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The impact of obesity on bone marrow hematopoietic stem cell function and acute immune response

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Abstract of the Dissertation

The impact of obesity on bone marrow hematopoietic stem cell function and acute immune response

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Obesity is associated with immune dysfunction due to impaired wound healing and response to infection. In obesity the accumulation of adiposity extends beyond the visceral depots and into the bone marrow (BM) niche where the immune system is regulated by hematopoietic stem cells (HSC). In this dissertation we evaluate how excess adiposity and changes to the bone marrow microenvironment impact hematopoiesis and HSC function. Using a mouse model of diet-induced obesity (DIO), we found that engraftment and repopulating capabilities of healthy HSCs is not hindered when they are introduced into an obese condition with excess fat following bone marrow transplantation (BMT). DIO mice exhibit increased BM engraftment of donor cells with a greater dependence on donor stem cell repopulation compared to lean mice. Long-term repopulation led to enhanced BM hematopoiesis in DIO indicated by increased B, T and myeloid cells with increased recruitment to extra-medullary visceral fat pads, suggesting that BM cells are partially fueling adipose tissue inflammation. Following acute radiation exposure DIO mice presented higher susceptibly to BM damage evidenced by greater suppression of BM lymphocytes, trabecular bone volume fraction (BV/TV) and greater numbers

of apoptotic cells compared to lean mice. This damage to the marrow is partially to blame for the greater dependence on donor cell engraftment in obesity. We also investigated the use of low intensity vibrations (LIV) as a countermeasure to the effects of obesity. LIV has been shown to suppress adiposity through its influence on BM mesenchymal stem cells. The application of LIV in an estrogen deficient model of obesity suppressed the encroachment of marrow adipose tissue (MAT) but was not effective at normalizing immune populations in the absence of estrogen. Additionally, LIV application for 7w during the development of obesity partially protected DIO mice from acute radiation exposure by preserving trabecular BV/TV and suppressing MAT. While implications of the enhanced HSC functionality in obese mice warrants further investigation, this study demonstrates that obesity induced inflammation likely drives BM HSC behavior and that subtle mechanical signals have the potential to protect the marrow niche and could possibly influence HSC activity in obesity.

Global Hypothesis: A systemic fat burden impairs the bone marrow (BM) niche altering hematopoietic stem cell (HSC) ability to engraft and repopulate following bone marrow transplantation. Mechanical signals will protect the marrow niche from excessive adiposity and damage caused by acute stress.

Hypothesis 1: The devastation of the bone marrow microenvironment as a result of systemic estrogen depletion can be protected by low intensity vibrations.

Sub-Hypothesis 1: Systemic estrogen withdrawal will favor excessive adipocyte infiltration of the bone marrow in conjunction with suppressed immune and stem cell populations. LIV will suppress MAT following OVX and maintain BM immune status.

Specific Aim 1: Characterize the BM niche following estrogen depletion and the impact of mechanical signals in the form of low intensity vibrations (LIV) on the niche. 8w old, female, C57Bl/6J mice will be ovariectomized (OVX) to induce systemic estrogen imbalance and adipose accumulation. BM will be evaluated 8w post OVX and 6w post LIV treatment. BM will be characterized for hematopoietic cellularity through flow cytometry (FACS), marrow adiposity through histological sectioning and trabecular architecture through ex vivo μ-CT.

Hypothesis 2: The impact of obesity on HSC behavior can be tested by its ability to rescue a lethally irradiated obese mouse compared to that in a lean mouse.

Sub-Hypothesis 2: HSC engraftment in obese mice will be impaired following transplantation compared to that in a lean mouse.

Specific Aim 2: To develop a model for testing the impact of obesity on hematopoietic stem cell behavior, HSC engraftment following BMT will be tested with three different doses of HSCs. 7w old, male, C57Bl/6J mice will be placed on a high fat diet (HF; 60% kcal from fat) or regular diet (RD; 10% kcal from fat) for 7w. Mice will undergo total body irradiation followed by BMT with one of three doses of HSCs from donor CD45.1 mice: low dose (LD) 500 cells, medium dose (MD) 1000 cells, high dose (HD) 4000 cells. HSC engraftment will be characterized longitudinally every 4w in peripheral blood (PB) and at 16w in BM through FACS.

Hypothesis 3: HSC lineage commitment following BMT in obese hosts will be disrupted compared to that in lean hosts. BM HSC populations suppressed by DIO will result in greater damage to BM niche following irradiation, one that can be protected by application of LIV.

Sub-Hypothesis 3.1: Healthy HSCs transplanted into an obese condition with excess fat will have biased lineage allocation favoring myelopoiesis over lymphopoiesis compared to that in a lean condition, which will positively correlate with presence of MAT and inflammation.

Specific Aim 3.1: Determine the impact of diet-induced obesity on the lineage commitment of transplanted HSCs following BMT. Concurrent with specific aim 2, RD and HF animals transplanted with the same number of HSCs will be evaluated for repopulation of immune cells in the BM following BMT through FACS. Bone marrow status will also be characterized in terms of level of adiposity and inflammatory status through histological sectioning and protein/gene analysis.

Specific Aim 3.2: Determine the baseline marrow phenotype immediately before and after BMT and the ability of LIV to protect marrow cellularity following irradiation in DIO mice. A set of DIO mice similar to that in specific aim 2 and 3.1 will be subject to LIV treatment upon initiation of the diet for 7w. Cellularity will be evaluated through FACS and histological sectioning. Immune status will be further evaluated by protein/gene analyses.

Sub-Hypothesis 3.2: Irradiation following 7w of DIO will suppress marrow hematopoietic cells to a greater degree than that seen in RD lean control animals. LIV will protect the marrow phenotype of DIO mice and therefore protect hematopoietic cells following irradiation to levels similar to that of RD animals.

Table of Contents

List of Figures

hoc indicated significance if $<0.05. one-way ANOVA with tukey post hoc indicated significance if #p<0.05 compared to RD-IR.

List of Abbreviations

ANOVA: analysis of variance

B220: B cell marker

BAT: brown adipose tissue

BM: bone marrow

BMI: body mass index

BMT: bone marrow transplant

BV/TV: bone volume/total volume, bone volume fraction

CD4: helper T cell marker

CD8: cytotoxic T cell marker

CD45.1: congenic marker found on all leukocytes from CD45.1 mouse

c-kit: CD117, hematopoietic stem and progenitor cell marker

cMAT: constitutive marrow adipose tissue

DIO: diet induced obesity

FACS: fluorescence activated cell sorting

Gr-1: myeloid, granulocyte marker

HD: high dose

HF: high fat diet (60% kcal from fat)

HF-IR: high fat - irradiated

HSC: hematopoietic stem cell

IL-1: interleukin-1

IL-6: interleukin-6

LD: low dose

LIV: low intensity vibrations

LIV-IR: low intensity vibrations - irradiated

LSK: lineage-, Sca-1+, c-kit- cells

Mac-1: myeloid, monocyte marker

MA: marrow adipocytes

MAT: bone marrow adipose tissue

MD: medium dose

MSC: mesenchymal stem cell

OVX: ovariectomy, a surgical procedure for removal of ovaries

PB: peripheral blood

RD: regular diet (10%kcal from fat)

RD-IR: regular diet – irradiated

rMAT: regulated marrow adipose tissue

Sca-1: stem cells antigen 1 marker for HSCs

Tb.N: trabecular number

Tb.Sp: trabecular spacing

Tb. Th: trabecular thickness

TNF-α: tumor necrosis factor- alpha

WA: white adipocytes

WAT: white adipose tissue

μ-CT: micro-computed tomography

Dedication Page

This dissertation is dedicated to my mother Uma Raman and husband Kevin Maloney. For staying on the phone with me for hours calming me down during my QE to the constant supply of food especially during the last few weeks leading up to my defense; you two have consistently been my rocks and I hope I make you proud. Love you both!

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Two of the biggest lessons I learned during my graduate studies were 1: you can almost always count on your hypotheses to be incorrect and 2: it is neither possible nor fun to conduct scientific research without the help and collaboration of a team of people. Admittedly, I came into the Ph.D. program at Stony Brook with a slight air of cockiness. I had just graduated from a very difficult undergraduate program and had years of summer research internships under my belt. However, I was quickly put in my place as I embarked upon the most intellectually difficult chapter of my life. In the past 5.5 years I have encountered so many amazing people in both my professional and personal life, without which I would certainly not have completed my Ph.D. Therefore, I would like to take this opportunity to acknowledge them.

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Thank you again to everyone and I sincerely apologize if I forgot to include anyone.

Chapter 1 Introduction

The Obesity Epidemic

Obesity rates are rising worldwide, especially in the United States where more than one-third (78.6 million) of adults and 17% of children are considered obese [3]. This overall prevalence is only projected to increase, reaching 42% by 2030 [4, 5]. An increase in the morbidity and mortality associated with being obese whether it is a result of nutrition (i.e. high caloric intact) or exacerbated by hormonal imbalances (i.e. estrogen deficiency in menopause) is of major clinical concern. Obesity is associated with a myriad of comorbidities including type 2 diabetes [6], heart disease [7], stroke [8], metabolic syndrome [6], cancer [5] and increased susceptibility to infectious disease [9]. Just in 2008, these cumulatively resulted in obesity related medical spending of approximately $147 billion in the United States [10]. Consequently, doctors are faced with new challenges in treatment, diagnosis and prognosis as we continue to learn more about the epidemiology of obesity. Extensive research in the last few years has clearly evidenced that obesity is a condition of chronic and mild inflammation. This inflammation has also been attributed to insulin resistance, the basis for the development of diabetes [11]. The mechanism of obesity-induced inflammation is thought to originate in the adipose tissue, which accumulate and hypertrophy with higher energy intake and lack of exercise [1, 11].

The lack of effective treatments for obese patients is also exacerbating the problem. Diet and exercise are often the first avenue of treatment, however, it is not an option for many patients especially those who are elderly or bedridden. For people who are capable of implementing these lifestyle changes often lack compliance, hindering any beneficial effects that might occur [12]. Pharmacologic interventions have been mostly ineffective as there are still difficulties translating pre-clinical research to pharmacy and bedside [13]. Bariatric surgery is currently the most effective treatment for obesity, however it is associated with a myriad of post-surgical complications [14]. Altogether, there is still a need for not only a better understanding of the disease but also more effective treatments for curbing the epidemic from progressing any further.

Adipose Tissue

Adipose tissue exists in different forms, the most well studied being white and brown. White adipocytes (WA) found in white adipose tissue (WAT) are generally known for their energy storing capabilities in the form of triglycerides. Brown adipocytes (BA) found in brown adipose tissue (BAT) essentially have the opposite function: thermogenesis or the use of energy to produce heat. Brown adipocytes store triglycerides in small vacuoles with multiple large mitochondria occupying most of the cell space [15]. Structurally white adipocytes are circular in shape and consist of one lipid droplet that takes up almost 90% of the cell space with a variable number of mitochondria. While BAT is found in limited quantity in adult humans, WAT is distributed throughout the body, especially within the abdomen. Therefore, white adipocytes are heavily studied in the setting of obesity. Their expansion in visceral depots is correlated to a higher risk of diabetes and metabolic syndrome [16]. The notion of white adipose tissue has exponentially grown over the past several decades, and is now also recognized as a metabolically active endocrine organ.

The endocrine function of WAT was first established by the discovery of leptin, a hormone or adipokine mainly secreted by white adipocytes [17]. Leptin is primarily involved in the regulation of food intake. However, leptin can also influence numerous endocrine and physiological functions such as energy homeostasis, thermogenesis, reproduction, hematopoiesis and skeletal growth [18-23]. The development of leptin deficient (ob/ob) and leptin receptor deficient (db/db) mouse models has also helped establish the strong relationship between leptin, obesity and type 2 diabetes [18]. For some time, leptin treatment was even considered as a potential treatment for obesity [24]. That idea was soon disregarded after discovering that obese patients rather than lacking leptin actually develop a resistance to it. Since the discovery of leptin, more than 50 other distinct adipokines have been documented. The most notable in the study of obesity include: cytokines, TNFα, IL-6; growth factors, TGFβ and proteins of glucose homeostasis, adiponectin [16]. In addition to the secretory abilities, WA are capable of communicating with other cells and tissue systems either directly or through paracrine signaling. In fact, co-cultures of adipocytes with skeletal muscle showed direct signaling between the cell types [25]. In addition to adipocytes, WAT also consists of an array of immune cells. These

resident immune cells include M2 macrophages, eosinophils, invariant natural killer T cells, innate lymphoid cells, T-helper type 2 cells and regulatory T cells [26]. Lean adipose tissue balance is maintained by the collective action of all these cells.

In obesity, WAT accumulation is a result of both an increase in number and size of adipocytes. This expansion causes cellular stress, which is thought to arise from hypoxia and oxidative stress. As a result, adipocytes secrete increasing levels of adipokines that promote inflammation, including leptin, monocyte chemotactic protein 1 (MCP-1), IL-6 and tumor necrosis factor-α (TNFα). Leptin is shown to act on T cells by promoting type 1 helper T cells differentiation and preventing that of type 2. MCP-1, IL-6 and TNFα recruit and activate monocytes and M1 macrophages, which engulf dying and dead adipocytes [27]. Lipolysis, or the release of free fatty acids is also present in obese adipose tissue and can activate a pro-inflammatory response by resident and recruited immune cells [26]. The collective response of these immune cells in obesity results in local (in visceral WAT) as well as systemic chronic inflammation and complications like insulin resistance (Fig 1).

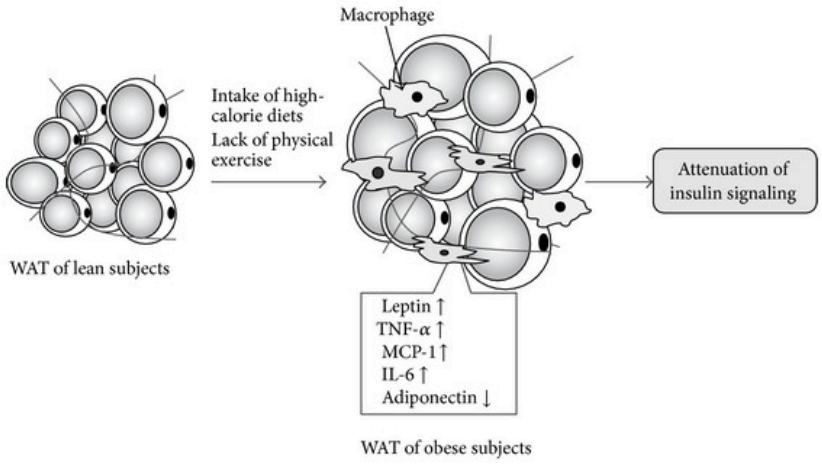


Fig 1: Schematic of adipose tissue in lean and obese subjects. Obese subjects have larger and more adipocytes, infiltration of macrophages and increased secretion of pro-inflammatory factors that can induce insulin resistance. Adapted from Sakurai et al. [1]

Marrow Adipocyte Tissue (MAT)

Anatomically distinct adipose depots differ in their impact on and progression of the comorbidities associated with excess adiposity as seen in conditions such as menopause or

obesity [5, 28, 29]. A less understood type of adipose tissue is found in bone and termed as marrow adipocyte tissue (MAT). Marrow adipocytes (MA) are present in physiological states and have been largely understudied due to their unique location making access and analysis difficult. MAT can be found in the long bones, vertebra and the phalanges and approximately occupies 70% of adult bone marrow [30]. MAT is distinct from other adipose tissue due to their response to stress, diet, physiological roles, gene expression and origin. Lineage tracing experiments have demonstrated that MA is derived from osterix-positive mesenchymal progenitors, and thus more closely related to osteoblasts and chondrocytes [31]. However, these adipocytes do share some traits with that of WA and BA. Like WA, MA can store energy in the form of triglycerides in single lipid droplets [32]. However, their gene expression profile often shows similarities with that of both WA and BA [33]. Interestingly, while WAT decreases with starvation, MAT increases [34], however in conditions such as obesity or age, WAT and MAT both increase. These and other contradictory and unique relationships continue to puzzle scientists today and have paved the way for a lot of new research efforts.

More recently, it has become well accepted that MAT itself exists in two distinct forms which can be identified by their anatomical position. “Constitutive MAT”, (cMAT), is primarily found in the distal tibia and tail in rodents and develops at birth. “Regulated MAT”, (rMAT), naturally accumulates with age in proximal tibia, femora and vertebrae. It has been shown that rMAT can be regulated by external factors such as diet, drugs, age, endocrine and metabolic factors [35] [34] [36] [37], while cMAT does not exhibit the same phenotype. Due to the close proximity of MAT to bone as well as other cells within the bone marrow (i.e. hematopoietic cells), several researchers have investigated their relationship to the bone and hematopoietic systems. rMAT is often, however not always, associated with low bone mass, classically seen in osteoporosis. However, due to contradictory reports, the relationship between bone and MAT is still being studied. The relationship of MAT to hematopoiesis is more poorly understood. cMAT has been shown to negatively impact hematopoiesis and HSC behavior [29], however the impacts of rMAT or even MAT that arises due to pathology on hematopoiesis and HSCs is still unknown.

In aging, it is understood that there is a natural increase in marrow adipogenesis as seen in animals and humans. In fact, in humans MAT is roughly proportional to age where, a 30-year-old would have 30% MAT and 70-year-old would have 70% MAT[38]. While this conversion of the marrow phenotype is a natural phenomenon, increased marrow adiposity is observed in several pathological conditions such as osteoporosis, starvation and even obesity. These can also be paralleled by changes to the surrounding bone marrow cells, such as the impaired functionality of stem cells associated with aging. As we are starting to understand the endocrine like function of adipocytes, their role in the pathogenesis of disease is of interest to explore.

Like WAT, MAT is also a secretory tissue and has been shown in *in vitro* studies that they have the ability to secrete adipokines including leptin, adiponectin and IL-6 [22, 39, 40]. Due it its metabolic activity and the fact that it shares its niche with so many important cells, including hematopoietic cells, osteoblasts, mesenchymal stem cells and endothelial cells, relationship with and influence of MAT with these other systems are important to fully investigate. Additionally, disruptions to the bone marrow niche such as the accumulation of excess MAT, presence of adipokines and pro-inflammatory markers and significant bone loss might play important roles in the development of diseases like obesity.

Bone Marrow

The bone marrow is a unique environment that modulates both the bone and blood systems. It provides cellular niches that supports the proliferation, self-renewal and maintenance of stem cells specifically hematopoietic and mesenchymal stem cells. Distinct niches have been identified that support various functions within the bone marrow. Cell-cell, paracrine and autocrine interactions within the niches control the fate selection of the stem cells. The osteoblastic niche is found in the endosteal region and is composed of osteoblasts, reticular cells, fibroblasts, adipocytes and other stromal cells of mesenchymal origin. Together they support quiescent and undifferentiated hematopoietic stem cells (HSCs)[41]. Bone forming osteoblasts secrete several hematopoietic cytokines, including granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), interleukin-6 (IL-6), interleukin-1 (IL-1), interleukin-7 (IL-7), CXC-chemokine

ligand-12 (CXCL12), Notch ligands, WNT ligands, thrombopoietin and angiopoietin 1[42]. Alterations in the expression of these factors as well as the number of osteoblasts have been shown to correspond with changes in the HSC pool[42, 43]. Changes to the HSC pools can therefore also impact immune systems in physiological and pathological states.

The central region consists of endothelial cells, forming sinusoids making the vascular niche. This niche promotes the recruitment of stem cells for proliferation, differentiation and migration through the sinusoidal wall. Endothelial cells express cell-surface markers that allow migration of HSCs and their progeny, the immune cells, through the vasculature between the bone marrow and extra-medullary sites[44]. The factors secreted by endothelial cells that influence hematopoiesis include G-CSF, GM-CSF, M-CSF, stem cell factor (SCF), IL-6 and FMS-related tyrosine kinase 3 ligand (FLT3L)[45]. Cell homing is also facilitated through molecules secreted by endothelial cells including E-selectin and CXC-chemokine ligand-12 (CXCL12)[44, 45]. Additionally, the perivascular cells within the bone marrow have also shown to regulate HSC function as they too express chemokines such as CXCL12 among others[41]. The influence of these factors as well as the close approximation of these cells to HSCs supports the idea that the vasculature is important to maintain the functional capacity of HSCs in the marrow.

While the complexity of the marrow microenvironment cannot be described in complete detail in this introduction, the evidence of multiple cellular niches and types of cells as well as their influence on stem cells is important for understanding how disease states such as those associated with obesity might disrupt the niche and its components. As described above, the increased formation of marrow adipocytes in obesity inherently disrupts the bone marrow niche. It is still not understood whether the adipocytes have direct influence on HSCs, whether other niche components influence the formation of adipocytes or whether extra-medullary factors influence the disruption of the niche. Therefore, conclusions regarding the bone marrow must consider the influence and contribution by the multitude of marrow components especially how each might be altered in diseases states.

Hematopoietic Stem Cells (HSCs)

Hematopoietic stem cells, primarily found within the bone marrow, are the ultimate population that gives rise to the immune system by the production of all the blood cells that maintain it. Blood cells consist of red blood cells and platelets that carry oxygen and help prevent bleeding, respectively, and white blood cells that maintain innate and adaptive immunity. As pictured in Fig 1, white blood cells are primarily subdivided into those of the myeloid lineage (granulocytes, monocytes etc.) and lymphoid lineage (T and B lymphocytes). Generally, B-lymphocytes produce antibodies and T lymphocytes recognize and kill foreign cells to collectively make up adaptive immunity, while myeloid cells are involved in fighting infections, bacteria and other parasites to comprise innate immunity. The average human requires one hundred billion new hematopoietic cells each day [2]. This is directly dependent on the presence and function of HSCs. Interestingly, however HSCs in the bone marrow are quite rare, comprising only about 0.001% of all cells within the marrow [2, 46]. Today, HSCs and their progeny can be detected quite easily with fluorescence activated cell sorting (FACS) through each cell population's unique cell surface markers. This technique has vastly advanced the study of HSCs and hematopoiesis (i.e. process of blood cell production) as we are able to purify and identify even the rare primitive HSCs from large mixed cell populations. The idea of the HSC and its role in immune cell generation was originally demonstrated through irradiation and bone marrow transplant (BMT) studies in the 1960's [47]. Even today, the assays that truly define HSCs are reconstitution assays where irradiated mice are injected with distinguishable HSCs or mixed populations of HSCs which repopulate the hematopoietic system of the mice [2, 46, 48, 49]. We know that hematopoiesis originates within the bone marrow; consequently, the status of the bone marrow, especially disturbances to the marrow caused by adipose burdens, can ultimately influence HSCs and immune function.

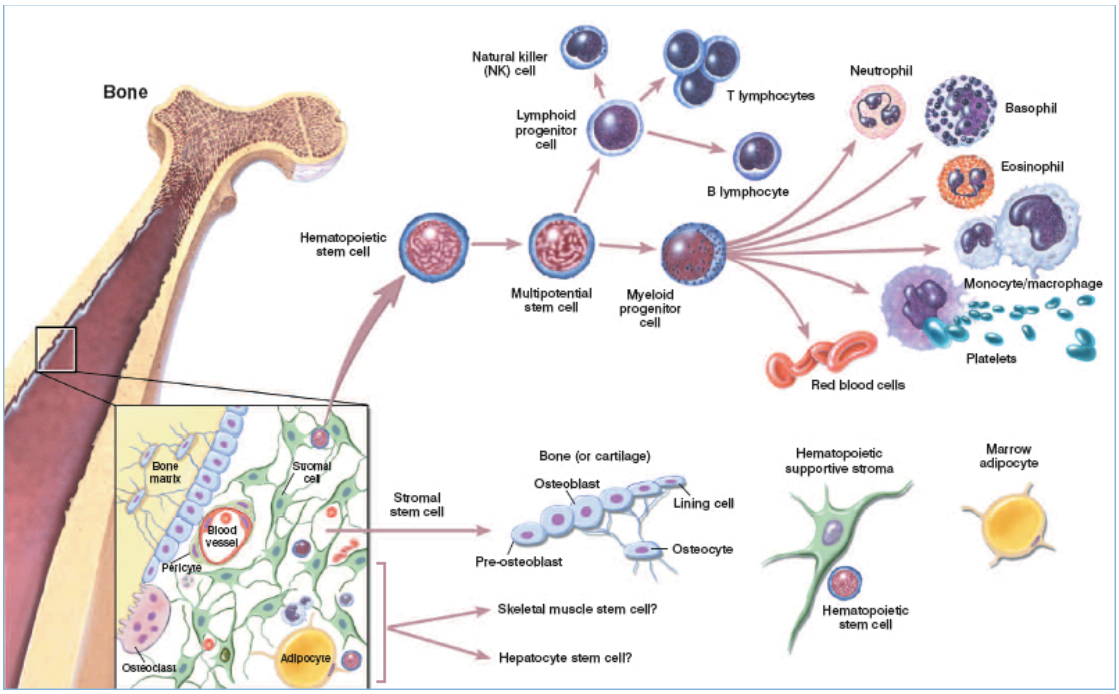


Figure 1.2: Hematopoietic and mesenchymal stem cell differentiation. Adapted from Doman et al. [2]

Diet Induced Obesity (DIO)

Primary causes for obesity include overconsumption and a sedentary lifestyle. The Centers for Disease Control and Prevention define obesity as having a body mass index (BMI) greater than 30, which is mainly a result of a high amount of body fat. With the lack of appropriate and effective treatments for obesity, it is important that models used to study the disease either *in vitro* or *in vivo* are a good representation of the human condition. Choosing the appropriate research model is dependent on the specific hypotheses and is critical for making conclusions about treatments and or processes that will have an impact in clinical practice. Common models for studying obesity and obesity related disorders (i.e. diabetes) include the ob/ob mouse, db/db mouse, the Zucker rat and the diet-induced obesity mouse model [13]. The ob/ob, db/db and Zucker mouse and rat models are a result of mutations in specific genes related to adipose tissue such as leptin, leptin receptors or the fatty gene, respectively. These models are helpful for eliciting severe and targeted phenotypes that are useful especially for testing pharmacological treatments where specific mechanisms and pathways are of interest. The DIO model is a closer representation of the human pathogenesis of obesity, as the mouse models result from a higher gradual intake of calories, affecting multiple systems in the process. The

C57BL/6J mouse model is the most commonly used mouse strain for DIO, as it is obesity-prone, resulting in increased adiposity, hyperinsulinemia, and hyperglycemia [13, 50]. The diet by which obesity is induced is also important to consider. A diet high in fat and carbohydrates, where the fat source is from lard, has been shown to closely mimic the human diet [51]. The 45% and 60% kcal from fat diets from the "Van Heek" series from Research Diet, are commonly used to induce obesity. The differences between the 45% and 60% kcal diet is the lard content. Both diets induce obesity however, to differing degrees, where the 60% is more severe and induces obesity in much shorter times than when using the 45% kcal diet. Pharmacological treatments have shown that both diets result in similar responses to treatment as seen in the human condition [52, 53], further validating the diet and use of DIO for studying obesity.

Ovariectomy (OVX)

Ovariectomy is the surgical procedure by which the ovaries are removed. Ovariectomy in rodents is a common research model to study the effects of menopause and post menopause in women as it mimics the loss of ovarian estrogen production that causes women to become menopausal. The ovariectomized mouse model has been highly studied especially in research pertaining to post-menopausal osteoporosis. The high prevalence of post-menopausal osteoporosis is shown by recent data published in the Journal of Bone and Mineral Research indicating that in the U.S. 10.2 million adults have osteoporosis [54], majority of whom are women past menopause, who lose almost 20% of their bone density within the first five to seven years following the decline in estrogen levels [55, 56]. This osteoporotic bone phenotype as seen in the OVX rat and mouse, results in a rapid loss of trabecular bone due to imbalances in bone remodeling, favoring increased bone resorption over formation [57]. In addition to bone loss, the loss of estrogen production, elicits an increase in body weight, making women more susceptible to becoming obese. The prevalence of obesity in women increases as they approach menopause, specifically in American women after the age of 40. The prevalence reaches 65% between the ages of 40-59 and 73.8% in women over the age of 60 [58]. OVX mice display similar increases in body weight, including increased abdominal adiposity as in menopausal women [59]. Studies have also shown that estrogen plays a role in fat metabolism and adipose tissue behavior [58],

however much is still be learned on the development of adiposity and obesity during menopause, emphasizing the need to use OVX models for research.

Exercise Benefits

Exercise is in general attributed to the betterment and maintenance of physiological function [60]. It's influence on weight loss and suppression of fat; especially make it a common treatment modality for obese patients [61]. Epidemiological studies have shown that exercise can prevent and improve obesity and related complications such as diabetes. A study by Helmrich et al. showed that among 5,990 male students, the risk of developing diabetes is reduced by 6% for every 500 kcal increase in weekly exercise over a period of 14 years (REF). Exercise also protects from cardiovascular disease, reduces blood pressure and bad cholesterol, improves insulin sensitivity [62], and protects from non-alcoholic fatty liver disease [63]. In obesity WAT is considered one of the critical tissues leading to the pathogenesis of obesity related diseases. Due to its increased secretion of adipokines and the immune dysregulation that is initiated, one of the major preventative strategies for obesity is the reduction of WAT and secretion of abnormal levels of pro-inflammatory cytokines.

Exercise is thought to break down triglycerides in adipocytes through the secretion of catecholamines from the adrenal medulla and sympathetic nerves [64]. This is likely also a reason why exercise can normalize the secretion of adipokines in obese rats as shown by decreased leptin mRNA and increased adiponectin in visceral adipocytes following treadmill running [65](REF). Other mechanisms have also been proposed in addition to the secretion of catecholamines, including the suppression of oxidative stress and improvement of hypoxia in WAT following exercise. Studies have shown lower levels of lipid peroxidation in rat WAT and increased expression of antioxidant enzymes (i.e. manganese superoxide dismutase) following exercise which has been correlated with decreased WAT mass and levels of pro-inflammatory adipokines [1, 66, 67]. Additionally, increased blood flow and thus oxygen to WAT that follows exercise is thought to positively influence the inflammatory changes within WAT [68, 69].

While there has been a substantial amount of evidence investigating the influence of exercise on WAT, little has been shown with marrow adipocytes, especially those that form in

obesity. In a recent study by Styner et al, high fat fed mice presented with increased bone marrow adiposity. This adiposity was further suppressed by 6w of voluntary wheel running exercise [37]. The accumulation of adipocytes is a dynamic process, which as evidenced by Styner's data, is reversible under certain conditions. The fact that exercise can also induce the formation of trabecular bone within the marrow while suppressing MAT [70], suggests a possible influence on marrow stem cells towards a biased lineage commitment favoring that of bone and away from fat. Notwithstanding that, adipocyte progenitors might be a distinct population from those of osteoblastic progenitors, mechanical signals introduced by exercise might still impart an influence on the fate of stem cells that suppresses that formation of fat in models of obesity and diabetes.

In addition to its effects on fat, exercise has also been shown to influence the hematopoietic system. Young, healthy individuals exhibit increased hemoglobin, hematocrit, platelets, side-population cells and IL-6 levels following exercise, suggesting the potential enhancement in the mobilization of stem cells [71]. Other studies have shown in mice exposed to exercise training leads to an expansion of the HSC pool that are more activated and differentiated, while still maintaining normal function [72]. Mechanical signals in general impart positive influences on the HSC niche in the bone marrow. Indeed, the skeleton is highly responsive to mechanical signals as evidenced by the deterioration of bone in the lack of mechanical loads. And while the HSC niche is supported by bone both structurally and by cellular interactions, the influence of mechanical signals on the skeleton might also influence hematopoietic activity in both physiologic and pathologic states.

Low Intensity Vibrations (LIV)

Although mechanical signals delivered through high impact (strains >1000$\mu\varepsilon$) exercises with fewer cycles (<1000 cycles per day) such as resistance training are anabolic to the skeleton, studies show that low impact signals (strains <100$\mu\varepsilon$) delivered over many repeating cycles (>1 x 10^5) might also impart similar benefits [73]. Using this idea of high frequency and low magnitude stimuli, the work presented here investigates the use of low intensity vibrations (LIV) as a surrogate for the traditional concept of exercise. These signals, which offer strains less than 5$\mu\varepsilon$,

have been shown in both human and animal models and in physiological as well as pathological conditions to positively influence bone health through the promotion of bone formation (REF). For example, in rat models of hind-limb unloading, used to study the impacts of immobility that would arise in bed-ridden or microgravity exposed subjects who lose significant bone mass, ten minutes of exposure to LIV normalized bone formation rates to that of control levels (REF). The ability of LIV to improve fracture healing was also evidenced by its ability to heal a 3mm osteotomy in a sheep tibia (REF). Studies investigating possible mechanisms for LIV's control over the skeleton led to the hypothesis that stem cells (i.e. MSCs & HSCs) within the bone marrow microenvironment could be directly targeted by the mechanical signals, influencing their differentiation towards bone tissue. This hypothesis was further supported by findings that LIV suppresses abdominal fat formation [74-77]. Given that MSCs can give rise to osteoblasts and adipocytes, in vitro and in vivo studies demonstrated that LIV biases MSC differentiation towards osteoblastogenesis and suppress differentiation towards adipogenesis (REF). Stiffening of the cytoskeleton in mesenchymal stem cells through the activation of the mTORC2 complex prevents adipogenesis by preserving β- catenin signaling and therefore stimulating osteogenesis [78]. While there are significant studies exploring the mechanical controlling of MSCs, the influence of LIV on bone marrow HSCs is still largely undefined. One in vivo study LIV treatment in diet induced obese mice restored B lymphocyte populations in part through the biasing of HSC away from osteoclastic activity and toward lymphopoiesis [79]. Overall we see a positive influence of LIV on the bone marrow microenvironment.

Chapter 2 Bone loss and marrow adipogenesis that parallels estrogen deficiency is suppressed by low intensity vibrations

Specific Aim 1: Characterize the BM niche following estrogen depletion and the impact of mechanical signals in the form of low intensity vibrations (LIV) on the niche.

Hypothesis 1*: Systemic estrogen withdrawal will favor excessive adipocyte infiltration of the bone marrow in conjunction with suppressed immune and stem cell populations. LIV will suppress MAT following OVX and maintain BM immune status.*

Introduction

Since the early studies of Fuller Albright, estrogen has been recognized as a major systemic regulator of bone metabolism [80], with its withdrawal, as seen during menopause in women, leading to accelerated bone loss [81]. Recent research has expanded our understanding of estrogen's regulation of bone remodeling, providing the foundation for treatment strategies for post-menopausal bone loss, including estrogen replacement, anti-resorptive (i.e. bisphosphonates) or anabolic (i.e. parathyroid hormone) therapy, each intended to reestablish the balance between bone formation and resorption [82, 83]. Non-drug approaches, including exercise, are geared toward reducing the risk of fractures by preventing loss of bone, may not be feasible for all subjects and have not been sufficient to combat the severity of bone loss following menopause [83, 84].

In addition to the loss of bone, menopause is often followed by significant gains in adipose tissue, especially visceral adiposity, and a concomitant increase in inflammatory cytokines, insulin sensitivity [85-87] and other comorbidities related to obesity [88]. The exact mechanisms for the development of obesity in menopausal women are not yet fully understood. However, it is now clear that estrogen and estrogen receptors play a role in fat development and metabolism. Both subcutaneous and visceral adipocytes express estrogen receptor α (ERα) [58, 89, 90], the lack of which leads to gains in visceral adiposity, insulin resistance, glucose tolerance and an overall obese phenotype [91]. In parallel with an increase in visceral adipose depots, some studies have shown that estrogen deficiency is permissive to increased adipocyte encroachment into the bone marrow [92, 93], possibly augmenting the marrow microenvironment which could disrupt bone marrow hematopoiesis. Several studies have demonstrated effects on hematopoiesis caused by the loss of ovarian function [94-96]. These studies primarily focus on

the relationship of hematopoiesis with OVX-induced bone loss, claiming that the expansion of hematopoietic progenitors is activated to aid in the up regulation of osteoclastogenesis. Although this might be true, it is important to also consider the relationship between MAT and their relationship to bone and hematopoiesis in the absence of ovarian function. With a better understanding of their relationship MAT could also be a target for treatment of obesity related comorbidities in menopausal and post-menopausal women.

Countermeasures for post- menopausal bone loss have largely focused on pharmaceutical interventions including estrogen replacement, anti-resorptive (i.e. bisphosphonates) or anabolic (i.e. parathyroid hormone) therapy, intended to re-establish the balance between bone formation and resorption [83, 97]. Non-drug approaches, including exercise, are geared toward reducing the risk of fractures by preventing bone loss however are not feasible for all subjects, and have not been sufficient to combat the severity of bone loss following menopause [98]. Additionally, methods for preventing post-menopausal weight gain are scarce with the change in lifestyle as the primary recommended approach. With the benefit of subtle mechanical signals in suppressing adiposity in models of diet-induced obesity [99] while also promoting bone growth [100], here we investigate the use of low intensity vibrations for counteracting the effects of estrogen deficiency. Additionally, due to the influence LIV shows on bone marrow cells [100, 101], it would also be of interest to evaluate if its influence on the marrow microenvironment could also impact the hematopoietic compartment that maintains immunity.

To that end, in this first specific aim, we will characterize the bone marrow niche in terms of trabecular bone, MAT and immune cell phenotype and find relationships between these systems in an estrogen deficient model of osteoporosis and adipose burden. Additionally, we will also evaluate the impact of mechanical signals in the form of low intensity vibrations on each of these systems.

Methods

Animals: All animal procedures were reviewed and approved by the Stony Brook University's Institutional Animal Care and Use Committee. 8w old female C57BL/6J mice underwent an ovariectomy (OVX, n=20) or sham surgery (age-matched control, AC, n=10) at The Jackson

Laboratory (Bar Harbor, ME, USA). Animals were delivered to Stony Brook University 1w after surgery, where they acclimated for an additional week. The LIV intervention began 2w post OVX surgery, for a total of 6w. The ovariectomized mice were divided into two groups, ovariectomized + sham LIV (OVX, n=10) and ovariectomized + LIV treatment (LIV, n=10). Intact sham surgery animals served as the age-matched controls (AC, n=10) and also received sham LIV. Mice were single housed (to prevent experimental variability) and fed a standard rodent chow diet (LabDiet Prolab RMH 3000, Purina Mills LLC, St. Louis, MO, USA) with food and water *ad libitum* for a total study period of 6w. Facility conditions were maintained under a 12h/12h light/dark photoperiod at 21° C.

Mechanical stimulation: The LIV group was subject to vertically oscillating low intensity vibration (0.3 *g* at 90 Hz, where *g* is earth's gravitational force = 9.81 m/s^2). Mice received LIV for 15 min/d for 5-d/w for a total of 6w. Mice were placed into a partitioned container (one mouse per partition), centered on the oscillating LIV system. AC and OVX were sham vibrated with the same handling and container, but without activating the LIV device.

µCT assessment of trabecular phenotype: All mice were imaged *in vivo* pre and post LIV treatment. *In vivo* parameters of bone microarchitecture were measured before (t=0w) and after (t=6w) LIV treatment, 2w and 8w post-OVX, respectively. Animals were anesthetized (2% isoflurane) and secured in a custom made bed while scanning in the vivaCT 40 (Scanco Medical, Basserdorf, Switzerland). Transverse sections of the tibia were measured at a voxel size of *17.5* µm. Trabecular bone was assessed at the proximal metaphysis of the tibia (490µm distal to the growth plate). A well-established script[102] was used to measure bone volume fraction (BV/TV) and trabecular thickness/separation/number (Tb.Th., Tb.Sp., Tb.N.). This script calculates BV/TV of trabecular bone by identifying all voxels within the endosteal region above a globally (across samples) defined threshold that is considered bone, which is then normalized to the volume of the endosteal region within the proximal tibia metaphysis region evaluated. Trabecular architecture is evaluated by calculating trabecular number (Tb.N.), separation between trabecular struts (Tb. Sp.), thickness of trabecular struts (Tb.Th.). In brief, these 3D architectural

parameters are calculated using the sphere-fitting method, that on the basic level fits the largest possible sphere within each voxel of the object, to calculate separation and thickness.

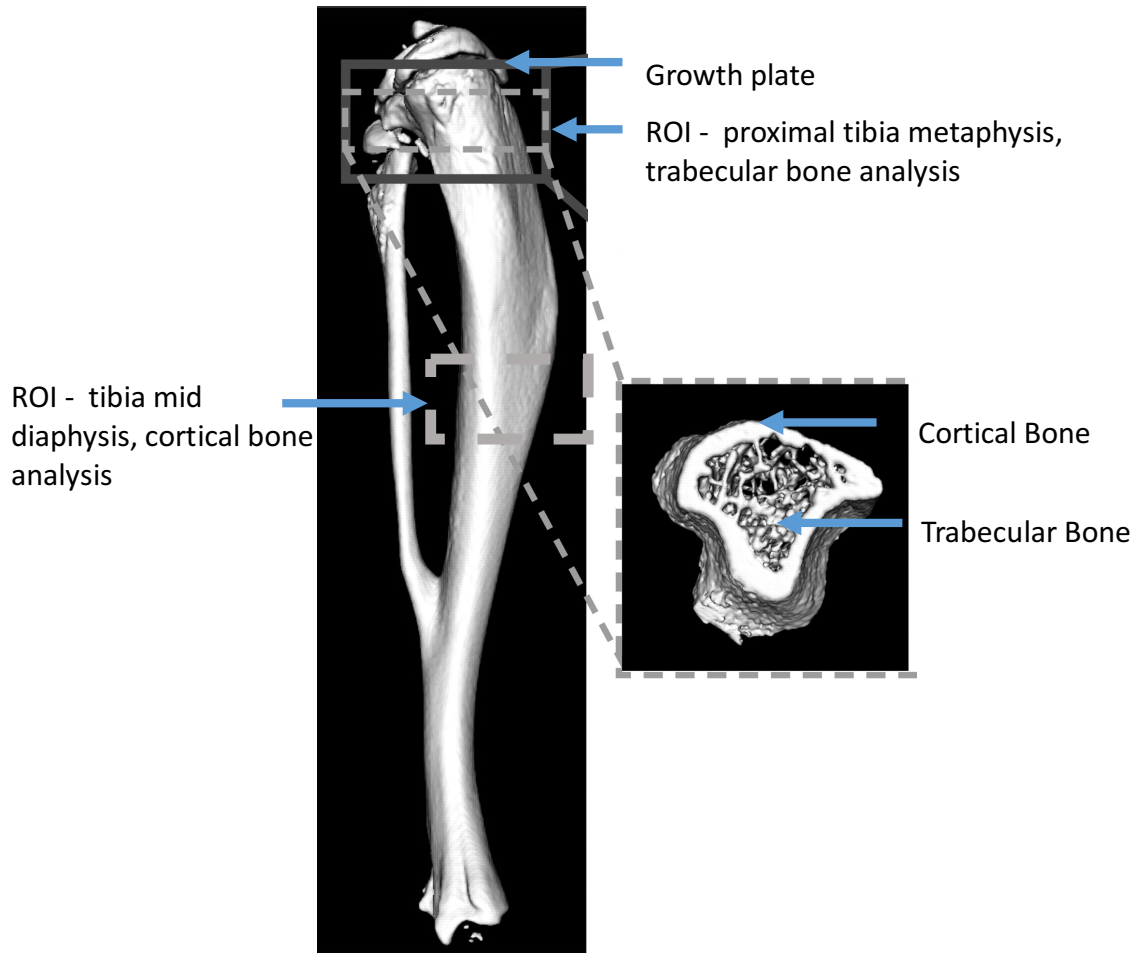


Fig 2. Schematic of micro-CT scanning regions in the tibia. Region of interest (ROI) at the proximal region was evaluated for trabecular bone analysis while the ROI at the mid diaphysis was evaluated for cortical bone and marrow cavity analysis.

Biochemical characterization of bone turnover: Serum from mice that were fasted overnight but given free access to water was collected at euthanasia for measurement of biochemical markers. Total Alkaline Phosphatase (ALP; Biovision Inc., Milpitas, CA, USA) measurements were performed using ELISA kits according to the manufacturer's recommendations. Serum tartrate-resistant acid phosphatase (TRAP5b) was measured with a TRAP ELISA kit (MouseTRAP™ Assay, Immunodiagnostic Systems LTD, Boldon, Tyne & Wear, UK). Both ELISAs were evaluated with a standard curve according to manufacturer's protocol.

Adipocyte encroachment into the bone marrow: Post-sacrifice, tissues were extracted from all animals and the right tibia fixed in 10% formalin, dehydrated in serial concentrations of 70%, 95% and 100% isopropyl alcohol and embedded in 85% poly (methyl methacrylate) (PMMA), 15% N-butyl phthalate and 2% Benzoyl Peroxide. Lateral, sagittal sections of the proximal metaphysis were cut at *8* μm with a sledge microtome (Leica, Bannockburn, IL, USA). Sections were approximately taken as close to the center of the tibia as possible as the depth into each bone was accounted for during the sectioning. Sections stained with Wright Giemsa and imaged at 10x on a fluorescent microscope (Carl Zeiss Microscope). Adipocytes were counted in the space immediately below the growth plate, within an arbitrary area that was kept consistent for each sample. Adipocytes, which appeared as adipocyte ghosts on a blue and purple background (marrow), were quantified using a semi-automated program (NIH ImageJ). All samples were blinded and analyzed by a sole researcher.

Assessment of adipocyte differentiation in the bone marrow: Immediately upon sacrifice, bone marrow from the left femur was flushed and preserved in RNA-later (Qiagen, Valencia, CA, USA) until analysis, which stabilizes and protects cellular RNA. RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and quantified with a spectrophotometer (NanoDrop; Thermo Scientific, Wilmington, DE, USA). A high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) was used to convert to cDNA. Real-time PCR (StepOnePlus, Applied Biosystems) and the TaqMan gene expression assays (Applied Biosystems) were used to evaluate gene expression levels involved adipogenesis (PPARγ)[103]. Beta-Actin was used as the housekeeping gene for normalization of all samples and the delta CT method for calculating relative expressions of the genes of interest.

Flow cytometric assessment of hematopoiesis in bone marrow: Immediately after euthanasia, bone marrow was extracted from the right femur and tibia. Samples were lysed with 1X pharmlyse (BD Biosciences, San Jose, CA) and filtered using a 40 μm filter. Cell counts were performed using an automated cell counter (Scepter, Millipore Billerica, MA). Single cell suspensions with 2 million cells each were stained for identifying hematopoietic primitive and progenitor populations, and leukocytes. Hematopoietic progenitors were identified by their

expression of Sca-1^+, c-Kit^+ and $Lineage^-$, commonly referred to as KLS cells. Among these progenitors, side population (SP-HSCs), considered the primitive HSCs, were distinguished by staining with an analog of the commonly used Hoechst 33342 stain, Vybrant DyeCycle. Leukocytes were stained in one tube per sample for single plot analysis. These included B cells based on B220 expression, T cells based on both CD4 and CD8 expression and myeloid cells based on both Mac-1 and Gr-1 expression. All antibodies were purchased from BD Biosciences.

Statistics: All data are presented as mean ± SD. Statistical significance was evaluated by performing a one-way ANOVA with Tukey post hoc tests between groups. Tests were performed using SPSS software and significance was met when $p < 0.05$.

Results

Confirmation of ovariectomy and increased body mass

Ovariectomy was confirmed at euthanasia by uterine hypoplasia. Uterine mass was -82% lower in OVX mice ($p<0.05$) compared to intact controls; AC. LIV had no impact on the uterus. Additionally, OVX mice were significantly heavier at the conclusion of the study (12.5%, $p<0.05$) compared to intact controls. Application of LIV did not impact body weight, as there were no differences between OVX and LIV. The LIV group was similarly 11.9% ($p<0.05$) heavier than AC.

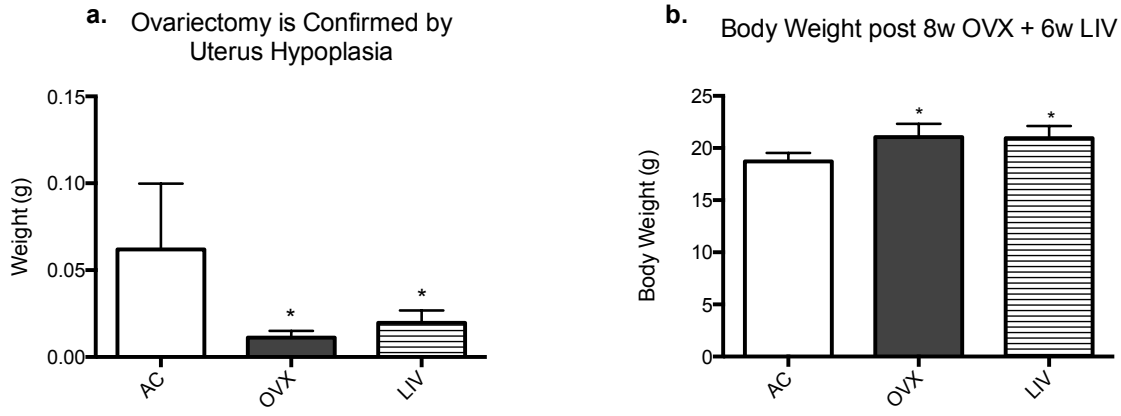


Figure 2.2. a) Uterus weights confirm successful removal of ovaries in both OVX animals compared to intact AC. b) Body mass shows both OVX and LIV were significantly heavier than AC 8w and 6w post OVX and LIV, respectively. Significant if *$p < 0.05$ compared to AC.

Ovariectomy devastates the trabecular bone phenotype

Baseline *in vivo* measurements at t=0w (i.e. 2w post OVX, prior to LIV treatment) showed a dramatic -40% ($p<0.05$) reduction in trabecular BV/TV in OVX compared to AC. Longitudinal *in vivo* μ-CT measurements between the baseline (t=0w) and end (t=6w) time-points within each group showed that animals experienced natural, age-related changes in trabecular BV/TV, with a -5%, -9% and -9% (all $p>0.05$) decrease in AC, OVX and LIV mice respectively. Trabecular thickness, spacing and number were reduced by 6% ($p<0.05$), increased by 7% ($p>0.05$) and reduced by 7% ($p<0.05$) compared to AC, respectively. No differences were observed between OVX and LIV after the application of the stimuli. Trabecular architectural parameters showed similar age-related changes to BV/TV with no changes with LIV treatment.

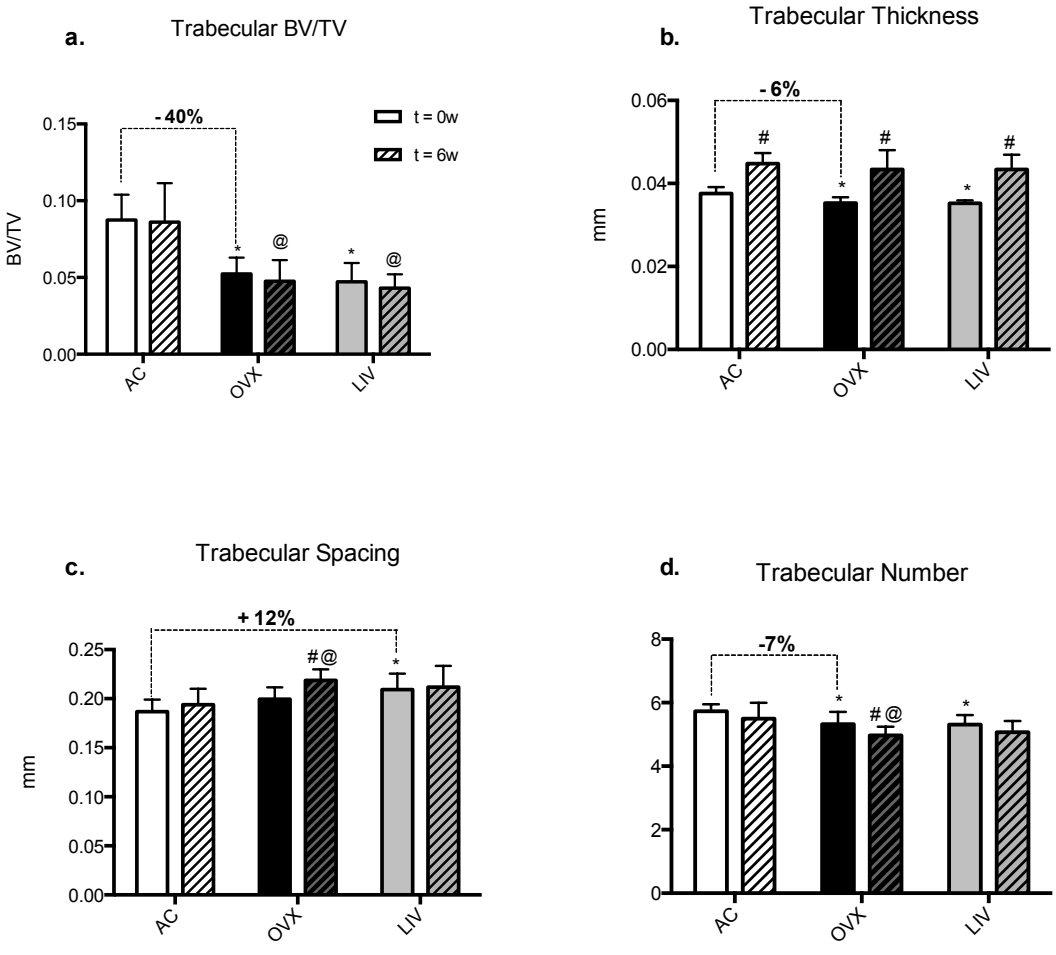


Figure 2.3. *In Vivo* μ-CT analysis showed that within two weeks of OVX, (t=0w, pre-LIV treatment), mice had a severe loss of trabecular bone with a further deterioration of trabecular architecture. At the end of the study, (8w post

OVX) 6w of LIV treatment did not have a dramatic effect on trabecular bone. Mice continued to lose bone during the 6w study, contributed to by both age related and estrogen deficiency related bone loss. *p<0.05 vs. AC groups at t=0w, @p<0.05 vs. AC groups at t=6w, #p<0.05 vs. 2w baseline control.

LIV promotes osteoblast but not osteoclast activity

Biochemical analysis of bone turnover as measured in the serum of ovariectomized animals showed deficiencies in both ALP and TRAP5b, a marker of bone formation and resorption respectively, in OVX animals compared to AC (-15%, p> 0.05 and -31%, p≤ 0.05, respectively). While TRAP5b levels were similar between LIV and OVX, circulating ALP levels of LIV animals were, +26% (p≤0.05) greater than that measured in OVX. The 8% greater value of ALP in LIV compared to AC was not significantly different.

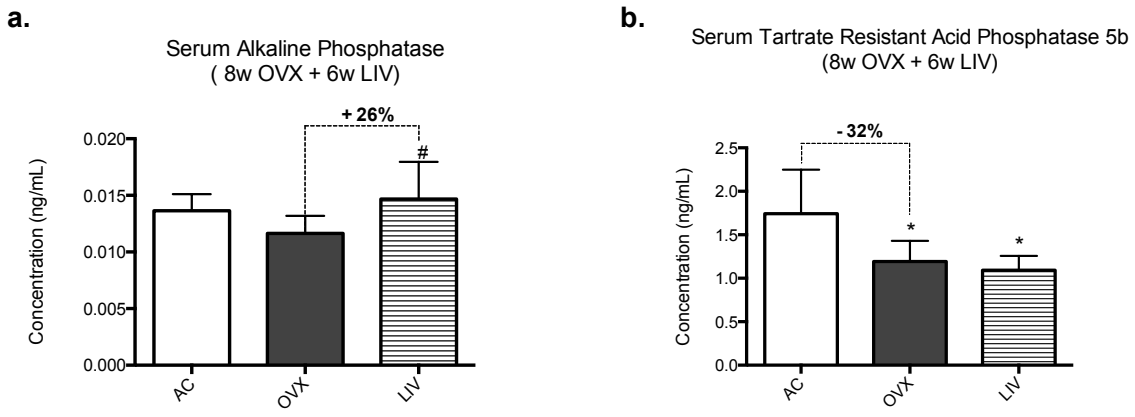


Figure 2.4. Serum Alkaline Phosphatase (ALP) was a) slightly reduced in OVX compared to AC, after 8w OVX, while LIV had a significant increase in ALP levels compared to OVX. b) Serum Tartrate resistant acid phosphatase 5b (TRAP5b) was significantly reduced in both ovariectomized groups compared to AC. LIV treatment did not have an affect on TRAP5b concentrations. *p<0.05 vs. AC groups, #p<0.05 vs. OVX

Ovariectomy leads to excessive adipocyte infiltration into the bone marrow, which is suppressed by LIV

8w following estrogen depletion total adiposity in the proximal marrow space of the tibia was +136% (p<0.05) greater in OVX compared to AC. Additionally, individual adipocyte size was +107% (p<0.001) greater in OVX over AC while number of adipocytes per marrow space showed similar trends to that of adipocyte size with +24% increase in OVX compared to AC. In contrast, adiposity in the marrow of LIV mice was -55% (p< 0.05) lower than that measured in OVX with no statistical significance to AC. Mean individual size of adipocytes in the marrow of LIV was only +39% (p>0.05) greater than control (-33%, p<0.05, smaller than OVX) and number of cells being

reduced -38% in LIV compared to OVX (p>0.05). To further evaluate adipogenesis in the marrow, gene expression of PPARγ, a transcriptional regulator of MSC adipocyte differentiation, was evaluated in a mixed cell population of bone marrow aspirates taken from the femur upon sacrifice. Using RT-PCR analysis a +28% (p>0.05) increase was found in OVX compared to AC and only a -5% (p>0.05) decrease in LIV compared to OVX.

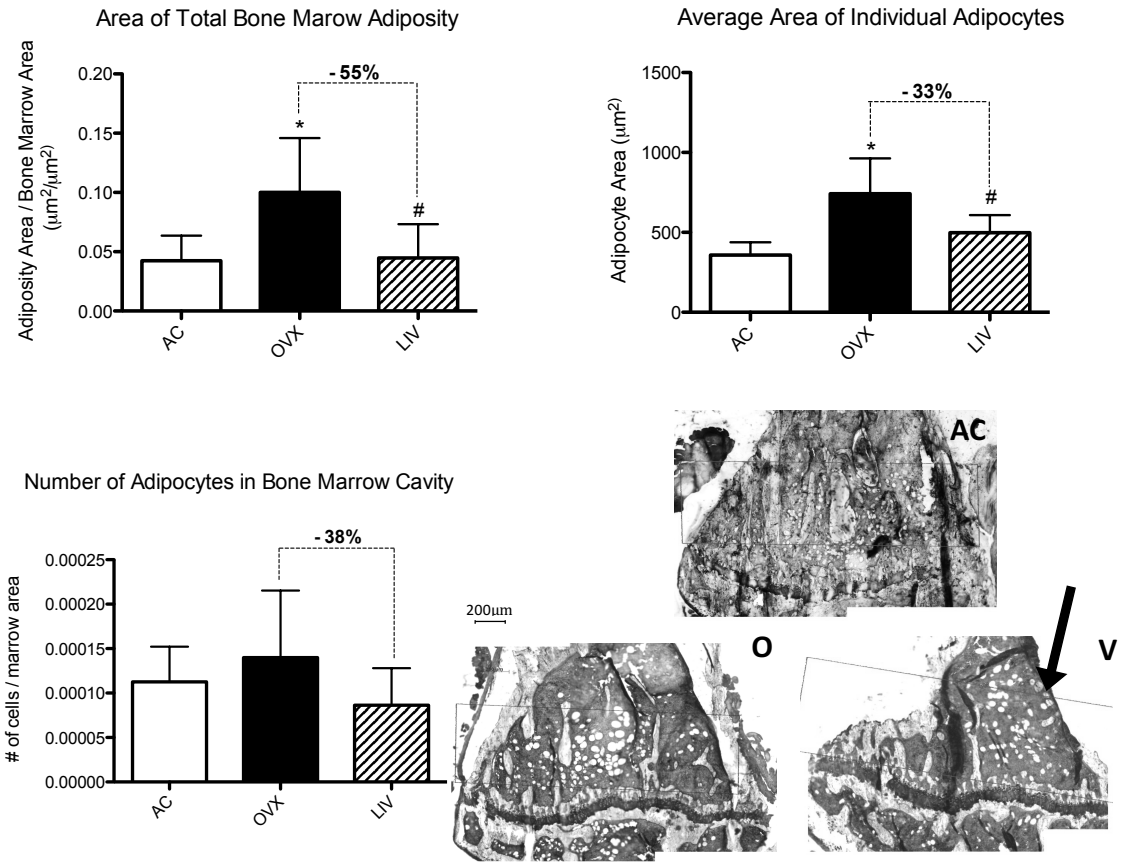


Figure 2.5. Histomorphometry and staining with wright-giemsa of the bone marrow in the tibial metaphysis showed a) significantly increased total adipocyte area and b) individual adipocyte area in OVX compared to AC after 8w OVX + 6w LIV. c) Number of adipocytes showed increasing trends in OVX compared to the AC group. LIV was able to significantly reduce total adipocyte area by reducing the size of individual adipocytes as well as trending towards normalized numbers of adipocytes in the bone marrow cavity. d) Representative proximal tibia histological sections, where the arrow points toward an adipocyte ghost. *p<0.05 vs. AC groups, #p<0.05 vs. OVX

Figure 2.6. PPARγ gene expression in bone marrow extracts from the left femur. Data presented as mean % fold change with SD. Significance was reached when *p<0.05 with a one-way ANOVA and tukey post hoc.

Ovariectomy leads to an impaired hematopoietic phenotype that is not protected by LIV

Hematopoietic cells in the bone marrow were disrupted following long-term estrogen depletion. B lymphopoiesis was increased by 35.1% and 30.7% ($p<0.05$ for both) in OVX and LIV, respectively, compared to AC. By contrast, T cells show an opposite trend, of decreasing proportion in OVX (21.9%, $p>0.05$) and LIV (52.4%, $p<0.05$), compared to AC. Myeloid cell proportions are reduced by 17% and 17.2% ($p<0.05$ for both) in OVX and LIV, respectively, compared to AC. Total leukocyte proportions, which includes B, T and myeloid cells show a slight increase of 3.3% ($p<0.05$) in OVX animals compared to AC with no differences with the application of LIV. Lastly, KLS+ HSCs, show no significant differences between groups with a slight 3% and 6% decrease in proportion in OVX and LIV, respectively, compared to AC. Primitive SP-HSCs also do not show significant differences but show a very slight increase by 10% and 6% in OVX and LIV, respectively, compared to AC.

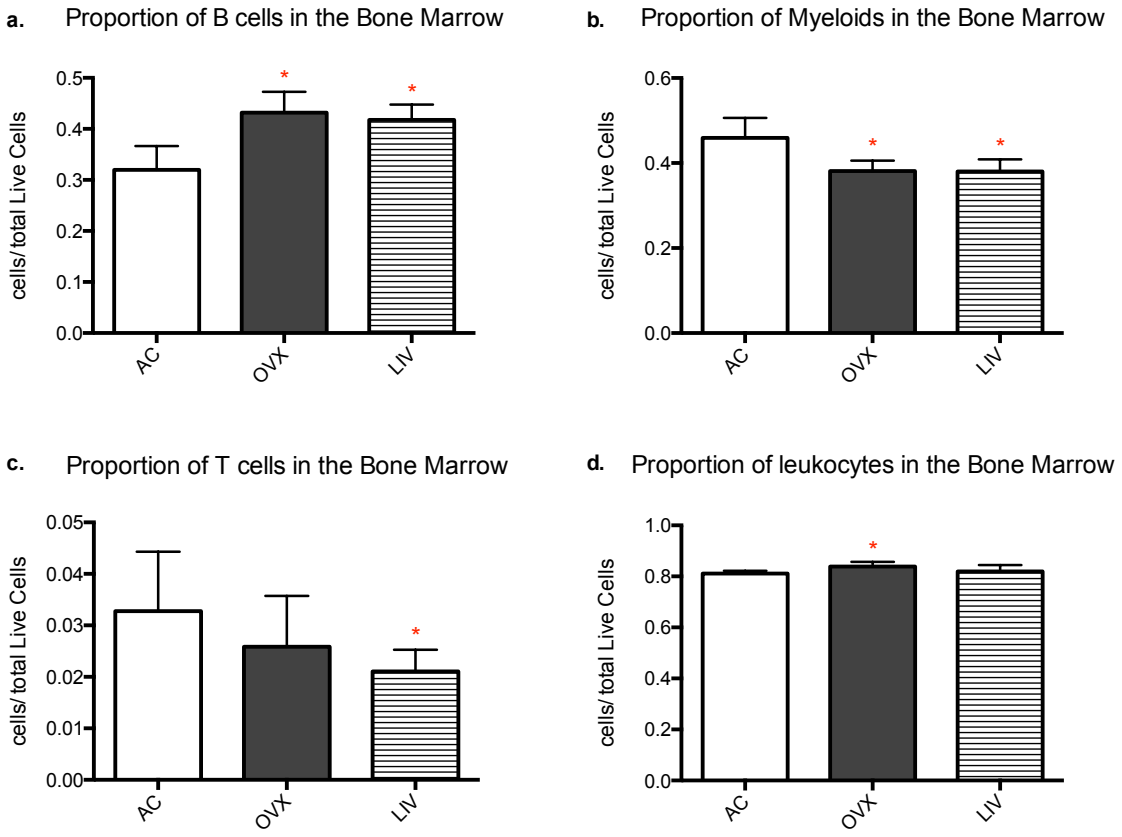


Figure 2.7. Hematopoietic phenotype in bone marrow. Proportions of cells were calculated as number of cells per total number of live cells that were sorted. a) B cell (B220+) proportions were greater in both OVX and LIV compared to AC, b) myeloid (mac-1+, gr-1+) proportions were also reduced in both OVX and LIV and c) T cell (CD4+, CD8+) proportions were significantly reduced in LIV but not OVX. d) total leukocyte populations (B & T cells + myeloids) show a very slight but significant increase in OVX compared to AC. * indicates significance when $p<0.05$ compared to AC.

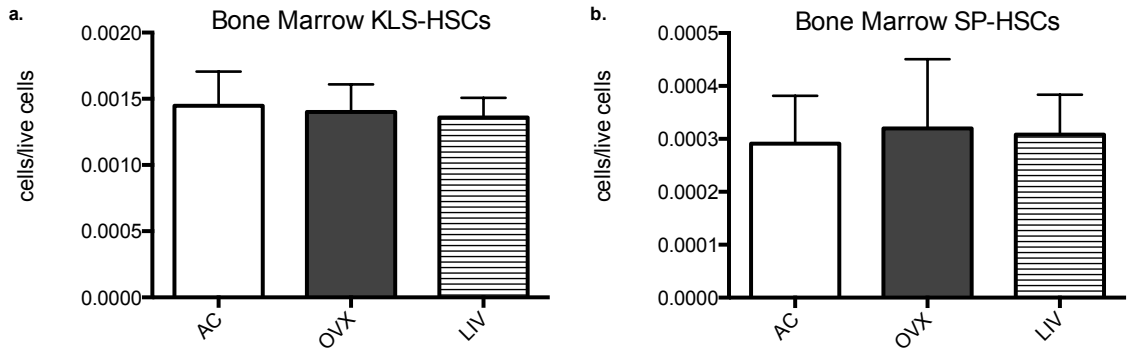


Figure 2.8. a) ckit+, sca-1+ and lineage- (KLS) progenitor cell proportion out of all live cells sorted via flow cytometry from bone marrow extracts 8w post OVX and 6w post LIV. b) Side Population (SP) stem cell proportion out of all live cells. Neither population shows significant differences between AC, OVX or LIV. Significant if *$p<0.05$.

Discussion

The bone marrow niche is supported by the close interaction of multiple compartments including bone, adipose tissue and hematopoietic cells to maintain homeostasis [104]. Estrogen depletion by ovarian removal results in a dramatic alteration of the marrow niche. We see a severe loss of trabecular bone paralleled by an increased marrow fat accumulation. This confirms our hypothesis and also supports the phenotype seen by others showing increased marrow fat often occurring concurrently with a loss of bone [77, 105, 106]. While LIV could not recover the severe bone loss; there was evidence that these subtle stimuli could mitigate adipocyte accumulation in the marrow, suggesting that LIV can protect a marrow from excessive marrow adipogenesis. Additionally, we saw an alteration of hematopoietic cells, favoring B lymphopoiesis and reductions in T lymphocytes and myeloid cells. Interestingly, hematopoietic stem and progenitor populations showed no differences following 8w of estrogen deficiency or with application of LIV; disproving our hypothesis that hematopoiesis would be altered as a result of suppressed stem cell pools and could be normalized with mechanical signals. These data might lead us to speculate that stem cell function rather than suppression of the population is contributing to the disrupted hematopoiesis. Considering the close proximity in the marrow; bone, fat and hematopoietic tissues cannot be considered distinct entities but rather considered as one system in order to understand their relationship and response to systemic stresses such as OVX, obesity or mechanical signals.

The OVX mice were significantly heavier than intact controls following 8w of estrogen removal in spite of dramatic reduction in trabecular bone as seen in the tibia, implying that the increased weight is most likely a consequence of increased fat. Unlike other studies that have shown LIV's anabolic impact on bone mass [74, 107], the fact that the majority of bone lost in the OVX mice took place before LIV treatment began, might explain the lack of response seen here after 6w of treatment. The devastation to bone was likely too severe for the subtle LIV signals to recover.

The abrupt cessation of estrogen by ovariectomy not only severely compromised trabecular bone volume but also allowed the encroachment of excess adipocytes into the

marrow. By 8w post OVX, the marrow compartment in the proximal tibial metaphysis of OVX showed a 136% ($p<0.05$) increase in total adipocyte area and a 107% ($p<0.05$) increase in size of individual adipocytes as compared to controls. That the number of adipocytes per unit area showed only an increasing trend of 24% ($p>0.05$) suggests that this encroachment is achieved through an aggregate of size and number of fat cells. Considering that homeostasis of the bone marrow is maintained by constant interaction amongst resident cells including osteoblasts, adipocytes, hematopoietic and mesenchymal stem cells [104], it is not surprising that disruptions in this balance can be detrimental to many processes and organ systems beyond bone. Relative to the skeleton, a biased commitment of mesenchymal stem cell fate selection towards adipogenesis comes at the expense of osteoblastogenesis, leading to an altered ratio of fat to bone cells [108] and contributing to the inability of bone formation to keep pace with resorption. Although the osteoblast-adipocyte system is believed to be a net closed system, it is plausible for systemic stresses (i.e. estrogen deficiency, radiation, diet induced obesity, etc.) to suppress the stem (i.e. MSC) and progenitor pool ultimately altering the marrow microenvironment. While not studied here, identifying the effects of estrogen deficiency on MSC populations could shed light to the above and corroborate the hypothesis that retaining the marrow phenotype will help protect the bone structure by maintaining the balance between the stem/progenitor pools.

Interestingly, LIV mitigated the encroachment of adipose tissue into the marrow space of OVX animals, with 55% ($p\leq0.05$) smaller total adipose area, 33% ($p\leq0.05$) smaller adipocytes and 38% ($p>0.05$) fewer adipocytes per area of marrow space compared to that of OVX. An upward trend of gene expression of PPARγ, a master regulator of adipogenesis, in the bone marrow supports the measured increase in adiposity observed in OVX marrow. However, the small differences between OVX and LIV in PPARγ expression would not seem to explain the large differences in adipose encroachment between these groups. That only the *size* of adipocytes, and not the number, was statistically significant between OVX and LIV might partially explain this finding. Additionally, biochemical analysis of osteoblast activity was interestingly up regulated with LIV, together with the adipocyte data alludes to a possible biasing of osteoblastogenesis at the expense of adipogenesis.

While LIV was able to suppress the accumulation of adipocytes in the marrow cavity, it was not able to maintain the distribution of immune cells (B cells, T cells and myeloid cells) that were disrupted by estrogen deficiency. As we have previously learned, estrogen deficiency results in a transient accelerated increase in bone turnover followed by a slower and sustained bone loss. The increase in bone turnover leads to bone loss due to enhancement of osteoclastic resorption, which exceeds that of osteoblast driven formation. Multinucleated osteoclasts are of hematopoietic origin and are primarily formed by the fusion of myeloid progenitors of monocyte/macrophage lineage [109]. The suppression of myeloid cells seen here, suggests that the initial push towards the enhanced formation of osteoclasts possibly exhausted the pool of myeloid progenitors. This also suggests the inability of the HSCs to maintain a normal myeloid cell population. Furthermore, consistent with our findings, estrogen deficiency is known to increase B lymphopoiesis [95, 110]. Interestingly, $B220^{+}$ B cells sometimes share a common progenitor with osteoclasts and thus might be acting as an additional source for osteoclastogenesis in this case [110, 111]. The effect of estrogen deficiency on T cells is however more controversial, as some claim that it elicits an increase in T cells which potentiate osteoclastogenesis by producing cytokines such as TNF-α [112, 113], while others report no affect or reductions in T cells in the marrow [95, 114]. Our results are consistent with the latter however due to these inconsistencies in the literature further analysis is needed.

Estrogen receptors are found on both bone and immune cells [115], thus absence of estrogen, can have direct impacts on these cell populations in the marrow. However, the dramatic changes to the marrow niche such as decreased trabeculae and increased adiposity can have added indirect influences on the immune cells as well. These relationships are important to investigate because these aspects of the marrow niche (i.e. bone and adiposity) can be potential targets for treating/reducing the risk of illnesses during menopause.

Our hypothesis was further disproved after evaluating hematopoietic stem cell pools in the bone marrow. The suppression of myeloid cells and T cells was not a result of suppressed stem cell pools as both hematopoietic progenitors and stem cells showed no differences between

intact and OVX animals. This might reveal a dysfunction, not in the number of stem cells but in the stem cells' ability for proper hematopoietic lineage allocation.

Evidence has shown that there is a negative correlation between MAT and BM HSCs, where regions of the marrow with greater adiposity are correlated with reduced HSCs and HSC activity [29]. While that may be true in physiological states, excess adiposity as a result of disease might have a different impact. It is well established now that adipose tissue both visceral and in the marrow secrete hormones, cytokines and fatty acids that can have profound effects on the marrow microenvironment [22, 116]. Adipocytes, especially WA, are critical for maintaining whole-body energy homeostasis through triglyceride storage. During times of energy shortage (i.e. starvation) adipocytes release free fatty acids (i.e. starvation) and secrete adipokines to maintain lipid and glucose metabolism [117]. Additionally, the lack of or redistribution of WAT, as seen in lipodystrophy, can lead to metabolic disorders [118]. Thus it is conceivable that increases in adiposity might not pose a potential danger as long as they maintaining normal functional capacity. However, the phenotypic shift of adipocytes (both WAT and MAT) in disease characterized by an expansion of cell size and secretion of abnormal levels of adipokines increases the risk for the development of several severe complications and immune dysregulation. Studies show that in obese conditions, the adipose tissue contributes to chronic inflammation [116, 119, 120]. Although the contribution of marrow adipocytes to this inflammation is much less understood, the fact that they behave in much the same way to WA in terms of their secretion (i.e. of leptin, adiponectin, TNF-α), dynamic response (i.e. in size and number) to stresses such as OVX or obesity and that they can be controlled by mechanical signals, suggests that they may directly or indirectly influence HSC behavior in the marrow.

In conclusion, we saw from this specific aim that estrogen depletion resulted in excessive adiposity in the bone marrow microenvironment and was associated with the deterioration of trabeculae and a disruption of immune cell populations. While we did not see a complete suppression of immune cells, the bone marrow did exhibit a biased B cell phenotype suppressing T cells and myeloid populations, suggesting impaired immunity in the OVX mice and possibly translating to a higher risk for illness in post-menopausal women. HSC evaluation suggested that

the impaired immunity be a result of an abnormal lineage biasing of stem cells, rather than a lack of stem cell pools. It is plausible that in the OVX mice, soluble factors such as pro-inflammatory cytokines are secreted by the high number of adipocytes to influence stem cell behavior and contribute to the dysregulation of immune cells in the marrow. Interestingly, LIV was able to suppress OVX-induced bone marrow adiposity with some evidence of osteoblast activity, indicating that even subtle mechanical signals can influence the marrow niche towards higher order tissues (i.e. bone). Earlier intervention or longer durations of LIV might result in more significant tissue level promotion of bone, in turn maintaining the marrow niche and possibly also immunity through influencing HSC lineage allocation. Thus LIV shows potential as a treatment modality for not only post-menopausal osteoporosis but also reducing risk of obesity related comorbidities that might arise due to an impaired immune system.

Limitations

Estrogen has a wide impact on a multitude of systems in the body including but not limited to the maintenance of bone, muscle, adipose tissue, immune cells and even neural systems [80, 92, 121-124]. This is one of the major limitations for addressing the main hypothesis of this proposal. We are interested in the effects of an adipose burden on the bone marrow niche, characterized by bone, fat and hematopoietic tissues, and while OVX induces a systemic adipose burden, the fact that estrogen plays such a crucial role in so many functions prevents us from making specific conclusions on just the impact of fat. Additionally, although ovariectomy is a standard model for assessing post-menopausal effects such as bone loss and fat gain, the abrupt cessation of estrogen production results in rapid and severe changes that are otherwise more gradual in humans [57] (i.e. trabecular loss following 2w of OVX). Using a model such as diet-induced obesity, where gonad function remains intact, will allow for a more controlled system. Furthermore, the absence of cytokine or protein expression profiles in the marrow is recognized as an additional limitation of this proposal. Data showing pro-inflammatory cytokines such as TNF-α and IL-6 or adipokines such as leptin or adiponectin in the marrow would strengthen the hypothesis that marrow adipocytes in OVX mice are contributing to the immune regulation through the secretion of these molecules. Lastly, although we do see bone deterioration through in vivo μ-CT 2w post OVX, we do not know the marrow phenotype at this early stage. These data

would confirm whether LIV in fact protected OVX marrow from adipocyte accumulation or rather recovered an already fatty marrow. Lastly, all flow cytometry data presented in this specific aim are presented as proportions of cells out of all bone marrow cells. Unfortunately, due to technical complications, absolute numbers were not attainable. Although proportions are the best way to assess lineage allocation; given that OVX animals did gain a significant amount of weight compared to controls, absolute numbers might be valuable especially for comparing HSCs and progenitors.

Chapter 2 Summary:

1. Ovariectomy leads to rapid and severe loss of trabecular bone that cannot be recovered by LIV.
2. Ovariectomy leads to increased marrow adipogenesis that is suppressed by LIV.
3. Ovariectomy leads to disproportionate bone marrow immune populations that are not impacted by LIV.
4. Long-term estrogen depletion and LIV does not impact hematopoietic progenitor and stem cell populations.

Chapter 3 Increased HSC engraftment in obese mice following BMT

Specific Aim 2: To develop a model for testing the impact of obesity on hematopoietic stem cell behavior.

Hypothesis 2.1: *The impact of obesity on HSC behavior can be tested by its ability to rescue a lethally irradiated obese mouse compared to that in a lean mouse.*

Hypothesis 2.2: *HSC engraftment in obese mice will be impaired following transplantation compared to that in a lean mouse.*

Introduction

It is well established that obesity is a risk factor for the development of several illnesses and complications such as cardiovascular disease, type 2 diabetes and cancer and is also responsible for high rates of morbidity and mortality worldwide. Obesity is characterized by a state of chronic inflammation that can be seen within the adipose tissue as well as the liver, pancreas, muscle and hypothalamus [119, 125] Innate as well as adaptive immune cell populations are affected by obese conditions and contribute to the pathogenesis of obesity induced inflammation. As the primary site of immune cell production, the bone marrow is also home to hematopoietic stem cells that give rise to all the blood cells that make up the immune system. Primitive HSCs remain in a dormant state for most of one's lifetime, only working to maintain the progenitor HSCs (which differentiate to mature hematopoietic cells) as needed to maintain homeostasis. Stressors to the system such as radiation or inflammation have been shown to activate the progenitor HSCs leading to their expansion, altering their activity which could possibly lead to the exhaustion of this pool in the bone marrow. For example, exposure to *Escherichia coli* infection causes the expansion of HSCs and mice infected with *Pseudomonas aeruginosa* and *Mycobacterium avium* present with defective hematopoietic stem cell activity [126-128]. In fact, bone marrow cells derived from these mice show poor engraftment upon transplantation. In addition to these acute inflammatory responses, states of chronic inflammation have also been shown to expand the progenitor HSC population, as seen in mouse models of atherosclerosis [129], a chronic inflammatory disease of the arteries, and diet induced obesity [130].

Over the past several decades, many have reported on the effects of obesity on HSC populations. Studies have shown that 20 weeks on a 60%kcal high-fat diet as well as in genetic models of obesity such as the ob/ob mouse leads to increases in HSC progenitor populations concurrently with increased leukocytosis [130, 131]. Additionally, we have seen that short term, 1 week exposure to 60% kcal of diet results in a transient reduction in primitive HSCs followed by a later increase [130]. While we see reports on the changes in apparent stem cell populations, several also report on the imbalance of leukocytes caused by obesity. Reports in literature are contradicting, some indicating suppressed B lymphopoiesis while others showing increased B cell populations [23, 132]. Reports have also indicated an activation of pro-inflammatory T cells [133] while others report that of thymic aging [134]. The exact impact on the innate and adaptive immune cells is clearly still controversial and warrants further research. Here we investigate the immune dysfunction caused by obesity through its impact on HSC function. It's possible that obesity leads to inherent alterations to the stem cells resulting in the dysregulation of immunity. However, environmental factors *in vivo* must also play a role in driving the behavior of these stem cell pools. Thus while the chronic inflammatory state in obesity might directly impact HSC by causing changes to its intracellular organelles, it is possible also that alterations in the HSC environment as a result of excess adiposity, cytokine environment or other non-hematopoietic cellular compositions may lead to indirect changes to the HSC. For example, bone marrow from healthy human donors, cultured with serum containing the hepatitis B virus, indicated, through colony forming assays, a suppression of hematopoiesis specifically through the effect of the virus on hematopoietic progenitors [135]. Similarly, Studies have shown that HSC behavior is strongly influenced by other cells within the marrow microenvironment. For instance, HSCs in close proximity to osteoblastic cells and the cytokines that they secrete are required to maintain HSC quiescence, an important stem cell property [136]. Additionally, bone marrow macrophages have been proven crucial to prevent migration of HSCs into the blood stream and for retaining the population within the marrow niche [137]. Quiescence and migration, both of which play a role in maintaining normal immune activity or even possibly in suppressing an immune response.

The primary cause of chronic inflammation in obesity stems from the excess adiposity which not only expands within visceral depots but also within crucial organs such as the bone

marrow, which is home to the hematopoietic stem cell. Additionally, since it is well recognized that adipocytes both, WA and MA, have endocrine like function [138] it becomes rather important to understand how obesity induced changes to the HCS niche, such as excess MAT, are related to HSC function and therefore immunity. To study this, in the following experiment we will use *in vivo* reconstitution assays, as they are the "golden standard" method for evaluating HSC function and behavior [49, 139]. For this method, normal mice are first lethally irradiated to ablate their immune system. The animals are then rescued by bone marrow transplant with specific numbers of stem cells that must engraft into the host and repopulate all the blood cells lost due to radiation to rebuild the body's immune system. The ability of the stem cell to first engraft and then contribute to all the lineages required can then tell you about the behavior and functionality of the stem cell.

While this method is primarily used to test stem cells from experimental mice in their capacity to repopulate a normal irradiated system, we are interested in how healthy stem cells respond in an experimental condition (i.e. diet induced obesity) compared to that in a control system. Because this method requires the use of bone marrow transplantation with lethal irradiation, we must also consider if the diet induced obesity condition, which is the primary response of interest will be overshadowed by the severity of the irradiation and the response of the system to it. Because the nature of this assay is dependent on the number of stem cells you introduce into the system, it is first important to create an appropriate model that would allow us to primarily test the impacts of the obesity condition. Our experimental design will therefore test three different doses of HSC transplants in obese versus lean mouse models to find if there is an optimal dose at which we are able to clearly see differences in the obese and lean conditions. The primary endpoint for finding the optimal model will be the percent of engraftment of donor HSCs, as this is the first response of the stem cell before it is able to repopulate the system. There has been some evidence in literature that suggests a negative relationship between adiposity and HSC engraftment. Naverias et al. showed, in mice genetically engineered to lack the ability to produce adipocytes (Azip mice), that a lack of adipocytes enhances HSC engraftment when compared to mice with physiological levels of "healthy" fat in the system (both systemically and in the bone marrow) [29]. Thus based on these findings and

those that suggest chronic inflammation to impair HSCs, we hypothesize that healthy HSCs introduced into obese animals following irradiation will have impaired engraftment compared to that in a lean irradiated mouse.

Experimental Design

In this specific aim, we will be using a model of diet-induced obesity in male mice to establish a systemic adipose burden. Although, an ovariectomized mouse model was used in SA1, we felt that the hormonal influence of estrogen added complexity to interpreting the data. Similarities in the obese phenotype of male DIO and OVX mice allows for the extrapolation of data between the two models to a certain extent. Male mice in general have a greater propensity to develop obesity than female mice of the same strain [59]. Ovarian hormone, estrogen, exhibits a protective effect against obesity in females, one that can be reversed with ovariectomy [59]. Stubbins *et al.* showed that OVX and DIO male mice both tend to have a higher accumulation of abdominal adiposity, which is also contributed to by increases in adipocyte size. When they are both exposed to a high-fat diet, both male and OVX mice exhibit increased marrow adiposity,[132] increased leptin mRNA and higher incidence of glucose intolerance [59]. Additionally, marrow phenotype in both OVX and male mice exposed to high fat diet, exhibit suppressed trabecular bone mass concurrently with increased marrow adiposity [79, 140]. Estrogen however influences an array of systems in the body [94, 121, 124] making it difficult to find relationships between adiposity and hematopoietic cells without considering the direct and or indirect effects of estrogen on these systems. In order to remove this extra variable, the animal model was changed to a diet-induced obesity model.

In brief, DIO was established in young male C57BL/6 mice with a 60%kcal high fat (HF) diet while lean controls were fed a 10%kcal regular diet (RD) for a total of 7w. At this point, animals were exposed to lethal irradiation and 24h following, BMT were given with one of three different doses of HSCs from donor (Cd45.1) mice combined with accessory cells from donor CD45.2 mice (same as recipient). Following BMT, animals continued with either their respective RD or HF diets. Engraftment of the donor CD45.1 cells was evaluated in peripheral blood longitudinally every 4w for 16w. Bone marrow engraftment was evaluated at 16w. (Fig 2.1)

Three doses of stem cells were tested: a low dose of 500, medium dose of 1000 and a high dose of 4000 Lineage- Sca1+ c-Kit+ (LSK) cells. The doses were determined from studies that used competitive repopulation assays. However, doses were also adjusted to account for the fact that the HSC population did not compete with another set of bone marrow in this experiment. A recent study by Naveiras *et al* injected 100 HSCs competitively in "fatless" AZIP mice for testing the functionality of HSCs derived from anatomically different regions of the mouse vertebrae.[29] We felt that 100 HSCs would not be sufficient to induce stem cell engraftment, especially because we did not sort primitive HSCs, but rather a more diverse population that included several progenitors (i.e. LSK+ cells), where only about one tenth of the population would have been HSCs [141]. The high dose was based on a study by Singer *et al* who injected 4000 LSK+ cells from DIO animals to test the behavior of DIO HSCs compared to those derived from lean mice [130]. Thus, after careful consideration of the literature these doses were tested to provide an appropriate range that we felt would provide meaningful results.

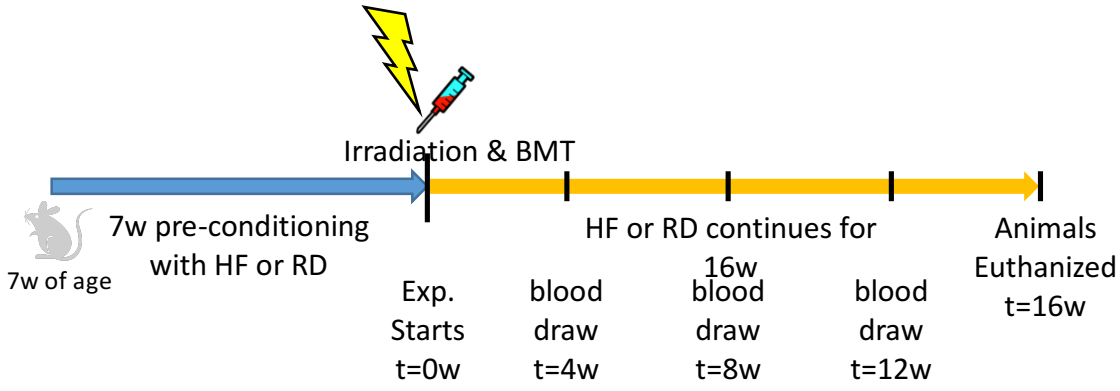


Figure 3. Experimental Timeline for SA2 and SA3.1. All experiments started with animals at 7 weeks of age. Animals were fed either the high fat diet or regular diet for 7 weeks prior to bone marrow transplantation. Following BMT, blood was drawn every 4 weeks for a total of 16 weeks when animals were euthanized. During this period, animals remained on their respective diets that they were on prior to BMT.

Methods

Animal Model: 6 week old male C57BL/6 mice purchased from The Jackson Lab, Bar Harbor, ME were allowed to acclimate for a week prior to starting the experiment. Animals were single housed on a 12h light-dark cycle with ad libitum access to food and water throughout the duration of the experiment. At 7 weeks of age, animals were started on either a 60% kcal from fat (HF, n=16) or 10% kcal from fat (RD, n=21) diet (TestDiet, "Van Heek" series) for a total of 7

weeks in order to establish an obese or lean phenotype, respectively. Body weight and food consumption were monitored weekly. After 7 weeks on the respective diets, animals underwent bone marrow transplantation.

Bone marrow transplantation: Mice were irradiated in a ^{137}Cs gamma-ray chamber and exposed to 12 Gy/1200rads (6Gy x2 separated by 4 hours) of total body lethal irradiation at 0.6 Gy/minute. Control mice that were fed the 10% lean diet were placed in an inactive chamber to serve as sham controls (C, n=6). 24 hours post irradiation; animals were rescued with bone marrow transplants, which consisted of donor cells from two sources. The first source of cells was acquired from 3 congenic strains of mice (male, 6 weeks of age) with the CD45.1 antigen (B6.SJL-Ptprc-Pepc^{b}/BoyJ). Whole bone marrow was collected from both tibia, femur and humeri of these mice. They were then processed and sorted for LSK^{+} cells through flow cytometry. Flow cytometric sorting was performed by the Flow Cytometry Core Facility at Stony Brook University Medical Center. The second source of donor cells was acquired from 6 male cd45.2 C57BL/6 mice (6 weeks of age) whose whole bone marrow was depleted of LSK+ cells using similar flow cytometry techniques. All recipient mice were then given via tail vein injection, 3 million total CD45.2 stem-cell-depleted bone marrow cells along with one of three doses of CD45.1+ LSK+ cells: low dose of 500 cells (LD, n=11), medium dose of 1000 cells (MD, n=10) and a high dose of 4000 cells (HD, n=10). HF and RD animals were each separated into three groups, so that there were 6HF and 5RD mice receiving a LD of CD45.1+ LSK+ cells (HFLD and RDLD, respectively); 5HF and 5RD receiving a MD of cells (HFMD and RDMD, respectively) and 5HF and 5RD receiving a HD of cells (HFHD and RDHD, respectively). Animals were then monitored daily and weighed every other day and continued the respective diets they were on prior to bone marrow transplants.

Longitudinal peripheral blood engraftment: At time points 4, 8 and 12 weeks, 100μl of blood was drawn from the facial vein for blood cell analysis through flow cytometry. At 16 weeks (conclusion of the study) 100μl of cardiac blood was extracted prior to animals being euthanized. Processing for analysis included red blood cell lysis (1X Pharmlyse, BD Biosciences), cell-counting using automated handheld cytometer (Scepter, Millipore) and staining for the CD45.1 antigen.

Bone marrow donor cell engraftment: 16w post irradiation, animals were euthanized and bone marrow was extracted from the femur and tibia for flow cytometric analysis. Cells were processed similarly to the blood and characterized for the CD45.1 antigen.

μ-CT analysis of trabecular and cortical bone quantity and quality: The tibiae were extracted at euthanasia, fixed in 10% neutral buffered formalin for 24h and stored in 70% ethyl alcohol until analysis. *Ex vivo* micro computed tomography of the proximal tibia metaphysis was conducted on the μ-CT-40 *(Scanco)* at a resolution of 12μm. The mid-shaft of the tibia was also scanned for cortical bone and endosteal volume calculations. An automated script (similar to that described in SA 1) was used to calculate trabecular BV/TV and architectural parameters as well as cortical bone quantity, see diagram and description in Chapter 2.

Statistics: All data are presented as mean ± SD. Student t-tests were conducted between RD and HF groups within the same dose. One-way ANOVA with Tukey post hoc was used to compare between doses. Graph Pad Prism and SPSS software was used to perform all tests and graph all data. Significance was met if $p < 0.05$.

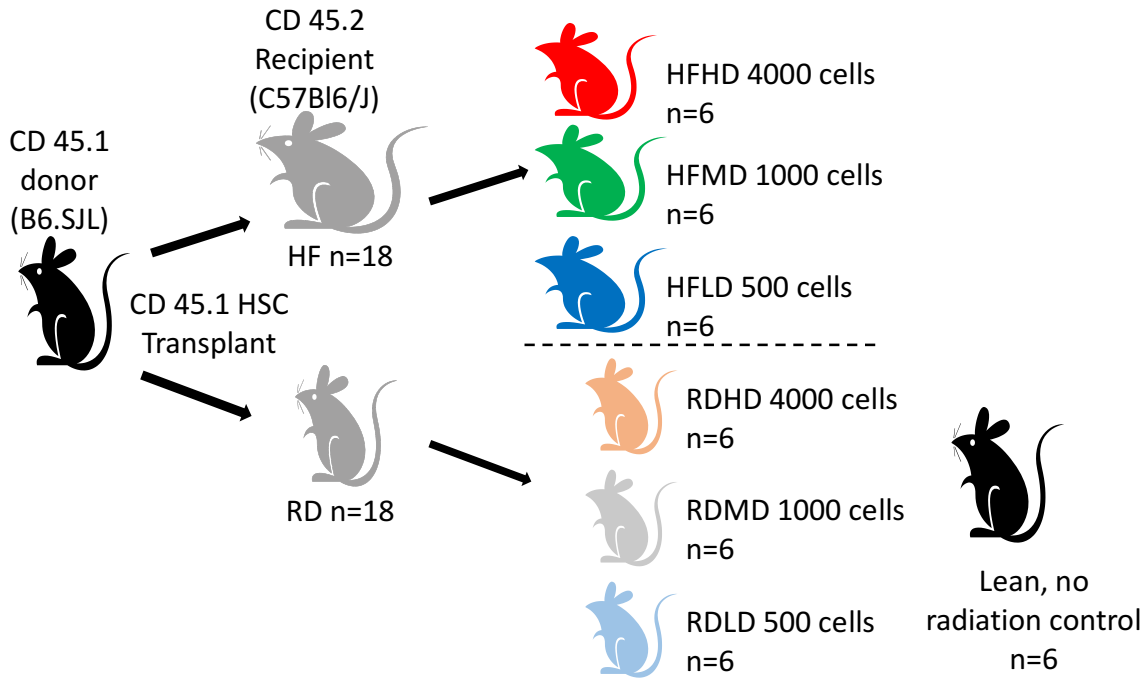


Figure 3.2. Experimental Groups for SA2 and SA3.1. CD45.1 HSCs were acquired from donor B6.SJL congenic mice and were transplanted into high fat or regular diet mice following lethal irradiation. Each HF or RD group was divided

into three dose groups: high dose (HD), medium dose (MD) and low dose (LD). A control group was also maintained throughout the experiment on a regular diet and received sham radiation and saline injections.

Results

DIO phenotype maintained post BMT with no adverse dose effects

After 7 weeks on the 60% kcal high-fat diet, all HF mice were significantly heavier than RD. The HF group was 29% ($p<0.05$) heavier than the RD group in body mass prior to irradiation (fig 3.3a). 16w post irradiation, pooled group of HF animals continued to be significantly heavier than the pooled group of RD animals. Differences between diets in the same dose show strong trends of increases in HF groups however not statistically significant (i.e. HFHD vs. RDHD; HFMD vs. RDMD; HFLD vs. RDLD; fig 3.3b). Gonadal fat pads of the HF mice in the HD and LD groups were also significantly heavier with a 165.9% and 175.3% increase than their respective controls. HFMD mice, although not significant still showed a 109.6% increase in wet weight compared to RDMD (fig 3.3c). There were no differences in body mass or fat pad mass between doses within the same diets (i.e. HFHD vs. HFMD vs. HFLD or RDHD vs. RDMD vs. RDLD). Similarly, primary immune tissues including the spleen and thymus (fig 3.3d and e) did not indicate any differences as a result of the different doses following the BMT. Diet differences were also not significant within doses except for a slightly elevated thymus weight in HFMD animals compared to that of RDMD ($p>0.05$).

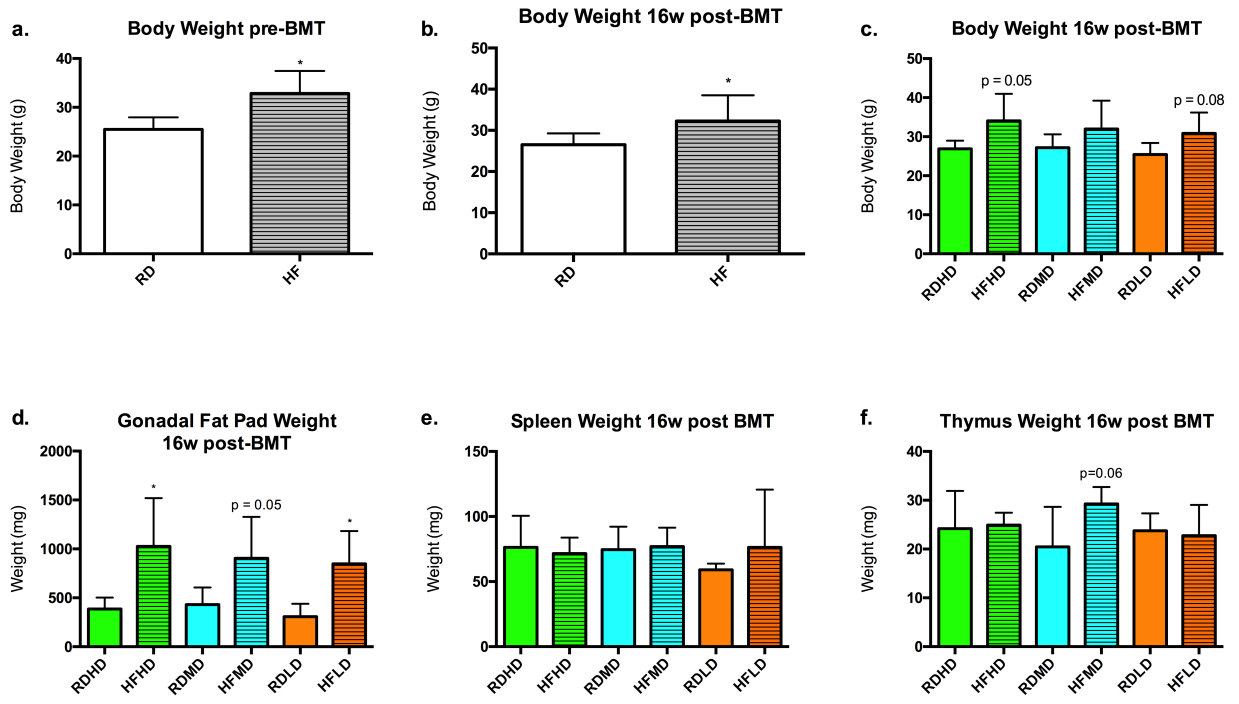


Figure 3.3. a) Body weight pre-BMT represents body weight after 7w on either lean 10%kcal or high fat 60%kcal diet. b) 16w post BMT, all HF animals pooled together are significantly heavier than all RD animals pooled. c) HFHD, HFMD and HFLD animals remained heavier than their controls ($p \geq 0.05$. Left gonadal fat pads were bigger in all HF animals indicating the presence of an adipose burden. Hematopoietic tissues, d) spleen and e) thymus did not show significant differences between diets in the same dose. Body weights, fat pad, spleen and thymus weights do no indicate any response to different doses of HSC transplants. *$p<0.05$ compared to RD

Successful peripheral & cardiac blood engraftment

Peripheral blood was drawn at 4, 8 and 12w post BMT to identify the engraftment level of donor cd45.1^{+} LSK^{+} cells between diets and between the different doses. We saw immediately, 4w after BMT that the engraftment generally corresponded to the injection density that each group received, whereby the animals that received the HD of cells had greater engraftment than the MD, who had more cells than the LD group (fig 3.4a). Among the HF animals, the HD group displayed 73.6% ($p<0.05$) and 94.4% ($p<0.05$) greater proportion of CD45.1^{+} cells at 4w compared to MD and LD, respectively, while the MD group had a 78.8% ($p>0.05$) greater proportion than that of LD. Among the RD animals, the HD group displayed 40% ($p>0.05$) and 83.5% ($p<0.05$) greater proportion at 4w compared to MD and LD, respectively, while the MD group had a 73% ($p>0.05$) greater proportion than that of LD. The HF animals maintained this engraftment pattern between doses throughout the study showing significant differences at 8w and 12w, and trends

at 16w in cardiac blood (fig 3.4b, c and d). The RD animals maintained similar trends but were not significant between the different doses at any point in the study.

When looking at engraftment differences between lean and obese animals within the same dose, the low and medium dose groups did not display a significant difference in proportion of CD45.1$^+$ cells between RD and HF groups throughout the study period. RDMD and HFMD showed consistently small differences in the proportion of CD45.1$^+$ cells in the blood with a 18%, 31%, 23% and 27% difference at 4, 8, 12 and 16w respectively (not significant). Differences between RDLD and HFLD were 35%, 75%, 74% and 13% for the same time points (not significant). The high dose of cells showed the greatest difference in CD45.1$^+$ cell proportion between diets. 4w after BMT HFHD had 90% ($p<0.05$) greater proportion of CD45.1$^+$ cells compared to RDHD. This trend remained consistent for the remainder of study. Although not statistically significant, percent differences between HFHD and RDHD increased to 135%, 122% and 103% at 8, 12 and 16w, respectively.

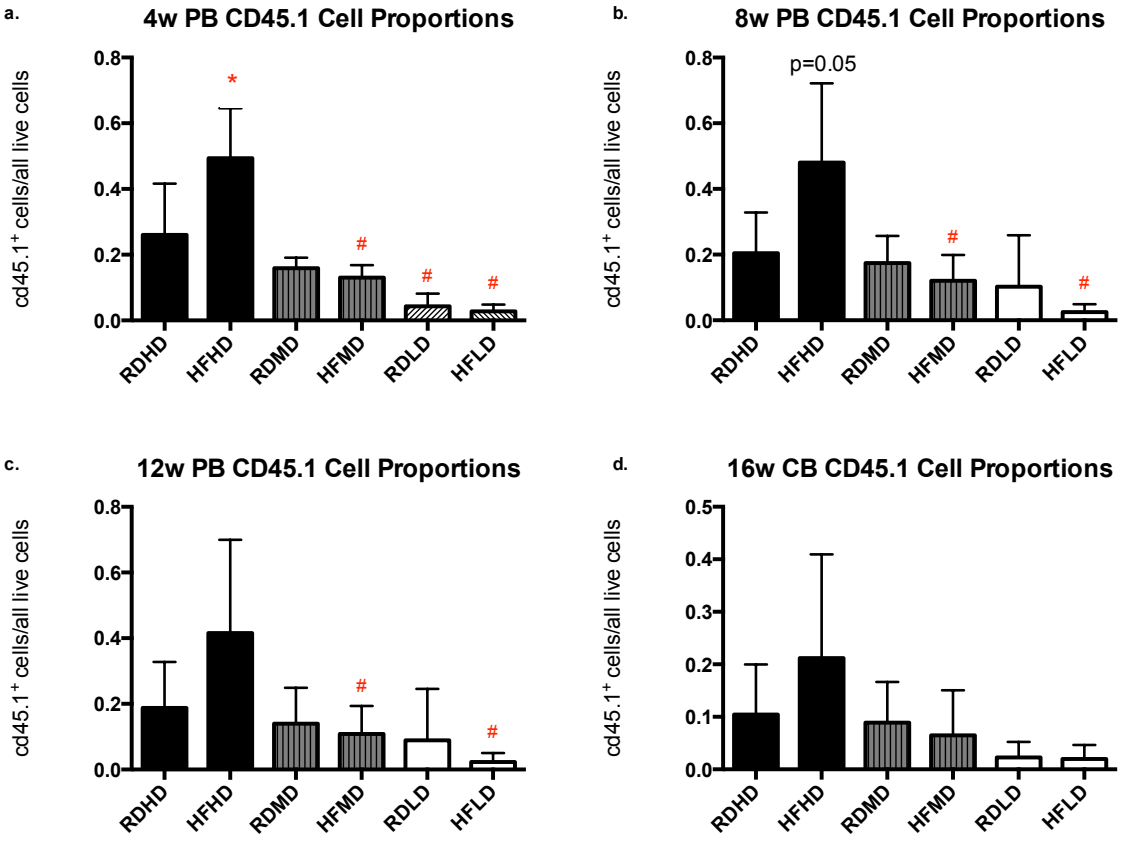


Figure 3.4: CD45.1$^+$ donor cell contribution to peripheral blood (PB) samples taken 4, 8, 12w post BMT and in cardiac blood (CB) at 16w. Graphs show the proportion of CD45.1$^+$ cells amongst all cells analyzed in flow cytometry. Student t-tests between diets of the same dose show significance if * $p < 0.05$. One-way ANOVA between doses of the same diet show significance if # $p<0.05$ compared to HD.

Long term CD45.1$^+$ cell engraftment in the bone marrow

At the conclusion of the study, 16w post BMT, bone marrow was evaluated via flow cytometry for level of contribution by the injected CD45.1$^+$ LSK$^+$ population, similar to that done in blood. Injection density was again, reflected in bone marrow even 16w post BMT. Among HF animals, HD was 81% and 97.7% significantly greater in proportion (fig 3.5b) compared to MD and LD, respectively. This is also reflected in total absolute numbers of CD45.1$^+$ cells (fig 3.5a). MD was 88% greater than LD in proportion, which was also similar to that in absolute numbers (both $p>0.05$). RD animals did not show the same pattern in HD, MD and LD groups. RDHD was 21.4% ($p>0.05$) and 46.7% ($p>0.05$) greater than RDMD and RDLD, respectively.

Differences between diets given the same dose showed similar trends to that seen in the blood. Medium dose groups (i.e. RDMD and HFMD) did not show a difference in proportion or absolute number of donor cells. RDLD showed greater proportions (85%, $p>0.05$) and absolute number (83%, $p>0.05$) compared to HFLD, but overall presence of donor cells in LD animals was very small. On the other hand, HD groups showed similar trends to that seen in the blood, with a 135% ($p=0.09$) and 247% ($p=0.09$) higher proportion and number of CD45.1$^+$ cells, respectively, in the HFHD compared to the RDHD groups.

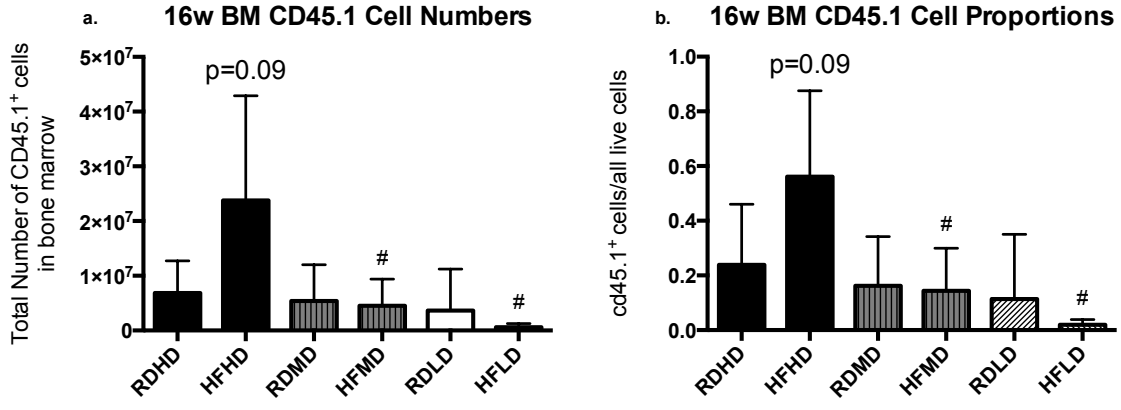


Figure 3.5: CD45.1$^+$ donor cells in bone marrow (BM) 16w post BMT. a) absolute numbers and b) proportions in bone marrow 16w post BMT. The absolute number of cells represents the total number of CD45.1$^+$ cells out of total

number of bone marrow cells extracted from the femur and tibia. Cell proportions represent all CD45.1^{+} cells from all live cells identified through flow cytometer. Student t-tests between diets of the same dose show significance if * $p < 0.05$. One-way ANOVA between doses of the same diet show significance if # $p<0.05$ compared to HD.

Trabecular bone damage to HF animals

μ-CT analysis of the proximal tibia metaphysis shows a significant loss of trabecular bone volume fraction in all HF mice regardless of dose with 42% ($p<0.05$), 59% ($p<0.05$) and 28% (n.s.) compared RDHD, RDMD and RDLD, respectively. Trabecular architecture showed some damage with an average of 13% reduction in trabecular number in HF groups compared to RD as well as an average 19% increase in spacing. Cortical BV/TV did not show any significant differences between any groups. Endosteal volume, which represents the average volume of the mid-shaft region of tibia shows slight, non-significant average increase of about 4% in HF animals compared to the RD.

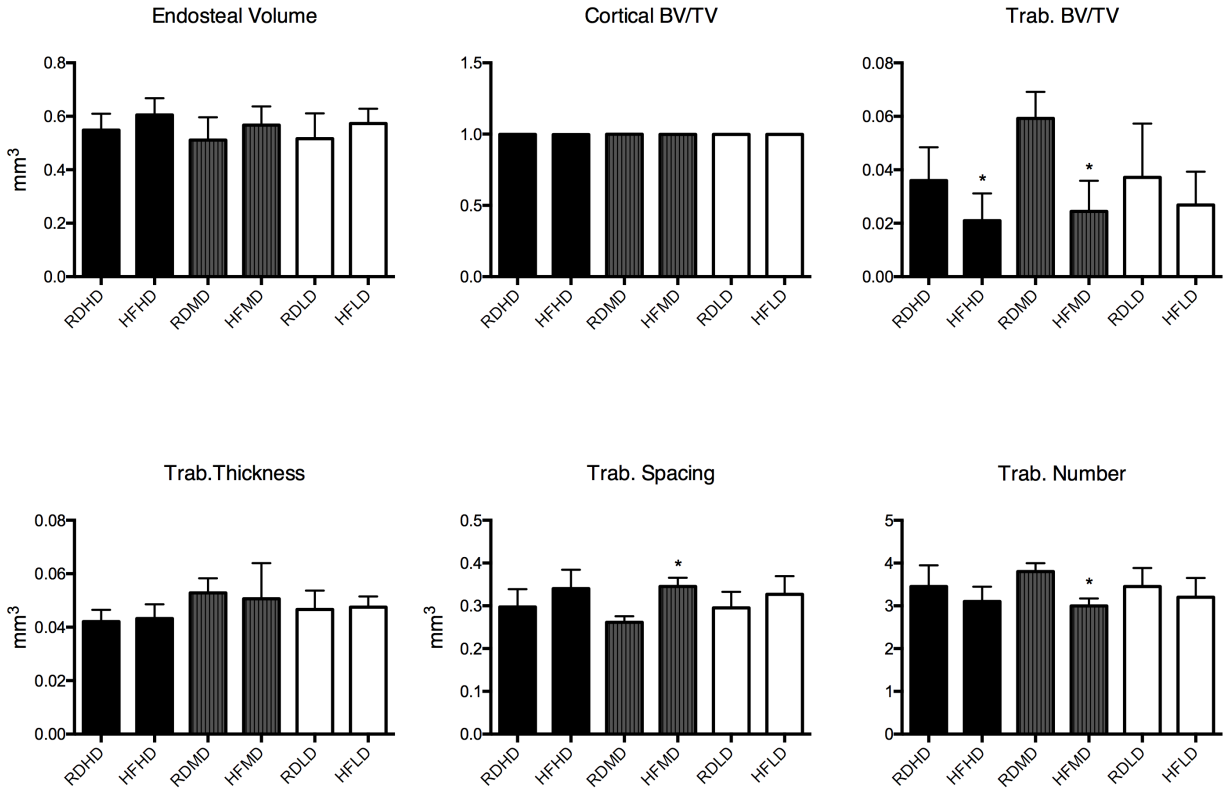


Figure 3.6 Cortical and Trabecular bone phenotype in the tibia. Endosteal volume and cortical BV/TV was evaluated in the mid-shaft while Trabecular bone volume fraction (BV/TV), spacing, thickness and number were evaluated in the proximal metaphysis. Student t-tests between diets with the same dose showed significance if *$p<0.05$.

Discussion

The engraftment of the HSCs post transplantation is very dependent on the function and composition of the bone marrow. The presence of cells such as osteoblasts, endothelial cells, and mesenchymal stem cells play a critical role in successful engraftment [142, 143]. These cells are also important for maintaining HSC function even after engraftment as mentioned earlier, emphasizing the dependence the HSC has on the whole bone marrow niche. Obesity induces significant alterations to the marrow niche. Pro-inflammatory cytokine production, especially of IL-1, IL-6 and TNFα are increased with high-fat diet [144]. Additionally, the increase in visceral adiposity is paralleled by increases in marrow adiposity in diet-induced obese mice [132]. These changes to the microenvironment along with other cellular as well as structural changes may modulate HSC behavior and consequently impair the immune system. HSC behavior has been previously shown to be primed by conditions of chronic inflammation to bias lineage commitment as well as their proliferative and self-renewal capabilities. For instance, mice with defective cholesterol efflux pathways have increased levels of growth factors in their bone marrow which causes HSC progenitors to expand and lead to leukocytosis following transplantation [145]. LSK+ HSCs from mice on a 45% kcal high-fat diet show reduced proliferation when removed from their obese in vivo environment. Additionally obese HSCs transplanted competitively with lean HSCs exhibit reduced reconstitution capabilities in lean irradiated animals [125]. These studies suggest that once the HSC and/or HSC progenitor is "primed" by the disease state, it's altered behavior cannot be reversed even when transplanted into a lean host.

While it may be true that HSCs become inherently "re-programmed" following long-term exposure to obesity [125, 130], it is still necessary to understand how a completely healthy HSC responds in obese conditions *in vivo* and whether the bone marrow niche mediates the response. We thus sought out to determine the engraftment and reconstitution capabilities of healthy HSC progenitors introduced into a pre-conditioned diet induced obese mouse. However, the first step towards this aim was to develop and validate an appropriate model that would be useful for looking directly at the impacts of the diet-induced obesity condition.

Three doses of CD45.1+ LSK+ cells were injected into obese or lean animals following total body irradiation. We saw from peripheral blood draws that as early as 4w following transplantation, donor cells had engrafted successfully into the animals and correlate with the initial injection density of stem cells. While HD and MD animals had between 20-50% donor engraftment, the LD animals exhibited only about 3% donor engraftment in both blood and bone marrow, regardless of their diet, even 16w following BMT. For this reason, LD animals were considered non-engrafted and not suitable for studying obesity effects on HSCs. When comparing MD and HD animals, it was interesting to see that as early as 4w and throughout the 16w experimental period, the HD animals consistently exhibited a stark difference in engraftment between RD and HF animals. Differences between doses within the same diet also showed that HFHD was 81% ($p<0.05$) greater than HFMD, while RDHD was not significantly different from RDMD as seen in the bone marrow. These trends were also consistent in the peripheral blood throughout the 16w test period. These data suggest a threshold of engraftment that is needed for recovery in regular diet animals. Even when presented with a greater number of cells as with the RDHD animals, percent engraftment did not significantly exceed that seen with RDMD animals, suggesting that many of the initial cells did not engraft and were perhaps discarded through circulation. However, it is difficult to make such assumptions because we do not know the absolute number of cells that actually engrafted immediately post transplantation. But, given the lack of dose dependence in RD animals, we can hypothesize that regular diet animals do not engraft all cells injected, especially over the assumed “threshold” of transplanted cells (i.e. the MD). As the primary purpose of the dosing experiments was to distinguish a diet impact, it was quite clear that when animals were given the high dose of cells, significant differences were seen between the RD and HF animals. The fact that we see differences also shows us that diet induced obesity primes the system in such a way that healthy HSCs respond even to the obese ‘environment’, characterized by excess adipose tissue and adipokines associated with obesity, and not just the stress from the lethal radiation dose. In other words, the effects of obesity are as caused by DIO are so significant that even after lethal irradiation, stem cells still respond to the obese condition. While there were no noticeable adverse responses of mice receiving the MD dose that would make them an unfit model, the chances of seeing differences in lineage

commitment due to obesity are greater when there is already a clear difference in engraftment among the HD groups.

Not only was there a diet impact of HSC engraftment post transplantation, the HF diet resulted in a 247% ($p=0.09$) increase in marrow engraftment of the donor cell population compared to that in RD animals receiving the same initial dose of cells. These data completely disprove our initial hypothesis. While it has been shown that obese stem cells transplanted into lean hosts have suppressed engraftment here we show in the reverse setup that the obese condition established by the rather robust 60% kcal diet did not negatively impact the engraftment of healthy stem cells post transplantation. While it may be argued that 16w following BMT is a long enough period to "prime" the initially *healthy* HSC, the fact that we see the increased engraftment in peripheral blood as early as 4w suggests that the LSK+ HSCs were responding to the diet-induced obese environment almost instantly. Additionally, because there were no signs of adverse effects to other tissues and that the only significant difference between RD and HF was visceral fat pad and body weight, we can assume that increased engraftment was indeed a response to the obese condition. In an effort to better understand the need for the expansion of donor cells in the HF animals, the marrow phenotype in terms of geometry and structure was further assessed.

The impacts of obesity on bone quantity are controversial, however the presence of excess adiposity has been shown to impact bone metabolism [146]. Some have shown that long term exposure to a high-fat diet results in a loss of trabecular quantity and architecture [79, 147]. Others have shown that diet-induced obesity can induce an abundant, thicker trabecular bone in an enlarged marrow cavity resulting in larger and stronger bones [148]. This led us to wonder if the increased engraftment of cells in the marrow of the HFHD animals was a result of a bigger marrow cavity. Micro-CT analysis of the mid-shaft region of the tibia (where BM cells were flushed and analyzed) did not reveal significantly larger marrow cavities in the HF animals compared to the RD, regardless of the dose. Additionally, the slightly bigger size (~4%) would not be alone sufficient to elicit such a stark difference in engraftment compared to the RD animals. Interestingly however, analysis of the trabecular bone compartment did indicate a significant

42% ($p<0.05$) and 59% ($p<0.05$) reduction in BV/TV in HF animals compared to RD in both HD and MD animals, respectively, with strong trends in LD animals. This significant loss of trabeculae might also play a role in allowing more cells into the marrow space as the lack of trabecular struts, as shown by significant reductions in trabecular number and spacing, would free up space in the cavity. While this hypothesis may be true, there are several aspects of the marrow that could have contributed to the increased engraftment seen in the HFHD animals such as cellular composition and cytokine expression. This will be analyzed and discussed in chapter 4 of this dissertation.

These data demonstrate that the obese condition characterized by excess visceral adiposity does not negatively influence the ability of HSC to engraft following total body irradiation. Our data further suggests that the greater loss of trabeculae combined with a moderate increase in marrow cavity volume might have contributed to the increased number of donor cells within the marrow. To better understand the other forces driving the expansion of the donor LSK+ cells and their contribution to the immune system in the obese condition, the stem cell differentiation and lineage commitment must also be evaluated. This will also enable us to understand whether an expansion of hematopoietic cells is advantageous to or negatively impacts the system.

Furthermore, these data highlight the importance of testing and developing appropriate experimental models in order to appreciate the questions being asked. Here we knew that a high-fat diet, even one as robust as the 60% kcal diet, was not as severe of an insult to the system as whole body ionizing irradiation. However, in order to use the HSC repopulation method, it became necessary to justify that the model answers questions regarding obesity rather than that of irradiation. While the high dose group might not be the only model, the fact that engraftment differences were seen with diets does make it a more interesting model to explore.

Limitations

One of the biggest limitations in comparing differences in HSC transplant doses arises from the method of transplant delivery. Although tail-vein cell delivery is a common method of transplantation, inconsistencies arrive due to the inevitable loss of solution (and cells) when

trying to locate the tail vein. While we tried to account for loss of solution, we still could not guarantee that each animal received the intended dose. Future experiments should adapt the retro-orbital or intra-tibial transplant delivery method as those show less loss of solution, thus able to guarantee more accurate dosage. Additionally, it would be advantageous to be able to evaluate exactly how many of the transplanted cells engraft immediately upon injection. Earlier time points, possibly 24h-7d following BMT, might be able to show us how many donor cells each animal actually started with. This would enable us to make better judgements on whether stem cells expand through proliferation, migration or differentiation to progenitors.

While we do see bone loss in HFHD animals, HFMD and HFLD animals also show significant loss but do not exhibit even trends towards greater engraftment, suggesting that size of the marrow cavity cannot be the only influence of increased engraftment. Chemokines, cytokines, as well as systemic demands must also play a role in the expansion. While we saw the reduction in trabecular bone, we cannot however comment on the mechanism behind the bone loss as we did not measure osteoblastic or osteoclastic activity. If bone loss was being motivated by an increase in resorption in the obese animals, it's likely that this could partially be influencing the expansion of hematopoietic cells, especially of pre-osteoclastic monocytes. Additionally, although we have body weight profile of the obese and lean animals, we do not have data showing increased adipose burden, thus while other studies can support the systemic and local (marrow) adipose burden, this study did not evaluate the initial phenotype of the animals upon transplantation.

Chapter 3 Summary:

1. Impacts of diet induced obesity on HSC engraftment can be identified by altering the dosage of transplanted stem cells following bone marrow transplant, where by a high dose of HSC transplants corresponds to increased HSC engraftment in diet induced obese mice compared to that in lean mice.
2. Diet induced obesity does not preclude from successful engraftment of transplanted HSCs.

3. Increased engraftment of HSC in bone marrow corresponds with increased loss of trabecular bone in diet induced obese mice.

Chapter 4 Obesity leads to expansion of bone marrow hematopoietic cells fueling visceral adipose tissue inflammation

SA 3.1: Determine the impact of a diet-induced obese condition on the lineage commitment of transplanted HSCs after lethal irradiation.

Hypothesis 3.1: *Healthy HSCs transplanted into an obese condition with excess fat will have biased lineage commitment favoring myelopoiesis compared to that in a lean condition.*

Introduction

Hematopoietic stem cells give rise to all the blood cells that make up the immune system, including red blood cells, platelets and leukocytes. Leukocytes consist of the myeloid cells, which include macrophages, granulocytes and monocytes, and lymphocytes which consist of the B and T cells. Collectively these cells help the body to fight against disease and infection. The body makes about 100 billion new hematopoietic cells every day, and is directly dependent on the activity and status of the hematopoietic stem cell [2]. Systemic stressors like inflammation or infection, can activate the expansion of primitive HSC pools to respond to immune system needs to replace populations that might have been lost due to these stresses. The activity of the HSC is largely orchestrated through the dynamic marrow microenvironment consisting of cytokines, bone cells, mesenchymal stem cells, endothelial cells, vasculature and other mature hematopoietic cells. Overstimulation of the HSC pool however can lead to their exhaustion and eventual failure of the hematopoietic system [128].

Obesity elicits stresses on the hematopoietic system through the development of systemic chronic inflammation that is thought to originate in the excess visceral adipose compartments [149, 150]. In obesity, as visceral white adipose tissue (WAT) expands, it becomes permissive to infiltration of macrophages, where at a time, 50% of WAT cells can comprise of pro-inflammatory macrophages [151]. This is a result of activation of bone marrow derived monocytes that migrate to sites of inflammation (i.e. adipose tissue) and differentiate into macrophages [152]. In addition to macrophages, there is also increased recruitment of the adaptive immune cells, B and T lymphocytes, to adipose depots [133, 153-155]. A variety of T cell subsets have been shown to infiltrate and contribute to adipose tissue inflammation. CD8+ T cells have been found to infiltrate adipose tissue prior to the accumulation of macrophages and whose deletion by antibodies improves obesity and diabetic phenotypes [156]. T_{regs} have also been

found in adipose tissue of obese mice, and are thought critical in the maintenance of adipose tissue inflammation [157]. Although the role of B cells is not yet fully understood, obese mice do present with increased numbers of select mature B cells within the inflamed adipose tissue. It is hypothesized that the secretion of autoantibodies and interaction with adipose tissue macrophages and T cells can exacerbate inflammation [158, 159].

While the association of visceral adipose depots and circulating immune cells has been largely studied, there is still much to be understood about the immune status in the obese bone marrow. And even more to learn about how changes to the marrow niche might influence HSC contribution to the systemic inflammatory condition. In a study by Singer et al., HSCs extracted from diet induced obese mice exhibited an increased propensity for generating adipose tissue macrophages when transplanted into lean irradiated hosts. This study suggests that HSCs, "primed" by the obesogenic environment, have biased myelopoiesis during engraftment and repopulation in irradiated animals who are not exposed to any obesogenic factors [130].

Since we know the importance of the marrow niche on HSC activity, we must also consider, adipose accumulation within the marrow and their influences on hematopoiesis and chronic inflammation. Few studies have investigated MAT in obesity in relation to HSC activity and immune regulation. In non-pathological conditions, Naveiras et al. demonstrated that areas of high bone marrow adiposity corresponded with reduced numbers hematopoietic stem cells and that marrow engraftment after irradiation is accelerated in the absence of MAT; implying that adipocytes negatively impact hematopoiesis [29]. In contrast, while MAT might negatively impact HSC progenitors, they have also been shown to protect primitive HSC pools through their secretion of adiponectin and TNF-α [160, 161]. Furthermore, in the SAMP6 mouse model of aging, a positive correlation was reported with myelopoiesis and increased MAT [162]. Obesity driven changes to the bone marrow have shown increased frequency of lymphocytes, both in B and T cells, in mice given a 45% kcal diet [23]. Studies with a more severe DIO (i.e. 60%kcal) have shown a suppression of B lymphopoiesis concurrently with increased MAT [132]. There is also some evidence of inflammation within the bone marrow as DIO mice exhibit increased mRNA expression of C-motif chemokine ligand 2 (CCL2) and cyclooxygenase-2 (Cox-2), both of which are

highly correlated with inflammatory conditions [116]. Collectively, the literature shows us that there must be a relationship between MAT and bone marrow HSCs and therefore immunity. However, the complexity of the marrow microenvironment as well as some of the discrepancies elicit the need for more research and understanding.

The following study, which is an extension of that done in chapter 3, evaluates the lineage commitment of hematopoietic stem and progenitor cells taken from healthy animals and introduced into obese *in vivo* conditions through the BMT repopulation assay.

Experimental Design

Concurrent with SA2, DIO was induced for 7w prior to BMT. As we learned from SA2, diet responses were clearly seen when animals were injected with a HD (4000 cells) of HSCs. Therefore, SA3.1 will only be looking at differences between RDHD, HFHD and also a regular diet fed, non-irradiated, saline injected, age-matched control, Control. Following BMT, animals continued the same diets they were on prior. Peripheral blood was drawn every four weeks. Animals were euthanized 16w post BMT and analyzed for bone marrow cellularity, morphology and mRNA expression.

Methods

Leukocyte analysis in blood and bone marrow: 100µl of blood was drawn from the facial vein every 4w. Bone marrow aspirates flushed with supplemented media (10% FBS, 1%Pen/Strep and DMEM, Gibco) from one tibia and femur were also collected at the conclusion of the study at t = 16w. Flow cytometry analysis was used to evaluate the three primary leukocyte sub-populations: B lymphocytes, T lymphocytes and myeloid cells which include monocytes and granulocytes. After red blood cell lysis (1X Pharmlyse, BD Biosciences, San Jose, CA), single cell suspensions (1 million cells/assay) of the blood and bone marrow samples were stained with the following surface markers: B220+ (APC, PE) for identifying B cells, CD4+ (PE) and CD8+ (PE) for identifying T cells, and Gr-1+ (APC) and Mac-1+ (APC) for identifying the granulocyte and monocyte population. Leukocytes derived from transplanted donor stem cells were identified by the expression of the CD45.1 hematopoietic cell surface marker. Antibodies were all purchased from BD Biosciences, San Jose, CA.

Bone marrow adiposity analysis: Bone marrow adiposity was evaluated in the proximal tibia metaphysis. After fixation with neutral buffered formalin, tibiae were decalcified in 10% EDTA (Ethylenediaminetetraacetic acid) for 2w at 4°C prior to embedding in paraffin wax. 9μm sections of the proximal tibia were stained with Wright-Giemsa and imaged on the Zeiss Microscope at 2.5X magnification. Using the NIH-ImageJ imaging software, a sole researcher blinded to the sample names, identified and quantified adipocyte ghosts to report number of adipocytes/bone marrow area, average size of individual adipocytes and total area of adiposity/bone marrow area.

mRNA analysis in bone marrow: Bone marrow aspirates from the femur were flushed with and stored in RNA-later. For RNA extraction, RNA-later was first carefully removed from samples by centrifugation followed by cell lysis with TRIzol (Invitrogen, Life Technologies), and isolated in an aqueous phase after chloroform separation. The Qiagen RNeasy kit was then used according to the manufacturer's instructions to extract purified RNA. RNA concentrations were measured with a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). RNA was then converted to cDNA using the Applied Biosystems' High Capacity cDNA Reverse Transcription kit at a concentration of 1 ng/μl assuming a 1:1 conversion from RNA to cDNA. For gene analysis RT-PCR was performed using Taqman gene expression assays from Applied Biosystems to measure the expression of the following genes: IL-1a (Mm00439620_m1) [163], IL-6 (Mm01295803_m1) [164], TNFα (Mm00443258_m1) [164], TGFβ1 (Mm01178820_m1) [165], C/EBPα (Mm00514283_s1) [166], Fabp4 (Mm00445878_m1) [167]. β-Actin was used as an endogenous control for calculating relative expression.

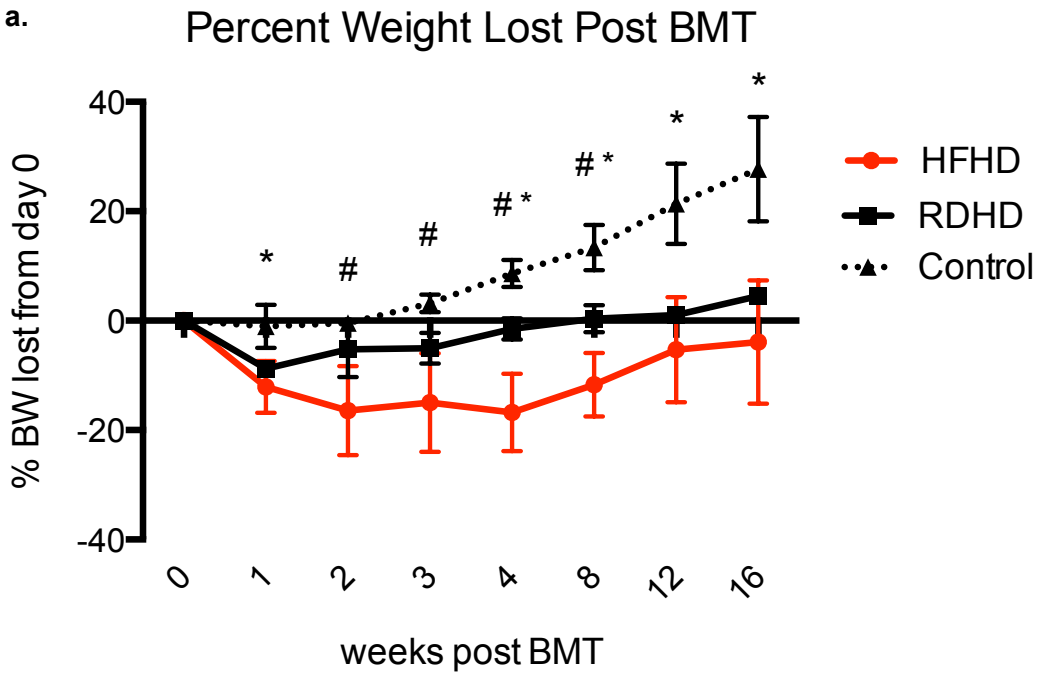
Hematopoietic cell analysis in gonadal fat: The left gonadal fat pad was collected, weighed and stored in supplemented media (10% FBS, 1%Pen/Strep and DMEM, GIBCO) at sacrifice. Samples were then digested in collagenase Type II followed by filtration through a 40μm cell strainer and finally ground with a syringe plunger. Cell pellets were then lysed (1X Pharmlyse, BD Bioscience, San Jose, CA) and finally stained in cell suspensions containing 1 million cells/assay for various leukocyte markers (refer to BM flow staining scheme above).

Statistics: All data are presented as mean ± SD. A one-way ANOVA with tukey post hoc was conducted between Control, RDHD and HFHD groups. Graph Pad Prism or SPSS software was used to perform all tests and graph all data. Significance was met if $p < 0.05$.

Results

Obesity delays recovery of bodyweight following BMT

The percent body weight lost from time of BMT showed that at 4w RD animals had almost recovered their initial body weight with only 1.5% from their initial weight while the HF animals were still indicating a 16.7% difference from their initial pre-irradiation weight ($p<0.05$, Fig 4a). Between 8 and 12w, RD animals had fully recovered their body weight to that of their pre-BMT weight. Contrastingly, HF animals were only starting to show signs of recovery between these times, where at 16w, they had only recovered 3.8% of the weight ($p>0.05$). Additionally, HF animals lost a greater percent of their body weight over all, with the lowest weight drop of 16.8% while RD animals lost a maximum of 8.8% of their body weight. Body weight profile (Fig 4b) indicated HF animals were significantly heavier than controls prior to BMT and remained significantly heavier 2w, 8w and 12w post BMT, while they trended higher than RD at 4w and 16w. The initial drop of about 10-15% in weight is expected and is a result of irradiation.



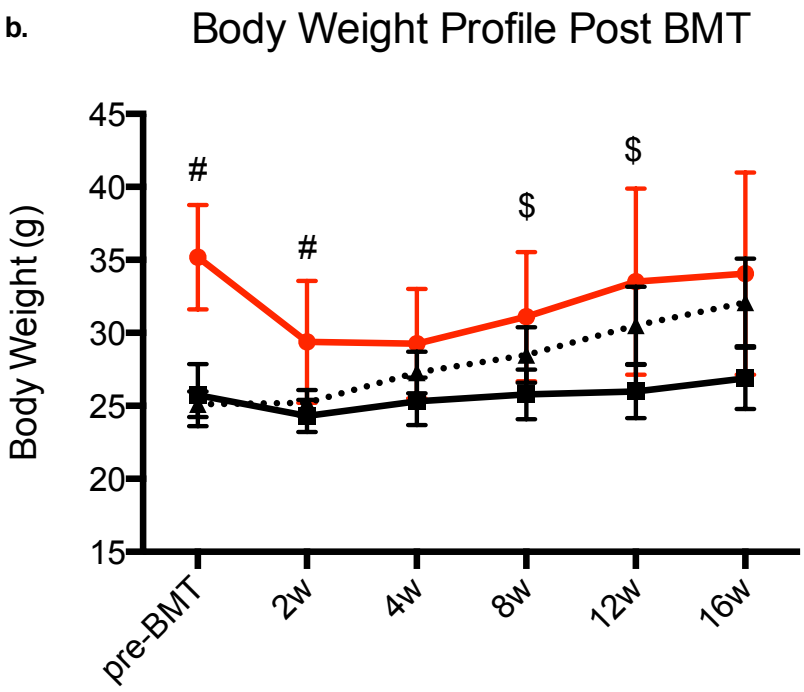


Figure 4. a) Percent body weight lost from day = 0, pre-BMT body weight. HFHD and RDHD both drop significant weight within a week after BMT. While RDHD begins to slowly recover after week 1, HFHD continues to drop weight until 4 weeks. A one-way ANOVA was performed at each time point between the three groups. b) Body weight profile showing HF mice were significantly heavier prior to BMT, 2w post BMT, 8w and 12w compared to RD. * for $p<0.05$ compared to Control, # for $p<0.05$ compared to HFHD, $ for $p<0.05$ between RDHD and HFHD

Obesity delays peripheral blood B lymphocyte reconstitution following BMT

Reconstitution of blood cells in the periphery was longitudinally evaluated every 4w following total body lethal irradiation. Flow cytometry analysis showed us that at 4w B and T lymphocytes in the RD animals had recovered to Control levels with a 0.5% and 15% (both, $p>0.05$) difference, respectively. While the HF animals displayed a delay in the recovery of B lymphocytes with a 53% (n.s.) deficit and slight recovery of T lymphocytes by 15% (n.s.) compared to Controls. Interestingly at 8w lymphocyte populations increase in RDHD and HFHD,183% and 83% (both n.s.) compared to Controls before normalizing to Controls by 12w. At 12w we however see that the HF animals have not fully recovered their B cells with a 20% deficit compared to Controls while RD has fully recovered (4%, $p>0.05$). Myeloid cells which include Mac-1+ monocytes and GR-1+ granulocytes were significantly increased in both irradiated groups compared to controls, with RD having 65% ($p>0.05$) greater numbers than the HF animals at 4w.

Myeloid cells in RD animals gradually normalized to Control levels from 4w to 12w but remain about 43% (n.s.) higher than Controls. HF animals on the other hand, gradually increased in number from 4w to 8w before normalizing back to the Controls at 12w.

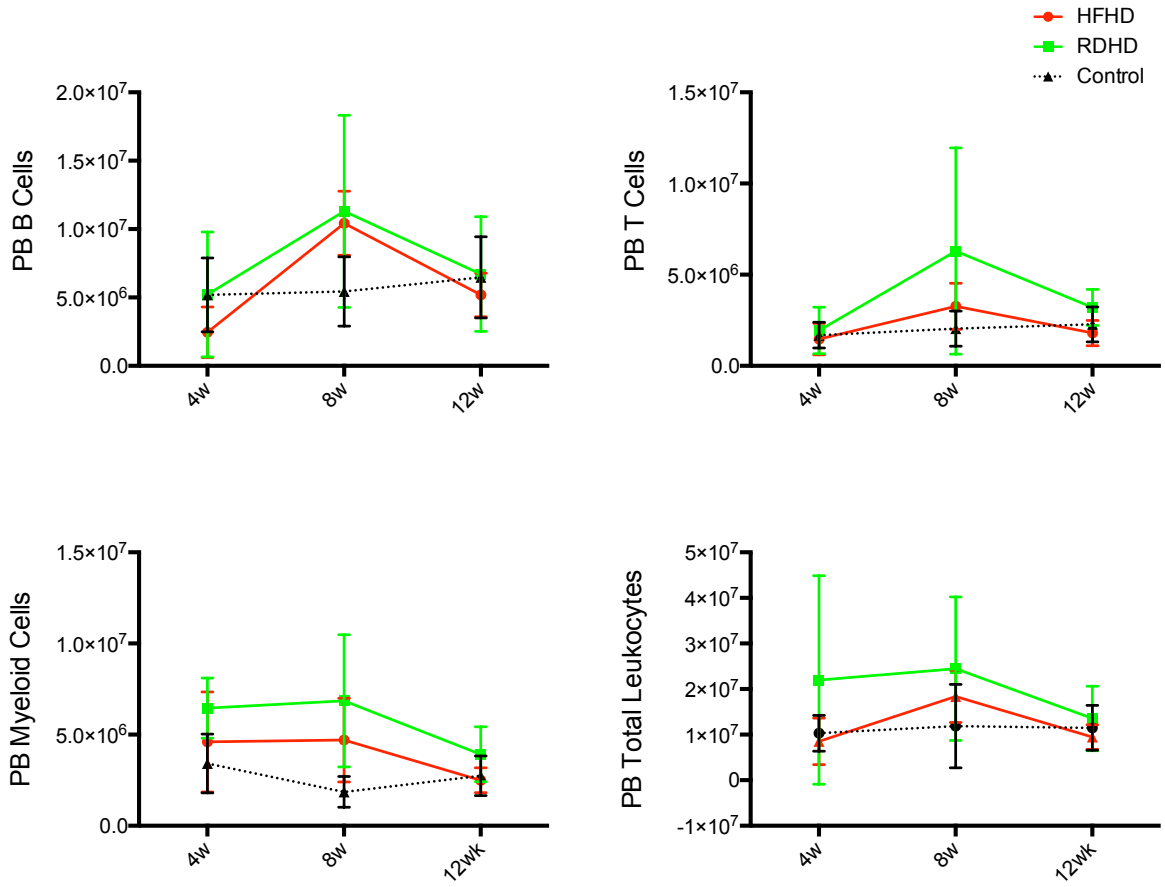
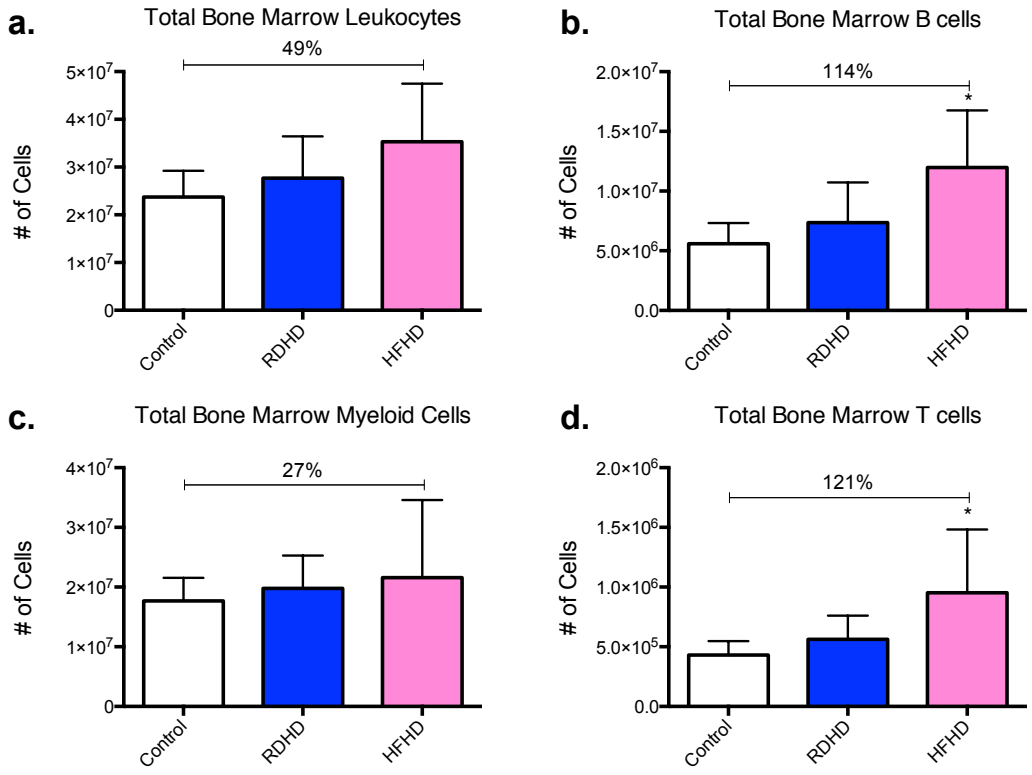


Figure 4.2 Longitudinal analysis through flow cytometry in 100µl of peripheral blood. B Cells were B220+ and were not fully recovered in HFHD at 4w, T cells were CD4+ and CD8+, Myeloid cells were Mac1+ and Gr1+. Leukocytes include both lymphocytes and myeloid populations. Lymphocyte populations increase at 8w post BMT in RDHD and HFHD. Leukocytes are normalized to controls by 12w.

Obesity induces increased bone marrow lymphopoiesis following BMT

Flow cytometric analysis of bone marrow hematopoietic cells at 16w showed that HF animals had a 28% ($p>0.05$) and 49% ($p>0.05$) greater number of total leukocytes (including B cells, T cells and myeloid cells) compared to RD and Control, respectively. More specifically, HF animals exhibited significantly greater numbers of lymphocytes compared to Controls, with 114% ($p<0.05$) greater B cells and 121% ($p<0.05$) greater T cells. Myeloid cell populations in HF exhibited a 27% (n.s.) increase compared to Controls. While HFHD animals do not exhibit

significantly greater total bone marrow cells, they show a trend toward 27% and 51% (both n.s.) increase compared to RDHD and Control, respectively. In an attempt to see if there was truly an increase in B and T cells or if this was a result of an overall increase in cellularity of HFHD animals, we looked at proportions of these lineages in relation to total cells. Proportions of B and T cells were again increased in HFHD compared to RDHD by 30% ($p<0.05$) and 26% (n.s.), respectively. While myeloid cell proportion was reduced by 11% (n.s.) in HFHD compared to RDHD.



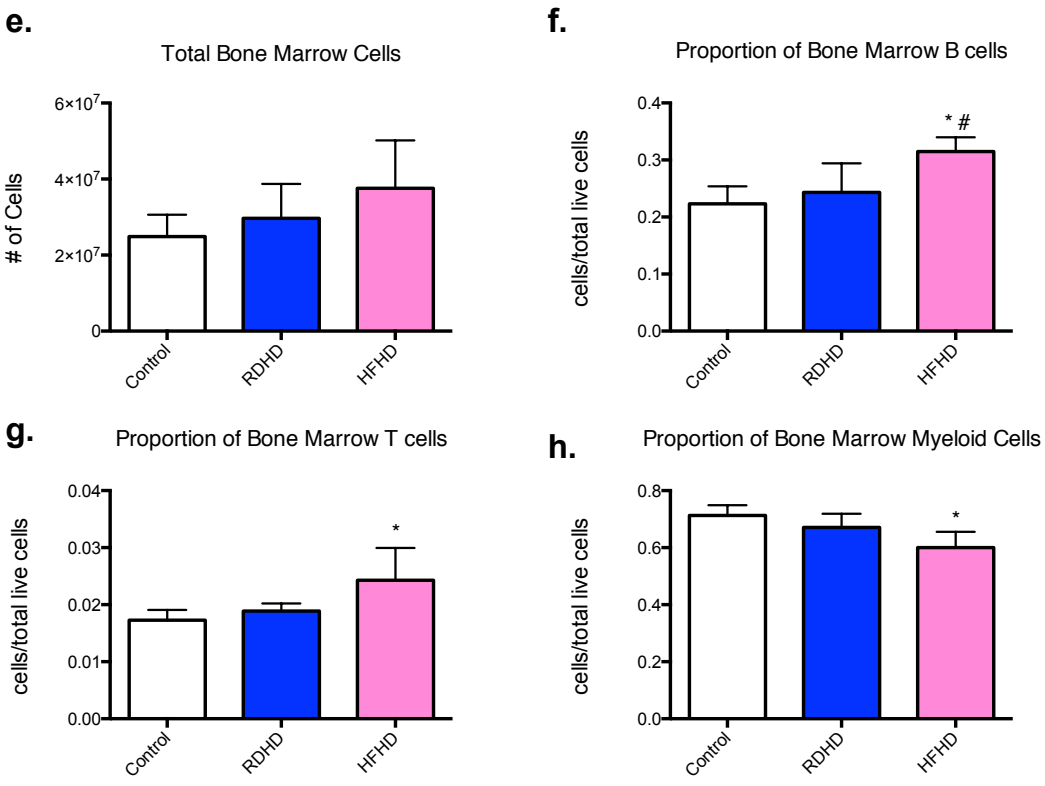


Figure 4.3. a-e) Absolute numbers of leukocyte populations out of all extracted BM cells from femur and tibia shafts. a) total leukocytes include B cells, T cells and myeloid cells. b) total number of B cells (B220+) is increased in HFHD c) total number of myeloid cells (Gr1+ & Mac1+) is increased d) total number of T cells (CD4+ & CD8+) e) total bone marrow cells acquired from Septer Counter f-h) proportions of B, T and Myeloid cells out of total BM cells ONE Way-ANOVA with tukey post hoc test shows significance compared to Controls if *p<0.05 and compared to RDHD if #p<0.05.

Obese mice have greater dependence on donor cell reconstitution

Donor CD45.1^{+} cell contribution to leukocyte populations was greater in HF animals compared to RD, in bone marrow. HFHD animals had 250% (p=0.07) more donor derived leukocytes compared to those of RDHD animals in the marrow. Host derived leukocytes had a 42% (p>0.05) greater presence in RDHD compared to HFHD in BM. Additionally, significant differences only existed between donor and host populations via Student t-tests in the Control and RDHD group. Similar trends were seen in the different leukocyte populations. Although, no differences were seen in host B and T lymphocytes, myeloid cells of the host population were suppressed in HF compared to Control (p<0.05) and reduced by 50% (p>0.05) compared to RD.

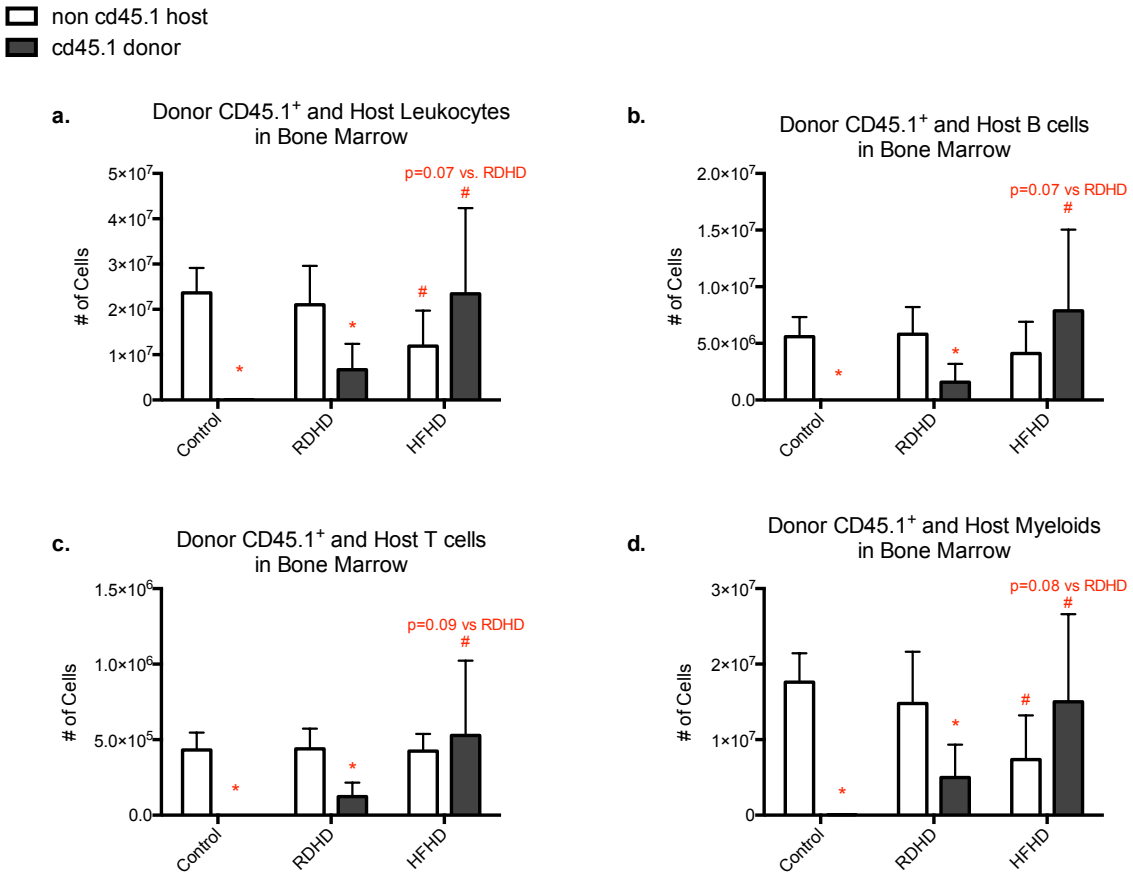


Figure 4.4: Number of a) leukocytes derived from host cells versus those from donor cd45.1^{+} stem cells in the bone marrow. b) B lymphocyte and c) T lymphocyte host derived populations are not significantly suppressed. d) Host derived myeloid populations are however suppressed. Student T-tests between donor and host cell populations in each group reveals significant differences when *p<0.05. One Way ANOVA and tukey post hoc between groups within host and donor populations show #p<0.05 compared to Control

Obesity exacerbates marrow adipocyte encroachment following BMT

Giemsa stained sections of the proximal tibia evaluated for the presence of marrow adiposity indicated that 16w following BMT, RDHD animals experienced a 99% (n.s.) increase in the presence of marrow fat compared to Controls. Animals on the high fat diet experienced an alarming 151% (p<0.05) increase compared to Controls. This dramatic change in marrow phenotype of both the RD and HF animals was realized through increases in number of adipocytes, with 82% (n.s.) greater numbers of fat cells in RDHD and 101% (p<0.05) greater numbers in HFHD compared to Controls. While the average adipocyte area was on 6% (n.s.) different in RDHD compared to Control, the HFHD animals displayed adipocytes 26% (n.s.) greater in size than those in the Control animals, which partially contributed to increased total adiposity

in these obese animals. In an effort to further corroborate the increased presence of fat in the obese animals, mRNA levels of Fabp4 and C/EBPα, was evaluated within the marrow. Both adipocyte related genes exhibit increased expression in HFHD animals compared to the Control group, with an almost 7-fold ($p<0.05$) increase in Fabp4 and 2-fold ($p<0.05$) increase in C/EBPα expression.

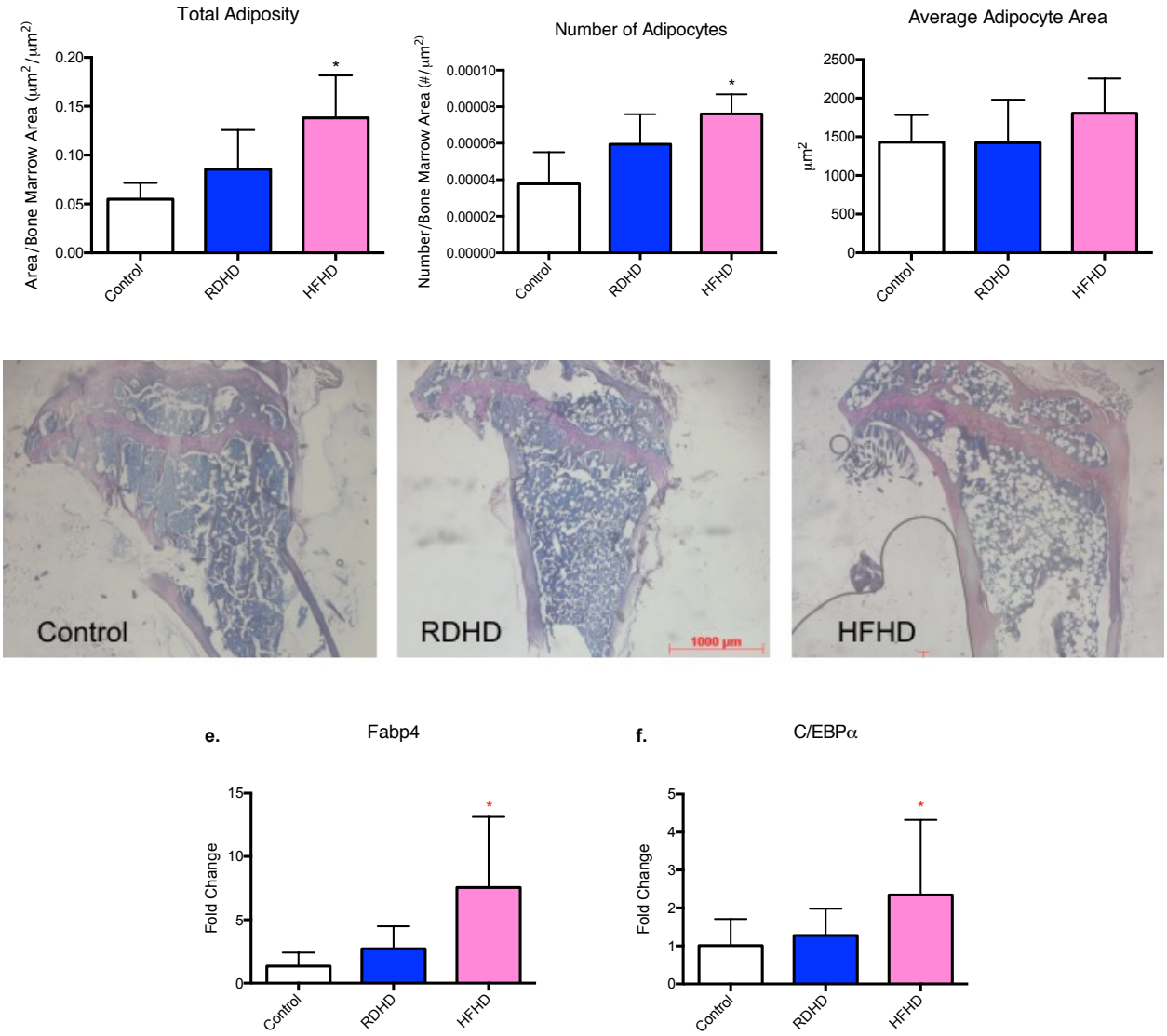


Figure 4.5. Wright-Giemsa stained sections of the proximal tibia metaphysis. a) Total Adiposity per area of bone marrow analyzed b) Number of individual adipocytes per area of bone marrow analyzed c) Average area of adipocytes analyzed d) representative micrographs of Control RDHD and HFHD sections where white spots represent the adipocyte ghosts e) Adipocyte related gene expression e) Fabp4 and f) C/EBPα in bone marrow cells flushed from the femur. One Way ANOVA and tukey post hoc between groups show significance if *$p<0.05$ compared to Control.

Cytokine profile does not demonstrate inflammation in BM microenvironment

Bone marrow cells flushed from the femur were evaluated for mRNA expression of genes known to promote inflammation and be secreted by adipocytes (WA and MA). These included IL-1, IL-6, TNFα and TGFβ and were measured in relation to house-keeping gene β-actin. Data from RT-PCR exhibited no statistically significant differences between any groups for these genes. IL-1, which is a pro-inflammatory cytokine previously shown to be increased in diet-induced obesity,[144] showed a 58% (n.s.) reduction in RDHD compared to Control and was increased by 3% (n.s.) in HFHD.

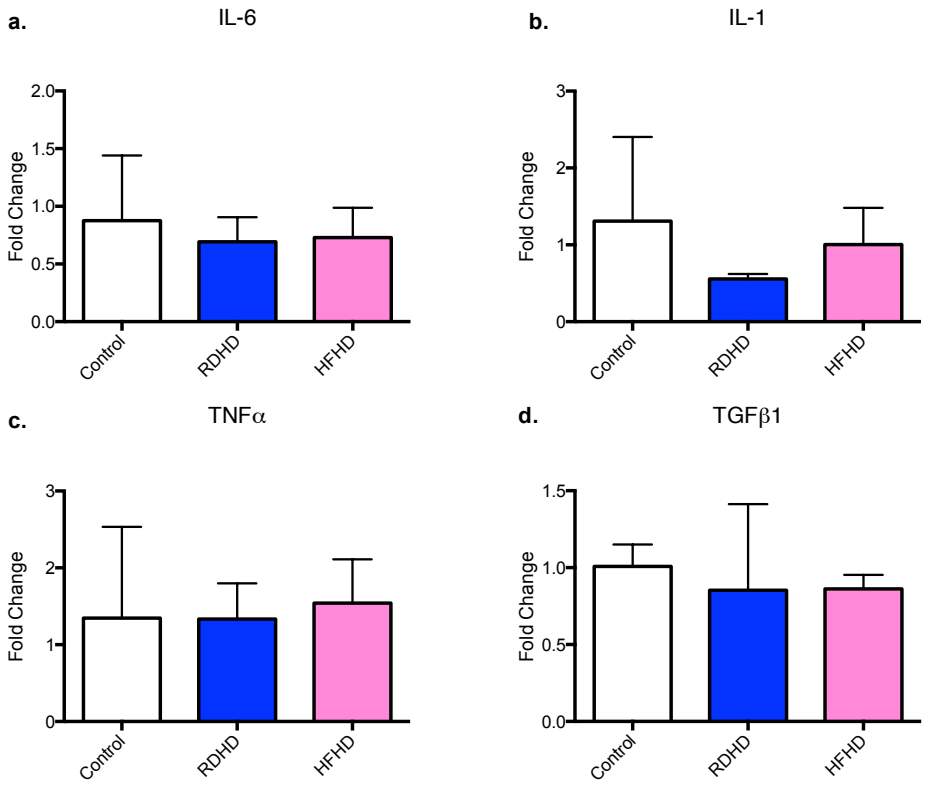


Figure 4.6. Bone marrow cells flushed from the femur were analyzed for the expression of pro-inflammatory genes. Gene expression was not significant between any groups. a) Interleukin-6 b) Interleukin-1α c) Tumor Necrosis Factor α d) Transforming growth factor β

Obesity promotes donor cell recruitment of leukocytes to adipose tissue

Gonadal fat pad cellularity indicated a significant increase in donor cell contribution in HFHD animals compared to RDHD. Total CD45.1+ cells had a 260% ($p<0.05$) greater presence in HFHD visceral WAT than in RDHD. Given that gonadal fat pads were significantly larger in DIO

animals (refer to Chapter 3) indicating greater number of overall cells, the proportion of total cells in the fat pad are also presented and indicated a similar 158% ($p<0.05$) greater contribution in HFHD than in RDHD. Additionally, analysis of the total hematopoietic cell phenotype within visceral WAT indicated significant infiltration of T lymphocytes (87%, $p<0.05$) and myeloid cells (64%, $p<0.05$) compared to Control animals. The HFHD also trended higher than RDHD animals with 37% and 31% (both n.s.) greater T lymphocytes and myeloid cells, respectively.

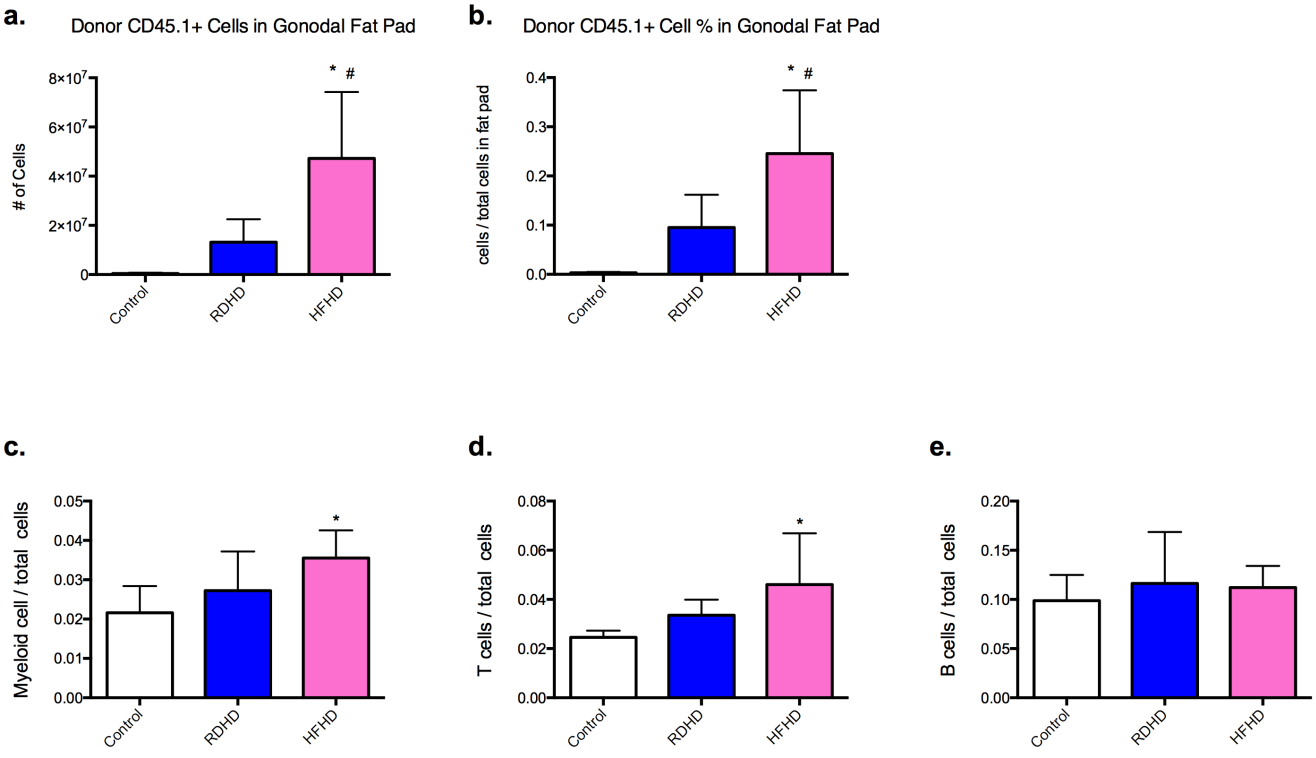


Figure 4.7. Left gonadal fat pads were processed for analysis of leukocyte infiltration through flow cytometry. a-b) total number as well as percentage of donor CD45.1+ cells in fat pad compared to total cells in the fat pad exhibit increased presence in the HFHD animals. c-e) proportions of T, B and Myeloid cells relative to total cells in fat pad. T and myeloid cells are greater in HFHD compared to Controls. One-way ANOVA and tukey post hoc test show significance if *$p<0.05$ compared to Control and #$p<0.05$ compared to RDHD.

Discussion

In chapter 3 we saw an increase in engraftment of donor cells in the high-fat animals compared to the regular diet animals following BMT. This was a relatively early response, seen just 4w after BMT and persisted throughout the 16w recovery period. While the pre-conditioned obese phenotype did not hinder the engraftment of cells, HFHD animals did exhibit a greater loss

of body weight compared to the RDHD animals following irradiation. This loss was not surprising as the HFHD animals had more weight to lose compared to the RDHD animals. Also, given the increased sensitivity of adipose tissue to radiation [168] and the fact that HF animals have significantly more visceral and subcutaneous adiposity [132], it is feasible that there is a greater loss of adiposity as a result of radiation. This loss of adiposity was also one they were not able to gain in the same manner they did pre-BMT. Prior to BMT, HF animals gained 53% of body weight in 4w. However, from 4w post BMT, the point when HF animals start gaining weight, till 8w post BMT, HF animals only gain about 6% in body weight. This stark contrast emphasizes the impaired recovery of body habitus and delay in recovery of as a result of the obese condition compared to the lean mice, even while they continued to consume the 60% kcal diet post BMT. While RDHD had completely recovered their original weight between 4 and 8w post BMT, HFHD had only begun to show signs of recovery during this time, without ever fully recovering their pre-BMT weight in the 16w period. These data suggest that obese animals are more susceptible to irradiation damage and that continued consumption of the high-fat diet is an additional stress, which might be contributing to the delayed recovery process.

In addition to the impaired body habitus recovery, HFHD animals exhibited delayed reconstitution of B cells in peripheral blood at 4w. Although not significant, peripheral blood analysis of leukocytes showed a 53% deficit of B cells in HFHD compared to Controls, while RDHD animals re-established their peripheral B lymphocytes by this time. These data imply three things: first, an impairment in hematopoiesis or the inability of HSC progenitors to catch up to control levels; second, repopulation by HSC progenitors shifted towards allocation to other leukocyte populations and away from B lymphopoiesis; or third, B cell recruitment to the peripheral blood was just lagging in high-fat mice. An impaired recovery was also observed by others whereby high-fat mice challenged by a myelo-suppressive agent, exhibited lower recovery of LSK+ and myeloid progenitors compared to regular diet controls. This was attributed to impaired hematologic regeneration [169]. Although the progenitor populations never fully recover in these animals, in our study we see by 8w that the HF animals not only recover their leukocytes but we see a considerable jump in leukocytosis in both irradiated groups in all three major cell types (B, T and myeloid cells). It's possible that this increase is a result of an over compensation

of hematopoiesis early following ablation which happened to be visible only at 8w, since by 12w all leukocytes drop back down to Controls. These data are slightly reminiscent of the transient response of progenitor and primitive HSCs reported by others in DIO, emphasizing that hematopoiesis is dynamically responsive to the stress of obesity or even just to the diet [125, 130, 132]. These data also highlight a missed opportunity for evaluating the bone marrow immune phenotype at the 8w time point. It's plausible that there was increased recruitment of cells from BM for repair of other hematopoietic tissues, such as the spleen or thymus. These data were not collected here; however future studies might consider an end point at 8w.

The bone marrow phenotype was however evaluated 16w post transplantation and exhibited a significant increase in hematopoiesis in HFHD animals compared to Controls. The expansion of hematopoietic cells was notably seen in lymphocytes as both B and T cells were significantly increased. The lack of differences between the diet groups was likely due to the impact of high doses of radiation causing slightly non-significant increases in hematopoiesis in RDHD animals as well. While the increased lymphocyte count in HFHD may be due to the slightly increased but non-significant total number of bone marrow cells in these animals, proportions of B and T lymphocytes in relation to total cells were also increased. Additionally, we see that myeloid cells, whose apparent population was not significantly increased in HF animals, did exhibit a significant reduction in their proportion likely due to the increase in lymphocytes, indicating that the enhanced lymphopoiesis is in fact a true increase since other BM cell populations (i.e. myeloid cells) did not significantly change. These data support reports by others, especially Trottier et al., who reported a dysregulated hematopoiesis in high-fat fed mice with reports of enhanced lymphopoiesis in the bone marrow attributed to the presence of increased leptin concentrations [23]. Further, it is important to recognize that in this study even though the HF animals were ablated of their immune cells with radiation and transplanted with *healthy* HSCs, their pre-conditioned obese phenotype combined with continued high fat diet consumption influenced increased B and T lymphopoiesis within the marrow. Whether, this is a direct result of differentiation by the transplanted *healthy* HSCs can be answered by distinguishing their specific CD45.1 cell surface marker which is expressed by all their hematopoietic progeny.

The uniqueness of this experiment arises from the fact that we were able to track the transplanted LSK+ HSCs, which consisted of a mixed population of progenitors and primitive cells, in their contribution to the hematopoietic phenotype established in the marrow of the HF and RD animals following BMT. The data shows that donor CD45.1 cells had an overall greater contribution to the immune phenotype (i.e. lymphocyte and myeloid cells) in HFHD animals compared to RDHD, even though both groups received the same number of initial donor cells. Not only do these data correspond with the increased engraftment seen in HFHD animals in chapter 3, they also indicate that the stem cells were functional in their capacity to differentiate and contribute to the hematopoietic recovery while being stressed by the high caloric diet and radiation effects. Furthermore, it is notable that while HFHD animals exhibited increased hematopoiesis, a majority of those cells arose from the direct differentiation of transplanted HSCs. These data suggest that while there was inherently nothing different about the transplanted stem cells in either diet group, HFD was essentially inducing changes in the BM microenvironment that drove the differentiation of the HSCs and expansion of the hematopoietic system. A recent study by Luo et al. came to similar conclusions after finding that BMT repopulation experiments demonstrated identical repopulation activity of BM from DIO and RD mice in RD hosts [169]. Taken together with our data influences of DIO on BM microenvironment as a whole elicits greater changes to hematopoiesis than the intrinsic impairment of the stem cell pool themselves.

The dependence on donor cell hematopoiesis in HF animals might also stem from the fact that host cells, primarily myeloid cells in this study, were significantly suppressed in HFHD compared to those in RDHD animals. We have seen from previous studies that a high fat dietary insult can lead to an aged leukocyte phenotype, characterized by depletion of B and T lymphocytes corresponding with an adipocyte rich marrow niche.[132] Marrow adipocytes are considered an energy source as well as an endocrine signaling factory through their secretion of free fatty acids, signaling molecules and growth factors [116, 138, 161, 170]. Their close approximation to hematopoietic cells in the marrow suggests that they could impart local, paracrine and cell-cell signaling, ultimately having a profound impact on hematopoiesis and possibly the chronic inflammatory state linked to obesity. Herein, we saw increased MAT in HFHD

animals compared to Controls 16w following BMT. Considering that radiation is in itself an inducer of MAT, it is difficult to ascertain MAT due to DIO versus radiation. It is likely that because we do not see significant differences between the RDHD and HFHD animals, that the fat accumulation in HFHD is a combined result of both high-fat and radiation and that the DIO exacerbated the accumulation of the MAT post BMT.

Interestingly while Navaries et al. claimed that areas of high "constitutive" marrow adiposity impairs hematopoietic activity [29] our data shows that increases in "regulatory" MAT does not correlate with impaired HSC activity. As a reminder, investigations spanning back to the 1970's by Tavassoli, have suggested the existence of two types of marrow adipocytes, one that is constant and preserved (constitutive) and one that responds to systemic stresses such as hemolysis (regulatory) [171]. While we did not precisely differentiate that the marrow fat identified here is regulatory MAT, studies have shown, at least in mice, that regulatory MAT is present in the proximal tibia and femur and this MAT responds to changes in nutritional status [172]. Additionally, as it relates to HSCs, many have reported during the development of marrow adiposity during DIO, there is also a suppression of hematopoiesis [79, 131, 132, 169]. However, in our study we show that HSCs forced in a "likely" fatty marrow support and perhaps drive hematopoiesis in HF animals.

In an attempt to better understand additional factors influencing the increased marrow hematopoiesis, we looked further into the cytokine expression in the marrow. Pro-inflammatory cytokines such as IL-6, IL-1 and TNFα are known regulators of hematopoiesis [144, 173]. While it is well established that WAT has active endocrine function, little research has been done on the secretory capabilities of MAT, especially pathological MAT. *In vitro* studies have shown that BM cells differentiated to adipocytes in culture, secreted the cytokines, IL-6, MIP-1α, G-CSF and GM-CSF [39]. Ex vivo primary human MAT from the iliac crest have also been shown to secrete IL-6 and G-CSF [32]. One study using corn-oil (CO) based diet induce obesity found that CO whole mouse femurs exhibited increased expression of IL-1, IL-6 and TNFα [174]. Collectively these data suggest that marrow adipocytes are capable of secreting cytokines and mediating inflammation in the marrow. Data from marrow aspirates from HFHD femurs in our study showed no signs of

inflammation as IL-6, IL-1, TNFα and TGFβ were not significantly different between any groups. While this does not explicitly suggest that the adipocytes are not secreting factors, we can conclude that inflammatory markers within the marrow are probably not having a big influence on hematopoietic activity. It's also possible that limitations in the mRNA-extraction protocol precluded the precise evaluation of these markers. During bone marrow cell flushing from the long bones, many cells are lost, especially those close to the bone surface. Additionally, fat cells are especially easier to lose as they are difficult to separate from the RNA-later solution supernatant. Thus, the presence of marrow inflammation cannot be completely discarded.

Whereas inflammation was not significant in the bone marrow, leukocyte infiltration of visceral adipose tissue was indicative of adipose inflammation in HFHD animals. Not only was there increased presence of total lymphocytes and myeloid populations, donor CD45.1 LSK+ cell contribution in HFHD fat pads was significantly greater than in the RDHD animals. Contribution of donor bone marrow cells to fat depots was also recently reported in a clinical trial of patients receiving allogenic BMT. The study showed that transplanted bone marrow derived cells contributed to subcutaneous WAT cell populations to a greater degree in patients who were considered obese [175]. It's very likely the same is occurring in our study and that the increased demand of hematopoietic cells in extra-medullary fat depots was contributing to the increased hematopoiesis seen in the marrow. A recent study by Nagareddy et al. showed that factors secreted by DIO visceral adipose tissue can signal the proliferation and expansion of HSC progenitors in the bone marrow [131]. Additionally, necrosis, adipocyte death and lipolysis are factors commonly associated with obesity and expanding adipose tissue. It is suggested that the products released through these processes (i.e. free fatty acids) can also elicit recruitment of leukocytes to the fat pads [131, 153, 176]. It is then not surprising that recruitment of leukocytes would be greater in obese rather than lean mice. The increased adiposity we see in both WAT and MAT in the obese mice is consistent with the increased leukocyte populations in the both of these sites.

In conclusion we have seen through this repopulation study that obese animals displayed delayed recovery following BMT as seen by their slowed weight gain and their suppressed

repopulation of blood leukocytes compared to RDHD mice. We have also demonstrated that repopulation of hematopoietic cells in obese mice leads to enhanced hematopoiesis by donor derived LSK+ HSCs within the bone marrow compared to that in lean mice. This increased hematopoiesis is accompanied by a slight bias toward lymphopoiesis and is hypothesized to be a partial result of suppressed host leukocyte populations in the marrow. These data could be further evidence of the greater damage to the obese condition, whether in response to BMT or one that is perpetuated by the high caloric diet during the hematopoietic recovery. The push for increased marrow hematopoiesis could also be attributed to the increased infiltration of donor-derived hematopoietic cells in visceral adipose tissue, in that the demand for extra-medullary hematopoiesis is greater causing bone marrow HSCs to be activated. The adipose tissue inflammation present in the visceral fat pads is likely being fueled by hematopoietic stem cells in the bone marrow. It is also important to note that while the HSCs originally transplanted in all irradiated animals were from healthy donors, their behavior in the HF and RD animals was starkly different. Whether the presence of the adipose tissue inflammation upon transplantation influenced the alteration of the HSCs towards the development of increased pro-inflammatory immune cells or whether other factors in the obese mice led to the recruitment of immune cells to the fat pads is unknown. Nevertheless, the altered behavior of the HSCs in the DIO system emphasizes the impact of environmental factors, whether local or systemic, to their engraftment as well as lineage commitment. While we see the long term repopulating activity and phenotype of the DIO and lean mice following BMT, the data presented here prompted an investigation into the early and acute impacts of obesity and irradiation exposure and whether their baseline phenotypes also drove the enhanced donor hematopoiesis.

Limitations

The limitations of the mRNA extraction protocol used in this study impeded the precise evaluation of cytokines secreted specifically by the bone marrow adipocytes. Although whole bone marrow was flushed from the femur, most adipocytes were likely lost when discarding the RNA-later supernatant to collect the pelleted cells. This might explain the lack of differences between groups in pro-inflammatory cytokine gene expression. Additionally, even with proper acquisition of the marrow fat cells, leukocytes also secrete these cytokines which would further

prevent the ability to distinguish the exact contribution of these bio-factors from MAT when analyzing a mixed marrow cell population.

Since we have seen that hematopoietic cells can have very transient expression, having only long term data of the bone marrow phenotype might have prevented making more connections with peripheral and marrow hematopoiesis. Because we saw such an interesting jump in peripheral hematopoiesis at 8w, data showing the longitudinal reconstitution of the marrow phenotype would also to see how the overall recovery of the animals related to its hematopoietic recovery. Additionally, we know that the bone marrow is composed of multiple cellular niches and that multiple cellular interactions support the function of HSCs. Information regarding the status of other cell types (i.e. osteoblasts, mesenchymal stem cells) within the marrow would allow us to better understand if the HSCs were compromised in their ability to properly "reboot" the system or if the supportive capacity of the marrow niche was already compromised in the obese irradiated mice.

Lastly, we cannot disregard the serious consequences of exposure to the high doses of radiation. Even though RDHD and HFHD animals were subjected to similar handling and doses, responses to the system at least early on are primarily due to the damage caused by ionizing radiation. It's also possible that the high doses used here might even overshadow many of the changes caused by the high-fat pre-conditioning period. Even when evaluating the long-term phenotype, many tissues and cells can become permanently damaged or altered by radiation [47]. Future studies might change the radiation dosage to a sub-lethal one to see if cells still engraft and can be tracked. Although the animals showed evidence of recovery, diet differences are not the only drivers of the hematopoietic phenotype we are seeing and should be properly accounted for when translating data to the clinic.

Chapter 4 Summary:

1. Diet induced obese mice have delayed recovery following bone marrow transplantation as indicated by delayed body weight recovery and peripheral blood B-Cell reconstitution.
2. Diet induced obese mice present increased bone marrow hematopoiesis with bias towards B and T lymphopoiesis.

3. Diet induced obesity exacerbates the presence of marrow adipocytes following bone marrow transplantation.
4. Diet induced obesity increases the hematopoiesis by donor transplanted HSCs.
5. Adipose tissue inflammation in obesity influences the recruitment of donor transplanted bone marrow HSCs to the visceral adipose depots.

Chapter 5 Increased damage to bone marrow microenvironment following acute irradiation in obesity

SA 3.2: Determine the marrow phenotype immediately before and after HSC transplantation and the ability of LIV to protect marrow cellularity following irradiation in DIO mice.

Hypothesis 3.2: *Irradiation following 7w of DIO will suppress marrow hematopoietic cells to a greater degree than that seen in RD lean control animals. LIV will protect the marrow phenotype of DIO mice and therefore, protect hematopoietic cells following irradiation to levels similar to that of RD animals.*

Introduction

Obesity is associated with a plethora of complications such as type II diabetes, hypertension, and atherosclerosis making it a complicated condition to characterize and therefore treat. While these complications may not all be present in every obese patient, chronic systemic inflammation is suggested to be a common factor in all forms of obesity. In chapter 4 we investigated the role of obesity in the development of immune cell subsets following bone marrow transplant and found that obesogenic environments (i.e. systemic adipose accretion and high caloric diet) potentiate enhanced hematopoiesis by HSC stem and progenitor cells. While BMT allowed us to track stem cell function in obese mice, we also discovered through the assay that being obese delays recovery from severe insults such as irradiation and puts a greater dependence on transplanted cells for repopulation.

Bone marrow or hematopoietic stem cell transplants are used to replace impaired and restore healthy bone marrow immune function. It is a primary treatment for bone marrow malignancies such as leukemia, lymphoma or myeloma. (NIH-National Cancer Center) According to the National Marrow Donor Program (NMDP) an estimated 7000 people had allogenic stem cell transplants for blood cancers in 2010. With the rise of the obesity prevalence and the increased risk of cancer among obese patients [177-179], the probability that more obese patients will require BMT will inevitably increase. Currently in the clinic, morbidly obese patients are not even considered for BMT due to the higher risk of complications [180]. One of the main concerns arises from the need to use higher radiation doses (and even chemotherapy) in order to target cancer cells through a greater amount of tissue. However, the higher the radiation exposure also results in greater damage to surrounding organs, which can be fatal [181]. While

BMT may never be safe for morbidly obese patients, there is still a high population of overweight and obese patients requiring BMT and a lack of data available to properly treat this population. Thus new considerations have to be investigated for better treatment and treatment implementation.

Therefore, in this study we look at the impacts of acute radiation exposure in obese and lean mice. This will not only uncover the early response of obese conditions to severe systemic stresses but also help to better understand the long term immune phenotype that was evidenced in Chapter 4. Specifically, it will help answer if changes seen in the long term following BMT are a response to the status of the animal at the time of transplantation or whether the continued consumption of the high caloric diet drove the engraftment and reconstitution that we saw. An additional aspect of this study was to investigate an intervention for the prevention or protection from obesity induced systemic alterations.

Strategies for treating the effects and comorbidities related to obesity are largely lacking. Exercise is associated with weight loss and also known to improve immune health, thus is a common treatment modality for overweight and obese patients [182]. Exercise intensity is positively correlated to weight loss, however the added risk of higher intensity exercise in obese patients also results in treatment with less intense and effective exercise regimens to prevent injury [182, 183]. However, diet and exercise are not always enough especially in cases of sustained obesity [184]. Clinicians are still not fully aware of the biological adaptations made in obese individuals that make it difficult for them to achieve and maintain weight loss. Interventions such the use of low intensity vibrations, while not having the ability to reverse obesity per se, does show potential in protecting from the complications associated with metabolic and hormonal imbalances. For example, the use of LIV in a model of post-menopausal osteoporosis proved more successful at preventing trabecular bone loss in mice rather than restoring it once the bone loss had already persisted [79, 185]. Additionally LIV has shown its influence in curbing fat accumulations in several tissues including visceral cavities [100], hind limb muscles [122] and bone marrow (Chapter 2) The mechanism behind LIV's impact has been attributed to the mechanical biasing of bone marrow mesenchymal stem cells that give rise to

bone and fat tissues [100], among others, which are two systems impacted by obesity. By promoting the formation of osteoblastogenesis, it is suggested that adipogenesis is in turn suppressed [101]. Therefore, in the current study, LIV will be administered concurrently with the start of the diet in an attempt to prevent alterations to immune and marrow health caused by diet-induced obesity. Moreover, we will also test the impact of the LIV intervention in the response of obese animals following acute radiation exposure.

Experimental Design

A similar animal model as used in SA 3.1 was used. 7w old male B6 mice were placed on either a 60% kcal HF diet (n = 30) or 10% kcal RD (n = 20) for 7w. A subset of HF animals (n = 10) were exposed to LIV treatment protocol (described previously) immediately upon beginning the diet for the duration of study. The remainder of the animals will be sham LIV handled. At the conclusion of the 7w, 10 HF sham LIV handled (HF-IR), 10 RD sham LIV handled (RD-IR) and the HF with LIV treatment (LIV-IR) will be exposed to 12 gy (6gy x 2) of gamma-radiation. The remaining HF and RD animals will receive sham irradiation. 24 hours post irradiation all animals will be euthanized without any rescue cells.

Methods

Animal model: 6w old male C57BL/6 mice purchased from The Jackson Lab, Bar Harbor, ME were allowed to acclimate for a week prior to starting the experiment. Animals were single housed on a 12h light-dark cycle with ad libitum access to food and water throughout the duration of the experiment. At 7w of age, animals were started on either a 60% kcal from fat (HF, n=30) or 10% kcal from fat (RD, n=20) diet (TestDiet, “Van Heek” series) for a total of 7w in order to establish an obese or lean phenotype, respectively. A subset of HF animals (LIV, n=10) began LIV treatment simultaneously with the start of the diet. While the rest of the animals received Sham LIV. Body weight and food consumption were monitored weekly. Following 7w of diet, LIV group, a subset of n = 10 of RD animals and a second subset of n= 10 of HF animals were subjected to total body lethal irradiation, while the remaining animals were sham irradiated (irradiation protocol below). Following 24h of irradiation or sham irradiation, the animals were sacrificed and tissues collected

for further analysis. Fasting blood glucose levels were also measured using a glucometer 1d prior to irradiation and again just prior to animal sacrifice.

Total body irradiation: The irradiated animals named, RD-IR, HF-IR and LIV-IR were irradiated in a ^{137}Cs gamma-ray chamber and exposed to 12 Gy (6Gy x 2, separated by 4 hours) of total body irradiation at a rate of 0.6 Gy/minute. The non-irradiated groups, RD and HF, were placed in an inactive chamber to serve as sham controls. 24 hours post irradiation; animals were euthanized.

LIV protocol: The LIV-IR group was subject to vertically oscillating low intensity vibrations (0.3 *g* at 90 Hz, where *g* is earth's gravitational force = 9.81 m/s^2). Mice received LIV for 30 min/d for 5 d/w for a total of 7w. Mice were placed into a partitioned container (one mouse per partition), centered on the oscillating LIV system. All other groups were sham vibrated with the same handling and container, but without activating the LIV device.

Flow cytometric analysis: Bone marrow was flushed with supplemented media (10% FBS, 1%Pen/Strep and DMEM, Gibco) at sacrifice until processing later that day. 100μl of blood was collected through cardiac puncture also at sacrifice. Both tissues were processed for flow cytometric analysis using previously described methods, briefly samples were lysed for red blood cells, bone marrow cells were filtered using a 40μm strainer, pelleted cells were counted using an automated cell counter. Bone marrow cells were separated in 1 million cell aliquots for each assay while all blood cells were used for leukocyte staining. Bone marrow cells were stained for HSCs progenitors (Lin-, Sca-1+, c-kit+), leukocytes including: B cells (B220+), T cells (CD4+, CD8+), Myeloid cells (Mac1+, Gr1+) and MSCs (Sca-1+, CD105+, CD90.2+). Blood samples were stained with the same leukocyte markers.

Ex vivo μ-CT analysis of trabecular bone: The right tibia was extracted, fixed in 10% neutral buffered formalin for 24h and then stored in 70% ethyl alcohol for several weeks until scanning. Trabecular bone analysis was done on the proximal tibia metaphysis on the μ-CT 40 (Scanco) with a voxel size of 12μm (55kV, 145μA). The region of interest began approximately 490μm below the growth plate. Cortical bone parameters were evaluated at the mid-shaft of the tibia at the same resolution. A well-established script, used in Chapters 2 and 3.1, was used to measure

cortical and trabecular bone volume fraction (BV/TV), endosteal volume, trabecular thickness, trabecular spacing and trabecular number.

Bone marrow adiposity: Following micro-CT analysis, the right tibia was decalcified in 10-14% EDTA for two weeks at 4°C. The fully decalcified bones were then processed and embedded in paraffin wax according to a protocol tested in the lab. 9 μm sections of the proximal metaphysis were evaluated for marrow adiposity following Wright-Giemsa staining. Images were taken using the Zeiss inverted microscope at a magnification of 2.5X. Adipocyte ghosts were quantified using NIH ImageJ for area occupied by adiposity, adipocyte number per area and adipocyte size which were done a sole blinded scientist.

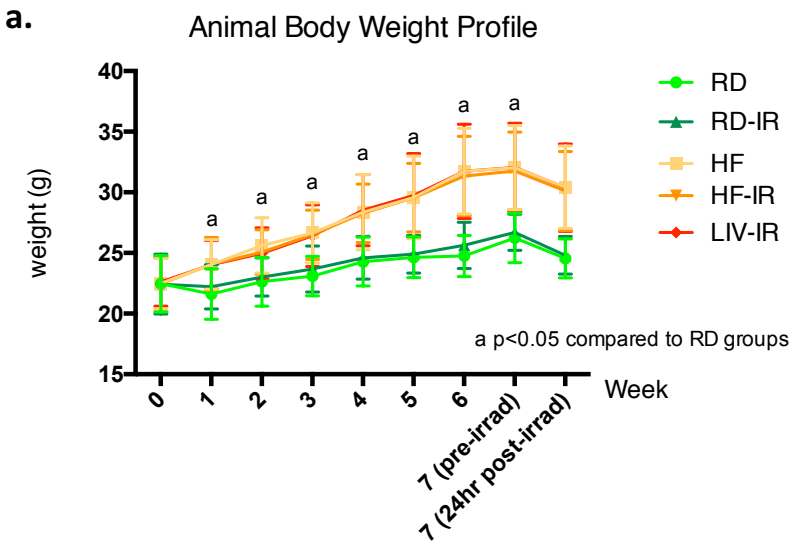
RT-PCR gene analysis: Bone marrow from the left tibia was flushed with RNA-later solution at sacrifice. For RNA extraction, samples were centrifuged several times before discarding supernatant. Pellets were then processed using the RNeasy Mini Kit to obtain purified RNA. Following quantification and purity verification using a spectrophotometer (NanoDrop, Thermo Scientific, DE, USA), a high capacity cDNA reverse transcription kit (Applied Biosystems, CA, USA) was used to convert to cDNA. All cDNA samples were diluted to a concentration of 1ng/μl using DNase-free water. Expression levels of TNF-α and IL-6 genes, both associated with inflammation (see section 3.1) was carried out using the Taqman gene expression assay (Applied Biosystems, CA, USA) on the StepOnePlus PCR machine (Applied Biosystems, CA, USA). Beta- Actin was used as the endogenous gene control and used to calculate relative expression of the target genes.

Statistical analysis: All data are presented as mean ± SD. Statistical tests and graphs were done using Graph-Pad Prism software. A two-way ANOVA with tukey post hoc was used to test differences between RD, HF, RD-IR and HF-IR to look at the diet and irradiation effects. One-way ANOVA with tukey post hoc was used to test differences between irradiated groups, RD-IR, HF-IR and LIV-IR. An unpaired student t-test was used between RD and HF groups. Significance was found if $p<0.05$ for all tests.

Results

Obesity phenotype is achieved with 7w on high fat diet

Animals on the high fat diet were significantly heavier than the regular diet controls in just 1w following the diet (+10%, $p<0.05$, Fig 5a). The HF animals remained heavier than RD throughout the duration of the study. LIV had no significant impact on body weight. At the conclusion of the study, following 24h of total body irradiation, all animals lost about 5-7% weight due to stress from radiation, with no significant differences in weight drop between diets. Fasting glucose measurements taken one day prior to irradiation indicated that all HF animals had significantly increased circulating glucose levels, indicative of pre-diabetic symptoms. Additionally, fat pad wet weights taken at sacrifice also confirmed the development of an obese phenotype, where by both HF, HF-IR and LIV-IR animals has significantly heavier gonadal fat pads compared to RD and RD-IR. There were no noticeably acute effects of irradiation on the fat pads.



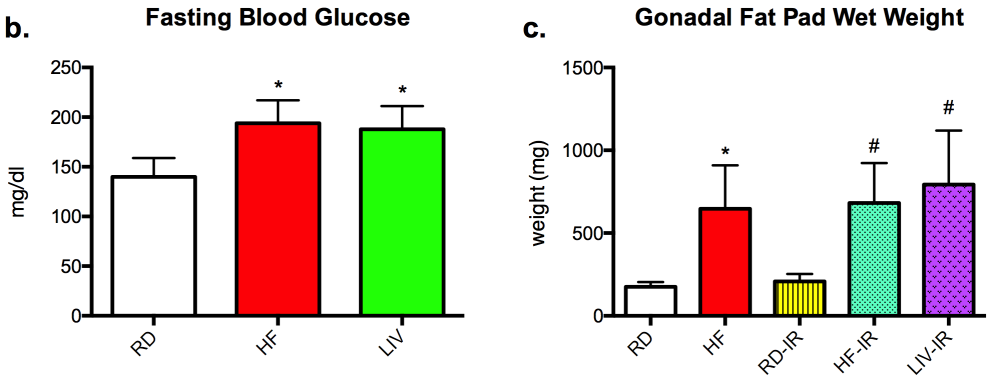


Figure 5. Obesity Phenotype exhibited through a) body weight following 7w on the 60%kcal diet. b) fasting blood glucose levels taken during euthanasia show pre-diabetic signs in both HF and LIV. c) gonadal fat pad weights are significantly increased following diet and do not show changes following acute radiation exposure. Student T-Test between RD and HF shows significance if *p<0.05. one-way ANOVA with tukey post hoc shows significance if #p<0.05 compared to RD-IR.

Irradiation cripples circulating leukocyte population regardless of diet

Cardiac blood leukocytes evaluated through flow cytometry show no significant differences between the RD and HF groups (fig 5.2), indicating that 7w on the high fat diet did not significantly alter circulating leukocyte populations as previously shown by Adler et al. Irradiated groups also do not show any impact of either diet or LIV on any leukocyte populations within the blood. However, we do see severe impacts on blood leukocytes 24h following total body irradiation compared to non-irradiated RD and HF groups. Leukocyte populations rapidly declined mainly due to a drop in T and B Cells. 2way ANOVA analysis showed significant declines in B cell populations by 92% and 93% (both, $p<0.05$) in RD-IR and HF-IR, respectively compared to RD and HF, respectively. T cell populations were reduced by 94% and 95% (both, $p<0.05$) in RD-IR and HF-IR, respectively compared to RD and HF, respectively. Interestingly, irradiated animals exhibited an expansion in myeloid populations compared to non-irradiated mice. RD-IR animals exhibited a 106% increase ($p<0.05$) while the HF-IR animals exhibited a non-significant 63% increase compared to RD and HF non irradiated animals, respectively.

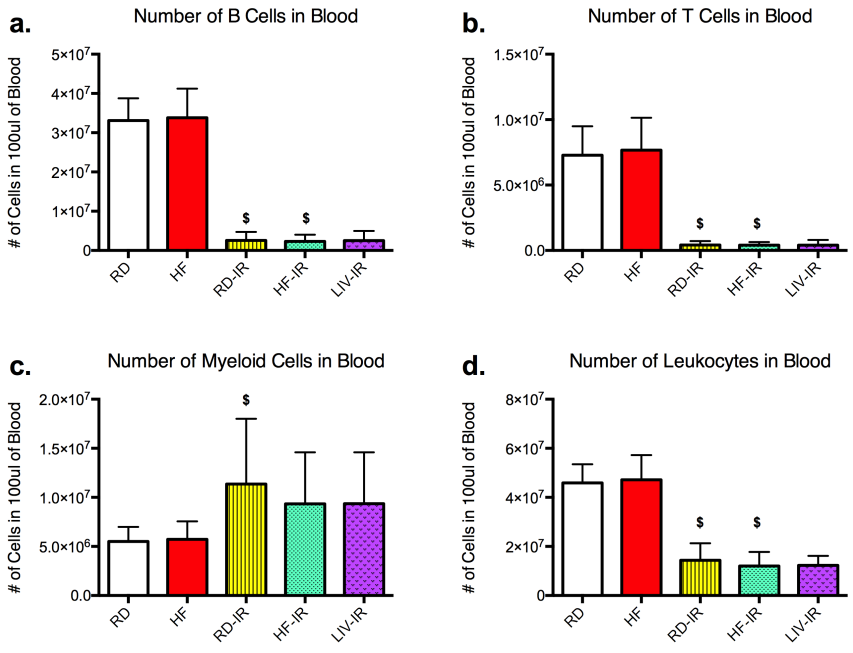


Figure 5.2. Cardiac Blood Leukocyte show no significant differences between diet. Radiation induces expansion of myeloid cells while lymphocytes drop a) B Cells b) T Cells c) Myeloid Cells and d) total leukocytes, which include B, T and Myeloid Cells. two-way ANOVA between non irradiated and irradiated RD and HF animals show significance if $p<0.05.

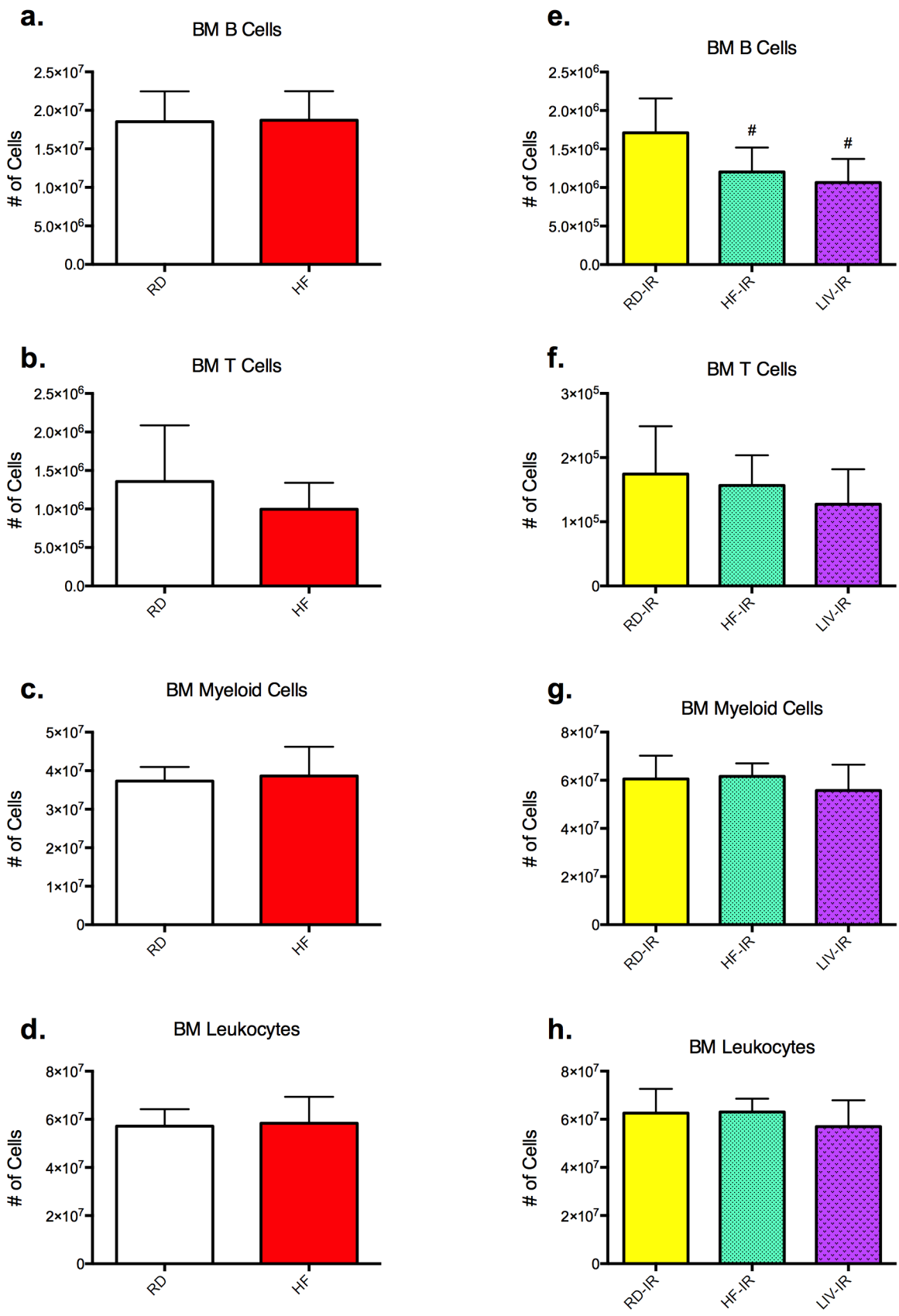
Bone marrow B Cells in obesity are more susceptible to irradiation damage

Bone marrow leukocyte populations did not show significant differences following 7w on a high fat diet. B Cells, Myeloid cells and total leukocyte populations were not different between RD and HF. T cells, although not significant, showed a 27% decline in HF animals compared to RD. 24h following irradiation, irradiated animals followed similar trends as that seen in the blood, with tremendous declines in B and T Cells and an expansion of the myeloid pools. Interestingly, the impact of the high fat diet on marrow leukocytes was prominent only in irradiated animals. B Cell populations in HF-IR and LIV-IR were significantly reduced compared to RD-IR by 30% and 37% (both, $p<0.05$). T Cell populations showed a 26% and 36% (both, n.s.) decline in HF-IR and LIV-IR groups, respectively compared to RD-IR. Myeloid cells and total leukocytes however did not show any differences between the irradiated animals. In addition to mature hematopoietic cell analysis, HSC progenitors, characterized as c-kit+, sca1+ and lin- (KLS cells), were also identified in the marrow cavity. These KLS cells were significantly suppressed by high fat diet,

with HF animals exhibiting 21% ($p<0.05$) decline compared to RD. Following irradiation however, this population fell and showed no differences between RD-IR, HF-IR or LIV-IR.

Obesity accelerates damage to bone marrow cells following irradiation

Annexin V staining of bone marrow cells 24h following lethal irradiation revealed increases in both RD and HF groups as was expected due to the high dose of irradiation exposure. Interestingly however was the greater deficit seen in HF animals compared to that in RD at this time point. While RD-IR animals revealed at 10% (n.s.) increase in early apoptotic cells (annexin v positive) compared to RD, HF-IR revealed a 52% ($p<0.05$) increase compared to the non-irradiated HF animals. Similarly, late apoptotic cells (annexin v and PI positive) increased by 18% (n.s.) in RD-IR compared to RD while increasing 159% ($p<0.05$) in the HF-IR animals compared to the HF.


a.
BM B Cells
# of Cells
2.5×10^7
2.0×10^7
1.5×10^7
1.0×10^7
5.0×10^6
0.0
RD
HF
b.
BM T Cells
# of Cells
2.5×10^6
2.0×10^6
1.5×10^6
1.0×10^6
5.0×10^5
0.0
RD
HF
c.
BM Myeloid Cells
# of Cells
5×10^7
4×10^7
3×10^7
2×10^7
1×10^7
0
RD
HF
d.
BM Leukocytes
# of Cells
8×10^7
6×10^7
4×10^7
2×10^7
0
RD
HF
e.
BM B Cells
# of Cells
2.5×10^6
2.0×10^6
1.5×10^6
1.0×10^6
5.0×10^5
0.0
#
#
RD-IR
HF-IR
LIV-IR
f.
BM T Cells
# of Cells
3×10^5
2×10^5
1×10^5
0
RD-IR
HF-IR
LIV-IR
g.
BM Myeloid Cells
# of Cells
8×10^7
6×10^7
4×10^7
2×10^7
0
RD-IR
HF-IR
LIV-IR
h.
BM Leukocytes
# of Cells
8×10^7
6×10^7
4×10^7
2×10^7
0
RD-IR
HF-IR
LIV-IR

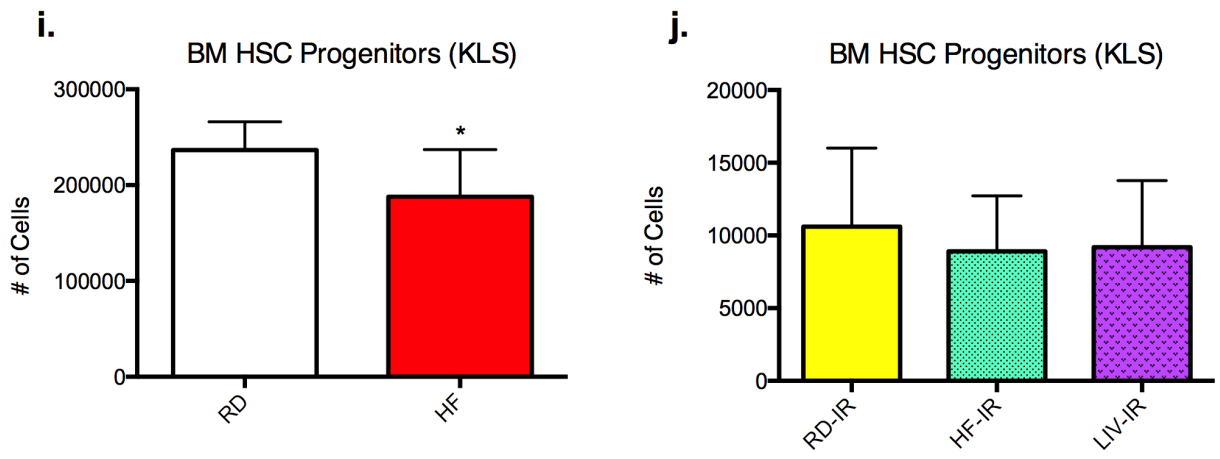


Figure 5.3. Bone marrow leukocyte and HSC progenitor populations are shifted with diet and radiation. a-d) 7w of DIO did not significantly impact B, T, myeloid cells and total leukocyte populations. e-h) 24h following irradiation B cells were suppressed in HF-IR, T cells dropped in all groups and myeloid cells increased. i) DIO significantly suppressed ckit+sca1+lin- HSC progenitors while j) irradiation suppressed progenitor pools in all groups. Student T-Test between RD and HF showed significance is *p<0.05 and one-way ANOVA with tukey post hoc showed significance if #p<0.05 compared to RD-IR.

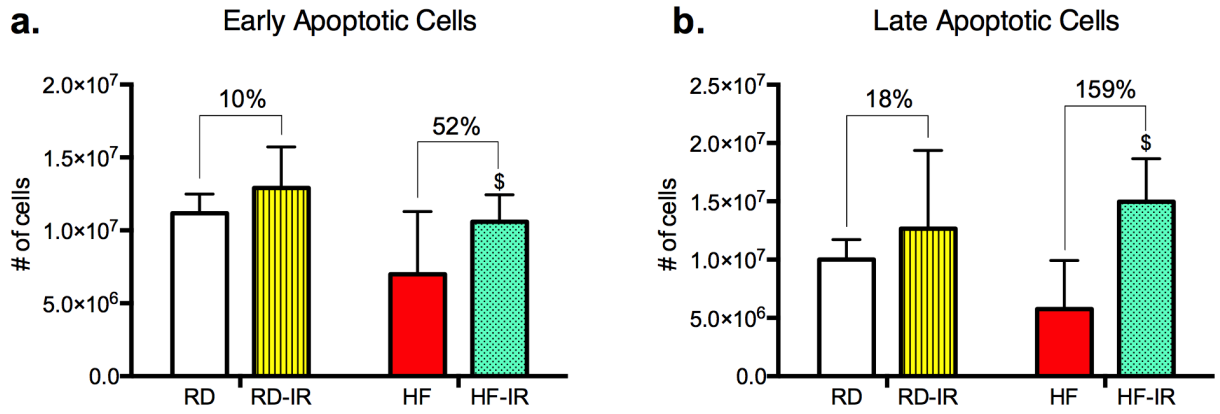


Figure 5.4. Apoptotic cells increased in obese bone marrow following irradiation. a) early apoptotic cells are positive for annexin V and b) late apoptotic cells are positive for annexin V and propidum iodide. two-way ANOVA with tukey post hoc indicated significant differences if $<0.05.

Bone marrow adiposity increased by high fat diet is suppressed by LIV

Giemsa stained sections of the proximal tibia were quantified for bone marrow adipocyte ghosts. The HF animals exhibited a significant 114% ($p<0.05$) increase in marrow adiposity compared to RD resulting from significantly increased size of individual cells (68%, $p<0.05$) and a moderately increased cell number (18%, n.s.). Irradiated HF animals, HF-IR, showed similar strong trends of increased adiposity compared with RD-IR, with a 127% and 103% (both n.s.) increase in

total adiposity and adipocyte number, respectively, compared to RD-IR. Effects of acute radiation exposure did not show statistical significance and were moderate compared to that of the diet. Trends between the RD and RD-IR groups exhibited a 47% reduction in cell number with a 23% increase in cell size resulting in a 34% reduction in total adiposity. However, trends between HF and HF-IR, while having a 30% decrease in total adiposity, realized a 24% decrease in cell size but only a 10% reduction in cell number. Interestingly, animals exposed to LIV prior to irradiation exhibit a 44% (n.s.) decline in total adiposity with 39% (n.s.) fewer adipocytes and no differences in cell size compared to HF-IR.

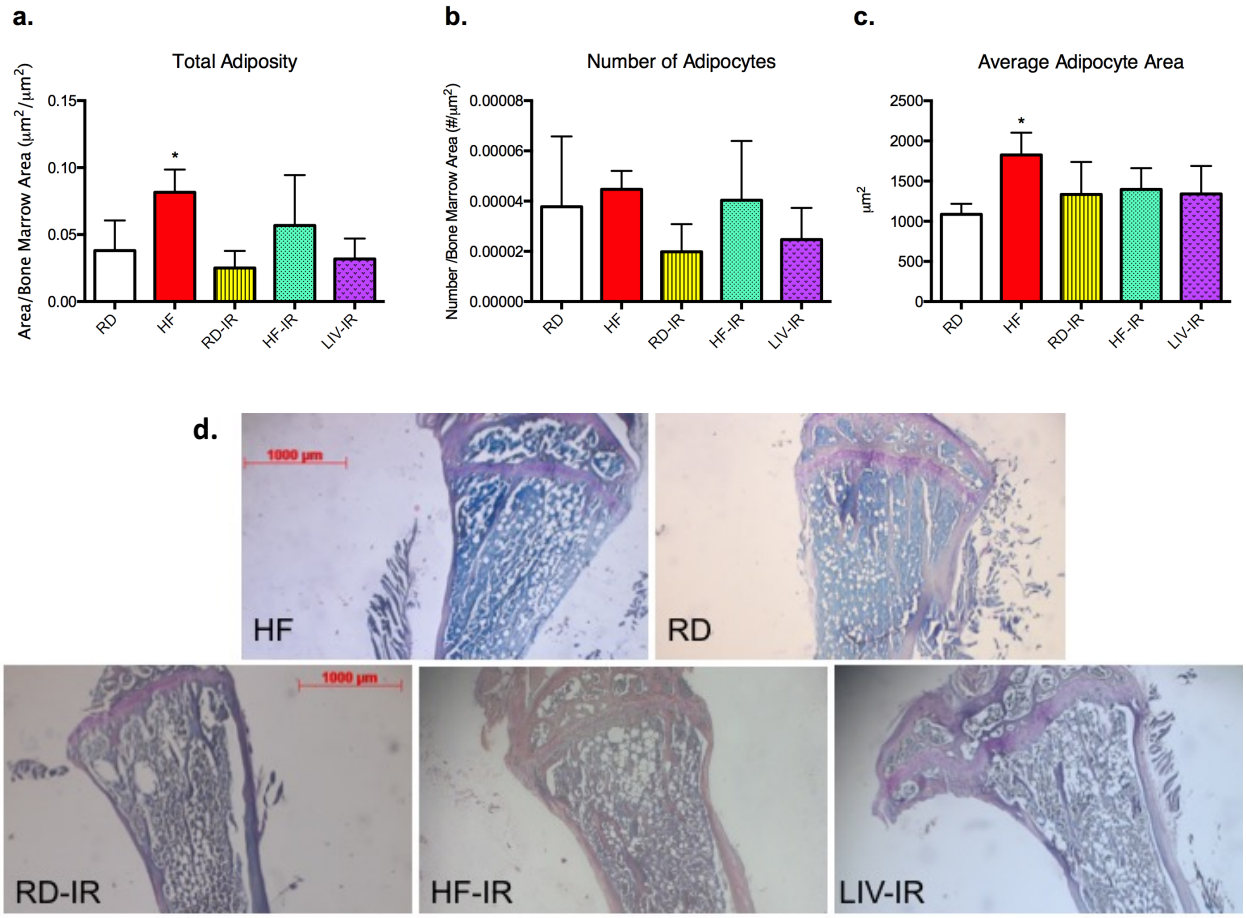


Figure 5.5. Bone marrow adipocytes quantified in the proximal tibia are increased in DIO and suppressed after LIV. a) Total adiposity includes total area occupied by all adipocytes per area quantified. b) number of adipocytes counted in the same area of interest c) average area of all adipocytes occupying area of interest. d) representative images of giemsa-stained sections of proximal tibia. Student T-test between RD and HF shows significance if *p<0.05.

Evidence of inflammation in obesity and suppressed by LIV

Inflammatory cytokines measured through RT-PCR in bone marrow aspirates presented evidence of inflammation within HF animals compared to RD with a 51% (p=0.09) and 53% (p<0.05) increase in TNFα and IL-6, respectively. Irradiated animals on a high fat diet showed no differences in TNFα compared to RD-IR however, those subjected to LIV do display a 38% (n.s.) decline in expression compared to HF-IR animals. IL-6 expression on the other hand was significantly reduced in both irradiated HF animals compared to RD-IR, with a 50% (p=0.07) and 46% (n.s.) decline in HF-IR and LIV-IR, respectively. Although not statistically significant, the impacts of radiation were prominent in TNFα expression in RD and RD-IR mice, where the expression in RD-IR was 76% (n.s) greater than RD. HF-IR mice showed a greater difference in IL-6 expression, with a 68% (n.s) compared to HF animals.

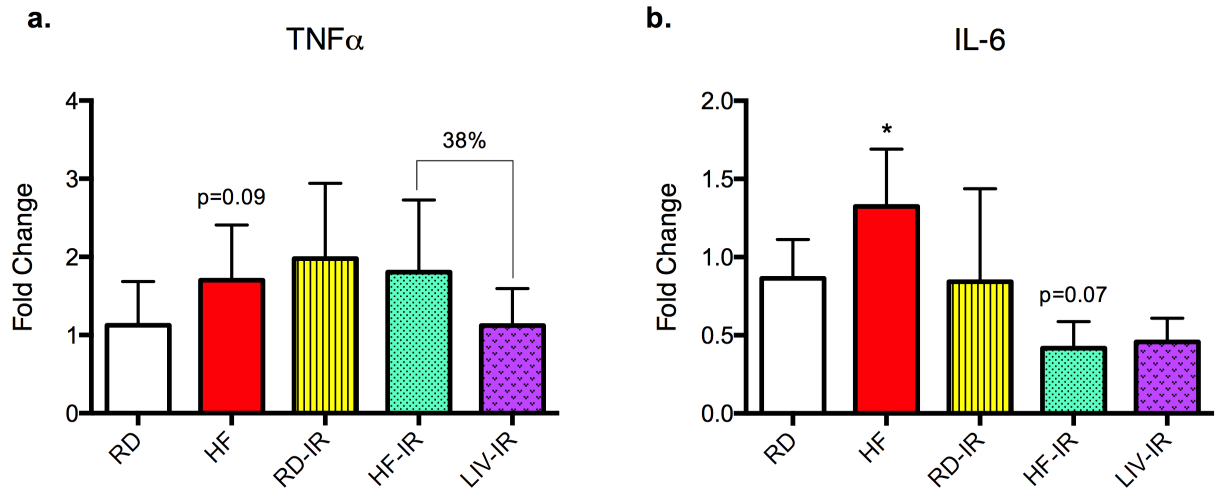


Figure 5.6. Inflammatory marker mRNA expression in bone marrow indicates increased inflammation in HF animals. a) TNF-α mRNA expression and b) iL-6 mRNA expression. Student T-test between RD and HF indicate significance if *p<0.05.

Trabecular bone in obesity is more susceptible to damage and protected by LIV

Ex Vivo micro-CT analysis of the proximal tibia metaphysis revealed no statistically significant differences in trabecular BV/TV following 7w on a high fat diet although did show a trend toward a 15% (n.s.) decline compared to RD animals. HF animals also revealed a 5% (p<0.05) decline in trabecular thickness compared to RD with no significant differences in trabecular spacing or number. Interestingly however, 24h following lethal irradiation, HF-IR

animals exhibited a significant 29% ($p<0.05$) decline in trabecular BV/TV compared to RD-IR, with damage to trabecular thickness and number, 10% ($p<0.05$) and 14% ($p=0.06$) declines, respectively. Treatment with LIV for 7w prior to irradiation showed a partial protection of the trabecular BV/TV with a 14% (n.s.) increase compared to HF-IR. This similar trend is realized in trabeculae architectural parameters as well.

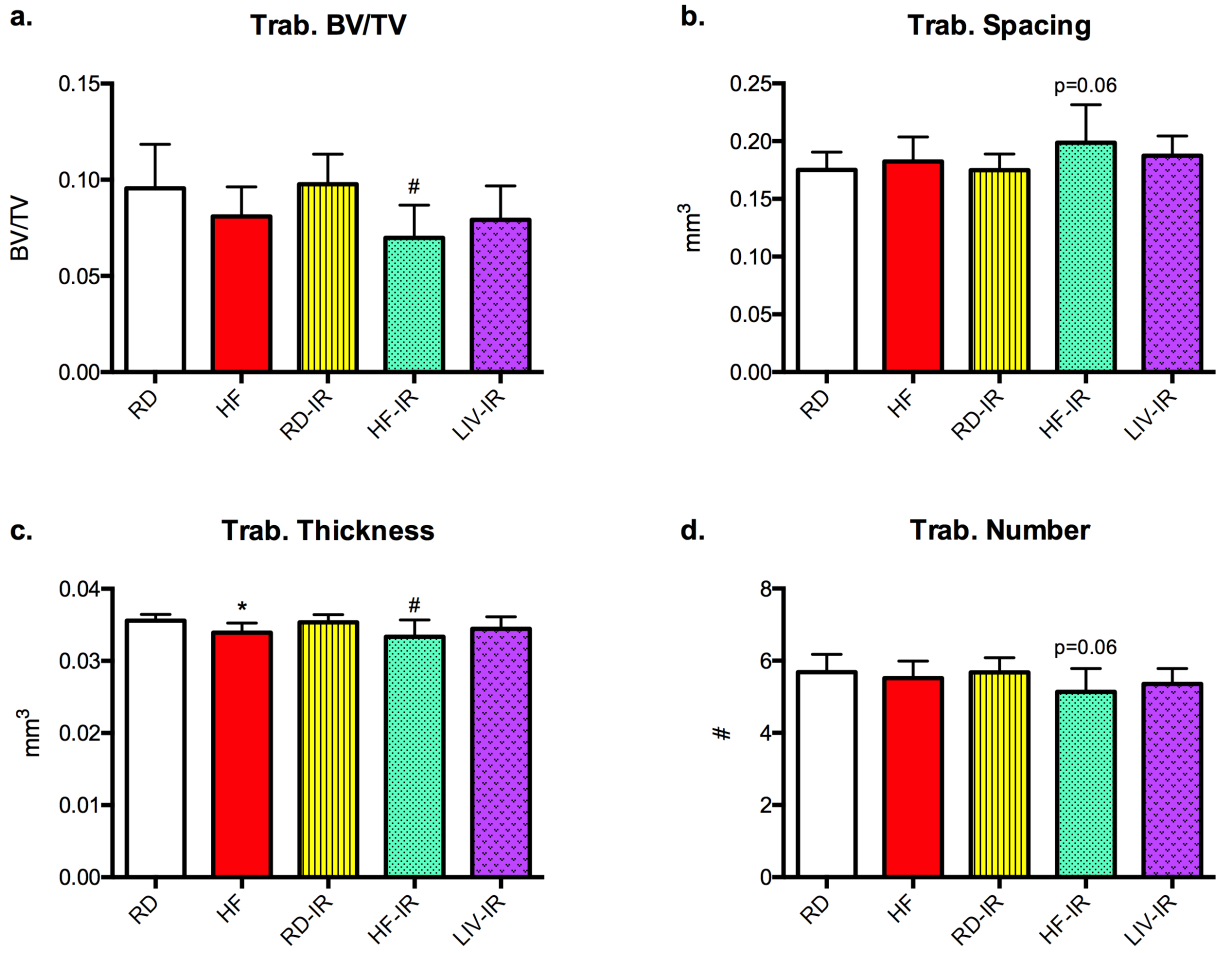


Figure 5.7. ex vivo Micro-CT analysis of trabecular bone shows decline in BV/TV in obese mice following irradiation. a) trabecular BV/TV b) trabecular spacing c) trabecular thickness and d) trabecular number. Student T-test between RD and HF is significant if *$p<0.05$. one-way ANOVA with tukey post hoc is significant if #$p<0.05$.

Suppressed bone marrow mesenchymal stem cell population in obese irradiated mice

Quantification of mesenchymal stem cells within the bone marrow through flow cytometric analysis indicated no significant differences in this population as a result of diet between RD and HF animals. However, 24h following radiation exposure, the population

significantly increased in RD-IR compared to RD by 68% ($p<0.05$) however we do not see a similar expansion of MSCs in HF-IR compared to HF. HF-IR animals present 34% increase ($p>0.05$, half of that seen with RD-IR. Differences between the irradiated groups indicated a significant 26% and 30% (both, $p<0.05$) decline in MSC population in HF-IR and LIV-IR compared to RD-IR, respectively. LIV-IR did not significantly differ from the HF-IR animals.

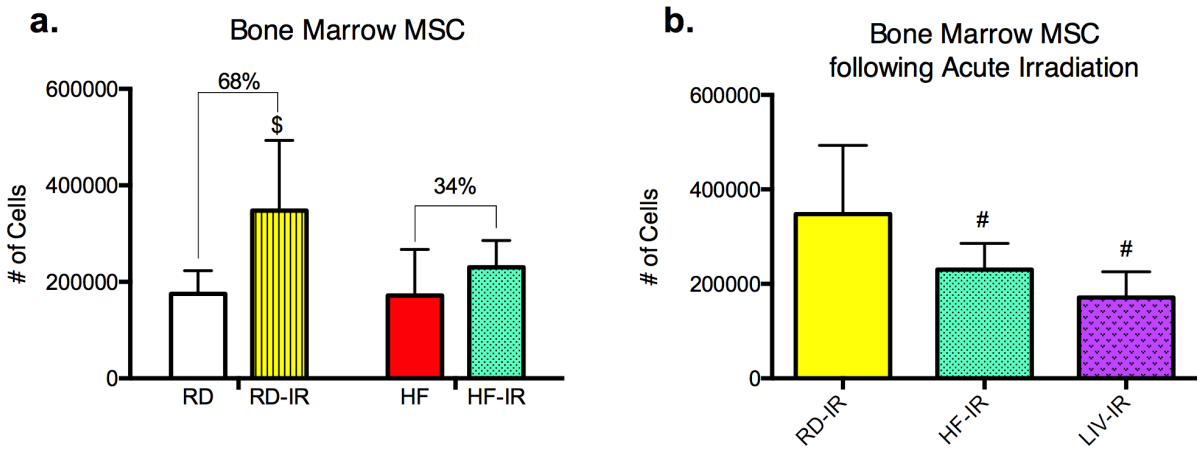


Figure 5.8. Mesenchymal stem cell population in bone marrow. a) Difference between irradiated and non-irradiated animals are great with RD. b) among the irradiated animals both HF-IR and LIV-IR have suppressed MSC pool compared to RD-IR. two-way ANOVA with tukey post hoc indicated significance if $\$<0.05$. one-way ANOVA with tukey post hoc indicated significance if $\#p<0.05$ compared to RD-IR.

Discussion

It is now well established that obesity is associated with immune dysfunction. With reports of disproportionate immune cell populations accompanied by increased inflammatory cytokine expression, the obese condition also impacts the host's ability to respond to hematologic stresses such a radiation or infection [23, 29, 79, 119, 132, 144, 186]. Reports looking at the influence of obesity when infected by the influenza virus, showed increased mortality, impaired wound healing and altered immune response by delayed pro-inflammatory cytokine expression compared to the response by lean infected hosts [187, 188]. Additionally, obesity also alters the way in which the body responds to antibodies aimed at fighting infectious disease [186, 189]. Here we set out to investigate the response of an obese condition to acute exposure to high doses of ionizing radiation. We also aimed to better understand whether the initial

immune response might influence the way in which hematopoietic stem cells engraft and repopulate following transplantation in obese and lean animals, seen in Chapter 3 and 4.

In the previous chapter we saw that following BMT, obese mice presented with delayed body weight recovery and B cell reconstitution in the blood. Here following acute exposure to high doses of radiation with no rescue transplant, obese mice exhibited greater deficits to immune cellularity, trabecular bone quantity and general marrow health compared to their lean irradiated counterparts. Analysis of the specific leukocyte lineages indicated significantly suppressed B lymphocytes in irradiated obese animals. Since differences in B cells were not significantly different between non-irradiated RD and HF animals, the suppression was indeed a response to radiation. While mature hematopoietic cells are sensitive to and expected to die from radiation, the fact that differences were present between the diet groups suggests that the response to radiation is altered in the obese animals. Additionally, it is notable that the influence of obesogenic factors was not exhibited in peripheral blood leukocyte analysis and only seen in the bone marrow. This might be indicative of greater diet induced changes to the marrow microenvironment that likely contributed to the further crippling of B cell populations in HF-IR mice following irradiation. Previous studies have suggested that diet-induced obesity specifically hinders B cell development [132]. Additionally, we see that HF animals exhibit suppressed HSC progenitor pools even without radiation. We also see a significant up-regulation of myelopoiesis in response to the acute inflammation due to radiation. Taken together, it is likely that as many HSC progenitors died from radiation damage, the few that did survive were primarily contributing to the demands of the myeloid cells, favoring myelopoiesis at the expense of both B and T lymphocytes. Apoptotic cells in the marrow were also consistent with and possibly contributed to the reduction in B cells. Here we saw significantly greater numbers of early and late apoptotic cells in irradiated obese mice than in irradiated lean mice, compared to their non-irradiated HF and RD controls, respectively. Given this, we can infer that a greater number of B cells were going into apoptosis in high-fat fed mice causing the significantly greater deficit seen following radiation. However, since we cannot distinguish between specific apoptotic cell types, it is difficult to prove this to be true.

It has been well established that ionizing radiation exposure severely damages bone quantity and quality [190, 191]. It has also been suggested that in addition to increased osteoclastic activity, physiochemical deterioration of the bone matrix likely contributes to the bone loss [190]. The impact of DIO on bone has been controversial, in that some studies indicate a protection of bone as a result of increased loads [192] while others show suppressed bone quantity due to obesogenic factors [193]. Herein, 7w of a high caloric diet did not result in a statistically significant reduction in trabecular bone volume. However, short exposure to lethal doses of gamma radiation resulted in significant damage (29%, $p<0.05$) to the trabecular bone of DIO mice compared to irradiated lean mice. While lean animals did not exhibit alterations to bone phenotype following radiation, DIO animals exhibited a 14% (n.s.) decline after irradiation compared to their non-irradiated HF controls, again emphasizing the greater susceptibility of obese trabecular bone to radiation-damage. Interestingly, when evaluating cellular composition in the marrow we found that while lymphocyte populations significantly declined, myeloid cells, including osteoclast forming monocytes were significantly elevated in all irradiated animals 24h after being irradiated. It is well established that osteoclastic activity is increased following radiation, thus it is not surprising to see this increase in osteoclast progenitors in irradiated animals.[194] However, bone loss from osteoclastic activity is not clearly visible until at least 7d once resorption beings [190, 195], indicating that the decline in BV/TV of the obese animals in this study is primarily a result of matrix deterioration. Therefore, while bone quantity was not visibly damaged in HF animals with no radiation exposure, perhaps material properties would be inferior to that of the lean animals, making them more susceptible to damage by irradiation.

Interestingly, the low intensity vibration intervention delivered to the obese mice for 7w prior to whole body irradiation, showed a partial protection of the trabecular bone volume fraction compared to the HF-IR and RD-IR groups. The salutary effect of mechanical signals has been evidenced in others models of metabolic and adipose burdens as well [79, 185]. LIV's impact on bone quantity has been in part associated with a bias of bone marrow mesenchymal stem cells towards osteoblastogenesis at the cost of adipogenesis [100]. While we don't have evidence of increased osteoblastogenesis we do see an adipose phenotype in the marrow that is consistent with this relationship. In the proximal tibia metaphysis, in conjunction with reduced trabeculae,

we also see significant accretion of marrow adipocytes in HF (114%, $p<0.05$) and HF-IR (127%, n.s.) animals compared to RD and RD-IR, respectively. This increase was a concomitant increase in number and size of individual adipocytes. However, while preserving trabecular quantity, HF animals exposed to LIV prior to irradiation (LIV-IR) also exhibit suppressed accumulation of MAT, with fewer adipocytes occupying the marrow space. The lack of the marrow phenotype prior to irradiation nor the comparison to a lean irradiated and vibrated control precludes precise assessment of LIV's protection from DIO or from acute irradiation. However, given the severity of the irradiation dosage, its negative effects on the marrow niche, and the absence of previous evidence of its protection again ionizing radiation, suggests that LIV's salutary effects might have taken place prior to irradiation exposure. A closer look at the bone marrow mesenchymal stem cell population, progenitor of fat and bone cells, indicated a significant reduction in both HF-IR and LIV-IR compared to RD-IR. Unfortunately, we cannot make any claims regarding mechanical biasing of MSCs by LIV in this model due to the greater influences of ionizing irradiation. However, it is notable that the MSC population increased following irradiation in both RD-IR and HF-IR compared to non-irradiated RD and HF, respectively. Moreover, increases in RD-IR animals are significantly greater, almost double, than that seen in HF-IR compared to RD and HF, respectively. MSCs are generally considered more radio-resistant under conditions that would be lethal to hematopoietic cells [196]. Additionally, their immuno-modulatory effects promote their migration towards sites of inflammation [197, 198]. The current study might be indicative of impaired response of obese MSCs, either those residing in the marrow or those migrating from the periphery to the acute hematologic stress. Whether the MSCs in the obese mice are not able to proliferate or migrate would be interesting to explore. The possible impairment in MSCs, either in their proliferation, migration or differentiation can also impact the interaction between MSCs and HSCs. If the interaction is disrupted in an obese animal, it is likely that the repopulation capabilities of the HSCs that follow the acute stress can also become altered.

Additionally, mRNA analysis of pro-inflammatory cytokine, TNF-α, was upregulated in the irradiated animals. While this cannot explain the lack of expansion of MSCs in HF-IR, this increased inflammatory state might partially explain the increased presence of MSCs in RD-IR animals. However, the reduced numbers of MSCs in LIV-IR animals is consistent with reduced

TNF-α expression and MAT in LIV-IR marrow. Taken together LIV partially protected the obese bone marrow microenvironment from damage by irradiation through its preservation of trabeculae and suppression of marrow adiposity. While the mechanism is suggested to be through the biasing of MSCs, the impact of irradiation precludes this observation, suggesting that LIV's protective effects occurred prior to irradiation and were protecting from the DIO insult. Additionally, more current research suggests that MA might be formed from a distinct progenitor population different from those that give rise to WA [199, 200]. Thus while our analysis of MSCs is interesting, the relationship of this rather loosely defined population to MAT in our study must be taken into consideration.

In conclusion, we see that obese animals are more susceptible to bone marrow damage from acute radiation exposure. The significant reduction of B lymphocytes might be attributed to increased apoptotic cells in obese mice. This suppression could also be influencing increased engraftment and reconstitution by transplanted donor cells as seen in Chapter 3. Additionally, trabecular bone quantity was only significantly damaged in obese mice following radiation exposure which could be due to alterations to the physiochemical properties of bone due to DIO and radiation. The marrow phenotype was also characterized by greater adiposity in obese animals which persisted following irradiation. Interestingly, treatment with LIV prior to irradiation partially protected the obese animals from the radiation induced damage as seen in the partial protection on trabecular BV/TV and suppression of total marrow adiposity. The severity of the radiation insult however overshadows any means of elucidating the mechanism of LIV action. However, these data bring light to the fragility of the obese system especially the impaired response of the hematopoietic cells to acute systemic stress and the impaired structural network within the marrow niche (i.e. trabeculae and adiposity). Lastly, although low intensity vibrations cannot prevent damage induced by acute irradiation, it did prove to partially protect the marrow niche, which could possibly provide a healthier HSC niche for faster recovery in the obese animals following BMT.

Limitations

The reason for the high dose of radiation tested was to be able to provide a sort of baseline for the BMT transplant studies that took place in section 3.1. However, to get a better understanding of the response to radiation exposure, it might be more beneficial to look at the response after lower doses of sub lethal radiation. In this way, there is still a systemic stress caused by ionizing radiation but damage to the system is not as severe. Previous studies looking at a sub-lethal radiation dose of 5Gy still showed a 65% decline 2 days following exposure [191]. This study shows that even half the dose that we administered can still injure the system, but allow at least half the population a chance to survive and respond to the stress.

While we did look at the level of apoptotic cells, it would have been more beneficial to be able to distinguish the types of cells going into apoptosis following radiation. Future experiments might include the annexin v stain into each leukocyte staining assay. This could help us to understand why B cell populations were so much more sensitive to radiation in the HF animals than in the RD. Mesenchymal stem cells were distinguished based on positive expression of CD105, CD90.2 and Sca-1. While these markers have been used in literature to distinguish this population [201, 202]. Due to the lack of universally accepted markers to define bone marrow MSCs, MSC data presented here must be taken with a grain of salt. Currently the only accepted way to prove that they are truly MSCs, is by their adherence and differentiation capacity in vitro. Additionally, it would be interesting to investigate whether MSC's were in fact proliferating or whether cells were migrating into the marrow from the periphery, which can be accomplished by injecting mice with Bromodeoxyuridine (BrdU) prior to sacrifice.

Lastly, this experiment provided a rather preliminary look at the protective effects of LIV in the obese conditions response to acute radiation. Due to the severity of the radiation, it was difficult to ascertain possible mechanisms through which the mechanical signals exerted their influence. For a more robust analysis of LIV in vivo, an additional two groups could be incorporated: one looking at LIV's influence on a lean irradiated mouse and one looking at a HF mouse with no irradiation.

Chapter 5 Summary:

1. Obesity leads to greater damage to the bone marrow follow acute exposure to irradiation characterized by increased apoptotic cells, greater suppression of B-Cells, deterioration of trabecular bone and suppression of mesenchymal stem cells.
2. This damage might partially predetermine the outcome of HSC transplantation in obesity towards greater dependence on donor cell engraftment and repopulation as seen in chapter 3 and 4
3. LIV partially protects the bone marrow by protecting trabecular bone mass and suppressing marrow adipose accretion in obese mice following acute exposure to irradiation.

Chapter 6 Conclusions

In this work, we set out to investigate the impact of an adipose burden on hematopoiesis and hematopoietic stem cell function. The obesity epidemic has prompted many studies in trying to understand how the presence of excess adipose tissue affects important functions within the body such as neural systems [203], skeletal remodeling [79], cardiovascular function [149], and especially the immune system [119]. The increased risks of infection and illness suggest a negative correlation between excess adiposity and immune function and therefore the hematopoietic stem cells that maintain it. The accumulation of adipose tissue in morbid obesity spreads beyond visceral depots to internal organs (i.e. liver) [149], musculature [204], and the bone marrow [132]. Bone marrow fat, present also in physiological states, respond to high caloric intake and inactivity in much the same way as visceral adipose depots [30, 205]. While the impact of visceral adiposity is much more widely studied, the influence of MAT to immunity and specifically HSCs is still not fully understood.

In addition to understanding the relationships between MAT and HSC functionality, we also sought to investigate an intervention aimed at counteracting the alterations excessive adiposity elicits to the system. Low intensity vibrations (LIV) are a non-pharmacologic exercise surrogate. They are found to bias the differentiation of mesenchymal stem cells in the marrow promoting osteoblast formation while also suppressing the formation of fat cells [100]. In the first part of this dissertation work ovariectomized (OVX) mice were used as a model to induce a systemic adipose burden. We found that while OVX resulted in a significant MAT accumulation, 6w of LIV intervention was able to significantly suppress the amount of fat, especially by reducing the size of individual adipocytes. Additionally, OVX animals exhibited a 40% decline in trabecular quantity just two weeks after estrogen cessation, after which the LIV treatment began. Due to the severity of bone loss, LIV was not able to visibly restore trabecular bone already lost however serum alkaline phosphatase levels indicated increased osteoblast activity in LIV treated animals. This anabolic effect of LIV suggests an initial push towards restoration of bone, especially since bone remodeling has normalized by this time point in OVX mice. Sustained exposure to the treatment could potentially lead to some visible promotion of bone formation. Alterations to the LIV protocol (i.e. intensity/frequency/duration and refractory periods) might also “improve” outcomes. While the 15 minutes per day protocol was successful in promoting bone formation

in less severe models [100], the dramatic changes caused by estrogen depletion might make the system less responsive to such brief exposure to the mechanical signals. Additionally, recent evidence suggests that the application of refractory periods, where by the mechanical stimulus is delivered in two bouts separated by a few hours has shown to enhance the effects of the signal [101]. Timing of the start of intervention is also important to consider especially when contending with translation of such therapies to human. In fact, LIV was shown to prevent trabecular bone loss when applied immediately upon removal of the ovaries for just 2w, emphasizing its protective effects on the marrow niche. This work was published in Osteoporosis International in 2016 [185]. Thus while exercise so clearly provides benefits to the whole body, the work presented here suggests that in cases when exercise might not be an option, subtle mechanical signals might pose as a surrogate.

Furthermore, the increased marrow adipogenesis was accompanied by disruptions in immune cell populations in the marrow of OVX mice. The abnormally high proportion of B cells and deficiencies in T and myeloid cells, suggests an impairment in the ability of HSCs to regulate the generation of immune cells, which can also impact the way in which they respond to stress. A closer look at HSC (LSK+ and SP) populations in the marrow showed no negative impact of long term (8w) estrogen depletion on these stem cell pools. Thus it was hypothesized that environmental factors in the bone marrow emanating from the adipocytes, were altering the behavior of the HSCs activating B lymphopoiesis and suppressing differentiation of the other immune populations. It is important to point out that hematopoietic cells carry estrogen receptors and therefore can be directly influenced by the lack of estrogen signaling. This makes it quite difficult to differentiate hormonal influences versus that of the excess adiposity. The lack of influence by the subtle mechanical signals can also be explained by the stronger influence of estrogen on the system. To test whether HSC stem cell functionality was in deed affected by the adipose burden, either inherently or by environmental influence, the ability of the stem cells to engraft and reconstitute an ablated hematopoietic system must be assessed using bone marrow transplantation.

In an effort to avoid the influence of estrogen on HSC behavior, the second part of this work used a diet induced obesity model to specifically look at influences of excessive fat. Physiological levels of marrow adiposity have been negatively correlated with HSC presence as well as their engraftment post transplantation [29]. However, studies investigating the impacts of pathological MAT are scarce. A recent study indicated that HSCs derived from obese mice are "re-programmed" to promote myelopoiesis and the development of pro-inflammatory macrophages when transplanted into lean irradiated hosts [130]. Obesity however elicits a multitude of changes within the marrow including damage to the trabecular network [79] with increased osteoclast numbers [146], increased adipose accretion[132] as well as increased expression of pro-inflammatory cytokines [174]. Systemic changes induced by obesity such as excessive adiposity in visceral cavities, increases blood glucose, as well as chronic inflammation can also induce changes to bone marrow hematopoiesis and especially HSC functionality. To test this, HSCs extracted from healthy lean donors were transplanted into either lean or obese irradiated mice and were evaluated for their ability to engraft and reconstitute within those distinct environments.

First an appropriate model enabling us to see differences between lean and obese conditions had to be determined. In the clinic bone marrow transplants are a routine procedure for treating patients with hematologic malignancies. The number of stem cells to transplant into a patient is dependent on his or her weight and the type of cancer being treated [206]. (American Society for Blood and Marrow Transplantation) In this work, three doses of HSC transplants were tested. To our surprise, engraftment of HSCs was not hindered in obese animals no matter the dose delivered. In fact, we saw that the highest dose transplanted resulted in a significant engraftment in obese compared to lean animals. While this suggests an improved HSC response, we cannot explicitly claim that this has a positive or negative influence on hematopoietic recovery. Comparison of body habitus and survival with other doses revealed that even mice injected with the lowest dose which showed very little to no engraftment recovered from irradiation. Additionally, size of bone marrow cavities showed increasing trends in all HF animals compared to their respective RD controls, regardless of their dose, suggesting that a greater cavity volume does not always lead to greater engraftment. Thus size of the marrow cavity might also be highly

uncoupled with the engraftment of HSCs into the marrow. Knowledge of the precise number of engrafted cells immediately upon transplantation would also help to understand this, unfortunately we do not have these data. However, it is understood that not all cells engraft in the bone marrow and that some travel to other tissues damaged by radiation as well. Still, it is notable that the severity of the ablation overwhelmed any diet impacts as seen through cell engraftment until we crowded the marrow with a lot of stem cells. Not only does this indicate the importance of developing appropriate experimental models but also brings light to the insufficiency of calculating transplant dosage solely on body weight. With the rise of obesity and the multitude of conditions associated with it, other factors might also have to be considered. Thus, while the high dose is not necessarily the "best" dose for treating obese mice the fact that differences even existed between the lean and obese animals warranted further exploration of the high dose animal phenotypes.

Longitudinal analysis of the body weight and peripheral blood cell recovery exhibited a delay in obese animals. While RD animals lost weight for 1w before weight gain began, the HF animals continued to lose weight for an additional week and maintained this weight until approximately 4w following BMT. Because adipose tissue is sensitive to irradiation [168] and HF animals have significantly more of it, it is likely the drop in weight is a result of damage to adipose tissue. It's also possible the dramatic drop adipose tissue can disrupt energy balance and cause changes in metabolism to compensate for the loss, much like what is seen after abdominal lipectomy [207]. This could also impact hematopoietic recovery in the obese animals. Therefore, it would be useful in future studies to also track changes in subcutaneous and visceral adiposity through *in vivo* μ-CT analysis to understand if recovery of the obese animals is related to the changes in adipose tissue loss and expansion.

At 16w, the bone marrow exhibited increased hematopoiesis in obese animals with significant increases in lymphocytes. It is generally accepted that at 16w following transplantation mature hematopoietic cells are a product of the more primitive transplanted HSCs rather than the progenitor cells used initially to increase animal survival. Thus we can confidently claim that transplanted HSCs directly contributed to increased hematopoiesis in

response to the obesogenic environment they were in. Because the stem cells had a specific tag (CD45.1) we were further able to distinguish the cells differentiated from the donor HSCs. This further confirmed our suspicions evidencing the increased hematopoiesis in obese animals by the same heathy stem cells that were initially injected into both lean and obese animals. It should be noted however, that the initial donor cells were a mixed population of progenitor and primitive HSCs due to the inability to sort enough primitive HSCs for transplantation. Thus it is not possible to attribute the formation of all CD45.1 tagged leukocytes to primitive HSCs per se, even after waiting 4 months to analyze the bone marrow. With better methods of sorting cells, future studies should transplant only tagged primitive HSCs (i.e. SP-HSCS) for a true measurement of the stem cell's response to obesogenic conditions. Nevertheless, these data did indicate that despite ablating the host systems with lethal doses of radiation, reconstitution of the hematopoietic cells was altered in obese animals and resembles the inflamed state characteristic of obese conditions.

Additionally, the significantly increased presence of donor cells in extra-medullary sites such as the gonadal fat pads in the obese animals could be increasing the demand for hematopoietic cell formation in the bone marrow. Hematopoietic cells travel in and out of the marrow daily replenishing peripheral blood cells and responding to inflammation or injury when needed. Over the past few decades, obesity has become classically defined as a disease of chronic inflammation originating within the visceral adipose tissue through recruitment of peripheral leukocytes [149, 208]. In this work, we can see that expansion of the adipose tissue in obese animals concomitantly results in the recruitment of bone marrow leukocytes. This heightened demand signals the increase of bone marrow hematopoiesis, in order to feed the chronic inflammatory condition. In obese patients, chronic inflammation is most commonly defined by increased circulating leukocytes. The lack of increased peripheral blood cells in the obese mice of this study however does not suggest an inflammatory state. It's possible that because the animals were irradiated they have not fully developed the obese phenotype, and perhaps they never fully develop it after radiation exposure. Previous studies showing 6w on the 60% kcal diet presented alterations in the marrow but did not exhibit changes in peripheral blood leukocyte populations [132]. Thus marrow immune phenotypes don't always manifest in the peripheral

blood. Nevertheless, the expansion of adipose depots, presence of increased bone marrow leukocytes as well as the increased marrow hematopoiesis, does suggest an influence of the excessive adiposity on HSC functionality. These data also highlight that obesity disrupts other tissues and systems beyond the bone marrow. Inflammation is one of cornerstones of obesity related complications and the interaction of immune cells in extra-medullary sites might actually lead to the progression and development of the complications. Thus while we did show in this work that adipose tissue inflammation might influence processes within the bone marrow, it is important to investigate other tissue systems affected by obesity such as the liver and pancreas to understand their role in the progression of obesity.

The recruitment of immune populations to fat pads provokes ideas towards new targets for the treatment of obesity. Since we know that bone marrow cells are inclined to migrate towards these extra-medullary regions in obesity, priming of the bone marrow cells to promote anti-inflammatory activities might be a potential strategy to curb the adipose tissue inflammation in obesity. This would in effect also suppress inflammation-induced complications such as insulin resistance. Data suggests that mechanical signals can increase populations of immune-modulatory mesenchymal stem cells while also biasing their differentiation away from fat (REF). Additionally, low intensity vibrations specifically, have the ability to promote anti-inflammatory growth factors including insulin-like growth factor-1, vascular endothelial growth factor and monocyte chemotactic protein-1 while also influencing a less inflammatory phenotype in certain macrophages found in a wound healing model in diabetic mice [209]. Given this, an interesting future study might transplant bone marrow cells primed with LIV treatment either in vitro or in vivo before being introduced into obese animals, to test the ability of these primed cells to suppress inflammation compared to cells that are not primed.

The final study in this work was prompted by the fact that obese mice presented significantly more donor cell hematopoiesis in marrow, blood and fat tissue compared to that in lean animals given the same radiation dose and transplant. Whether the donor cell engraftment was a result of an initially suppressed system in obese animals or whether it was in response to the HF diet consumption that ensued following BMT and recovery was interesting to investigate. High doses

of whole body irradiation is meant to ablate the entirety of host hematopoietic cells in order to accept and engraft donor transplanted cells. However, we see from the experiment that some host cells do survive and contribute to the recovery of the animals. Interestingly, after acute exposure to the high doses of radiation, we saw significantly suppressed B lymphocytes in obese compared to lean animals. This supports the former hypothesis, in that the obese mice although exhibiting no difference in marrow B cells without radiation, are significantly more damaged after radiation than that seen in lean marrow conditions. Additionally, obese animals presented with significantly greater apoptotic cells in the marrow compared to lean animals. While it's possible that more B cells were going into apoptosis, it also brings light to the greater overall damage irradiation causes in obese animals. This was also evident in the trabecular network within the marrow. Radiation-induced bone loss is a well-established phenomenon and one that continues to be a concern in patients undergoing BMT [210]. However, we saw that in just 24h following exposure to high doses of radiation, there was a significant deterioration of trabeculae in obese animals compared to lean irradiated controls. These data evidence the accelerated damage to bone within the marrow compartment experienced by obese animals in response to systemic stresses like radiation. While we don't have information regarding the physiochemical properties of these bone, it would be interesting to analyze if obese bone present inferior bone matrix material properties that are more sensitive to damage by radiation. Taken together, the suppression of marrow lymphocytes and trabecular bone combined with the increase in apoptotic cells in obese mice is likely to have contributed to increased dependence on donor cell engraftment seen in the previous study. However, the overall increased hematopoiesis compared to lean animals is still not fully understood. It is hypothesized that the expanding adipose tissue and its increasing demand for peripheral leukocytes that is driving the marrow hematopoiesis evidenced following BMT.

It has been suggested that the accumulation of marrow adiposity resulting in obesity would prevent successful engraftment of HSCs either due to the lack of space or due to obesogenic factors secreted through marrow adipocytes [29, 130]. We showed in this work that obesity does not inhibit engraftment however it is still unknown the exact role marrow adipocytes play, if at all, in marrow engraftment and reconstitution. However, the fact that there is increased marrow

fat in parallel with increased hematopoiesis, and the fact that these cells are in such close proximity in the small marrow space, suggests a relationship must exist. This relationship might however only be correlative and that systemic disruptions caused by the high fat diet could be driving the chronic inflammation characterized by obesity. Inflammation, one of the primary pathological side effects of obesity is at the heart of most of the obesity- related life-threatening complications. Thus it is likely that inflammatory processes primarily taking place in extra-medullary sites (i.e. visceral fat pads) is inducing the filling of the marrow with adipocytes while also promoting increased hematopoiesis. While we saw that interventions such as LIV were able to curb adipose accumulation in OVX marrow, it was interesting to see that when LIV was delivered in parallel with the HF diet, we saw similar trends towards curbing adipose accumulation. Granted these animals were irradiated, a very clear trend was present between HF animals with and without prior LIV treatment, suggesting that mechanical signals were inducing changes to the obese marrow niche. These changes could potentially impact the way in which the obese animals recover if given a BMT. Additionally, TNF-α, a cytokine that is involved in inflammation and secreted by macrophages, CD8+ T Cells and even adipocytes, was suppressed in the marrow of LIV treated obese animals following irradiation. Although TNF-α is one of the mediators influencing cell apoptosis following irradiation, the fact that there are fewer adipocytes in the marrow of LIV treated animals might also coincide with the downregulation of TNF-α expression in the marrow. On a slightly different note, the response of obese animals to mechanical signals in general has shown mostly positive effects in that it leads to weight loss and suppressed adiposity, among others [1, 37]. However, when investigating their immune response to a mechanical stimulus such as exercise for example, it is different from that of a lean animal, as was reported by Kostrycki et al. [211] Thus while exercise is generally considered to improve health, the adaptations that obesity elicits to the system also change the way they respond to "good" stressors such as exercise. Future studies might include a lean irradiated LIV group to compare their response to this exercise surrogate to that of the obese mice.

While we did not see a positive impact of LIV on OVX bone in parallel with adipose suppression, the trabecular bone in these obese animals did show a partial protection following radiation compared to the irradiated HF animals receiving sham LIV treatment. Due to the fact

that there was no non-irradiated LIV animal group, the ability to investigate potential mechanisms for the influence on the bone and fat tissues was overshadowed by radiation effects. For example, previous studies have implied a possible mechanism through the biasing of bone marrow mesenchymal stem cells. Here MSC populations increased following acute radiation exposure, a possible response to inflammation as a result of increased cell death from irradiation. Nevertheless, even when faced with such a severe systemic stressor, 7w of LIV treatment had a positive influence on the marrow niche through partial protection of trabeculae and suppression of MAT. These alterations to the marrow niche could have indirect influences on engraftment and reconstitution of HSCs following BMT. We also have to consider the limitations that the flow cytometry method introduces as we try to unravel the reasoning behind the population shifts we see especially in stem cell populations. While immune cell identification with FACS is well established, methods to accurately identify MSCs and even primitive HSCs are continuously being developed. Therefore, our approximation of these cell populations might also contribute to our confusion in understanding why we see these population shifts at all, thus it is important to also investigate the biology and function of the cellular populations in addition to their presence.

This work demonstrated that obesity causes healthy HSCs to alter their behavior when engrafting and repopulating following ablation due to irradiation. Obesogenic factors whether from peripheral adipose tissue or those that are within the marrow influenced them to promote hematopoiesis in the bone marrow. The increased leukocytes in the marrow however does not necessarily translate into a better immune response. Obesity has been connected to the production of increased pro-inflammatory cytokines (as seen in this study), poor response to infections and vaccinations and also greater susceptibility to damage from radiation as evidenced by suppressed marrow lymphocytes and trabeculae and increases apoptotic cells. These findings are not normal for obese subjects and should bring attention to the abnormal status of the marrow niche. Thus while the enhanced hematopoiesis might be harmless to overall health in the obese animals, it could also contribute to immune malfunction and inflammation. Inflammation as a result of obesity is considered a major underlying contributor to insulin resistance, which, along with islet dysfunction, defines type 2 diabetes, a sequela to being obese. Adipose tissue inflammation results from an infiltration of immune cells and the activation of an

immune response. In fact, studies have shown that the infiltration, expansion and phenotype switch of macrophages in adipose tissue results in the activation of the cJun NH92)-terminal kinase (JNK) signaling pathway that can directly interfere with insulin signaling [212]. Additionally, a subset of T cells (Tregs), known to express anti-inflammatory cytokines, are downregulated in adipose tissue and consequently have been attributed to the development of inflammation-induced insulin signaling as well [213]. These are but a few examples of the roles of immune populations in directly inflammation and therefore insulin signaling. While this work did not evaluate diabetic indices, it is conceivable that the enhanced recruitment of immune cells in the adipose tissue, suggests that the bone marrow HSCs are contributing to the eventual development of inflammation-induced insulin signaling. Further understanding of the activity of the specific leukocyte populations and their contribution to the immune status would help test this hypothesis.

Lastly, we must consider, in humans, obesity is a life-long challenge and that the development of inflammation and therefore the associated complications occurs over a matter of years. In this work, obesity was induced only over a matter of weeks with a robust diet intervention, a diet which is not directly comparable to any such diet among people. The use of lethal radiation doses poses adds to the stresses any intervention must counteract. With this caveat in mind mechanical signals be it exercise or low intensity vibrations may be helpful in the longer term. Thus here the use of a mechanical intervention such as LIV, while not having an impact on hematopoietic cells directly, did show positive influences on the bone marrow niche. These subtle alterations could indirectly contribute to a more normalized repopulation in obese mice post transplantation. Additionally, in this experimental design obese animals continued their high caloric diet following BMT, which likely drove much of the changes that we see during the recovery process. To test whether LIV can truly protect and drive HSC repopulation towards a non-obese phenotype, the stress of the diet should be removed. In this way, we could test whether by ablating the system with irradiation and transplanting new healthy HSCS, the obese phenotype can be reserved. Perhaps in cases of morbid obesity where diet and exercise are not feasible and bariatric surgeries are far too complicated, HSC transplants might even pose as a potential therapy to combat obesity associated immune complications. With lower doses of

radiation combined with the LIV treatment, severe impacts of radiation could potentially be averted.

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