan [50ug/ml], VP-16 [50uM], methotrexate, vincristine, docetaxel or carboplatin. Following treatment, the cells were rinsed, media replaced and supernatants collected at 2, 4, 6, 8, 24 or 48hrs post-treatment and and IL-6 ELISA completed (eBioscience).

RNA Isolation and Real Time PCR

Total RNA was isolated using the Qiagen RNeasy kit following the recommendations of the manufacturer (Qiagen Inc., Valencia, CA). RNA concentration was determined by NanoDrop. To determine relative levels of IL-6 expression, real-time PCR was completed. All reactions were performed in triplicate using 50 ng of RNA per reaction and the one-step QuantiTect SYBR Green RT-PCT kit (Applied Biosystems, Foster city, CA). IL-6 gene primers (Real Time Primers, Elkins Park, PA) or the housekeeping gene beta-glucuronidase (*GusB*) (Real Time Primers) were used. Amplifications were completed using a 7500 real-time cycler (Applied Biosystems). The amplification conditions were 50°C for 30 minutes, 95°C for 15 minutes and 45 cycles of 94°C for 15 seconds, 55°C for 30 seconds, and 72°C for 45 seconds. The relative changes in gene expression were calculated using the Comparative Ct method[16].

Results

Melphalan treatment of BMSCs and HOBs results in diminished IL-6 protein.

To determine if melphalan disrupted IL-6 protein expression, BMSCs and HOBs were exposed to melphalan [50µg/ml] and an IL-6 ELISA completed using both cell supernatants and lysates. Melphalan treatment led to decreased IL-6 protein detected in supernatants and lysates of both BMSCs and HOBs. To determine if IL-6 protein detection was decreased due to expression of the soluble IL-6 receptor (sIL-6R), BMSCs and HOBs were treated with melphalan

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and a sIL-6R ELISA completed. No detectable levels of sIL-6R were detected in BMSCs or HOBs (data not shown). Finally, to evaluate whether IL-6 protein was being degraded more rapidly in melphalan treated populations of cells, BMSCs and HOBs were treated with melphalan alone or in combination with cyclohexamide [100ug/ml]. Cycloheximide exposure resulted in the expected decrease in IL-6 protein over 24 hrs and the addition of melphalan did not lead to a more rapid degradation of IL-6 protein (data not shown).

Melphalan treatment, compared to other chemotherapeutic agents, resulted in the most pronounced decrease in IL-6 protein.

Based on the previous data, we investigated if the melphalan-induced decrease in IL-6 protein was specific or if chemotherapeutic agents, in general, led to the same decrease. While all the different chemotherapeutic agents investigated led to significant changes in IL-6 protein compared to untreated cells, melphalan treatment consistently led to the most pronounced decrease in IL-6.

The IL-6 polymorphism does not affect how BMSC respond to melphalan.

An IL-6 polymorphism has been well described in autoimmune and inflammatory diseases[17-20]. To evaluate a potential correlation between of the G>C174 IL-6 SNP in BMSCs treated with melphalan to drug associated changes in IL-6 expression, primary BMSCs were first genotyped. Two representative cell lines of each SNP were treated with melphalan [50ug/ml] and an IL-6 ELISA completed. Regardless of genotype, all cell lines had decreased detection of IL-6 protein with melphalan treatment.

Melphalan treatment decreased IL-6 mRNA

Based on the observation that melphalan decreases IL-6 protein expression, we investigated changes in IL-6 mRNA expression with melphalan treatment. To determine relative

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IL-6 transcript levels, BMSC and HOB were treated with melphalan [50μg/ml] and real-time PCR completed. The levels of IL-6 mRNA transcripts increased initially with 4hrs of treatment, but overall, decreased with melphalan treatment at the 16hr time point. To evaluate if melphalan treatment altered the stability of IL-6 RNA, BMSCs and HOBs were treated with melphalan alone or in combination with Actinomycin-D or α-amanitin. Treatment of cells with Actinomycin-D or α-amanitin in combination with melphalan treatment did not decrease the stability of IL-6 mRNA (data not shown). Additionally, BMSC and HOB were left untreated or treated with melphalan [50ug/ml] and cellular fractionation and western blot completed to determine if there were any changes in the cellular localization of p65, c-jun or C/EBP-β as transcription factors previously described to be required for optimal IL-6 gene expression[8]. There were no changes in the cellular localization of these proteins with melphalan treatment compared to untreated cells, using Histone 3 (H3) and heat shock protein 90 (Hsp90) as fractionation controls (data not shown).

Recombinant IL-6 treatment restores IL-6 mRNA levels.

Treatment of BMSC and HOB with melphalan leads to a decrease in IL-6 mRNA. To evaluate if this decrease in mRNA could be restored, BMSC and HOB were treated with recombinant IL-6 alone or melphalan alone or in combination with recombinant IL-6. While melphalan decreased IL-6 mRNA as described earlier, the addition of recombinant IL-6 restored IL-6 mRNA expression.

***Recombinant IL-6 treatment restores IL-6 protein levels*.**

Based on the results that recombinant IL-6 restored IL-6 mRNA, we determined if the addition of recombinant IL-6 to melphalan treated BMSC and HOB would restore IL-6 protein expression. BMSC and HOB treated with melphalan alone had decreased IL-6 protein as described earlier, and the combination of melphalan and recombinant IL-6 led to significant

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increases in IL-6 protein, with the most pronounced increases at 24 and 48hrs post-treatment. Control experiments were completed to confirm that the recombinant IL-6 was completely was rinsed from the cells and not being detected in the ELISA as a residual factor (data not shown).

Discussion

In the current study we investigated the effects of chemotherapy on IL-6 expression in bone marrow stromal cells and human osteoblast as two representative supportive cells of the bone marrow microenvironment that influence stem and hematopoietic progenitor cell development[3;21-23] .While the target of dose-escalated chemotherapy or irradiation is a tumor cell population, it is clear that additional cells are also vulnerable to therapy. Successful stem cell or bone marrow transplantation following immuno-suppressive or myeloablative chemotherapy is dependent on the ability of diverse cellular components of the microenvironment to maintain their functionality, including secretion of soluble factors and expression of cellular adhesion molecules that are critical for the survival, proliferation, and differentiation of stem and immature progenitor cells[24-29].

Previously mentioned was the damage that bone marrow stromal cells are vulnerable during aggressive treatment. There has also been much literature describing the effects of osteoblast functional deficiencies and how they impact hematopoiesis. Work by Visnjic et al showed defects in hematopoiesis in mice where osteoblast deficiency was induced[30]. Another model of osteoblast damage was in a transgenic mouse model using the herpesvirus thymidine kinase gene under the control of a collagen alpha 1 type I promoter, allowing for lineage specific expression of the gene in osteoblasts. This targeting allowed for the specific ablation of osteoblasts by addition of ganciclovir. These mice lost lymphoid, myeloid and erythroid progenitors in the bone and had significantly decreased HSCs. When osteoblasts were allowed to recover, a coincident recovery in hematopoiesis occurred as well in the bone marrow. Chitteti

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et al showed that CFU expansion was increased when HSCs were cultured with osteoblasts[31]. This suggests that osteoblasts have critical roles in the regulation of hematopoiesis, most likely through both physical and soluble factors.

We have shown that melphalan treatment decreases IL-6 protein in the absence of intracellular accumulation, suggesting melphalan induced changes in BMSC or osteoblasts is not causing dysregulated secretion of IL-6 (Figure 1). IL-6 can signal through both its membrane receptor as well as a soluble receptor[32-34]. To confirm that the decrease in IL-6 protein was not due to decreased detection of IL-6, we evaluated if BMSC and HOB expressed the sIL-6R in culture and if the expression of the sIL-6R changed in response to melphalan. The sIL-6R was not detected in untreated cells or in cells treated with melphalan, suggesting the sIL-6R is not interfering with our detection of IL-6 protein in cell supernatants (data not shown). BMSC and HOB were treated with a variety of agents to determine if the effects of chemotherapy on IL-6 were specific to melphalan or if all chemotherapeutic agents of diverse classes resulted in comparable damage. Heterogeneity in the drugs examined is reflected by VP-16 being a topoisomerase II inhibitor, methotrexate an anti-metabolite, vincristine a tubulin inhibitor, docetaxel an anti-microtubule agent and carboplatin a heavy metal DNA binding agent. While all these agents led to significant changes in IL-6 protein compared to cells that were untreated, melphalan had the most pronounced decrease in IL-6 protein in both BMSC and HOB (Figure 2). Additional studies are required to determine if this effect is seen with other alkylating agents.

In addition to our studies evaluating different classes of drugs and their effects on IL-6 levels, we also investigated changes in IL-6 protein associated with an IL-6 polymorphism. The 174 G>C SNP has been well characterized in the setting of auto-inflammatory and autoimmune diseases and is somewhat controversial in whether or not the genotype is a prognostic factor[17-20;35]. In general, individuals with a genotype of G/C are thought to have "normal" levels of IL-6 in their serum and when presented with an immune challenge, IL-6 levels increase and then return

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to normal. Individuals with a G/G genotype are described as having high levels of serum IL-6 and hyper-respond when presented with an immune challenge, while individuals with a C/C genotype are described as having very low levels of IL-6 in their serum and have a minimal inflammatory response when presented with an immune challenge[20]. It was of interest to us if the genotype of an individual affected a person's response to chemotherapy. Two BMSC cell lines of each genotype were left untreated or treated with melphalan. Our data show that regardless of genotype, all cell lines had decreased IL-6 protein in response to melphalan (Figure 3). Additionally, the amount of IL-6 detected in untreated cells did not correlate with genotype as the BMSC genotyped as C/C had the highest levels of IL-6.

Based on observed changes in IL-6 protein in response to chemotherapy, we next investigated whether the decrease in IL-6 protein was due to changes in mRNA. Our data showed that melphalan decreased IL-6 mRNA in both BMSC and HOB (Figure 4), suggesting melphalan may be effecting IL-6 expression at a transcriptional level. We evaluated if melphalan treatment was decreasing the stability of mRNA through experiments using actinomycin-D and α-amanitin and determined that melphalan treatment was not affecting the stability of IL-6 mRNA (data not shown). We also investigated if any of the transcription factors known to positively regulate IL-6 transcription were being affected by melphalan treatment. BMSC and HOB were left untreated or treated with melphalan and cellular fractionation and western blot analysis completed looking at NF-κB (p65), AP-1 (c-jun) and C/EBP-β[8]. It was not evident that any of these transcription factors are being affected based on cellular localization however, this does not address binding efficiently to the DNA during melphalan treatment, which still needs to be investigated.

Finally, in an effort to evaluate if disrupted IL-6 expression can be restored, BMSC and HOB were treated with a combination of melphalan and rIL-6. While melphalan treatment alone decreases IL-6 protein as discussed previously, the addition of rIL-6 with melphalan began to restore IL-6 mRNA and protein levels (Figure 5 and 6). Administering rIL-6 to patients was

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previously attempted following bone marrow transplant, but the toxicity associated with rIL-6 treatment have prevented its use in a clinical setting[36]. However, the connection between melphalan and IL-6 in a clinically relevant setting may be the use of melphalan as first-line therapy in treatment of multiple myeloma (MM). IL-6, in the setting of MM, is a potent proliferative and survival factor[37] and many attempts have been made to decrease IL-6 in the bone marrow microenvironment through the use of proteosome inhibitors and anti-IL-6 neutralizing antibodies as part of the therapeutic strategy[38]. Melphalan, as part of a chemotherapy regimen for MM, may initially decrease IL-6 in the bone marrow microenvironment, resulting in myeloma cell sensitivity to chemotherapeutic agents. Work by Gupta et al and many others has shown that MM cells in contact with BMSC increase the secretion of IL-6 from the BMSC[39-41]. By showing that IL-6 levels increase with the addition of rIL-6 (Figure 5 and 6), this may, in part, mimic what is happening in an in vivo setting, where MM cells have the ability to restore IL-6 signaling in the microenvironment through cell contact, thus contributing to relapse of disease.

In conclusion, we have demonstrated that melphalan, more so than other chemotherapeutic agents tested in the current study, decreases IL-6 mRNA and protein with decreases not being due to instability of message. We have also shown that the addition of rIL-6 partially restores IL-6 mRNA and subsequently, IL-6 protein expression, possibly through stimulating IL-6 signaling pathways in BMSC or HOB. This finding could be important in the setting of MM, where IL-6 present in the microenvironment acts as a potent proliferative factor and contributes to progression of the disease. Additionally, understanding the decrease in IL-6 in the microenvironment caused by chemotherapy treatment is important for normal hematopoietic and immune system recovery following bone marrow or stem cell transplantation, as IL-6 affects both myeloid and lymphoid lineages of cell differentiaion. To increase the translational potential of these findings, the effects of melphalan *in vivo* need evaluated to determine if the effects on IL-6 protein are similar or if there is redundancy of the IL-6 family

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members that overcome this deficit during treatment. As previously mentioned, murine IL-6 knock-out models have been described as having hematopoietic deficits, suggesting that the IL-6 family members are not completely redundant and that optimal IL-6 levels are required to sustain hematopoiesis. Overall, understanding the effects of chemotherapeutic agents on a molecular level can provide insight as to why these agents work in disease settings such as MM as well as provide an understanding as to why patients receiving treatment with these agents as part of myeloablative regimens prior to bone marrow or stem cell transplantation have long term hematopoietic deficits.

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Figure Legends

Figure 1. Treatment of human BMSC and HOB with Melphalan decreases Interleukin-6 secretion. BMSC **(A,C)** and HOB **(B,D)** cells were seeded into a 96 well plate in triplicate and left untreated or were treated with melphalan [50 µg/ml] in complete media for 24 hours. After 24 hours, the media was replaced with complete media and supernatants **(A and B)** or cells lysates **(C and D)** in RIPA buffer were collected at the time points above. An Interleukin-6 ELISA was performed to quantitate changes in the levels of IL-6 protein secreted following chemotherapy.

Figure 2. Melphalan treatments causes the most pronounced decrease in IL-6 protein. A). BMSC and **B).** HOB were treated with different chemotherapeutic agents for 24hrs. After 24hrs, the cells were rinsed, the media replaced, supernatants collected at 24 and 48hrs post-treatment and an IL-6 ELISA completed. The concentration of drug used was the highest tolerable dose without a decrease in cell viability (data not shown). Melphalan treatment led to the most pronounced decrease in IL-6 protein in both BMSC and HOB.

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Figure 3. The IL-6 G>C174 SNP does not affect BMSC response to melphalan. BMSC were genotyped for the presence of the IL-6 polymorphism. Two cell lines from each genotype were treated with melphalan [50ug/ml] for 24hrs. Following treatment, cells were rinsed, supernatants collected 24hrs post-treatment and an IL-6 ELISA completed. All BMSC lines had decreased IL-6 protein, regardless of genotype.

Figure 4. Treatment of BMSC or HOB with melphalan decreases IL-6 mRNA expression. **A).** BMSC or **B).** HOB treated with melphalan (50μg/ml) for 4 or 16 hours. Following treatment, RNA was isolated and quantitated and real-time PCR completed to evaluate changes in IL-6 mRNA expression in chemotherapy treated cells as compared to their untreated controls treated controls. Melphalan treatment decreases IL-6 mRNA in both BMSC and HOB.

Figure 5. Addition of rIL-6 restores IL-6 mRNA expression in both BMSC and HOB. A). BMSC or B). HOB were left untreated or treated with rIL-6 [10ng/ml], melphalan [50ug/ml] or a combination of rIL-6 and melphalan for 4, 8, 18 or 24hrs. Following treatment, the cells were collected, RNA isolated and real-time PCR completed for IL-6 mRNA expression. Melphalan treatment alone decreased IL-6 mRNA, while the combination of rIL-6 with melphalan restored IL-6 mRNA levels.

Figure 6. Addition of rIL-6 restores IL-6 protein expression in both BMSC and HOB. A). BMSC and B). HOB were left untreated or treated with rIL-6 [10ng/ml], melphalan [50ug/ml] or a combination of rIL-6 and melphalan for 24hrs. Following treatment, cells were rinsed, media replaced, supernatants collected at 2, 4, 6, 8, 24 and 48hrs post-treatments and an IL-6 ELISA completed. Control experiments were completed to confirm the rIL-6 was being washed out and

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not detected by the ELISA assay (data not shown). rIL-6 combined with melphalan began to restore IL-6 protein expression at 24 and 48hrs post-treatment compared to melphalan treatment alone.

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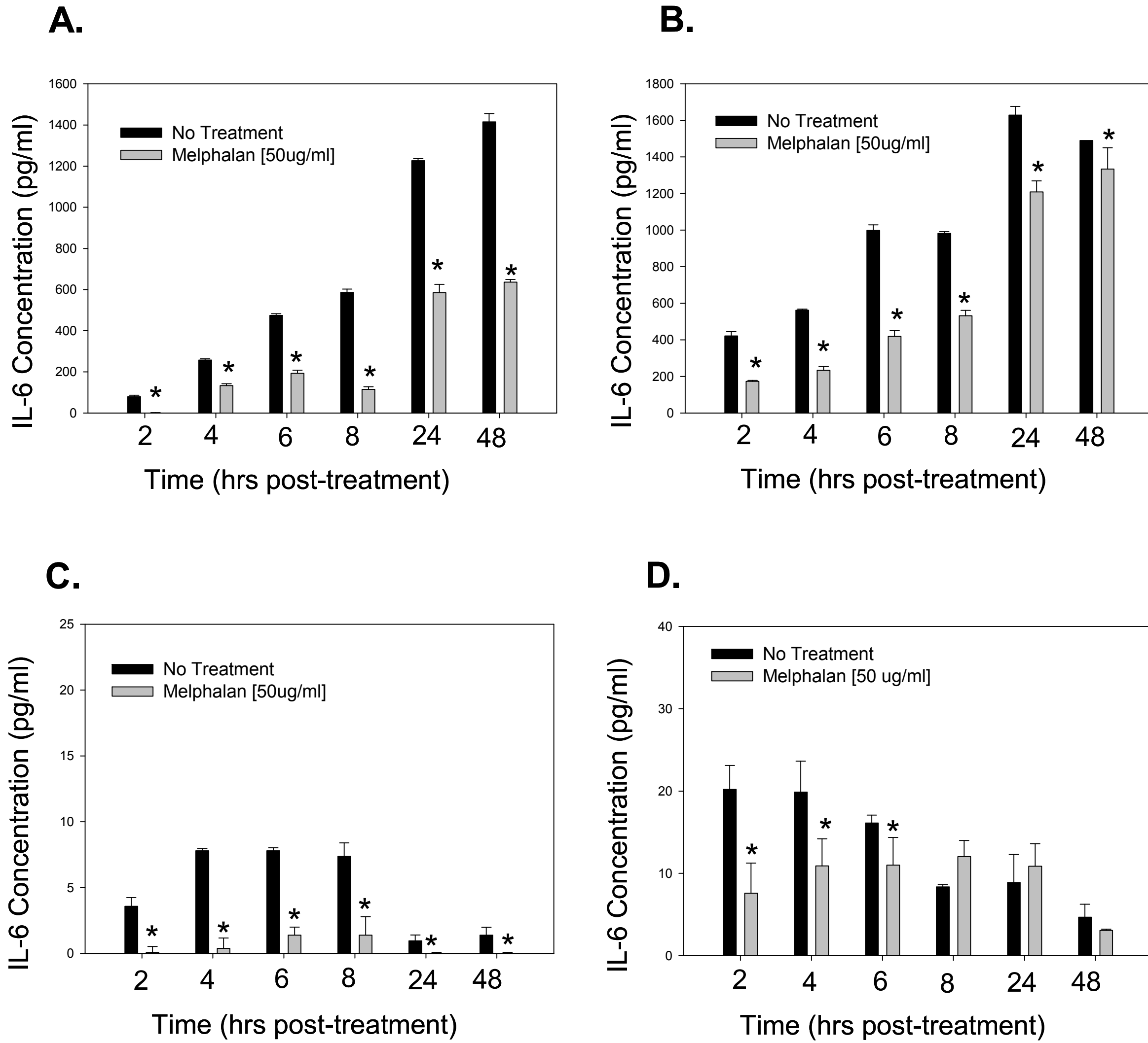

Figure 1. Treatment of human BMSC and HOB with Melphalan decreases Interleukin-6 secretion.

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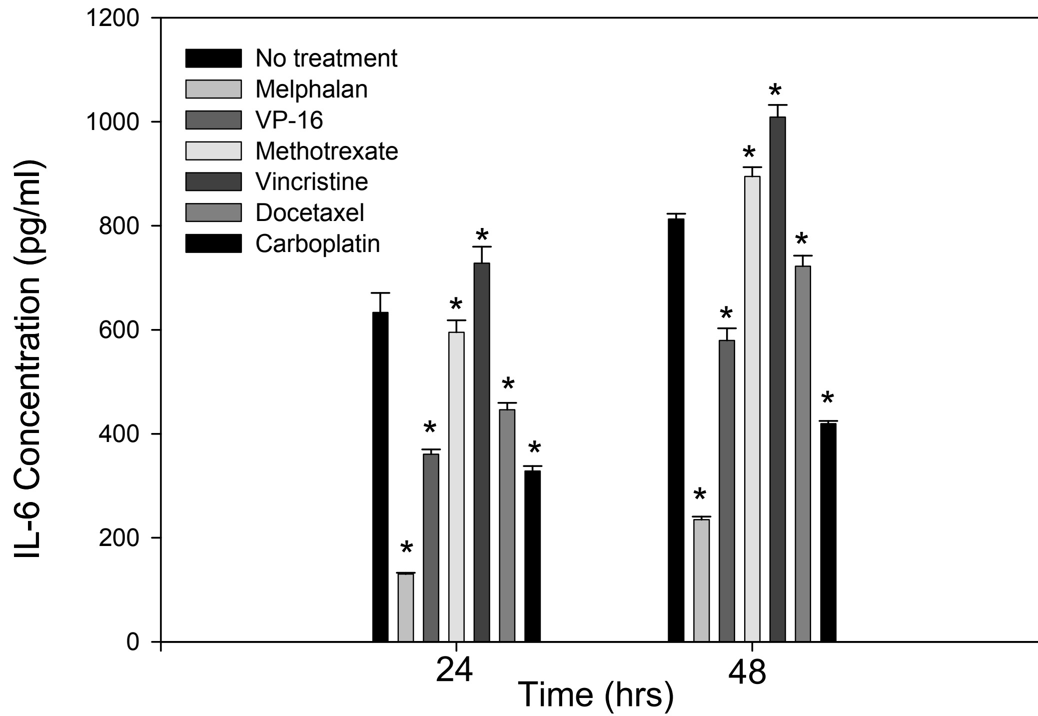
A.



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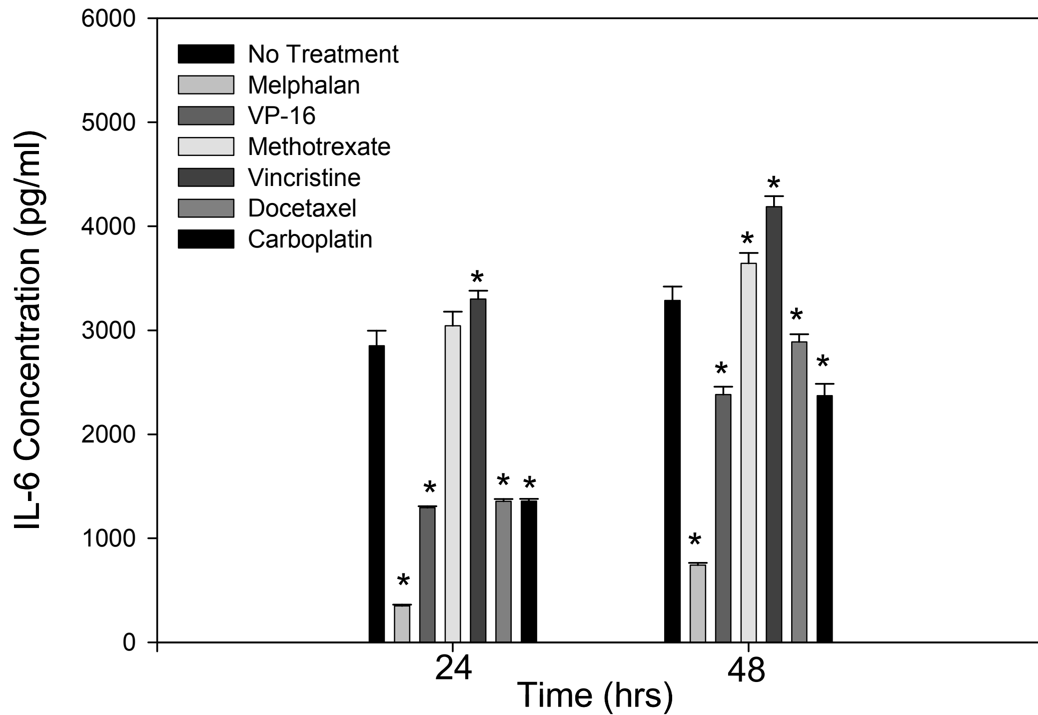

Figure 2. Melphalan treatments causes the most pronounced decrease in IL-6 protein.

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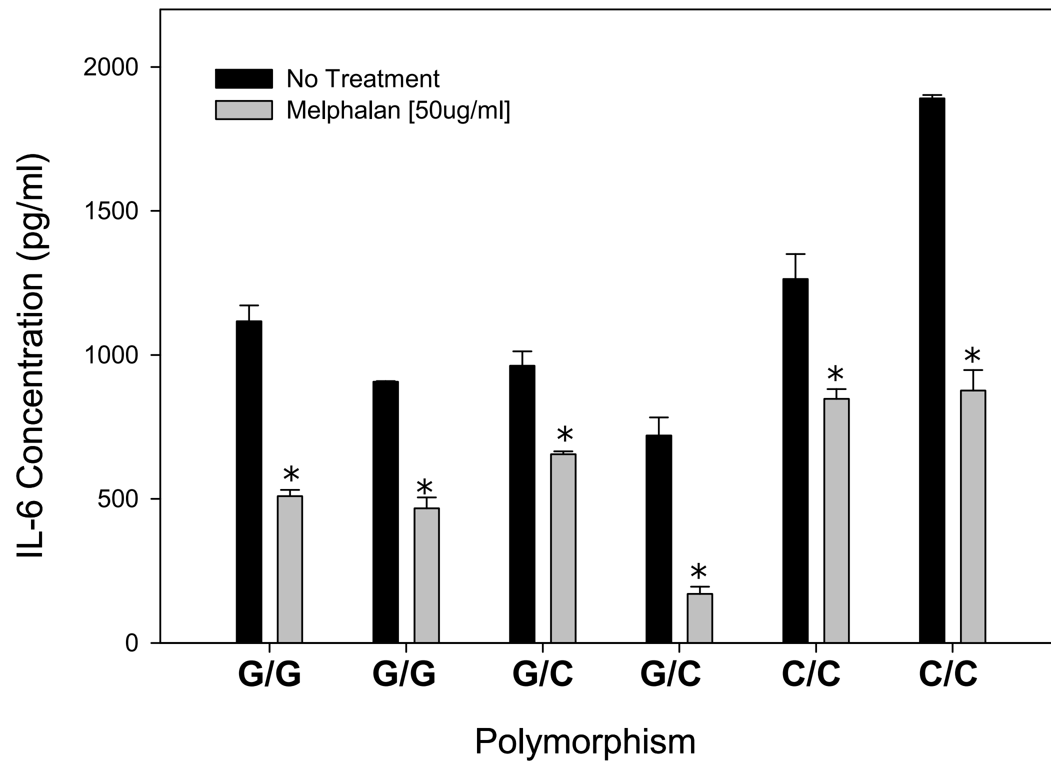

Figure 3. The IL-6 G>C174 SNP does not affect BMSC response to melphalan.

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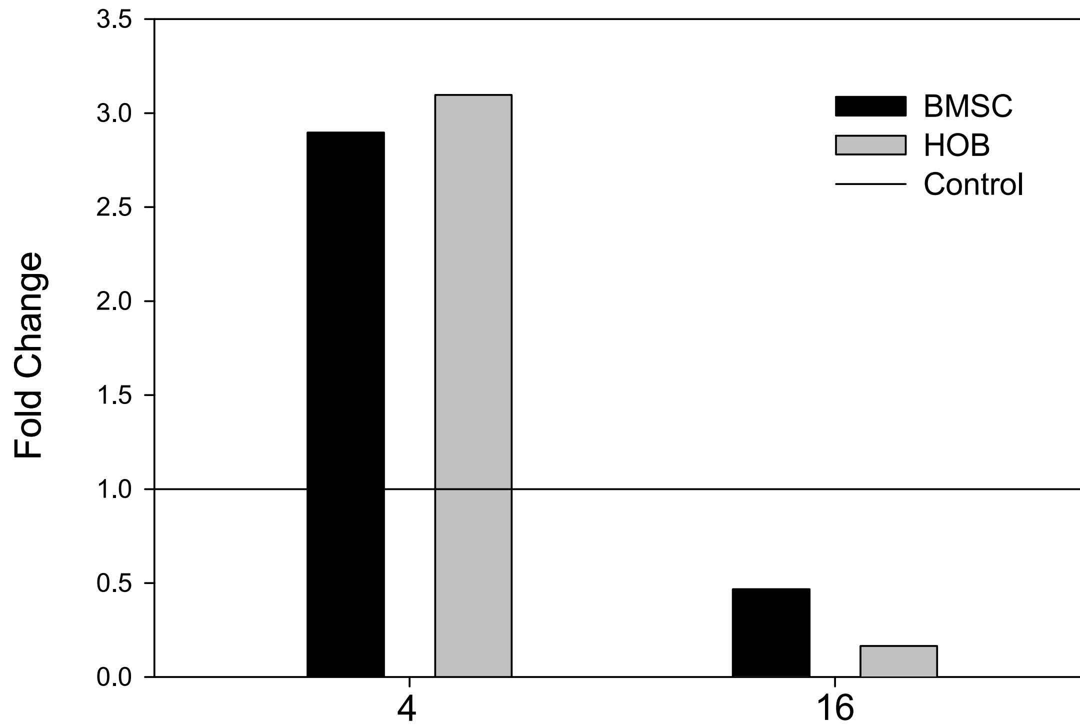

Figure 4. Treatment of BMSC or HOB with melphalan decreases IL-6 mRNA expression.

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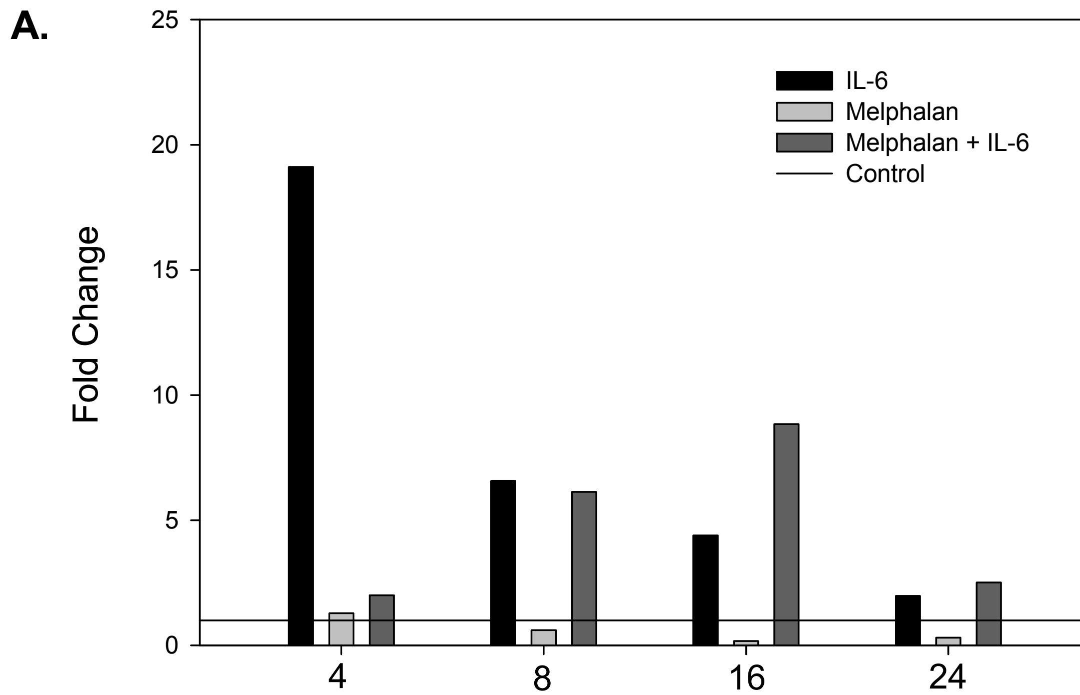

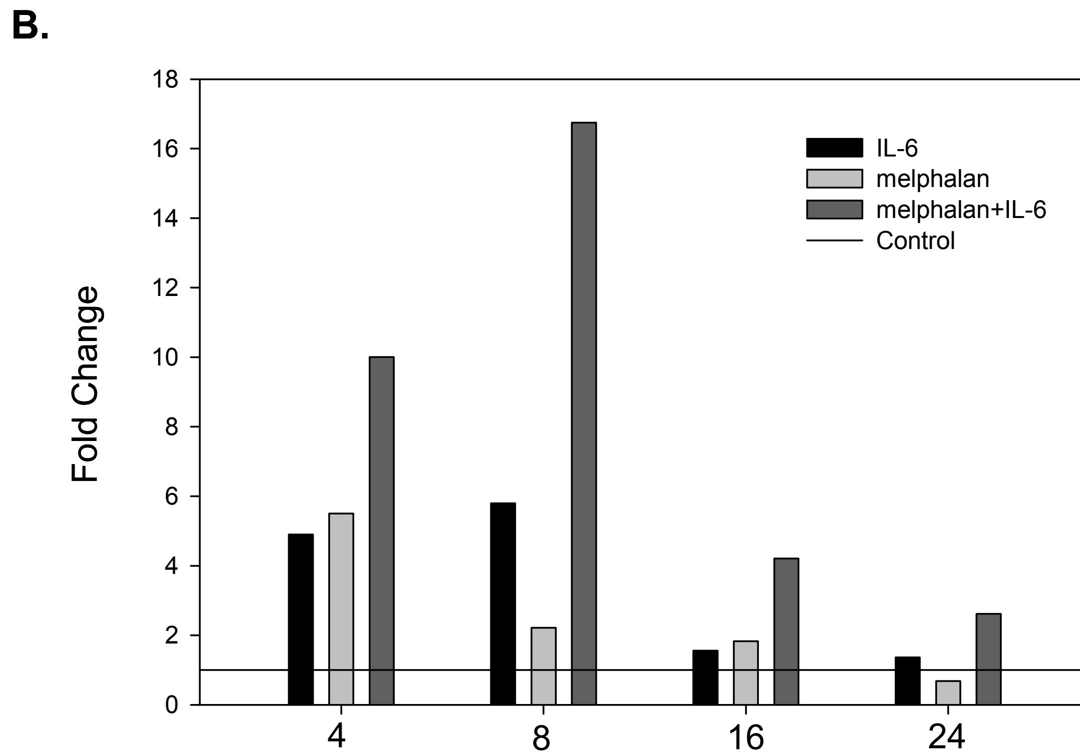

Figure 5. Addition of rIL-6 restores IL-6 mRNA expression in both BMSC and HOB.

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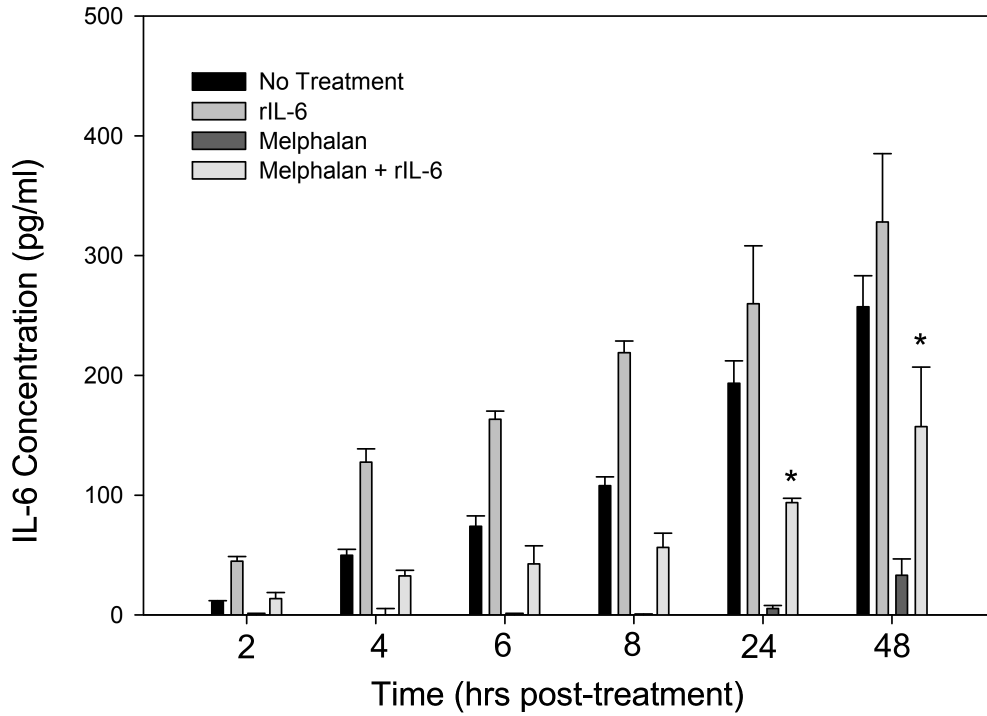
A.



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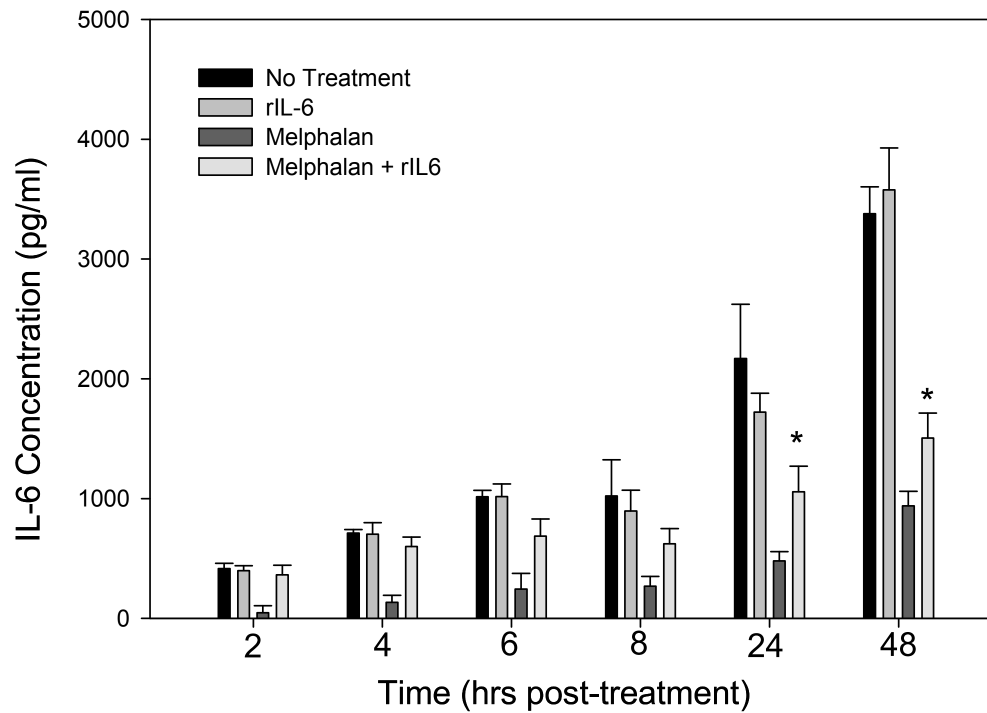

Figure 6. Addition of rIL-6 restores IL-6 protein expression in both BMSC and HOB.

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Chapter V

General Discussion

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The idea of using bone marrow transplantation to cure disease was first described in 1951 when Lorenz et al described how using an animal's own marrow allowed for survival following total body irradiation[1]. The first use of this procedure in humans occurred in 1959 when Thomas and colleagues treated a leukemia patient with total body irradiation and subsequently provided that patient with an infusion of bone marrow from an identical twin[2]. These studies and many other provided the groundwork for using total body irradiation, later in combination with chemotherapy treatment, and bone marrow transplant for treatment of disease. Currently, bone marrow or hematopoietic stem cell transplant is used for a variety of diseases such as multiple myeloma, leukemia, lymphoma, autoimmune diseases, aplastic anemia etc[3]. The goal of most of these therapies is eradication of the tumor population, creating space for the transplanted cells. While hematopoietic stem cell transplantation has a mortality rate of less than 2 percent for autologous transplant and less than 10 percent for allogeneic transplant, about 40 percent of patients that receive allogeneic transplants die from complications related to the transplantation including GVHD, veno-occlusive disease and infection, emphasizing the need to reduce toxicity of conditioning regimens[3].

While advances have been made in therapies to eradicate tumor, significantly less research has been done to address damage that occurs to the hematopoietic microenvironment following myeloablative chemotherapy regimens. Studies describing BMSC have shown that damage by chemotherapy and radiotherapy can affect the ability of the BMSC to self-repair and leads to decreased numbers of functional immune system cells in the blood, with deficits persisting years after transplant[4;5]. Additional studies observed that at 1 year post transplant 61% of patients have subnormal values in one or more hematopoietic lineages[6]. Further, Nieboer *et al*. showed that at 5 years post transplant 15% of the patient population analyzed had low values in one or more hematopoietic cell lineages[7]. Galotto et al completed retrospective studies of patients that received allogeneic bone marrow transplants and determined patient BMSC did not recover to the levels of control patients, even after 12 years,

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as measured by colony forming units-fibroblast (CFU-F) frequencies, suggesting that the damage of the structural, hematopoietic supportive cells of the bone marrow can be sustained[8]. Osteoblasts, another important structural component of the bone marrow in addition to BMSC, have also been studied for their importance in maintenance of hematopoiesis. These studies have included transgenic models where osteoblast deficiency was induced by actively targeting and ablating the osteoblasts[9] or using models where the osteoblast niche was manipulated by knock-out of critical factors such as osteopontin or the osteoblast niche was increased through the use of parathyroid hormone, leading to decreased and increased hematopoiesis, respectively[10-12].

The broad goals of the work presented in this dissertation were to gain a better understanding of chemotherapy-induced damage occurring to the cells of the bone marrow microenvironment and how this damage would affect hematopoietic recovery through evaluation of an in vitro co-culture model.

Previous work by our lab has characterized damage to BMSC and has shown increases in active TGF-β following chemotherapy treatment[13]. TGF-β, as previously discussed, is an important factor in supporting and maintaining quiescent hematopoietic stem cells[14]. It has been proposed that this could lead to depletion of the stem cell pool. We evaluated the effects of chemotherapy on osteoblasts to determine how both direct and indirect chemotherapy exposure may lead to damage of the osteoblast population. As we discussed in Chapter 1, human primary osteoblasts were treated with melphalan and VP-16 and we evaluated changes in soluble factors, cellular adhesion molecules and overall support of both human embryonic stem cells and CD34+ bone marrow cells. Treatment of osteoblasts with chemotherapy led to slight increases in TGF-β mRNA and active TGF-β, evaluated by phosphorylation of Smad2. Additionally, chemotherapy treatment led to decreases in CXCL12 mRNA and protein and decreased chemotaxis. We also evaluated the effects of recombinant TGF-β (rTGF-β), to mimic both autocrine and paracine signaling associated with chemotherapy-induced changes.

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Treatment of osteoblasts with rTGF-β led to increased TGF-β mRNA and protein and decreased CXCL12 mRNA and protein, coincident with decreased chemotaxis. To determine functional changes in the ability of damaged osteoblasts to support stem cells, hESC and CD34+ bone marrow cells were cultured on osteoblasts pre-treated with chemotherapy or rTGF-β. In both circumstances, hESCs did not retain Oct-4 expression, a marker of pluripotency, and CD34+ cells had deficits in their ability to differentiate into different hematopoietic cell lineages. These data suggest that osteoblasts are a population of cells that are vulnerable to chemotherapy damage, and hematopoiesis could be negatively affected through damage to the microenvironment. One way to help restore this environment might be through the use of co-transplantation of MSCs as a mechanism to "rebuild" the microenvironment. Additionally, one could speculate that factors leading to the differentiation of osteoblasts, BMSC and the other cell types of the microenvironment could be administered to stimulate differentiation of MSCs already present in the microenvironment to functionally supportive cells.

Another soluble factor investigated in the studies presented was Interleukin-6 (IL-6) as a factor important in the differentiation of both myeloid and lymphoid lineages[15]. Initially, we expected that chemotherapy treatment of BMSC or osteoblasts would lead to increases in IL-6, as it has been a factor associated with acute graft versus host disease[16]. We actually determined that chemotherapy treatment, melphalan specifically, led to decreases in IL-6 protein and mRNA in both BMSC and HOB (Chapter 3). Compared to other chemotherapeutic agents evaluated in this study, melphalan had the most pronounced effect. To evaluate if IL-6 mRNA and protein levels could be restored in an autocrine fashion, we combined melphalan treatment with recombinant IL-6. Both mRNA and protein levels were restored in these combination treatment groups. This was an important observation relevant to multiple myeloma (MM). In MM, IL-6 is a proliferative factor for the myeloma tumor cells and work by Gupta et al and Barille et al have shown that myeloma cells, directly in contact with BMSC and osteoblasts ,

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can upregulate IL-6 expression[17;18] and Westendorf et al described how myeloma cells themselves could secrete IL-6 and engage in autocrine signaling[19]. Melphalan, along with other agents, is first-line therapy for the treatment of MM. The decrease of IL-6 in BMSC and osteoblasts by melphalan could help to explain why this chemotherapeutic agent has effects in MM. By decreasing IL-6 in the microenvironment, the myeloma cells may be more sensitive to chemotherapy. By showing in vitro that that addition of rIL-6 could restore IL-6 levels, this may, in part, explain how in vivo, the tumor is able to restore IL-6 secretion in the bone marrow microenvironment allowing for relapse of disease. The cytokine profile and recovery dynamics *in vivo* is important in understanding how prolonged the decrease in IL-6 and other hematopoietic cytokines is. Studies by Testa et al have looked at hematopoietic growth factors (IL-3, SCF, IL-6, IL-8 and GM-CSF) in patient serum pre and post-chemotherapy, and have shown how these factors increase or decrease following chemotherapy[20]. The chemotherapeutic agents used for these studies were busulphan and cyclophosphamide, so similar experiments in patients treated with a melphalan regimen would be useful in understanding differences in cytokine recovery profiles with different chemotherapy regimens to determine optimal schedules for infusion of hematopoietic stem and progenitor cells.

Additional studies presented in this proposal related to IL-6 have a focus on the neuroendocrine modulation of the bone marrow[21]. Studies have shown that NGF has roles in colony formation[22;23] and in a model using stromal cells isolated from the thymus, it was shown that NGF increased IL-6 expression[24]. The role of BDNF in hematopoiesis was also studied using a BDNF knockout mouse and it was demonstrated that the number of B lymphocytes both in the spleen and the bone marrow were decreased[25]. These data suggest that neurotrophins have much broader paracrine effects than just in the central nervous system. In chapter 2 of this dissertation, we have shown that primary BMSC express neurotrophin receptors and that these receptors can actively signal following stimulation with NGF or BDNF, and using

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phosphorylated Akt as a read-out of signaling. Treatment of BMSC with NGF or BDNF also led to increases in both IL-6 mRNA and protein and we determined that the mechanism of this increase of IL-6 by neurotrophins was through the MAPK pathway. One could speculate that injury to the central nervous system, possibly by total body irradiation as part of a myeloablative pre-transplant regimen, could lead to the increase of the neurotrophic factors. This potential increase in neurotrophins could have paracrine effects in the bone marrow, leading to increases in IL-6. Increases in IL-6 are associated with a number of inflammatory diseases such as systemic lupus erythematosus and rheumatoid arthritis[26] and IL-6 has also been implicated in acute graft versus host disease[16] so it is possible that increased neurotrophins could lead to dysregulated hematopoiesis.

In conclusion, the data presented here have allowed for an appreciation of not only the complexity of the bone marrow microenvironment, but also the dynamic nature of the supportive cells, namely BMSC and HOB, and how chemotherapy affects other cell populations in addition to the tumor population. While many studies have been done showing BMSC are affected by chemotherapy, very few, if any studies have attempted to understand the mechanisms by which that damage is occurring to another critical component, marrow osteoblasts. Understanding the mechanisms by which chemotherapy damages the supportive cells of the bone marrow cells can help with the development of therapies that could help restore the microenvironment following aggressive chemotherapy, like the use of MSCs as a way to help "rebuild" the microenvironment. While in vitro models provide a way to study mechanisms of damage, it is critical to complete additional experiments in an *in vivo* model to further understand if the effects of chemotherapy described are also present in vivo or if redundant signaling pathways blunt these effects. Collaborations with clinicians are also vital to ensure that basic science questions are being addressed in such a way that they are translatable to a clinical setting.

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