EXERCISE HORMESIS FROM MUSCLE TO BONE MARROW

THE ADAPTIVE RESPONSE TO EXERCISE TRAINING: IMPLICATIONS FOR RADIATION PROTECTION AND BONE MARROW TRANSPLANTATION

By

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ABSTRACT

Radiation is a prominent source of environmental oxidative stress that can have deleterious consequences for health. Despite its well-known negative effects, radiation is commonly employed clinically for disease treatment and diagnosis. Bone marrow transplantation (BMT), used in the treatment of a variety of diseases, is preceded by a myeloablative regimen that usually involves radiation. Mortality associated with BMT is quite high and the aggressive radiation pre-treatment regimen contributes to these high rates of mortality. Interventions that inhibit the negative consequences of irradiation and promote BMT success would have significant implications for public health. Exercise-induced adaptations in numerous body tissues have been associated with amelioration of a variety of pathologies, particularly those associated with oxidative stress, and an overall improvement in health. Whether these adaptations can protect from damage induced by an external source of oxidative stress, such as a high dose of radiation, or promote BMT success is unknown. The purpose of this thesis was to determine if the adaptive response to exercise training could inhibit the negative effects of irradiation in skeletal muscle and bone marrow, and promote BMT success. To apply these adaptations to BMT, we examined the response of hematopoietic stem cells (HSC) and their niche to exercise. We report that muscle from exercise trained mice exhibits an enhanced response to radiation characterized by increased antioxidant and mitochondrial

metabolic enzyme activity. Extending these findings to cells in the bone marrow, we demonstrated that exercise training inhibited radiation-induced genotoxicity and cytotoxicity. With respect to BMT, exercise training increased HSC quantity with no effects on HSC function; however, preconditioning BMT recipients with exercise training resulted in improved probability of survival and enhanced hematopoietic regeneration. Collectively, results from the studies presented herein suggest that exercise training may be a successful therapeutic intervention to inhibit the damaging effects of radiation and improve BMT outcomes.

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ABBREVIATIONS

7AAD	-	7-Aminoactinomycin D
ANOVA	-	Analysis of variance
ARS	-	Acute radiation syndrome
ATP	-	Adenosine triphosphate
BAX	-	Bcl-2 associated X protein
Bcl-2	-	B-cell lymphoma 2
BMT	-	Bone marrow transplantation
BSA	-	Bovine serum albumin
Caspase	-	Cysteine-aspartic acid protease
CAT	-	Catalase
CFLAR	-	CASP8 and FADD-like apoptosis regulator
CLP	-	Common lymphoid progenitor
CMP	-	Common myeloid progenitor
CON	-	Control
COX	-	Cytochrome c oxidase
CS	-	Citrate synthase
CT	-	Computed tomography
CuZnSOD	-	Copper Zinc superoxide dismutase
CXCR4	-	C-X-C chemokine receptor type 4
DAPI	-	4’,6-diamidino-2-phenylindole
DMEM	-	Dulbecco’s modified Eagle’s medium
DMSO	-	Dimethyl sulfoxide
Dmtf1	-	Cyclin-D-binding myb-like transcription factor 1
DNA	-	Deoxyribonucleic acid
DNP	-	2,4-dinitrylphenylhydrazine
DSB	-	Double strand break
EDTA	-	Ethylenediaminetetraacetic acid
EGR1	-	Early growth response protein 1
ELISA	-	Enzyme-linked immunoassay
ETC	-	Electron transport chain
EX	-	Exercise trained

Fas-L	-	Fas-Ligand
FBS	-	Fetal bovine serum
FITC	-	Fluorescein isothicyanate
FLICA	-	Fluorescent-labeled inhibitors of caspases
Flt3	-	Fms-like tyrosine kinase 3
FoxOs	-	Forkhead box proteins
GFP	-	Green fluorescent protein
GMP	-	Granulocyte/macrophage progenitor
GPx	-	Glutathione peroxidase
H_2O_2	-	Hydrogen peroxide
HDR	-	High dose radiation
HSC	-	Hematopoietic stem cells
HSCT	-	Hematopoietic stem cell transplant
G_0	-	G zero phase
G_1	-	Gap 1 phase
G_2/M	-	Gap 2 phase/mitosis
G-CSF	-	Granulocyte colony-stimulating factor
γH2AX	-	Gamma histone 2AX
GM-CSF	-	Granulocyte-macrophage colony-stimulating factor
IFN-γ	-	Interferon-gamma
IL-1Rα	-	Interleukin-1 receptor alpha
IL-3	-	Interleukin-3
IL-6	-	Interleukin-6
IL-8	-	Interleukin-8
IL-10	-	Interleukin-10
LD	-	Lethal dose
LFA-1	-	Lymphocyte function-associated antigen 1
Lin	-	Lineage
LRC	-	Long-term repopulating cell
LSK	-	Lineage negative, Sca-1 positive, c-Kit positive
LT-HSC	-	Long-term repopulating hematopoietic stem cell
Mac-1	-	Macrophage-1 antigen

MAD1	-	Mitotic arrest deficient 1
M-CSF	-	Macrophage colony-stimulating factor
MFI	-	Mean fluorescent intensity
MN-RET	-	Micronucleated reticulocyte
MnSOD	-	Manganese superoxide dismutase
MPBC	-	Mobilized peripheral blood cells
MPP	-	Multipotent progenitor
MSC	-	Mesenchymal stem cell
NF-κB	-	Nuclear factor-kappaB
NT	-	Non-trained
PBS	-	Phosphate buffered saline
PE	-	Phycoerythrin
PGC-1α	-	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI	-	Propidium iodide
PTH	-	Parathyroid hormone
PVC	-	Polyvinyl chloride
RBC	-	Red blood cell
ROI	-	Region of interest
ROS	-	Reactive oxygen species
RPMI	-	Roswell Park Memorial Institute medium
S	-	Synthesis phase
Sca-1	-	Stem cell antigen-1
SCF	-	Stem cell factor
SCL	-	Stem cell leukemia protein
SDF-1	-	Stromal cell-derived factor-1
SED	-	Sedentary
SEM	-	Standard error of the mean
SLAM	-	Signaling lymphocyte activation molecule
SOD	-	Superoxide dismutase
SP	-	Side population
ST-HSC	-	Short-term repopulating hematopoietic stem cell

TBS	-	Tris-buffered saline
TBST	-	Tris-buffered saline-Triton
TGFβ1	-	Transforming growth factor beta 1
Tie2	-	TEK tyrosine kinase
TNF-α	-	Tumor necrosis factor alpha
TPO	-	Thrombopoietin
UCB	-	Umbilical cord blood
VCAM1	-	Vascular cell adhesion molecule-1
VEGF	-	Vascular endothelial growth factor
VLA-4	-	Very late antigen-4
WT	-	Wild-type

DECLARATION OF ACADEMIC ACHIEVEMENT

Chapter 2

Publication

De Lisio M, Kaczor JJ, Phan N, Tarnopolsky MA, Boreham DR, Parise G. Exercise training enhances the skeletal muscle response to radiation-induced oxidative stress. *Muscle Nerve*. (2011) Jan;43(1):58-64. Doi:10.1002/mus.21797.

Contribution

GP was the principal investigator of the study. MD, DRB and GP conceived and designed the experiments. MAT, DRB and GP contributed new reagents/materials/analytical tools. MD conducted the mouse exercise training. NP conducted the high dose radiation challenge. MD, NP and GP collected tissue. MD and JK conducted experiments. MD analyzed the data with assistance from JK and GP. MD wrote the manuscript. MAT, DRB and GP supervised the experimental work, interpretation of the data and edited the manuscript. All authors approved the manuscript prior to final submission.

Chapter 3

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Contribution

GP was the principal investigator of the study. MD, DRB and GP conceived and designed the experiments. DRB and GP contributed new reagents/materials/analytical tools. MD conducted the mouse exercise training. NP conducted the high dose radiation challenge. MD, NP and GP collected tissue. Experiments were conducted by MD, NP and technicians from DRB's lab. MD analyzed the data and wrote the manuscript. GP edited the manuscript. GP and DRB supervised the experimental work, interpretation of the data and edited the manuscript. All authors approved the manuscript prior to final submission.

Chapter 4

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Contribution

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Chapter 5

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Contribution

GP was the principal investigator of the study. MD and GP conceived and designed the experiments. GP contributed new reagents/materials/analytical tools. MD collected tissue. MD and JB conducted the experiments. MD analyzed the data and wrote the manuscript. GP and JB edited the manuscript. GP supervised the experimental work and interpretation of the data. All authors approved the manuscript prior to final submission.

Chapter 1: General Introduction

1.1 INTRODUCTION

The biological effects of radiation and stem cell biology are two separate but related fields. The first stem cells were discovered by a radiation biologist and a hematologist studying the mechanisms responsible for radiation protection [1]. These seminal experiments led to the use of bone marrow transplantation (BMT) as a clinical intervention to treat radiation exposure and a myriad of diseases. Today, radiation and BMT have widespread clinical use from disease diagnosis to treatment. Despite their common application, radiation and BMT do not come without risk. Mitigating the harmful effects of radiation exposure and improving success rates for BMT are two popular areas of research with important implications for public health.

Exercise has unquestionable health benefits. Large bodies of research exist demonstrating the beneficial effects of exercise on cognitive function, the cardiovascular system, immune function, and muscle health. Given these widespread effects of exercise, it is no surprise that exercise is suggested for the prevention and treatment of many pathologies. Nevertheless, all of the health effects of exercise have yet to be realized. Specifically, the effects of exercise in the context of radiation and BMT are poorly understood, and the present thesis will investigate these effects. The following sections will review the relevant literature related to the effects of radiation at both the cellular and whole body

level. It will go on to describe characteristics of hematopoietic stem cells (HSC),their interactions with the niche and their role in BMT. It will conclude with a review of literature regarding exercise hormesis, the effects of exercise on hematopoietic stem/progenitor cells and the potential role of exercise in improving BMT outcomes.

1.2 RADIATION EXPOSURE RISK AND PREVALENCE

From Roentgen's discovery of the X-ray in 1895 to the development of the 64 slice CT scan in 2004 [2], the application of radiation in medical technology has had important implications for public health. The widespread use of radiation clinically is evidenced by an increase in the per capita radiation dose from medical sources from 18% in 1980 to 54% in 2006 [2]. Doses from medical procedures can range from 0.001 - 30 mSV for diagnostic X-rays and CT scans on the low end, to doses from 1 – 200 Gy for cancer treatment at the high end [2]. This increase in medical exposure has led to questions concerning the biological effects of this exposure and to the development of interventions to mitigate these effects. The following sections will discuss the effects of acute radiation exposure at the cellular level, as well as the response of whole organ systems and tissues.

1.2.1 Cellular Effects of Radiation

At the cellular level, radiation primarily exerts its effects via induction of oxidative stress. Gamma rays from a source of ionizing radiation react with

intracellular water molecules resulting in the formation of reactive oxygen species (ROS) [3,4]. ROS are highly unstable and will react with and damage nearby cellular macromolecules, or with other intracellular oxygen-containing molecules resulting in the propagation of ROS production [5] (Figure 1). The highly reactive nature of ROS makes them extremely short-lived and difficult to study directly; therefore, biomarkers have been established to evaluate ROS-induced damage that focus on specific targets of ROS such as lipids, proteins, and DNA [5]. Elevated ROS production with large amounts of damage to cellular macromolecules causes cells to enter a pathological state of oxidative stress and basic cellular function is impaired. Mitochondria are particularly susceptible to irradiation and ROS, due to their highly aerobic nature [4,6]. Indeed, electron micrographs reveal swollen mitochondria with damaged inner membranes [6], which is likely related to increased mitochondrial ROS production [4,7,8] following irradiation. Furthermore, damage to mitochondrial membranes results in the release of apoptosis-promoting proteins, such as cytochrome c, from the mitochondrial matrix initiating the apoptosis cascade within cells [8]. Therefore, cells exposed to radiation experience oxidative stress, particularly within mitochondria, leading to damaged cellular macromolecules and apoptosis.

Both enzymatic and non-enzymatic antioxidants are natural cellular defense mechanisms to protect against ROS-induced damage and prevent oxidative stress. Cellular antioxidant enzymes are ROS-specific and localized

to various cellular organelles where ROS production is high. Superoxide dismutase (SOD), an antioxidant responsible for the conversion of the highly reactive oxygen radical to the less reactive hydrogen peroxide molecule, exists in two isoforms: manganese-SOD (MnSOD) localized to the mitochondria, and copper-zinc SOD (CuZnSOD) localized to the cytosol [5]. Although hydrogen peroxide is a less reactive ROS, it is longer lived and able to diffuse farther distances; therefore, two additional antioxidant enzymes: cytosolic glutathione peroxidase (GPx) and catalase (CAT) located primarily in peroxisomes, are responsible for the conversion of hydrogen peroxide to water [5]. Localization of MnSOD to mitochondria is important because the electron transport chain (ETC) is the primary source of intracellular ROS production during normal metabolism [9]. To maintain homeostasis, these antioxidant enzymes neutralize ROS produced during normal metabolism and maintain cells in a state of oxidative balance. However, an acute elevation in ROS, such as that following a high dose of radiation, results in a shift in balance between oxidant production and antioxidant protection leading to cellular oxidative stress and ultimately, cellular dysfunction and death [10].

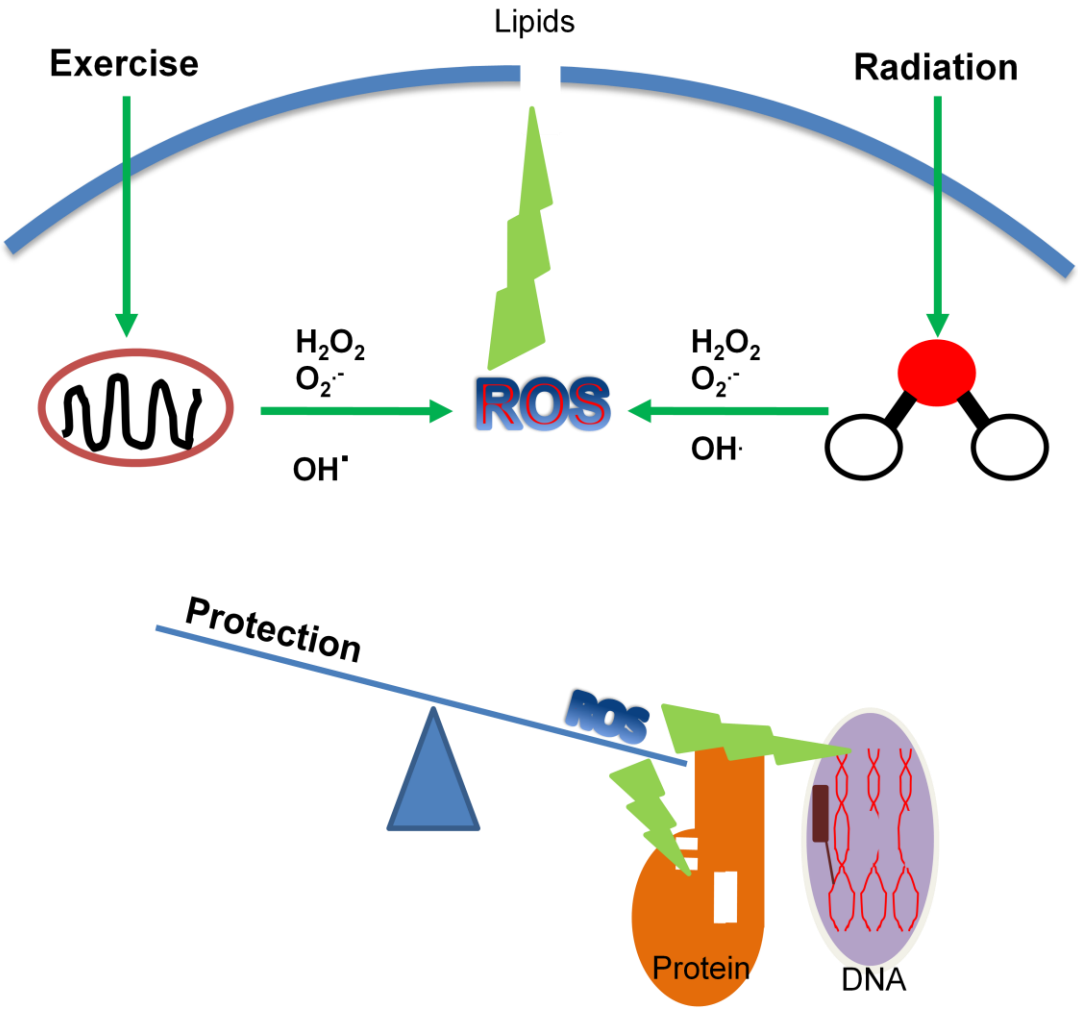

Figure 1. Cellular effects of exercise and radiation are ROS-mediated. Increased energy demand with exercise increases electron flux along the electron transport chain. Increased electron flux results in improper reduction of molecular oxygen and the formation of ROS (detailed below). Gamma rays from a source of radiation react with intracellular water molecules causing formation of ROS. Elevated levels of ROS exceed the cell's natural protective mechanisms causing the cells to enter a state of oxidative stress. Cellular oxidative stress is associated with damage to intracellular macromolecules including lipids, proteins and DNA.

1.2.2 Systemic Effects of Radiation

The acute response to high dose radiation exposure is characterized by systemic inflammation and pathologies in multiple organ systems. The most

susceptible tissues are those containing highly proliferative stem and progenitor cells such as hematopoietic stem/progenitor cells in the bone marrow, progenitors in crypts of intestinal villi and endothelial cells lining vessels in the central nervous system [11]. Determining the specific cause of death following acute radiation exposure is difficult due to the interaction of various systems [12], and recent data has suggested that the time of death following radiation exposure is no longer considered a reliable marker of which tissue or organ system failed [13]. The multi-organ failure that is associated with acute radiation syndrome (ARS) may be exacerbated by the systemic inflammation induced by radiation, referred to as a “cytokine storm” [14–16]. The acute increase in inflammation is mediated by inflammatory cell infiltration into various tissues including the lungs [7], brain [17], gastrointestinal system [18] and the skin [19]. The role of radiation-induced systemic inflammation in morbidity and mortality has led to the investigation of anti-inflammatory compounds as a treatment for acute radiation exposure [4]. It can be argued that the negative effects of radiation on HSC are at the root of all these potential organ system failures associated with acute radiation exposure. A direct effect of radiation exposure is the loss of HSC and their subsequent failure to reconstitute the hematopoietic system [4]. Indirectly, acute immune cell loss may result in death due to sepsis or infection if damage to endothelial cells lining vessels and epithelial cells of the intestinal villi results in hemorrhaging with internal bleeding and leakage of pathogens from the intestines into circulation [20]. For this reason, the primary treatment for acute radiation

exposure is stimulation of HSC proliferation treatment with hematopoietic growth factors, or hematopoietic stem cell transplant (HSCT) to regenerate the destroyed hematopoietic system [4].

The hematopoietic system is the most susceptible to acute radiation exposure. The lethal dose for 10%-90% (LD_{10-90}) of mice exposed to radiation due to bone marrow failure is 8.3-9.4 Gy, while nearly double that dose is required for LD_{10-90} due to GI failure [13]. Indeed a dose of less than 1 Gy can cause death in 33% of cells in isolated bone marrow fractions, with nearly complete ablation of isolated bone marrow cells exposed to 6.7 Gy [1]. Furthermore, significant effects on immune cells are realized with doses as low as 5 Gy resulting in total ablation of B and T cells [21]. In general, maximal hematopoietic cell death following irradiation in occurs approximately 12-16 days following exposure in mice exposed to 10 Gy [22], while in humans the response is even more rapid with various hematopoietic cell populations reaching their minimum between 1 and 10 days post-exposure [23]. Radiation induced hematopoietic cell death occurs via apoptosis [24–26], and is therefore associated with an up regulation of pro-apoptotic markers such as Fas-L, BAX and caspase-3 and a down regulation of Bcl-2 [27,28]. As such, hematopoietic cell death can happen directly in response to the radiation insult, or can be delayed until cells undergo their first cell division in a cell death process referred to as “mitotic catastrophe” [11,27]. Not only does radiation affect cells of the

hematopoietic system, but it also causes pathological consequences in the cells comprising the HSC niche in the bone marrow cavity. Radiation-induced damage to endothelial cells of bone marrow sinusoids causes them to enlarge, deform and leak blood into the marrow cavity [29–31]. Additionally, apoptosis of hematopoietic cells that normally fill the marrow cavity decrease the pressure gradient supporting the endothelial cells of the sinusoids further contributing to their leakiness resulting in severe hemorrhaging into the marrow cavity [23]. Finally, oxidative stress in the bone marrow persists long after irradiation, especially in long-lived cells such as HSC [32]. Given their susceptibility to radiation as well as the effects of radiation on their niche, interventions aimed at increasing the radiation resistance of cells associated with hematopoiesis are necessary.

1.3 OVERVIEW OF HEMATOPOIETIC STEM CELLS

The hematopoietic system includes all mature blood cells found mostly in circulation, as well as stem and progenitor cells found predominantly in the bone marrow. It has numerous functions including oxygen and nutrient delivery, pathogen removal through the immune response and blood clotting. The hematopoietic system follows a hierarchy from immature multipotent stem cells with high capacity for self-renewal, to more differentiated, lineage-restricted, highly proliferative progenitors to fully differentiated mature blood cells (Figure 2). The mature, fully differentiated hematopoietic cells are relatively short-lived with a

high rate of turnover [23]. The roughly 10 billion new blood cells required per day to maintain homeostasis are generated through the self-renewal, proliferation and differentiation of a small population of hematopoietic cells, the HSC in the bone marrow [33]. HSC are rare, at levels of only $1/10^4$ to $1/10^5$ whole marrow cells [34,35], multipotent and normally mitotically quiescent [36]. HSC exist in a hierarchy from the most primitive, rarely dividing HSC responsible for long-term reconstitution of myeloablated recipients (LT-HSC) to HSC with decreased capacity to self-renew but increased ability to expand responsible for short-term reconstitution of recipients (ST-HSC), and finally multi-potent progenitors (MPP) that are highly proliferative but still maintain the stem cell qualities of multi-lineage differentiation and self-renewal [37–40] (Figure 2). The capacity for HSC to regenerate the hematopoietic system is truly amazing. It has been estimated that a single HSC can produce 10^{15} progeny [41], a single HSC can regenerate the entire hematopoietic system of a myeloablated recipient [42,43], and HSC can be used in multiple rounds of transplantation outlasting the life-span of their original host [44]. HSC can undergo a number of cell fate decisions including self-renewal, proliferation and differentiation into more mature hematopoietic cells, migration from their niche in the bone marrow and apoptosis [37]. Given their high proliferative capacity, HSC cell fate decisions are tightly regulated by a number of intrinsic pathways and extrinsic factors from the niche. The following sections will focus on a historical overview of HSC and assays used to study

them, their immunophenotype, function, their interactions with their niche and their clinical role in BMT.

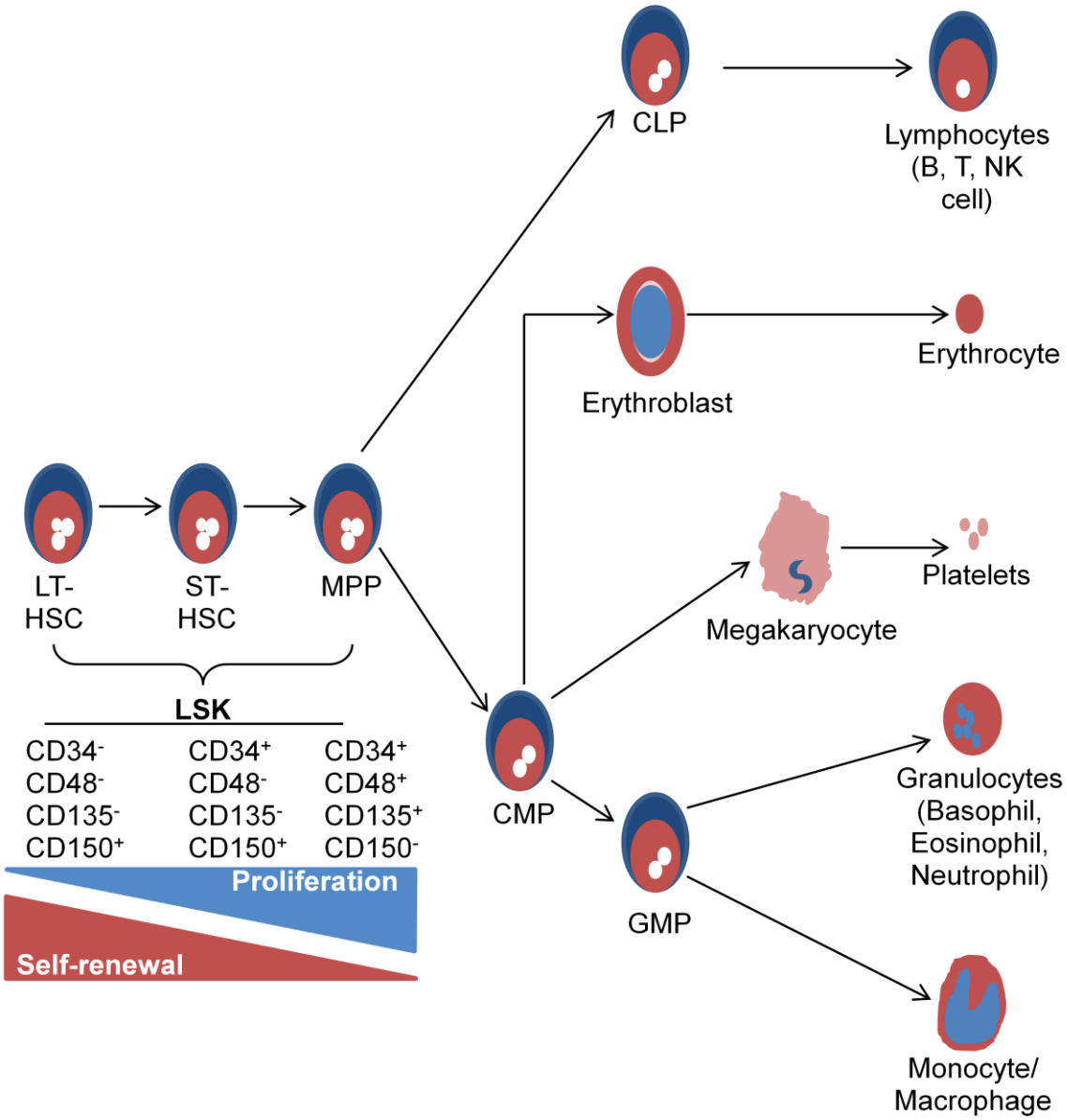

Figure 2. The hematopoietic system. The most primitive cells of the hematopoietic system are the hematopoietic stem cells (HSC). HSC exist in a hierarchy of the most primitive long-term reconstituting HSC (LT-HSC) to more differentiated short-term reconstituting HSC (ST-HSC) to the most differentiated multipotent progenitor cells (MPP). Self-renewal capacity decreases while

proliferative capacity increases as HSC become more differentiated. HSC in mice are as being negative for markers of mature blood cell lineages and positive for Sca-1 and c-Kit (LSK), and can be further fractionated based on their expression of the various cell surface antigens listed. The primary progenitor cells of each lineage are listed along with the various mature hematopoietic cell types. CLP=common lymphoid progenitor, CMP=common myeloid progenitor, GMP=granulocyte/macrophage progenitor. (Adapted from [45])

1.3.1 HSC: Immunophenotype

Identification of cell-surface markers to quantify and isolate adult HSC has been difficult, and no single marker has been established as a specific label of HSC (Figure 2). Advances in flow cytometry allowing for multi-colour labelling of HSC has led to the development of multiple markers used to highly enrich populations of bone marrow cells for HSC. In mice, the cell-surface markers most commonly used to identify a population of bone marrow cells enriched for HSC are the lineage markers in combination with Sca-1 and c-Kit, the so-called "LSK" markers [34]. LSK denotes the population of bone marrow cells that are negative for all mature blood cell lineage markers, usually a panel including Mac-1, Gr-1, B220, Ly76 and CD3ε, and positive for the stem cell marker Sca-1 and the stem cell factor receptor, c-Kit [34]. Although the LSK population is considered to contain all functional HSC [39,45,46], the LSK population can only be considered to be enriched for HSC as only 10% of the LSK population are true LT-HSC [47]. The LSK population is extremely rare, making up only approximately 0.08% of the entire bone marrow population [46] with Sca-1^+ and c-Kit^+ cells making up approximately 3-8% of lineage negative cells [35]. The LSK population can be further sub-divided into more specific HSC subpopulations

using additional markers (Figure 2) [45]. Aside from the LSK population, HSC selection can also be achieved by their ability to efflux the supravital dyes Rhodamine-123 [48] and Hoechst 33342 [49]. HSC identified by these dye efflux methods are referred to a side population (SP) cells because of their distinctive scatter patterns with flow cytometry [49]. Adding to the complexity of identifying HSC by their cell surface phenotype is that cell surface antigens used to identify HSC vary between species. In humans, HSC are commonly identified as SP cells from the bone marrow [50] or as $CD34^+$ cells in peripheral blood [51–55]. Therefore, these factors necessitate the use of up to 6 different markers if one wishes to distinguish LT- and ST-HSC for example [45]. As a result, the true definition of an HSC is based on a cell's functional capacity to regenerate the hematopoietic system of a myeloablated host [56].

1.3.2 HSC: A Functional Definition

The seminal experiments that led to discovery of HSC were conducted by Drs. James Till and Edward McCulloch at the University of Toronto using the bone marrow transplantation (BMT) assay. In these experiments, the hematopoietic system of myeloablated mice was reconstituted with bone marrow from donor mice, and colonies of transplanted cells were localized to the spleen shortly after transplant. It was determined that a stem cell source was responsible for blood cell reconstitution and marrow regeneration, and they coined the term "hematopoietic stem cells" (HSC) [1]. These experiments led to

the functional definition of HSC as cells capable of self-renewal and long-term, multilineage blood reconstitution of myeloablated hosts [56]. The BMT assay, or long-term repopulating cell (LRC) assay, became the gold standard assay for HSC analysis (Figure 3). Typically, this assay involves myeloablation of recipient mice by either treatment with chemotherapeutic agents or radiation to remove native hematopoietic cells. Recipients are then reconstituted with donor cells consisting of either whole bone marrow or a specifically isolated cell population mixed with a supporting population of progenitor cells that allow for survival acutely following the myeloablative strategy. Donor cells are distinguished from native recipient cells in the resulting chimera either by distinct cell surface markers (i.e. CD45.1 vs. CD45.2), mis-matched sex chromosomes (male Y chromosome-expressing cells into female recipient) or fluorescent label (typically green fluorescent protein (GFP)). Disadvantages of the LRC assay include the large number of recipient mice needed as well as the length of the assay as evaluations of donor-derived reconstitution of recipient blood need to be conducted at least up to 6 months post-transplant in order to evaluate the presence and function of the LT-HSC [47]. The advantages of the *in vivo* BMT assay are many as it allows the evaluation of a variety of functions of HSC as successful recipient blood reconstitution depends on the following criteria: (i) sufficient ST- and LT-HSC in the graft, (ii) successful homing and engraftment in the recipient bone marrow niche, (iii) proliferation and differentiation to regenerate the recipient hematopoietic system, and (iv) self-renewal to maintain the HSC

population [57]. Self-renewal can be further evaluated using the serial reconstitution assay where marrow from engrafted primary recipients is used as the donor cell source for reconstitution of secondary recipients. Only in this secondary reconstitution assay can it be proven that successful self-renewal divisions occurred in primary recipients as defective self-renewal and exhaustion of the HSC pool will result in defective engraftment in secondary hosts [45]. These functions cannot be determined in various *in vitro* assays developed and these *in vitro* assays may not identify long-term repopulating HSC [58]. Finally, the BMT assay has direct clinical relevance as it allows for the evaluation of the effects of various conditions on the success of hematopoietic stem cell transplantation (HSCT).

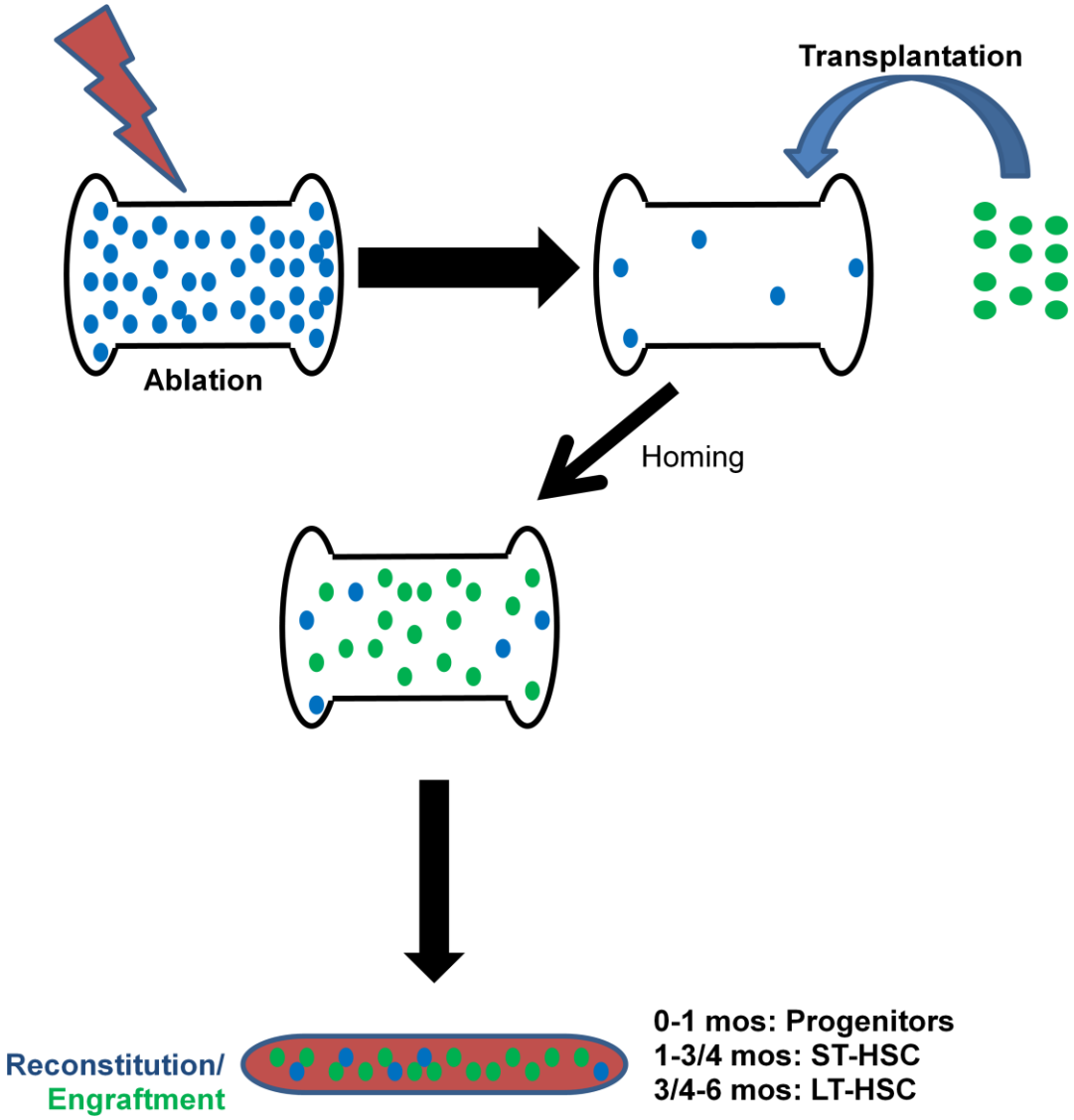

Figure 3. The BMT assay. Following ablation of recipient bone marrow via a hematopoietic-lethal dose of radiation, recipients are transplanted with donor marrow via intravenous injection. Donor marrow is distinguished from recipient marrow via fluorescent expression, differences in cell surface antigens or sex chromosome mis-matching. Donor marrow homes to the recipients' bone marrow cavity where it engrafts and regenerates the recipients' hematopoietic cells. Donor-derived engraftment is determined by assessing the percentage of donor-derived hematopoietic cells in circulation. Various time points following transplantation can be analyzed to identify specific donor-cell populations responsible for engraftment.

Under normal physiological conditions, HSC are slow cycling, and generally found in a state of mitotic quiescence *in vivo*. It has been estimated that most HSC will divide at least once every 4-12 weeks to maintain blood cell homeostasis [59,60]. Indeed, cultured HSC have an extended time of first cell division due to the process of exiting from quiescence and entering the cell cycle [61]. Mitotic quiescence may be a protective mechanism to maintain DNA integrity that becomes vulnerable during cell division [62]. Quiescence is tightly controlled in HSC by a number of cell intrinsic regulators of cell cycle entrance and progression. The cyclin dependent kinase inhibitors, $p21^{Cip1/Waf1}$, $p27^{kip1}$ and p57, as well as the tumor suppressor genes, *Dmtf1* and *MAD1,* are essential regulators of HSC quiescence as their ablation leads to hyper-proliferation with impaired self-renewal [63–66]. Since HSC are a long-lived, reserve cell population, intrinsic regulation of the quiescent state helps minimize DNA damage and prevents exhaustion of the HSC pool by excessive proliferation.

When the hematopoietic system is stressed and forced to regenerate, such as in the BMT assay, HSC must be able to exit the quiescent state, proliferate and maintain the HSC pool by undergoing self-renewal. HSC activation seems to be associated with decreased hematopoietic reconstituting potential. Indeed, actively cycling HSC have decreased engraftment potential compared to HSC transplanted in G0/G1 phase of the cell cycle [67]. Furthermore, a decrease in HSC repopulating ability is seen with serial

transplantation, a condition where HSC are repeatedly activated and forced to proliferate to regenerate the hematopoietic system of many hosts [68,69]. Additionally, oxidative stress in HSC, as would be induced by preconditioning regimens with the BMT assay, is a proliferative stimulus that if uncontrolled can result in exhaustion of the HSC pool [70]. Interestingly, antioxidant treatment can prevent exhaustion of the HSC pool in HSC with high levels of oxidative stress [70] suggesting that antioxidants may promote HSC quiescence. These data indicate that in response to hematopoietic ablation or oxidative stress induced in the BMT assay, HSC enter the cell cycle and begin to proliferate. At the population level, HSC proliferation is associated with differentiation to an overall more mature HSC phenotype that has decreased hematopoietic reconstituting capacity. To maintain the HSC pool in response to stress while enabling proliferation for hematopoiesis requires tight regulation between proliferation and self-renewal. Transcription factors regulating cell cycle progression and responsive to various forms of cellular stress, such as p53, SCL, FoxOs and EGR1 have been implicated in regulating the balance between quiescence and proliferation in HSC [70–73]. These data suggest that at the population level, the proliferative response of HSC to hematopoietic ablation must be tightly balanced with maintained self-renewal to maintain long-term function of HSC.

The importance of the balance between proliferation and self-renewal is further evidenced by *in vitro* models. The rarity of HSC has limited their clinical

applicability and has led to the exploration of ideal culture conditions to promote HSC expansion. Most of the approaches for *ex vivo* expansion of HSC have tried to replicate and enhance the natural *in vivo* environment of HSC [74]. For example, co-culturing HSC with different stromal layers [75] and altering extra-cellular matrix substrates *in vitro* [74], have demonstrated a 2-8 fold expansion of HSC *ex vivo* [75] , and have increased our understanding of important factors regulating HSC. The most common approach thus far has been to vary cytokine mixtures and concentration that regulate HSC function [75]. *In vitro* HSC expansion was accomplished via growth in culture base media containing Flt3 ligand, stem cell factor (SCF), thrombopoietin (TPO) and supplemented with either IL-3 or IL-6 [76]. Interestingly, while cultures treated with IL-3 and IL-6 both showed short-term HSC expansion *in vitro*, only cultures treated with IL-6 were capable of long-term HSC expansion and recipient engraftment, as well as secondary recipient reconstitution [76]. These results indicate an important role for maintenance of LT-HSC in culture by IL-6, and also highlight the primary limitation of studies expanding HSC *ex vivo*, namely impaired HSC function. Impaired HSC function in *ex vivo* expanded HSC manifests upon transplantation as impaired homing to the bone marrow cavity [77], increased apoptosis of transplanted cells [78] or exhaustion of the HSC pool from proliferation without self-renewal [76,79,80]. These drawbacks highlight the complexity of the interactions between HSC and their microenvironment as well as the importance of the *in vivo* environment in maintaining HSC function. Indeed HSC expansion

in culture is associated with decreased expression of cell surface adhesion molecules, and down-regulation of adhesion molecules was associated with decreased hematopoietic reconstituting ability [81]. These data further support the importance of the niche in HSC regulation and maintaining HSC function. The ability of physiological stimuli that expand HSC in their natural *in vivo* environment while maintaining their long-term repopulating capacity has not previously been explored.

1.3.3 HSC: Regulation by the Niche

In 1978, Schofield first referred to the HSC niche when he hypothesized that stem cell fate was determined by the cells they directly associated with in their microenvironment, and that maturation and differentiation of the cells occurred because stem cell progeny do not occupy the same niche as the original stem cell [82]. Since then, numerous studies have investigated the importance of the HSC niche demonstrating that interactions between HSC and their niche are vital for the regulation of HSC self-renewal and differentiation [45]. Within the bone marrow, HSC are believed to reside in two separate but related niches: the endosteal niche and the vascular niche [45] (Figure 4). Although, the importance of the HSC niche is well known, and the HSC niche has been long-studied, specific characteristics of the niche are just now being identified. A common approach has been to evaluate the function of HSC following alteration of factors

in the niche; however, little is known as to the role of physiological factors in regulating the interaction between HSC and their niche.

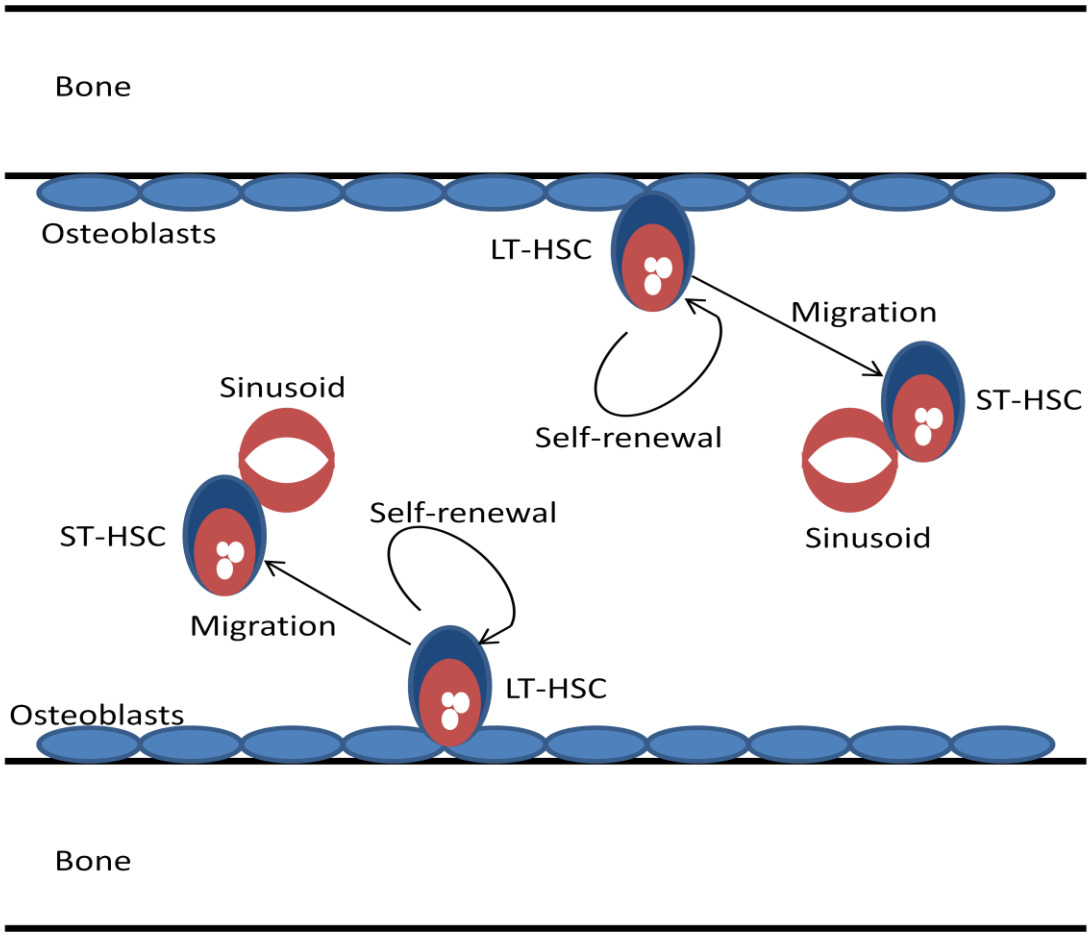

Figure 4. The HSC niche. In the bone marrow cavity HSC can be found associated with osteoblasts in the endosteal niche lining the inner surface of the bones or associated with endothelial cells of the bone marrow sinusoids in the vascular niche. HSC can migrate between the two niches. The endosteal niche is associated with more quiescent HSC with higher capacity for long-term repopulation, while the vascular niche is associated with more activated HSC primed for differentiation and release into circulation.

The inner bone surface outlining the bone marrow cavity makes up the endosteal niche. Lord and colleagues (1975) were the first to show HSC associated with the endosteum [83]. Later HSC were demonstrated to associate

directly with osteoblasts [84], specifically via homo-dimeric interactions between N-cadherin expressed on osteoblasts and HSC [85]. The relationship between osteoblasts and HSC was further demonstrated by studies where osteoblasts numbers were increased and HSC quantity underwent a concomitant increase [84,85]. Conversely, osteoblasts ablation resulted in decreased quantity of HSC, leading to a stress response that was associated with increased extra-medullary hematopoiesis [86,87]. Furthermore, HSC may regulate production of their own niche via interactions with mesenchymal stem cells (MSC) as HSC drive the differentiation of MSC along the osteogenic lineage with this function being enhanced during hematopoietic stress [88]. These data demonstrate the intricate association between HSC and the endosteal niche and the importance of the endosteal niche in regulating the HSC quantity.

The endosteal niche serves two related purposes: (i) HSC protection; and (ii) maintenance of quiescence. At steady state, the endosteal niche maintains HSC in a state of mitotic quiescence, and is home to the most primitive HSC [36,89]. This was eloquently demonstrated by Haylock and colleagues (2007), when HSC isolated from the endosteal region were co-transplanted in a competitive assay with HSC isolated from the central marrow region, endosteal HSC demonstrated greater long-term reconstitution of recipients [90]. The primary cellular component of the endosteal niche is the osteoblast which regulates HSC via direct cell to cell interactions or via secreted factors. Osteoblasts express N-

Cadherin, responsible for holding HSC in the endosteal niche via “homotypic” interactions with N-Cadherin expressed by HSC [85,89]. Osteoblasts also secrete factors that maintain HSC quiescence and regulate self-renewal. For example, osteoblasts release Angiopoietin 1, which interacts with its receptor Tie2 expressed by LT-HSC, to maintain their quiescence [91]. Cultured osteoblasts release hematopoietic cytokines such as G-CSF and IL-6 [92,93], suggesting a mechanism whereby N-cadherin maintains HSC in close association with osteoblasts which release factors that regulate HSC function.

Since the most primitive HSC reside in the endosteal niche, it is important that a second major function of the endosteal niche is to protect HSC from environmental stressors. The area lining the inner surface of the bones in the bone marrow cavity is hypoxic [94], protecting HSC from ROS-induced damage [95]. Oxidative stress has been shown to cause HSC exhaustion and hematopoietic failure [96]. The ROS-protective properties of the endosteal niche were highlighted in a study by Xie and colleagues (2009), where donor HSC were tracked in recipients preconditioned with radiation or not, and donor cells were found to specifically home to the endosteal niche in irradiated recipients, but homed to random bone marrow regions in non-irradiated recipients [97]. Since preconditioning recipients with radiation causes prolonged increases in marrow ROS [98], these data suggest that in an oxidative environment, donor HSC engraft in low oxygen regions of the marrow perhaps protecting them from

elevated ROS levels. It has been suggested that donor HSC only home to the endosteal niche in recipients pre-conditioned with radiation because the radiation destroys the vascular niche [57]. Contrary to this hypothesis, osteoblasts ; secrete SDF-1 following irradiation treatment [31]. The SDF-1 receptor, CXCR4, is expressed by HSC, HSC were shown to migrate specifically to SDF-1 *in vitro* [99], and deletion of SDF-1 impairs HSC engraftment upon transplantation [100]. Furthermore, transplanted Lin^- bone marrow cells, a HSC-containing fraction, home specifically to the endosteal region, whereas transplanted Lin^+ cells, a non-HSC-containing fraction, home to the central marrow region [101]. These data suggest that the endosteal homing of HSC may be a specific response to a signal released by osteoblasts, which may promote HSC engraftment in a more protected niche following radiation-induced damage. Taken together, these data demonstrate that the endosteal region is a protective environment for primitive HSC, and is the primary location for HSC homing following transplantation.

Although, the primary location of LT-HSC is the endosteal niche, the majority of HSC in the bone marrow are found in the vascular niche. The vascular niche is found in the central marrow region and is comprised of the endothelial cells of bone marrow sinusoids [102]. HSC in the vascular niche are slightly more differentiated than HSC from the endosteal niche [85,89,90,102], and have higher proliferative capacity [102]. HSC proliferation in the vascular niche is intimately related to differentiation as sinusoidal endothelial cells promote

HSC differentiation, especially along the megakaryocyte and myeloid lineage [103]. Vital to these processes is the SDF-1-CXCR4 signaling axis. SDF-1 is expressed by sinusoidal endothelial cells and via its interaction with HSC expressing CXCR4, it can regulate HSC homing, mobilization and adherence in the niche [104,105]. Together, these observations suggest that the vascular niche is home to a more proliferative, differentiated population of HSC, and the vascular niche regulates HSC transit between peripheral blood and the bone marrow.

To facilitate passage of HSC and mature blood cells from the marrow cavity to circulation the endothelium comprising marrow sinusoids is fenestrated [23]. The opening of these fenestrations is tightly regulated by pressure gradients within the marrow cavity. When blood flow to the marrow cavity increases, the pressure from the circulating blood cells expands the sinusoids, opening the fenestrations and allowing for release of hematopoietic cells [23]. The reverse process also occurs when blood flow in the marrow sinusoids decreases, or when pressure from increased hematopoietic cell production increases forcing the fenestrations between endothelial cells closed and preventing the release of hematopoietic cells from the marrow [23]. Passage through the fenestrations is further facilitated by proteins expressed on endothelial cells that promote HSC attachment [106]. Specifically, SDF-1, LFA-1 and VLA-4 expressed on endothelial cells activate HSC and results in their passage through the fenestrations between endothelial cells [107]. Taken

together, the available data suggest a model whereby primitive HSC are located in the endosteal niche where they are protected from stressors and maintained in a state of mitotic quiescence, then migrate to the vascular niche where they prepare for release into circulation by proliferating and differentiating into mature hematopoietic cells [45]. Once in the vascular niche, HSC can sense and receive signals from circulation that regulate their release [45], and can respond quickly to maintain hematopoietic homeostasis.

1.3.4 Hematopoietic Stem Cell Transplantation

Hematopoietic stem cell transplants (HSCT) originated from early studies that demonstrated protection from radiation and animal survival could be achieved by shielding the spleen and bone marrow from the radiation source [108], or infusing bone marrow from non-irradiated mice into irradiated mice [108,109]. These initial experiments led to the first reports of HSCT in humans reported in 1959 [110,111]. Today, HSCT is the most common form of stem cell therapy used clinically, and is employed in the treatment of a variety of hematological disorders including leukemias, autoimmune disorders and anemias. Based on the early literature demonstrating radioprotection by shielding of the mouse hematopoietic organs [108], early HSCT used bone marrow as their source of hematopoietic cells, and are referred to specifically as bone marrow transplants (BMT). Currently, the most common donor cell source for HSCT are mobilized peripheral blood cells (MPBC), with umbilical cord blood (UCB) as another source of

hematopoietic cells gaining in popularity [20]. Two broad types of HSCT exist: (i) autologous, where the patient's own cells harvested prior to the myeloablative strategy are used as the donor cell source; and (ii) allogeneic, where related or unrelated donors matched for histocompatibility provide hematopoietic cells to the recipient [20]. HSCT are preceded by preparative strategies that employ high doses of radiation or chemotherapeutic drugs to remove tumorigenic cells but also remove native hematopoietic cells [20]. Despite their widespread use, the latest research demonstrates that overall survival 5 years following transplant is less than 50% with treatment related mortality occurring in 41% of transplants [112]. Recently, milder preparative strategies have been designed to decrease mortality associated with HSCT; however, the use of these more mild preparative strategies is less common due to increased incidence of disease relapse [113]. Although it is known that factors such as donor [114] and recipient age [112], as well as disease progression in recipients [112] are related to HSCT success, a paucity of data exist regarding the role of physiological stimuli, such as exercise, in enhancing donor characteristics, or improving recipient outcome

1.4 THE ADAPTIVE RESPONSE TO EXERCISE TRAINING

1.4.1 Exercise Hormesis

Hormesis refers to the beneficial adaptations induced by low levels of a substance that would be harmful at higher doses. The term has been applied to

a variety of stimuli including, but not limited to drugs [115] and radiation [116]. For example, cells preconditioned with low levels of radiation prior to high dose exposure exhibit decreased DNA damage [117–119], increased cell survival [118,120,121], decreased cancer occurrence [122] and slightly increased time to induction for some forms of cancer [123]. These protective effects induced by preconditioning cells with low doses of radiation are mediated, in part, through elevated levels of antioxidant enzymes [124–126]. The term hormesis has also been applied to adaptations associated with exercise training [127,128] (Figure 1 - above). Oxidative phosphorylation for the production of ATP involves electron transport along the ETC, located in the inner mitochondrial membrane, in a series of redox reactions to the final electron acceptor, oxygen, to produce energy required for cellular function [129]. This process of electron transfer is not 100% efficient, and electrons that “leak” from the ETC react with oxygen and produce ROS [130], and this incorrect reduction of oxygen will occur ~2-5% of the time during normal metabolism [131]. During exercise, contracting muscles increase their demand for energy in the form of ATP. To meet this increased demand, electron flow along the ETC also increases resulting in increased leakage of electrons from the ETC enhancing ROS production [132]. The result of increased ROS production during exercise is cellular oxidative stress that is proportional to exercise intensity [133,134]. Indeed, intense exercise has been demonstrated to produce protein carbonyls [135], lipid peroxides [136] and DNA damage [137] (Figure 1 - above), and this ROS induced damage is exercise intensity [5] and

duration dependent [135]. Despite the detrimental cellular effects associated with high intensity, long-duration exercise; exercise training is associated with a myriad of health benefits, including those associated with minimizing oxidative stress and damage. This is best punctuated by the benefits of exercise in populations suffering from pathologies associated with oxidative stress [138–142]. This paradox can be explained by the adaptive response induced by exercise.

The theory of exercise hormesis postulates that each individual exercise bout in a training program causes mild oxidative stress triggering an adaptive response to diminish the stress of subsequent exercise bouts [127,128]. Specific aspects of the adaptive response to exercise training are responsible for exercise hormesis. These adaptations can be divided into two categories: (1) adaptations that inhibit ROS formation and (2) adaptations that repair damage caused by ROS. The primary cellular defense mechanisms against ROS are antioxidant enzymes. Indeed, exercise training increases antioxidant enzyme activity [143] and whole tissue antioxidant capacity [144]. Regarding repair of cellular macromolecules, DNA repair is of the utmost importance because DNA damage can result in cell death or transformation of healthy cells into cancerous ones. Importantly, exercise training increases DNA repair enzyme activity [145] and enhances removal of damaged nucleotides [146]. Collectively, these adaptations result in a reduction in DNA and protein damage in exercise trained animals

[146]. Over time, and with progressively increasing training intensities and durations, cellular defense and repair mechanisms increase improving the overall cellular capacity to deal with oxidative stress.

The protective adaptations associated with exercise training are not localized only to organs directly stressed by exercise such as skeletal or cardiac muscle. Many other tissues including the liver [144], brain [147], and spleen [148], also show similar adaptations with exercise. Relevant to the current thesis, exercise training has been shown to induce protective adaptations in peripheral blood cells. A number of studies from the lab of Dr. Hoffmann-Goetz have demonstrated a number of effects of exercise training and acute exercise in intestinal lymphocytes. In lymphocytes, acute exercise increases inflammatory markers and apoptosis [149–153] while exercise training has opposite effects [149,154,155] potentially via up regulation of anti-oxidant enzymes [150]. Data from other groups have also suggested that acute exercise induces oxidative stress in peripheral blood cells as indicated by acute increases in antioxidant enzymes [156–159] that were associated with increased damage to cellular macromolecules [160,161] and increased cell death [156]. Studies investigating global changes in gene expression using micro-arrays following an intense exercise bout support these findings with genes involved in the stress and inflammatory response being consistently up-regulated [162,163]. Conversely, exercise training has been found to cause an increase in antioxidant activity

[164,165] with increased DNA repair capacity in peripheral blood cells [164] which were associated with increased protection from oxidative stress [164]. Therefore, it is evident that the adaptive response induced by exercise is not limited to tissues directly stressed by exercise and that similar protective mechanisms are seen in various tissues. Although beneficial adaptations have been demonstrated in mature, fully differentiated cells, likely mediated by acute oxidative stress associated with each exercise bout, the protective effects of exercise on more primitive hematopoietic cells located in the bone marrow are unknown. Furthermore, hormesis in general, and specifically exercise hormesis refers to protection from a stressor induced by the response to lower levels of the same stressor; however, less information is known regarding the ability of the adaptive response to one stressor to protect against damage induced by an alternative stressor. Increased knowledge in this area would be particularly interesting with reference to exercise and disease prevention.

1.4.2 Exercise and HSC

A paucity of data exists on the effects of acute exercise or exercise training on HSC (Table 1). To date, the studies that have examined HSC, in response to exercise have focused on their quantification in peripheral blood, and the potential mechanism responsible for the observed effects. Acute exercise seems to increase peripheral blood HSC quantity; however, firm conclusions are difficult to make when comparing studies due to the wide range of subject

characteristics, markers used to define HSC and timing of analysis. With respect to acute exercise, Bonsignore and colleagues compared the quantity of HSC in peripheral blood, identified as $CD34^+$ cells, between sedentary individuals and those who competed in a half or full marathon [51]. HSC were unchanged compared to pre-race values immediately following the race in both half- and full-marathon competitors, and were significantly decreased 24 hours post-race [51]. Morici and colleagues, evaluated the quantity of HSC in peripheral blood immediately prior to and following an all-out rowing test in competitive rowers [52]. Although HSC, identified as $CD34^+$ cells, were increased in peripheral blood immediately following exercise in both males and females tested, the most primitive HSC population, $CD34^+CD38^-$ cells, were unaffected by the acute exercise stimulus [52]. Wardyn and colleagues, compared HSC content in peripheral blood in previously sedentary individuals versus habitual exercisers following an acute treadmill test [50]. The authors concluded that HSC, identified as side population (SP) cells, quantity in peripheral blood increased irrespective of training status and gender immediately post-exercise [50]. Upon closer inspection of the data, the overall increase in peripheral HSC seemed to be primarily due to the increase in trained individuals (~ 2 fold; 44.4 ± 51.0 post vs. 21.8 ± 21.8 pre) while a negligible increase was observed in sedentary subjects (30.0 ± 32.1 post vs. 29.5 ± 59.7 pre) [50]. The most comprehensive timecourse of HSC, identified as $CD34^+$ cells, appearance in peripheral blood was conducted by Möbius-Winkler and colleagues [55]. The authors demonstrated that cycling

at 70% of anaerobic threshold resulted in an approximately 3 fold increase in circulating HSC with 4 hours of continuous cycling that had returned to baseline 24 hours later [55]. Collectively, data from these studies suggest that in healthy adults, exercise duration, intensity and previous training status but not gender are important factors affecting HSC appearance in peripheral blood post-exercise, but more research is necessary to draw any firm conclusions.

The effects of age on the response of HSC to acute exercise have also been examined. Zaldivar and colleagues (2007), examined the response of early versus late pubertal boys to 20 minutes of high intensity cycling, and observed equal increases in peripheral $CD34^+$ cells in both groups [53]. At the opposite end of the aging spectrum, Thijssen and colleagues (2006) observed a similar relative increase in circulating HSC following an acute exercise stimulus in young and elderly individuals [54]. Therefore, age does not seem to play a role in the peripheral HSC response to acute exercise. Taken together, the acute increase in peripheral HSC quantity following exercise is duration dependent, but independent of age or previous training status, and the most primitive HSC populations are not increased in peripheral blood by exercise.

The effects of exercise training on basal circulating HSC quantity have been examined in few studies. Some studies have demonstrated that the quantity of circulating HSC does not change with exercise training [50,54,166]; however, under certain conditions, circulating HSC quantity was elevated in study

participants [51,166,167]. The lack of consensus among studies may be related to the type or intensity of exercise. Studies where no effect of training was observed used either self-described habitual exercisers [50], employed the mild training stimulus of treadmill walking [166], or employed non-weight bearing exercise [54]. Conversely, studies demonstrating an increase in circulating progenitors examined subjects whose exercise stimulus was more intense. For example, the only group that increased circulating progenitor quantity post-training in the study by Sandri and colleagues [166] exercised under conditions of lower limb ischemia, and the subjects in the study by Bonsignore and colleagues [51] were training for a marathon. Furthermore, recent data from our lab supports these findings demonstrating increased hematopoietic stem/progenitors following forced treadmill running in mice [167]. Importantly, weight bearing exercise, such as running, has been demonstrated to increase osteoblasts activity [168], and favoured MSC differentiation towards the osteogenic lineage over the adipogenic lineage while decreasing intra-marrow adipose accumulation [167]. Additionally, ischemia is a potent stimulus for HSC to enter circulation to participate in vasculogenesis [166]. Therefore, stimulation of the HSC niche with exercise by increasing osteoblasts quantity, or imposing a proliferative/mobilization stress on HSC as with ischemic exercise may be necessary signals responsible for the observed increases in these studies [51,166,167]. The only study to examine the effects of aging on the HSC response to training was conducted by Thijssen and colleagues (2006) and while basal levels of circulating HSC were higher in young

individuals, training did not increase circulating HSC in either young or old subjects [54]. Taken together, these data suggest that HSC quantity with training is not dependent on age, but is dependent on exercise type and intensity.

Table 1. Summary of studies examining the response of HSC to exercise.

Subjects	Status	Exercise	Cell Pop.	Result	Ref.
♂ (~41 yr)	SED/ ET	Marathon	CD34+ $CD34^{+}$/ $CD38^{-}$	Pre-Ex: 3-4x↑ in ET Post-Ex: ↔ 24h post-Ex:↓ Post-Ex : ↔	[51]
♂/♀ (16-22 yr)	ET	1000 m sprint on rowing ergometer	CD34 $CD34^{+}$/ $CD38^{-}$	Post-EX : 2x↑ ♂/♀: ↔ Post-Ex : ↔	[52]
♂/♀ (19-35 yr)	SED/ ET	Treadmill test to exhaustion	SP	Pre-Ex: ↔ Post-Ex:↑ 1.5x	[50]
♂ (~32 yr)	Active	4 h cycling @ 70% IAT	$CD34^{+}$	3x ↑	[55]
♂ Y (19-28 yr)/ O (67-76 yr)	SED/ ET	Training: Cycling 8wk Acute Ex: Max cycling test	$CD34^{+}$/ $CD45^{(low)}$	Pre-Ex:↔ Y/O Post-Ex:↑ Y/O and pre/post training	[54]
Patients (<75 yr): ischemic / Non-ischemic	SED	Treadmill Walking: 5 d/wk, 4 wk	CD34+	Post-ET: 5x ↑ (ischemic only)	[166]
Mice	SED	10 wk treadmill running	LSK	Post-ET: ↔	[167]

(♂=male, ♀=female, Y=young, O=old, SED=sedentary, ET=exercise trained, Ex=exercise, wk=week, ↑=increase, ↔=no change, ↓=decrease, Ref.=reference, IAT=individual anaerobic threshold)

1.4.3 Mechanisms Responsible for the Effects of Exercise on HSC

To investigate the mechanisms responsible for the increased appearance of HSC in circulation with exercise, investigations have focused on levels of various cytokines and growth factors involved in HSC proliferation and mobilization. Levels of G-CSF, involved in hematopoietic progenitor cell activation and mobilization from the niche [45], were increased in peripheral blood following a marathon [51,169] and also after an acute cycling test in adolescent boys [53]; however, levels of G-CSF did not changed following the acute rowing protocol employed by Morici and colleagues [52]. Another factor involved in hematopoietic progenitor mobilization and homing, SDF-1 [99,100], was increased in response to an acute cycling test in adolescent boys [53]. Although no correlation was observed between factors involved in hematopoietic progenitor cell mobilization and content in peripheral blood [51], VEGF, released in response to tissue hypoxia [170], was increased in response to acute exercise suggesting tissue hypoxia may be the stimulus for the observed increases in peripheral progenitor content [52]. This hypothesis is in agreement with the study by Sandri and colleagues (2005) who only observed an increase in peripheral HSC content with ischemic training [166]. The findings from the studies outlined above suggest that tissue hypoxia may be the primary stimulus for the observed increases in progenitor cell quantity with exercise. Another growth factor responsive to acute exercise with implications for HSC quantity is parathyroid

hormone (PTH). Acute exercise has been demonstrated to increase serum PTH levels [168], and injection of PTH was shown to increase osteoblast quantity resulting in a concomitant increase in HSC quantity [84]. Although it is difficult to compare pharmacological levels of a hormone to physiological increases of hormones induced by exercise, these data may point to another mechanism for increases in HSC content post-exercise where exercise-induced increases of PTH increase osteoblasts quantity in the HSC niche resulting in a concomitant increase in HSC quantity.

The inflammatory response to acute exercise may also regulate the appearance of HSC in circulation following acute exercise. Levels of IL-6 reported to be important for HSC self-renewal [76], increased in peripheral blood following both a half marathon [51], and a full marathon [51,169], and also in response to an acute treadmill test [50]. The increase in IL-6 post-exercise was dependent on the duration of exercise as the increase in IL-6 was greater following a full than a half marathon [51]. Conversely, no changes in IL-6 were observed in response to an acute maximal exertion rowing test [52] suggesting that the level of IL-6 in serum may be dependent on the type of exercise with weight-bearing exercise inducing a greater response. In general, the acute changes in IL-6 following exercise did not correlate to changes in circulating HSC quantity [50–52]. Interestingly, Möbius-Winkler and colleagues observed a peak in serum IL-6 levels immediately after 4 hours of cycling, following the peak in

circulating HSC, and coinciding with the decline in levels of HSC in circulation [55]. These data indicate that IL-6 may signal peripheral blood HSC to return to their niche in the bone marrow or enter damaged tissue to assist with repair. Alternatively, IL-6 may be an indicator of systemic inflammation that may negatively regulate HSC quantity. Along these lines, Bonsignore and colleagues (2002) demonstrated an inverse correlation between levels of the pro-inflammatory cytokine TNF-α immediately post-exercise, and circulating HSC quantity 24 hours post-exercise [51]. Therefore, inflammation associated with acute exercise decreases HSC quantity in peripheral blood. Whether this decrease in HSC quantity represents increased HSC cell death when exposed to increased inflammatory cytokines induced by exercise or removal of HSC from circulation to contribute to tissue repair is unknown [50].

The effects of training on hematopoietic growth factors are less clear with only one study specifically examining the levels of TNF-α, IL-6, G-CSF and Flt3 in trained versus sedentary individuals and did not observe any basal differences [51]. Recent data from our laboratory suggest that production of IL-3, M-CSF and GM-CSF is increased in skeletal muscle at the transcript level suggesting regulation of circulating cytokines by exercised muscle [167]. Exercise training is generally considered to be anti-inflammatory, reducing levels of the pro-inflammatory cytokines IFN-γ and TNF-α, resulting in a concomitant decrease in anti-inflammatory cytokines such as IL-10 [171–174]. Given the susceptibility of

HSC to dysregulation by inflammatory environments [15,16], the anti-inflammatory effects of exercise may be important for maintaining HSC quantity and quality. There is an obvious need for more research into the mechanisms responsible for the increased quantity of circulating HSC with exercise.

1.4.4 Exercise and HSC Function

Functionally, circulating HSC are believed to be involved in tissue repair [175]. For example, HSC have been demonstrated to contribute to muscle repair following acute exercise [176]. Therefore, elevations in circulating HSC content following acute exercise likely represents a physiological response to stress that promotes tissue repair and adaptation [176]. Elevated circulating levels of HSC with training may indicate an increased capacity for repair and adaptation in trained individuals. For example, the process of HSC contribution to tissue repair may be a more regular and rapid occurrence in trained individuals due to their higher basal levels of circulating HSC. Indeed, mononuclear cells (a fraction containing circulating HSC) isolated from patients with varying degrees of peripheral arterial occlusive disease who were exercise trained for 4 weeks, a model of exercise under conditions of ischemia, showed a higher propensity to form vascular networks in culture perhaps due to elevated levels of CXCR4 expression [166]. Conversely, Wardyn and colleagues (2008) estimated circulating HSC quality by the ratio of low SP to high SP [50], as it has been suggested that cells that are better able to efflux Hoechst dye are a higher quality

HSC [177]. No difference in the ratio of low SP to high SP was observed with acute exercise, and the authors concluded that acute exercise did not affect HSC quality [50]. This analysis only represents an estimation of HSC quality and not a direct measure. As such, there is an obvious need for more research into the effects of exercise on the more primitive HSC located within the bone marrow, and if exercise can improve HSC quality as well as quantity. Given their wide use in cell therapy and contribution to tissue repair, understanding the physiological effects of exercise on HSC may have wide-ranging implications for health.

1.4 .5 Exercise and BMT

Despite the evidence that certain physiological and lifestyle factors can influence transplantation success [114]; no data exist on the level of fitness or physical activity of donors, and whether or not this has an influence on transplantation success. With respect to BMT recipients, a number of studies have examined the effects of exercise training in the pre-, peri- and post-operative period surrounding BMT in humans [178]. In general, it is suggested that exercise programs are well-tolerated and can be undertaken by individuals with severely advanced disease states as demonstrated by an 85% compliance rate on average [178]. Exercise results in improvements in psychological parameters, as well as physiological parameters such as endurance capacity and fatigue levels, strength and body composition [178]. Particularly relevant to the present thesis, four studies have examined the effects of exercise on

hematological recovery post-BMT. The majority of studies demonstrated an attenuation of hematopoietic cell loss, specifically, decreased lymphocyte loss [179], an attenuation of dendritic cell loss [180] and also decreased time of neutropenia [181] in patients that underwent a regular exercise training program. A fourth study; however, failed to show any effect of exercise on lymphocyte and leukocyte quantity [183]. Interestingly, the studies showing a beneficial effect of exercise on peripheral blood cells employed an in-patient training program where exercise sessions began prior to BMT, continued through the pre-conditioning strategy and transplantation until hospital discharge [179–181]. Conversely, in the study by Hayes and colleagues (2003) where no effect of exercise was observed in blood cell quantity, patients underwent an out-patient training program where exercise training began the day patients were discharged from the hospital [182]. Together, these studies suggest that the timing of initiating an exercise training protocol in BMT recipients is an important consideration to maintain blood cell quantity, and that initiating exercise training prior to BMT may be optimal.

1.5 PURPOSE OF THE THESIS

The present thesis was undertaken to investigate the following four objectives. First, based on the extensive literature describing the adaptive response to exercise training, we wanted to determine the effects of radiation on skeletal muscle preconditioned with exercise training. We hypothesized that the

adaptive response to exercise training characterized by increased antioxidant enzyme activity and increased activity of the mitochondrial enzymes, citrate synthase and cytochrome c oxidase, would provide an enhanced response to radiation exposure with decreased macromolecular damage and increased antioxidant enzyme activity. Given the increased susceptibility of mitochondria to radiation, we focused on the response of this organelle to radiation with training by evaluating the activity of mitochondrial enzymes involved in energy production. Second, based on the literature describing the systemic effects of exercise and the increased sensitivity of the hematopoietic system to radiation, we were interested in investigating the potential protective effects of exercise training on primitive cells of the hematopoietic system in the bone marrow. To further elucidate the mechanisms responsible for the potential protective effects of exercise, we asked if the protective effects of training were mediated by acute stresses imposed by a single exercise bout. Our third objective was to establish if the effects of exercise training observed in mature cells of the hematopoietic system extend to the most primitive HSC. To investigate this question, quantification of HSC from the endosteal and vascular niches via multi-colour immunophenotyping was conducted, and evaluated HSC function using bone marrow transplantation (BMT) paradigms never previously employed in the exercise science literature. Finally, to investigate the potential for exercise training to promote recipient survival post-BMT, as well as the effects of exercise training on the HSC niche, we preconditioned recipients in the BMT assay with

exercise and evaluated various parameters related to survival, as well as short- and long-term engraftment. Together, the four studies presented herein describe the effects of exercise pre-conditioning prior to radiation exposure on skeletal muscle and hematopoietic cells, describe the effects of exercise training on HSC quantity and function, and investigate the potential for exercise training as an adjuvant therapy for BMT.

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2. Chapter 2

Exercise training enhances the skeletal muscle response to radiation-induced oxidative stress

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EXERCISE TRAINING ENHANCES THE SKELETAL MUSCLE RESPONSE TO RADIATION-INDUCED OXIDATIVE STRESS

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ABSTRACT: Overproduction of reactive oxygen species (ROS) can damage cellular macromolecules and lead to cellular dysfunction or death. Exercise training induces beneficial adaptations in skeletal muscle that may reduce cellular damage from exposure to ROS. To determine the response of exercise-conditioned muscle to acute increases in ROS, four groups of mice were used: non-trained (NT, $n = 12$); NT + high-dose radiation (HDR, $n = 3$); exercise-trained (EX, $n = 13$, 3 days/week for 10 weeks, 10 m/min to 18 m/min); and EX + HDR ($n = 3$/group). Quadriceps muscle was harvested 3–5 days following the last exercise bout in the training program for measurement of antioxidant enzyme and metabolic enzyme activity. Total superoxide dismutase (41%), and manganese sodium oxide dismutase (51%) activities were significantly increased in radiation-challenged EX mice as compared with unchallenged EX mice (all $P \leq 0.05$). No such increase was observed in NT mice. Citrate synthase (42%) and cytochrome *c* oxidase (38%) activities were both elevated in radiation-challenged EX mice as compared with unchallenged EX mice (both $P < 0.05$), and no such increase was observed in NT. We demonstrate that preconditioning skeletal muscle with EX enhances the response of antioxidant and mitochondrial enzymes to radiation.

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Cellular oxidative stress occurs when reactive oxygen species (ROS) production exceeds the capacity for antioxidant defense.[1] Minor perturbations in cellular redox homeostasis may elicit beneficial cellular adaptations that induce mechanisms that are protective against future oxidative stress, whereas large increases in ROS formation results in oxidative damage to cellular macromolecules and leads to dysfunction and death. For example, an exercise-induced increase in ROS[2] has been implicated as a potential mechanism underlying adaptations associated with exercise training, including increased antioxidant enzyme activity,[3] enhanced DNA repair capacity,[4] and decreased DNA damage, upon exposure to subsequent bouts of oxidative stress.[5] On the other hand, severe oxidative stress induced by exposure to high-dose radiation (HDR) can have deleterious consequences, including oxidative damage to lipids,[6] proteins,[6] and DNA,[7] and ultimately cell death.[8]

Previous studies that examined the response of trained and sedentary muscle to an acute oxidative stress have employed acute exercise[9–11] or ischemia–reperfusion[12] as an oxidative stress–inducing insult. Although these models may have high practical relevance with regard to exercise performance or disease prevention, conclusions regarding the specific response to ROS exposure are difficult to derive. For example, exercise training enhances mitochondrial efficiency[13]; therefore, at the same exercise intensity, intracellular ROS production in a trained animal is lower than in a sedentary animal. As a result, the isolated effects of intracellular ROS production cannot be determined. Furthermore, the use of ischemia–reperfusion models before and after exercise training is not ideal, because exercise is known to induce an increase in muscle vascularization.[14] Improved vascularization following exercise can increase oxygen supply to skeletal muscle and produce disproportionate ROS production between exercise and sedentary muscle.

In an attempt to circumvent these limitations and produce a consistent level of ROS between sedentary and trained muscle, we employed an HDR approach as our model to induce oxidative stress. Radiation-induced cellular damage is the result of formation of intracellular ROS, and only a small percentage is due to direct ionization of cellular macromolecules by gamma rays themselves.[15] Gamma rays create ROS via the ionization of water molecules to form the hydroxyl radical and hydrogen,[16] and similar models have previously been used to examine ROS-mediated damage in muscle.[6,15] In this model, intrinsic cellular changes that may have occurred during the course of the training period, such as increased efficiency of the mitochondrial electron transport chain (ETC) or increased vasculature, are controlled for by inducing cellular oxidative stress via an exogenous source, which enables the specific examination of the skeletal muscle response to an acute increase in ROS. The objective of this study was to

Abbreviations: AP-1, activator protein-1; CAT, catalase; COX, cytochrome *c* oxidase; CS, citrate synthase; Cu/ZnSOD, copper zinc superoxide dismutase; DNP, 2,4-dinitrylphenylhydrazine; EDTA, ethylene-diamine tetraacetic acid; ELISA, enzyme-linked immunoassay; ETC, electron transport chain; EX, exercise-trained; HDR, high-dose radiation; MnSOD, manganese superoxide dismutase; NT-κB, nuclear factor-kappaB; NT, sedentary; PVC, polyvinyl chloride; ROS, reactive oxygen species; SOD, superoxide dismutase

Key words: antioxidant, exercise, high-dose radiation, hormesis, mitochondria

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determine if the response of skeletal muscle to severe oxidative stress was altered by preconditioning with exercise training. Given the beneficial adaptations to exercise training, such as increased antioxidant enzyme activity, we hypothesized that skeletal muscle from exercise-trained animals would have an enhanced response to cope with acute oxidative stress induced by HDR.

METHODS

Animals and Exercise Training. Sixteen-week-old male C57Bl/6 mice were maintained on a 12-hour light:dark cycle and provided food (Harlan Lab Diets; Harlan, Indianapolis, Indiana) and water ad libitum. All procedures were conducted according to guidelines established by the Canadian Council on Animal Care with ethics approval from the McMaster University Animal Research Ethics Board.

Mice were randomly assigned to an exercise-trained (EX) or non-trained (NT) group. EX mice ($n = 16$) were trained on a motorized treadmill (Exer 6M Treadmill; Columbus Instruments, Inc., Columbus, Ohio) three times per week (Monday/Wednesday/Friday) for 10 weeks. The exercise training protocol was based on the training protocol used by Avula and Fernandes[17] and Mahoney et al.,[18] but the training intensities were modified to accommodate the capabilities of the mice in this study, and the training load was modified to produce a moderate intensity training stimulus. During weeks 1–3, mice were acclimatized to treadmill running with training sessions consisting of a 10-min warm-up at a speed of 8 m/min followed by a 25-min (week 1), 35-min (week 2), or 45-min (week 3) training period at a speed of 10 m/min and a 5-min cool-down at a speed of 8 m/min. During weeks 4–10, the intensity of the training period progressively increased from 10 m/min to 18 m/min and was always preceded by a 10-min warm-up and a 5-min cool-down. Mice were encouraged to run with a mild electric shock or hindlimb stimulation with the bristles of a paintbrush. Initially, 16 mice were included as non-trained controls (NT), which were handled in the same manner as exercise-trained mice but were not exposed to treadmill running; however, one NT mouse died during the intervention period for reasons unrelated to the study protocol. Thus, 15 mice were included in the NT group.

Radiation Challenge. A subgroup of mice from each group ($n = 3$ per group) received a non-lethal, high dose of radiation (HDR; 1 Gy), as previously described,[7] 3 days following the final exercise bout. Briefly, mice were placed two at a time in a polyvinyl chloride (PVC) tube (5 × 12.5 cm) equipped with holes to permit circulation of room air. While in the PVC tubes, mice were exposed to gamma radiation from a 137-Cs gamma-ray source at a dose rate of 0.279 Gy/min for 4 min, giving a total dose of 1.116 Gy ($\backsim$1 Gy). This particular dose of radiation was chosen because it is below the LD_{50} of gamma radiation in mice (5 Gy).[6] It has previously been employed to determine biomarkers of radiation exposure in a variety of tissues, and it produced a response similar to other non-lethal doses.[19] Unchallenged mice ($n = 12$ in NT, $n = 13$ in EX) received a "sham" irradiation in which they were placed in the same PVC tubes for an equal amount of time but were not exposed to the radiation source.

Muscle Harvest and Homogenization. Mice were euthanized via cervical dislocation over 2 days, and muscle was harvested. The majority of "sham"-irradiated mice were harvested on the first harvest day, 3 days following the final exercise bout, to avoid acute effects of the final training bout. On the second harvest day, 2 days following the HDR challenge, the remaining sham-irradiated mice and all irradiated mice from each group were harvested. On this day, all HDR-challenged mice and a subset of unchallenged mice were harvested to account for any variability that may have occurred across the 2 harvest days. On both harvest days, quadriceps muscle from each hindlimb were quickly removed, placed in sterile tubes, and immediately frozen in liquid nitrogen before being stored at −80°C. This time course was chosen based on the findings of two prior studies. Oh-Ishi and colleagues[10] suggested that the increased antioxidant activity following acute oxidative stress is due to increased protein content, not posttranslational modifications, and Lee and colleagues[19] found that Cu/ZnSOD protein levels were elevated 1–3 days following 1 Gy of whole-body irradiation in a variety of tissues.

Muscle samples were homogenized (1:25, wt/vol) in ice-cold homogenization buffer [50 mM phosphate buffer, 1 mM ethylene-diamine tetraacetic acid, 1.15% KCl, 0.1 mM dithiothreitol, protease inhibitor tablet (1/10 ml; Roche), pH 7.2 or 7.3] using a glass tissue homogenizer. Homogenates were centrifuged at 4°C for 10 min at 900*g*, or at 10,000*g* for protein carbonyl analysis. The protein content of the aqueous layer was determined colorimetrically via Bradford dye binding[20] using the Bio-Rad Bradford assay kit with colorimetric changes measured at 595 nm in a spectrophotometer (HP UV Visible Chemstation 8453). Protein fractions were flash frozen in liquid nitrogen and stored at −80°C until analysis.

Markers of Oxidative Damage. *Protein Carbonyls.* Protein carbonyls were determined as previously described[21] by measuring the reactivity of carbonyl groups with 2,4-dinitrophenylhydrazine (DNP) using an enzyme-linked immunosorbent assay (ELISA; Biocell PC test) according to kit instructions. Briefly, a 5-μl sample was mixed with 200 μl of DNP (diluted 1:10 in guanidine-HCl) and incubated in DNP for 45 min at room temperature. Five microliters of each DNP-treated sample was mixed with 1 ml of EIA buffer (supplied in kit), and 200 μl of this solution was aliquoted to a well of a 96-well plate. The plate was incubated overnight at 4°C. Samples were washed with EIA buffer and then blocked with diluted blocking solution (supplied in kit) for 30 min at room temperature. Samples were again washed with EIA buffer, then incubated with diluted anti-DNP–biotin antibody (supplied in kit) for 1 h at 37°C. After washing in EIA buffer, samples were incubated with diluted streptavidin–horseradish peroxidase for 1 hour at room temperature. For colorimetric detection, samples were incubated with Chromatin reagent (supplied in kit) for 5 min, after which the reaction was stopped with stopping reagent (supplied in kit). Immediately upon cessation of the reaction, absorbances were read in duplicate at 450 nm in a microplate reader (BioRad, Canada). Quantification of protein carbonyls from each sample was done using a standard curve obtained using oxidized protein standards (supplied in kit).

8-Isoprostanes. The concentration of 8-isoprostane in each sample was detected using the 8-isoprostane EIA kit (Cayman Chemical Co.), according to the manufacturer's instructions. Briefly, 50 μl of diluted sample (1:15 in ddH_2O) was incubated with 50 μl of 8-isoprostane–acetylcholinesterase (AChE) tracer (supplied in kit) and 50 μl of 8-isoprostane antiserum (supplied in kit) overnight at 4°C. Following five washes with wash buffer (supplied in kit), samples were incubated with 200 μl of freshly made Ellman's reagent (supplied in kit) in the dark with shaking for 95 min. Samples were analyzed in duplicate, and plates were read using a microplate reader (BioRad, Canada) at a wavelength of 412 nm. 8-Isoprostane concentration was quantified based on a standard curve produced using 8-isoprostane standards (supplied in kit).

Antioxidant Enzyme Activity. *Superoxide Dismutase.* Superoxide dismutase (SOD) was measured according to the technique of Flohe and Otting,[22] which measures inhibition of reduction of cytochrome *c* via the kinetic consumption of superoxide. In a 1-ml cuvette, 12 μl of sample and 970 μl of master mix [50 mM phosphate buffer, 0.1 mM EDTA (pH 7.8) with partially acetylated cytochrome *c* (25 mg/100 ml) and 5 μM xanthine] was combined with 17 μl of xanthine oxidase (0.2 U/ml) to initiate the reaction. The reactions were conducted in duplicate at 30°C for 2.5 min, and absorbance was measured at 550 nm in a spectrophotometer (HP UV Visible Chemstation 8453). One unit of SOD activity equaled the amount of enzyme that caused a 50% inhibition of cytochrome *c* reduction.

MnSOD activity was determined concurrently with SOD activity using the same samples. In a 1-ml cuvette, 25 μl of sample was incubated with 25 μl of KCN (0.2 M, made fresh daily) for 2 min at room temperature to inhibit Cu/ZnSOD.[23] To this solution, 940 μl of master mix (same as noted earlier) was added, followed by 16.5 μl of xanthine oxidase (0.2 U/ml) to initiate the reaction. The reactions were conducted in duplicate at 30°C for 3 min, and absorbance was measured at 550 nm in a spectrophotometer (HP UV Visible Chemstation 8453). Cu/ZnSOD activity was estimated by subtracting MnSOD activity from SOD.

Catalase. Catalase (CAT) activity, determined by the kinetic decomposition of H_2O_2, as previously described by Aebi,[24] was measured by combining 70 μl of sample with 920 μl of buffer [50 mM phosphate buffer, 5 mM ethylene-diamine tetraacetic acid (EDTA), and 0.05% Triton X-100 added fresh daily (pH 7.3)] and 10 μl of 1 M H_2O_2. Reactions were carried out in duplicate at 30°C for 2 min, and the absorbance was read at 240 nm in a spectrophotometer (HP UV Visible Chemstation 8453).

Metabolic Enzyme Activities. *Citrate Synthase.* The activity of citrate synthase (CS) was determined by combining 10 μl of sample, 870 μl of buffer [100 mM Tris-HCl, 5 mM EDTA, and 0.05% Triton X-100 added fresh (pH 8.1)], 100 μl of freshly made DTNB (1 mM), 10 μl of freshly made acetyl-coenzyme A (10 μM), and 10 μl of freshly made oxaloacetic acid (10 mM) to initiate the reaction. The reactions were conducted in duplicate at 37°C for 2 min, and absorbance was read at 412 nm in a spectrophotometer (HP UV Visible Chemstation 8453). CS activity was calculated using the extinction coefficient of 1.36 and reported as micromoles per minute per milligram of protein.

Cytochrome c Oxidase. Cytochrome *c* oxidase (COX) activity was determined by measuring the oxidation of reduced cytochrome *c* as described elsewhere.[25] Briefly, preparation of 2 mM reduced cytochrome *c* was conducted by dissolving cytochrome *c* with ascorbic acid (20:1) in phosphate buffer [10 mM (pH 7.0)]. In a 1-ml cuvette, 970 μl of buffer [20 mM phosphate (pH 7.2)] and 5 μl of

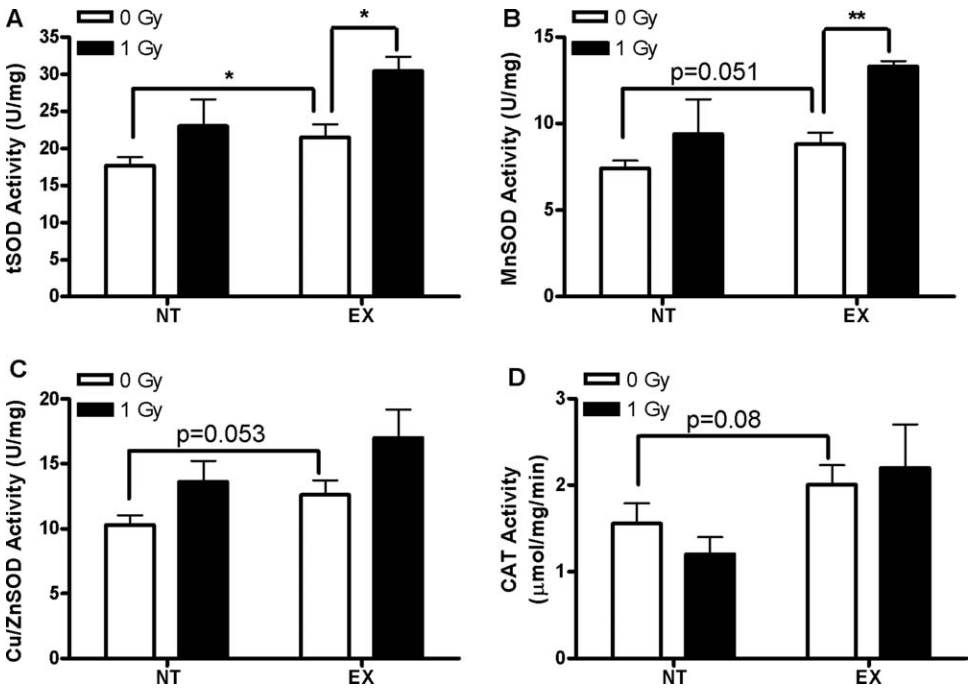

FIGURE 1. The effects of exercise training and an HDR challenge on skeletal muscle total SOD activity **(A)**, MnSOD activity **(B)**, Cu/ZnSOD activity **(C)**, and CAT activity **(D)**. Data are presented as mean ± SEM, with $n = 12/13$ in unchallenged NT and EX mice, respectively, and $n = 3$ in challenged NT and EX mice. $^{*}P < 0.05$; $^{**}P < 0.01$.

sample were mixed, and 20 μl of reduced cytochrome *c* were added to initiate the reaction. The reaction was conducted at 37°C for 2 min, and absorbance was read at 550 nm in a spectrophotometer (HP UV Visible Chemstation 8453).

Statistics. Statistics were performed using SigmaStat v3.1 statistical software (Systat). The following a-priori hypotheses were tested using planned comparisons:

1. Exercise training would increase antioxidant and mitochondrial enzyme activity.
2. Exercise-trained muscle would exhibit an enhanced response to HDR.

The first a-priori hypothesis was tested with a one-tailed *t*-test between unchallenged EX and NT. A one-tailed test was chosen because we hypothesized that exercise training would only increase antioxidant and mitochondrial enzyme activity, which is supported by many previous studies.[11,26–28] The second a-priori hypothesis was tested with two-tailed *t*-tests between challenged and unchallenged mice within EX or within NT. Data are reported as mean ± SEM, with $P \leq 0.05$ considered significant.

RESULTS

No significant differences for all outcome measures were observed between unchallenged samples harvested on harvest days 1 or 2; therefore, data from days 1 and 2 were combined for the unchallenged mice in both groups (NT and EX).

Markers of Oxidative Damage. No significant differences in levels of protein carbonyls (data not shown) and 8-isoprostane levels were observed following exercise training and HDR (data not shown).

Antioxidant Enzyme Activity. Total SOD activity was significantly increased with exercise training compared with NT (21%, $P < 0.05$; Fig. 1A). In the EX mice, total SOD activity increased significantly in response to the HDR challenge compared with unchallenged (41%, $P < 0.05$; Fig. 2A), whereas no such increase was observed in the NT mice (30%, $P = 0.08$). MnSOD activity exhibited a strong trend to increase in EX compared with NT (19%, $P = 0.051$; Fig. 1B). In the EX mice, MnSOD activity increased significantly in response to the HDR challenge compared with unchallenged (51%, $P < 0.01$; Fig. 2B), whereas no such increase was observed in the NT mice (27%, $P > 0.05$). Cu/ZnSOD activity also exhibited a strong trend to increase in EX vs. NT (22%, $P = 0.053$; Fig. 1C). Cu/ZnSOD activity did not increase significantly in response to HDR in either group (EX: 35%, $P = 0.11$; SED: 32%, $P = 0.068$; Fig. 1C). Catalase activity was unchanged in EX mice compared with NT (29%, $P = 0.08$; Fig. 1D), and there was no effect of the HDR challenge in either group.

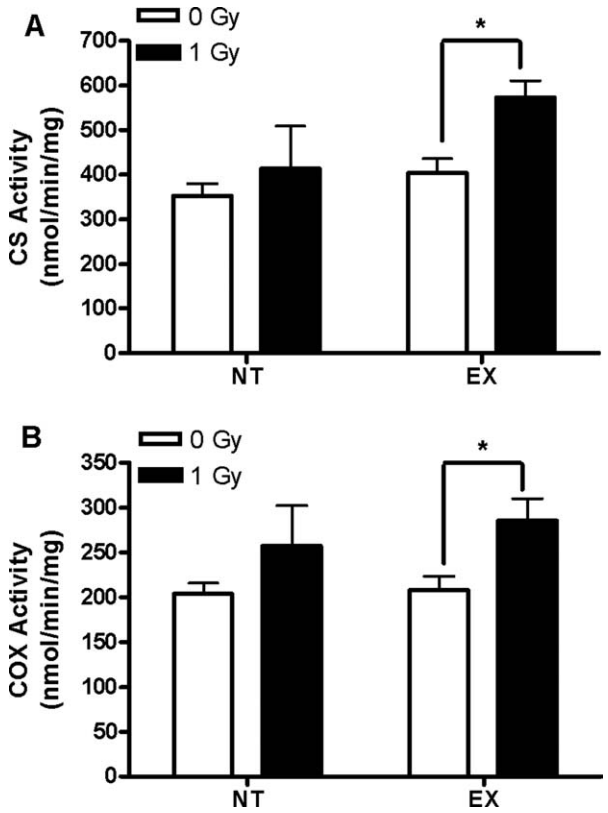

FIGURE 2. The effects of exercise training and an HDR challenge on skeletal muscle CS **(A)** and COX **(B)** activity. Data are presented as mean ± SEM, with $n = 12/13$ in unchallenged NT and EX, respectively, and $n = 3$ in challenged NT and EX for CS activity and $n = 11/12$ in unchallenged NT and EX, respectively, and $n = 3$ in challenged NT and EX for COX activity. *$P < 0.05$.

Metabolic Enzyme Activity. CS activity was not increased in EX compared with NT mice (15%, $P = 0.11$; Fig. 2A). In EX mice, CS activity increased significantly in response to HDR compared with unchallenged EX mice (42%, $P < 0.05$; Fig. 2A), and no such increase was observed in NT mice (18%, $P > 0.05$; Fig. 2A). No effect of exercise training was observed for COX activity. In EX mice, COX activity was increased significantly in response to HDR compared with unchallenged EX mice (38%, $P < 0.05$; Fig. 2B), whereas no such increase was observed in the NT mice (26%, $P > 0.05$).

DISCUSSION

In this study we have shown the response of skeletal muscle to exercise training and an acute oxidative challenge in the form of HDR. Exercise training increased antioxidant enzyme activity. Furthermore, the data suggest synergistic interactions between exercise and radiation, as the majority of these increases were accounted for by significantly enhanced enzyme activities within the exercise-trained group exposed to HDR. Significant increases in antioxidant and mitochondrial enzyme activities were not observed in non-trained animals. This suggests that the response of skeletal muscle to HDR is enhanced by preconditioning with exercise training.

It has been suggested that the acute elevation of ROS in skeletal muscle by HDR could rapidly consume all antioxidant defenses in skeletal muscle and proceed to damage cellular macromolecules until cellular defenses can recover.[6] Therefore, increasing basal levels of antioxidant protection may delay or prevent oxidative damage. Indeed, the exercise training protocol employed in this study enhanced skeletal muscle antioxidant activity, which is in accordance with numerous other studies.[11,26–28] Our data demonstrate increased antioxidant enzyme activity in trained muscle, which suggests that a higher level of ROS production would be required before antioxidant enzyme defenses were consumed in trained muscle.

Muscle, preconditioned with exercise training, augmented its antioxidant defenses in response to HDR, whereas sedentary muscle did not. Specifically, the exercise-trained mice displayed increased MnSOD activity in response to HDR, but non-trained mice did not. Previous studies that examined the responses of trained and non-trained muscle to an oxidative challenge did not show increases in MnSOD activity in exercise trained muscle, but they did observe increased catalase and glutathione peroxidase activities.[9,12] The differences in upregulation of specific antioxidants are likely due to the different methods of oxidative stress induction. Taken together, however, these data suggest that exercise training induces the capacity to rapidly upregulate antioxidant enzyme activity in response to acute insults, over and above that of sedentary muscle. Although not measured in this study, activation of nuclear factor-kappaB (NF-κB) and activator protein-1 (AP-1) signaling are the likely mechanisms for the enhanced antioxidant response in trained skeletal muscle. Zhou and colleagues demonstrated that cultured myotubes treated with paraquat or H_2O_2 increased both NF-κB and AP-1 activity in a dose-dependent fashion,[29] and these transcription factors have been shown to have upregulated antioxidant enzyme gene expression.[30]

CS and COX are among the most responsive mitochondrial enzymes to exercise training and are commonly used as markers of mitochondrial content and function (reviewed by Holloszy and Coyle[31]). In our study, basal COX and CS activity did not change with exercise training. These data support our contention that the training protocol was of moderate intensity. Despite a lack of basal changes in these mitochondrial enzymes, the exercise protocol did successfully induce cellular

adaptations, because these enzymes were responsive to HDR (CS: 42%; COX: 38%) only in the exercise-preconditioned muscle. These data demonstrate that adaptations acquired as a result of exercise training augment the capacity for skeletal muscle to respond to HDR. There are at least two potential explanations for the observed relationship between HDR and increased CS and COX activity. First, exercise training may upregulate basal levels of proteins involved in mitochondrial biogenesis, such as PGC-1α,[32] and produce a rapid increase in mitochondrial content following HDR. Alternatively, the observed increase in CS and COX activity may be due to posttranslational modifications of these enzymes induced by HDR. Exercise training may increase the amenability of these enzymes to post-translational modifications, which manifests in the synergistic response of HDR and exercise.

CS and COX may be elevated in trained muscle exposed to HDR to act indirectly as antioxidants that prevent further production of ROS.[33] It is known that electron leakage from the ETC is proportional to the amount of time electrons spend at each complex in the chain,[34] and ROS production is decreased with increased oxygen consumption by the ETC.[35] Therefore, increased COX activity may increase the rate at which electrons are shuttled down the ETC. This would reduce the time spent at each complex and thus reduce electron leakage and ROS production.[33]

HDR did not increase levels of skeletal muscle 8-isoprostanes or protein carbonyls in either group. Non-lethal doses of HDR, similar to the dose used in this investigation, have caused oxidative damage in bone marrow cells[8,36,37] and blood[8,38] following an HDR challenge, at the same time interval (2 days) as that used in this study. However, in tissues such as the liver[37,38] and plasma,[36] markers of oxidative damage were not elevated until at least 4 days following the HDR challenge. Together, these results demonstrate the differential susceptibility of various body tissues to HDR and suggest that the timing of muscle harvest in this study, 2 days following the HDR challenge, may have precluded us from observing elevated levels of oxidative damage in skeletal muscle. Therefore, the trend for an increase in carbonyls may represent an elevation, peaking later or earlier than the time examined in the present investigation. Our primary objective was to determine the possible protective mechanisms induced by exercise training; therefore, our primary outcome was antioxidant enzyme activity, given the role of antioxidants in neutralizing ROS. This time course was chosen based on findings from studies by Oh-Ishi and colleagues,[10] who suggested that the increased antioxidant activity following acute oxidative stress is due to increased protein content, not posttranslational modifications, and by Lee and colleagues,[19] who found that Cu/ZnSOD protein levels were elevated 1–3 days following 1 Gy of whole body irradiation in a variety of tissues. Therefore, 2 days post-HDR was chosen as the endpoint for our study.

The model employed in this study allowed us to focus specifically on the effects of elevated ROS and to control for any endogenous cellular changes known to occur with training, such as increased efficiency of the ETC[13] and increased vasculature.[14] Our results suggest that muscle preconditioned with exercise has an enhanced response to oxidative stress in the form of HDR. Higher basal levels of protection are present in trained muscle as a result of increased antioxidant enzyme activity with training. Furthermore, the enhanced upregulation of antioxidant activity in exercise-preconditioned muscle likely functions to prevent further production of ROS following HDR. Finally, exercise-preconditioned muscle exposed to an acute oxidative stress prevents endogenous formation of ROS by enhancing mitochondrial enzyme activity. These results offer important clues as to the mechanistic differences in the response of trained and sedentary muscle to oxidative challenges.

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3. Chapter 3

Exercise-induced protection of bone marrow cells following exposure to radiation

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Exercise-induced protection of bone marrow cells following exposure to radiation

Michael De Lisio, Nghi Phan, Douglas R. Boreham, and Gianni Parise

Abstract: The hormetic effects of exercise training have previously been shown to enhance cellular protection against oxidative stress. Therefore, adaptations to exercise training may attenuate the harmful effects of radiation induced by oxidative stress. Flow cytometric analysis of genotoxicity (γH2AX foci and micronucleated reticulocytes (MN-RET)) and cytotoxicity (apoptosis and percentage of reticulocytes) were conducted on bone marrow cells isolated from acutely exercised (Acute EX), exercise-trained (EX), and sedentary (SED) mice following 1 and 2 Gy radiation challenges in vitro. Acute EX increased the percentage of cells with activated caspase-3 and -7 (32%, $p < 0.001$) and γH2AX foci formation in response to 2 Gy radiation challenge (10%, $p < 0.05$). Exercise training significantly attenuated γH2AX foci formation and MN-RET production in response to 1 Gy radiation challenge (18%, $p < 0.05$ and 22%, $p < 0.05$, respectively). Exercise training also significantly reduced basal percentages of cells with activated caspase-3 and -7 and in response to radiation in bone marrow cells (11%, $p < 0.05$). These results suggest that oxidative stress caused by acute exercise induces an adaptive response responsible for the radioprotective effects of exercise training.

Key words: exercise, high-dose radiation, radiation protection, DNA damage, apoptosis, micronucleated reticulocyte, oxidative stress.

Résumé : D'après des études antérieures, les actions hormétiques de l'entraînement physique améliorent la protection cellulaire contre le stress oxydatif. Par conséquent, les adaptations à l'entraînement physique devraient atténuer les effets néfastes des radiations suscitées par le stress oxydatif. On analyse la génotoxicité (foci γH2AX et réticulocytes micronucléés (MN-RET)) et la cytotoxicité (apoptose et pourcentage des réticulocytes) des cellules de la moelle osseuse isolées chez des souris après une séance d'exercice (Acute EX), après un programme d'entraînement physique (EX) et chez des sédentaires (SED) soumis à des radiations de 1 Gy et 2 Gy in vitro. En réponse à une radiation de 2 Gy, les souris Acute EX présentent un plus fort pourcentage de cellules avec caspase-3 et -7 activée (32 %, $p < 0{,}001$) et incluant la formation de foci γH2AX (10 %, $p < 0{,}05$). L'entraînement physique atténue significativement la formation de foci γH2AX et la production de MN-RET en réponse à une radiation de 1 Gy (18 %, $p < 0{,}05$ et 22 %, $p < 0{,}05$, respectivement). En réponse à la radiation des cellules de la moelle osseuse et à l'entraînement physique, on observe aussi une diminution significative du niveau de base des cellules avec caspase-3 et -7 activée (11 %, $p < 0{,}05$). D'après ces observations, le stress oxydatif causé par une séance d'exercice enclenche une réponse adaptative suscitant ainsi les effets radioprotecteurs de l'entraînement physique.

Mots-clés : exercice, radiation à fortes doses, radioprotection, lésion de l'ADN, apoptose, réticulocyte micronucléé, stress oxydatif.

[Traduit par la Rédaction]

Introduction

Exposure of living organisms to ionizing radiation from cosmic and terrestrial sources is a natural occurrence; however, exposure from manmade sources is becoming increasingly common. The use of medical diagnostic imaging procedures in the past 25 years has increased dramatically (Mettler et al. 2008) and is expected to increase in the future with advances in new and combined modalities (Brenner 2010). There is growing interest to better understand the biological effects of these medical exposures and to develop strategies to mitigate any resulting harmful effects produced in patients (Brenner 2010). Therefore, it would be extremely valuable to develop radioprotective strategies that can decrease the potentially harmful consequences of high-dose overexposure in normal tissues following radiation therapy (early and late effects) and also reduce potential damage caused by too many diagnostic exposures.

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The damaging effects of low linear energy transfer radiation are primarily the result of oxidative stress caused by the production of reactive oxygen species from the radiolysis of water molecules (Prasad 1995). Enhanced production of reactive oxygen species, resulting in cellular oxidative stress, is associated with damage to proteins, lipids, and DNA. The accumulation of cellular damage can lead to dysfunction and has been linked to a myriad of diseases such as diabetes, cardiovascular disease and neurological disorders (Valko et al. 2007), as well as ageing (Harman 1956), and cancer (Marnett 2000). Therefore, identifying and developing radioprotectants could help in the management and protection of organisms from a host of diseases.

Many studies have focused on pharmacological agents (Devipriya et al. 2008) or nutritional supplements (Lemon et al. 2008*a*) as radioprotective interventions that enhance the cell's natural defense mechanisms with exogenous sources of free radical scavengers. A related area of research capitalizes on the hormetic properties of low doses of radiation to stimulate endogenous protective mechanisms (Wolff 1996; Feinendegen et al. 2007). The theory of hormesis has also been applied to exercise training (Calabrese and Nieman 1996; Radak et al. 2005, 2008; Ji et al. 2006). Like low doses of radiation, acute exercise causes cellular oxidative stress (Davies et al. 1982) and has been shown to cause apoptosis in lymphocytes (Hoffman-Goetz and Quadrilatero 2003; Mooren et al. 2004; Quadrilatero and Hoffman-Goetz 2004, 2005) and DNA damage (Hartmann et al. 1998; Tsai et al. 2001). In response to the oxidative stress induced by individual exercise bouts in a training program, cellular adaptations such as increased antioxidant activity (Venditti and Di Meo 1996; Avula and Fernandes 1999; Radak et al. 2005), DNA repair capacity (Radak et al. 2002, 2005), and decreased oxidative damage (Venditti and Di Meo 1996; Radak et al. 2002) have been observed. Traditionally, these adaptations have been associated with protection from oxidative stress in skeletal muscle (Alessio and Goldfarb 1988; Laughlin et al. 1990; Oh-ishi et al. 1997; Smolka et al. 2000); however, the beneficial effects of exercise have also been reported in tissues other than muscle, such as liver (Somani and Husain 1996; Venditti and Di Meo 1996; Nakamoto et al. 2007), brain (Somani and Husain 1996; Devi and Kiran 2004), lung (Somani and Husain 1996), and blood (Calabrese and Nieman 1996; Connolly et al. 2004; Büttner et al. 2007). In particular, trained individuals are resistant to DNA damage (Umegaki et al. 1998) and apoptosis (Avula et al. 2001; Peters et al. 2006) in peripheral lymphocytes when exposed to an oxidative challenge. Although the effects of exercise in mature, circulating blood cells have been examined, a paucity of data exist regarding immature blood progenitors residing in the bone marrow.

Given the wide-ranging tissue types that adapt to exercise, and the ability of exercise to enhance protection against oxidative stress, we hypothesized that exercise training would be protective against an oxidative challenge caused by a high-dose radiation challenge exposure. We focused on bone marrow cells, since they are highly sensitive to radiation such that failure of the hematopoietic system following high-dose radiation exposure is a primary cause of radiation lethality (Dainiak et al. 2003). Furthermore, direct study of cells from the bone marrow compartment has never been performed in the context of exercise training and modulation of radiation response.

Materials and methods

Animals

Adult male C57Bl/6 mice (Jackson Laboratory, Bar Harbor, Maine), aged 16 weeks (at the beginning of training), were used for the exercise training experiments, while 25-week-old male C57Bl/6 mice were used for the acute exercise experiments. No more than 5 mice were housed per cage (27 cm × 12 cm × 15.5 cm) and were provided food and water ad libitum. Mice were maintained on a 12 h light / 12 h dark cycle at 22 ± 2 °C. Ethics approval was granted by the McMaster University (Hamilton, Ont.) Animal Research Ethics Board, and experiments conformed to the guidelines of the Canadian Council on Animal Care.

Exercise training protocol

Mice were exercised trained (EX, $n = 10$) on a motorized treadmill (Exer 6M Treadmill, Columbus Instruments Inc., Columbus, Ohio) 3 days per week (Monday, Wednesday, Friday) for 10 weeks. The mice were allowed to acclimatize to the treadmill for the first 3 weeks with the following training protocol: warm-up at 8 m·min^{-1} for 10 min; training at 10 m·min^{-1} for 25 min (week 1), 35 min (week 2), and 45 min (week 3); cool-down at 8 m·min^{-1} for 5 min. For the remaining 7 weeks, mice were subjected to a progressive exercise protocol with the training portion of the protocol beginning at 12 m·min^{-1} for 45 min (week 4) and increasing to 20 m·min^{-1} for 45 min. The training portion of the protocol was always preceded by a 10 min warm-up at 10 m·min^{-1} and followed by a 5 min cool-down at 10 m·min^{-1}. We have previously shown that this exercise training protocol successfully increases antioxidant enzyme activities in mouse skeletal muscle (De Lisio et al., in press), and a similar training protocol has been shown to increase both antioxidant enzyme (Avula and Fernandes 1999) and base excision repair enzyme activities (Radak et al. 2007). Mice were encouraged to run using mild electric shock or hind limb stimulation with the bristles of a paint brush. Sedentary mice (SED) were handled in the same manner as exercise-trained mice but were not exposed to treadmill running. Mice from the SED group in the present study were also included in the control group for a study by Phan et al. (in review).

Acute exercise protocol

Prior to exercise, all mice were placed on the treadmill for 5 min at a speed of 8 m·min^{-1} to acclimatize them to treadmill stress. Four mice were randomly removed from the treadmill and served as unexercised controls (CON), while 4 mice remained on the treadmill and completed the acute exercise protocol (Acute Ex). Mice began running at 8 m·min^{-1}, and treadmill speed was increased by 2 m·min^{-1} every 10 min until the mice reached a speed of 16 m·min^{-1}. Mice then exercised at 16 m·min^{-1} for 30 min, then 18 m·min^{-1} for 20 min; therefore, the total time of the acute exercise protocol was 90 min. The nonexercised control mice (CON) were also used by Phan et al. (in review).

Radiation challenge

In vivo challenge

The in vivo radiation challenge took place 3 days following the final exercise bout in the 10-week training protocol and has been described previously (Lemon et al. 2008*a*; De Lisio et al., in press). Briefly, a subset of mice from each group (SED and EX) were irradiated with a Cs-137 γ ray source and received a whole-body dose of 1.116 Gy (approximately 1 Gy) at a dose rate of 0.279 Gy·min^{-1}. During irradiation, mice were placed in a polycarbonate tube and returned to their cage once the irradiation was completed. The remaining mice in each group were placed in the polycarbonate tubes for an equal amount of time to simulate the stress of the radiation challenge.

In vitro challenge

Five days following the final exercise bout of the 10-week training protocol, bone marrow was harvested from mice in each group that did not receive the in vivo high-dose radiation challenge and divided into 3 separate samples. Separate bone marrow aliquots from each mouse were exposed to 0 Gy (unchallenged), 0.969 Gy (~1 Gy) at a dose rate of 0.171 Gy·min^{-1}, and 1.938 Gy (~2 Gy) at a dose rate of 0.171 Gy·min^{-1} irradiations. Irradiations were administered with a Cs-137 γ ray source. Bone marrow from acutely exercised mice and their controls was harvested, divided, and irradiated in the same fashion 6 h following the acute exercise bout.

Sample collection and cell preparation

Mice were briefly anaesthetized with isoflurane, and blood was collected by cardiac puncture. Mice were then euthanized via cervical dislocation. Both femurs were dissected of muscle and fat, excised, and flushed with 1 mL of heparinized RPMI 1640 to remove the bone marrow. The cell suspension was disaggregated with a 23-gauge needle and placed on a water–ice slurry until the cells were counted. Cells were counted using the Z2 Coulter particle count and size analyzer (Beckman Coulter, Miami, Fla.), and the concentration was adjusted to 1×10^6 cells·mL^{-1} with ice-cold supplemented RPMI 1640 (10% fetal bovine serum (VWR International, Mississauga, Ont.), 2.5% HEPES, 1% penicillin–streptomycin, 1% L-glutamine). Cell suspensions were placed in a 37 °C water bath for the duration of the incubation.

γH2AX

The protocol for determination of γH2AX foci by flow cytometry has been previously described in detail (Lemon et al. 2008*a*). Briefly, following incubation for 30 min at 37 °C, a 500 µL aliquot of each bone marrow cell suspension was fixed in 70% ethanol for 60 min, then stored at –20 °C for future use. Cells were fixed 30 min following radiation exposure, as this time has previously been shown to correspond to the peak of γH2AX levels postradiation (Lemon et al. 2008*a*). Cells were washed in Tris-buffered saline (TBS; Trizma base plus NaCl, pH 7.4; Sigma–Aldrich, Mississauga, Ont.) then permeabilized with ice-cold Tris-buffered saline–Triton (TBST; 4% fetal bovine serum, 0.1% Triton X-100 (Sigma–Aldrich)) for 10 min. Cells were stained with anti-phospho-γH2AX (ser139) primary antibody (1:400, Upstate Cell Signaling, Charlottesville, Va.) for 2 h at room temperature. After washing, cells were incubated with AlexFluor 488 goat anti-rabbit IgG F(ab′)2 (1:500, Invitrogen Canada, Burlington, Ont.) secondary antibody for 1 h at room temperature in the dark. After washing, cells were resuspended in propidium iodide (PI; 1:60, Sigma–Aldrich) and run immediately on the Epics XL flow cytometer (Beckman Coulter, Mississauga, Ont.). Analysis was based on 5×10^3 cells from the low forward and side-scatter population, which is enriched for lymphocytes at all stages of development (Salzman et al. 1975; Hoffman et al. 1980; Terstappen et al. 1988, 1989). Each sample was analyzed in duplicate.

Apoptosis

The carboxyfluorescein FLICA caspase-3- and -7 apoptosis detection kit assay (Immunochemistry Technologies, Bloomington, Ind.) was used according to the manufacturer's instructions. Briefly, 10 µL of 30× FLICA solution was added to 300 µL aliquots of bone marrow suspension at a concentration of 1×10^6 cells·mL^{-1} and incubated for 6 h in a 37 °C, 5% CO_2 incubator. Aliquots were gently resuspended every 60 min. Cells were washed twice with 1× wash buffer (supplied) and resuspended in 400 µL of a solution of 0.5% 7AAD (Beckman Coulter) in wash buffer for the training study or 400 µL wash buffer for the acute study. Cells were put on ice and immediately run on the Epics XL flow cytometer. Percentages were determined from analysis of 2.5×10^4 cells. The gating strategy employed for the training study is shown in supplemental Fig. S2[2]. Briefly, the first gate (Fig. S2A[2]) was set to exclude all doublets with high forward and side scatter. Next, the percentage of cells expressing activated caspase-3 and -7 and either positive or negative for 7AAD was determined (Fig. S2B[2]). Total caspase-3 and -7 positive cells represent the combination of caspase-3 and -7 plus 7AAD+ and caspase-3 and -7 plus 7AAD–. The same gating strategy was performed in the acute experiments, except that 7AAD was not used. Levels of apoptosis were determined 8 h following radiation exposure. This length of incubation may contribute to the relatively high spontaneous (nonradiation-induced) levels of apoptosis reported in the present study and previously by our group (Lemon et al. 2008*b*); however, this period of incubation has previously been shown to correspond to peak levels of apoptosis postradiation (Lemon et al. 2008*b*).

Micronucleated and percentage of reticulocytes

The percentage of reticulocytes and the percentage of micronucleated reticulocytes (MN-RET) were determined using the method of Dertinger et al. (2007) using the MicroFlow PLUS kit (Litron Laboratories, Rochester, N.Y.). Dertinger et al. have previously shown MN-RET levels to be at their highest 43 h following 1–2 Gy radiation (Dertinger et al. 2007). Therefore, this assay was not performed in acutely exercised mice. Once collected, blood was immediately mixed with 350 µL of solution B (anticoagulant supplied

[2] Supplementary data for this article are available on the journal Web site (http://apnm.nrc.ca).

with kit), fixed in methanol (supplied), and stored at –80 °C for a minimum of 24 h. Samples were washed in ice-cold buffer (supplied) and incubated in 80 μL of an antibody cocktail containing RNase, FITC-conjugated CD71, and PE-conjugated CD61 on ice for 30 min, followed by 30 min at room temperature. Samples were treated with 1 mL of PI staining solution (supplied) just prior to running samples on the Epics XL flow cytometer. Cells that were double positive for CD71 and PI represented MN-RET, and cells that were CD71 positive only represent reticulocytes and were expressed as a percentage of total cells analyzed. CD61 was used as a platelet marker to differentiate between reticulocytes and platelets, as per manufacturer's instructions (Fig. S3)[2]. The percentage of reticulocytes was determined from analysis of 2×10^5 red blood cells, and the percentage of MN-RET was determined from analysis of 2×10^4 reticulocytes. All samples were analyzed at least in duplicate 43 h following radiation exposure. Certain samples were excluded from analysis because of technical problems with sample fixation causing scatter patterns in which the population of interest could not be reliably determined.

Statistics

Data were analyzed using the commercially available SigmaStat 3.1 software. Data are presented as means ± SE, with $p \leq 0.05$ considered significant. γH2AX and apoptosis data were analyzed using a 2-factor ANOVA with Tukey's post hoc test. Student's *t* tests were used to determine between-group differences in the absolute change in γH2AX, reticulocyte data, and acute H2AX data.

Results

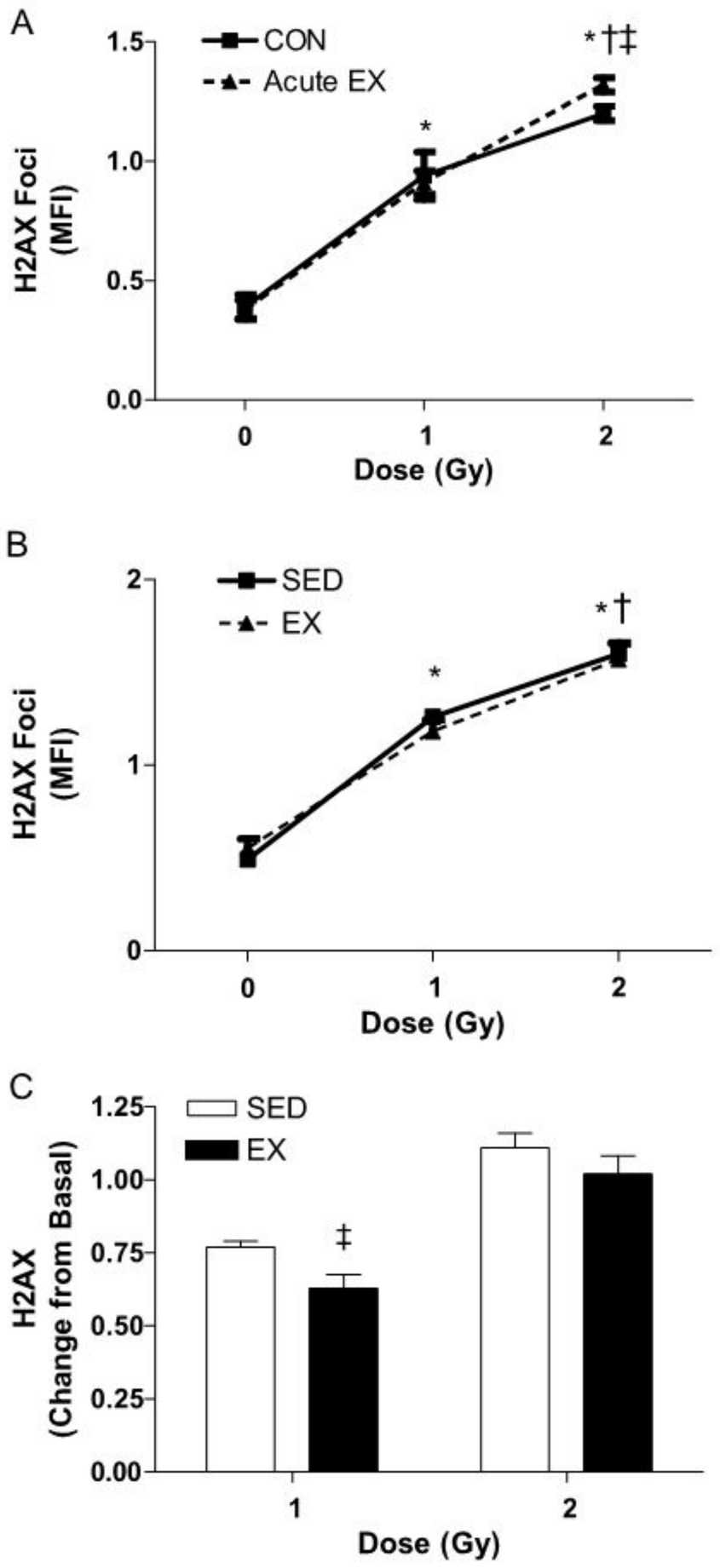
γH2AX Foci

Cells expressing phosphorylated γH2AX foci, a surrogate marker of DNA damage, were detected by flow cytometry (Lemon et al. 2008*a*) (Fig. S1[2]). Acute exhaustive exercise did not increase basal levels of γH2AX in the lymphocyte-enriched population of bone marrow. In vitro radiation of 1 and 2 Gy increased γH2AX foci formation in both acutely exercised and nonexercised control mice ($p < 0.001$ for both doses). Acutely exercised mice had significantly higher γH2AX foci formation in response to 2 Gy in vitro radiation compared with nonexercised controls (10%, $p < 0.05$, Fig. 1A). Exercise training did not increase basal levels of γH2AX foci, and γH2AX foci were significantly increased in both exercise-trained and sedentary mice exposed to 1 and 2 Gy radiation in vitro ($p < 0.001$, Fig. 1B). Exercise training attenuated the increase from basal levels in mice exposed to 1 Gy radiation in vitro (18%, $p < 0.05$, Fig. 1C), but the protective effect was abolished by the 2 Gy dose.

Apoptosis

Cells containing activated caspase-3 and -7, with or without 7AAD positivity, were identified by flow cytometry (Fig. S2[2]). The percentage of total caspase-3 and -7 bone marrow cells was significantly greater in acutely exercised mice compared withthat in nonexercised controls (32%, $p < 0.001$, Fig. 2A). The percentage of total caspase-3 and -7 tended to increase with radiation dose ($p = 0.052$, Fig. 2A). The percentage of total caspase-3 and -7 bone marrow cells

Fig. 1. γH2AX foci in a lymphocyte-enriched population of bone marrow cells from acutely exercised (Acute EX: 0 and 1 Gy, $n = 4$; 2 Gy, $n = 3$) and nonexercised controls (CON, $n = 4$) whose marrow was exposed to 0, 1, or 2 Gy radiation in vitro (A). γH2AX foci in a lymphocyte-enriched population of bone marrow cells from sedentary (SED, $n = 5$) vs. exercise-trained (EX, $n = 5$) mice whose marrow was exposed to 0, 1, or 2 Gy radiation in vitro (B). Increase in γH2AX foci in a lymphocyte-enriched population of bone marrow cells from exercise-trained (EX, $n = 5$) and sedentary (SED, $n = 5$) mice (C). Basal (0 Gy) values were subtracted to determine the absolute change in mean fluorescent intensity (MFI) caused by radiation. Values represent means ± SE expressed as MFI. *, $p < 0.001$ vs. 0 Gy; †, $p < 0.001$ vs. 1 Gy; ‡, $p < 0.05$ vs. CON and SED.



was significantly decreased in exercised-trained compared with that in sedentary mice (11%, $p < 0.05$, Fig. 2B). The percentage of total caspase-3 and -7 cells was significantly elevated from basal levels by each radiation dose in both sedentary and exercise-trained mice (1 Gy: 18%, $p < 0.05$; 2 Gy: 27%, $p < 0.001$, Fig. 2B). The percentage of cells

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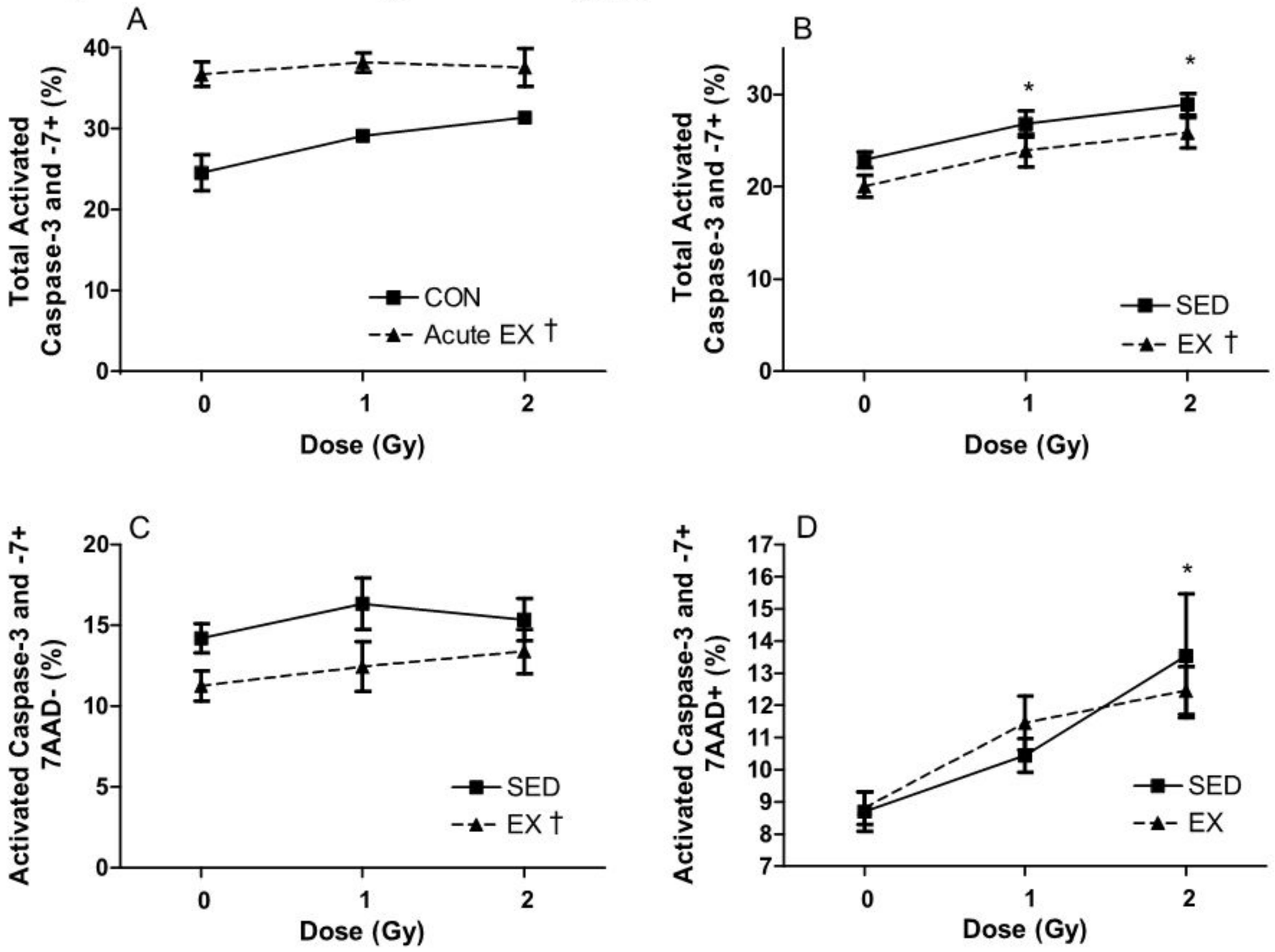
Fig. 2. The percentage of bone marrow cells with activated caspase-3 and -7 from acutely exercised (Acute EX, $n = 4$) and nonexercised controls (CON, $n = 4$) whose marrow was exposed to 0, 1, or 2 Gy radiation in vitro (A). The percentage of bone marrow cells with activated caspase-3 and -7 from exercise-trained (EX, $n = 5$) and sedentary (SED, $n = 5$) mice whose marrow was exposed to 0, 1, or 2 Gy radiation in vitro (B). The percentage of bone marrow cells with activated caspase-3 and -7 that did not stain positively for 7AAD from exercise-trained (EX, $n = 5$) and sedentary (SED, $n = 5$) mice whose marrow was exposed to 0, 1, or 2 Gy radiation in vitro (C). The percentage of bone marrow cells with activated caspase-3 and -7 that stained positively for 7AAD from exercise-trained (EX, $n = 5$) and sedentary (SED, $n = 5$) mice whose marrow was exposed to 0, 1, or 2 Gy radiation in vitro (D). Values are expressed as percentage of total cells analyzed and presented as means ± SE. *, $p < 0.05$ vs. 0 Gy; †, $p < 0.05$ vs. CON and SED.



expressing activated caspase-3 and -7 and negative for the DNA dye 7AAD were significantly elevated in sedentary mice compared with that in exercise-trained mice (24%, $p < 0.05$, Fig. 2C). The percentage of cells expressing activated caspase-3 and -7 and 7AAD were not different between sedentary and exercise-trained mice; however, the percentage of cells positive for both markers was elevated with 2 Gy of radiation relative to that of nonirradiated samples (0 Gy; 48%, $p < 0.001$, Fig. 2D).

Micronucleated reticulocytes

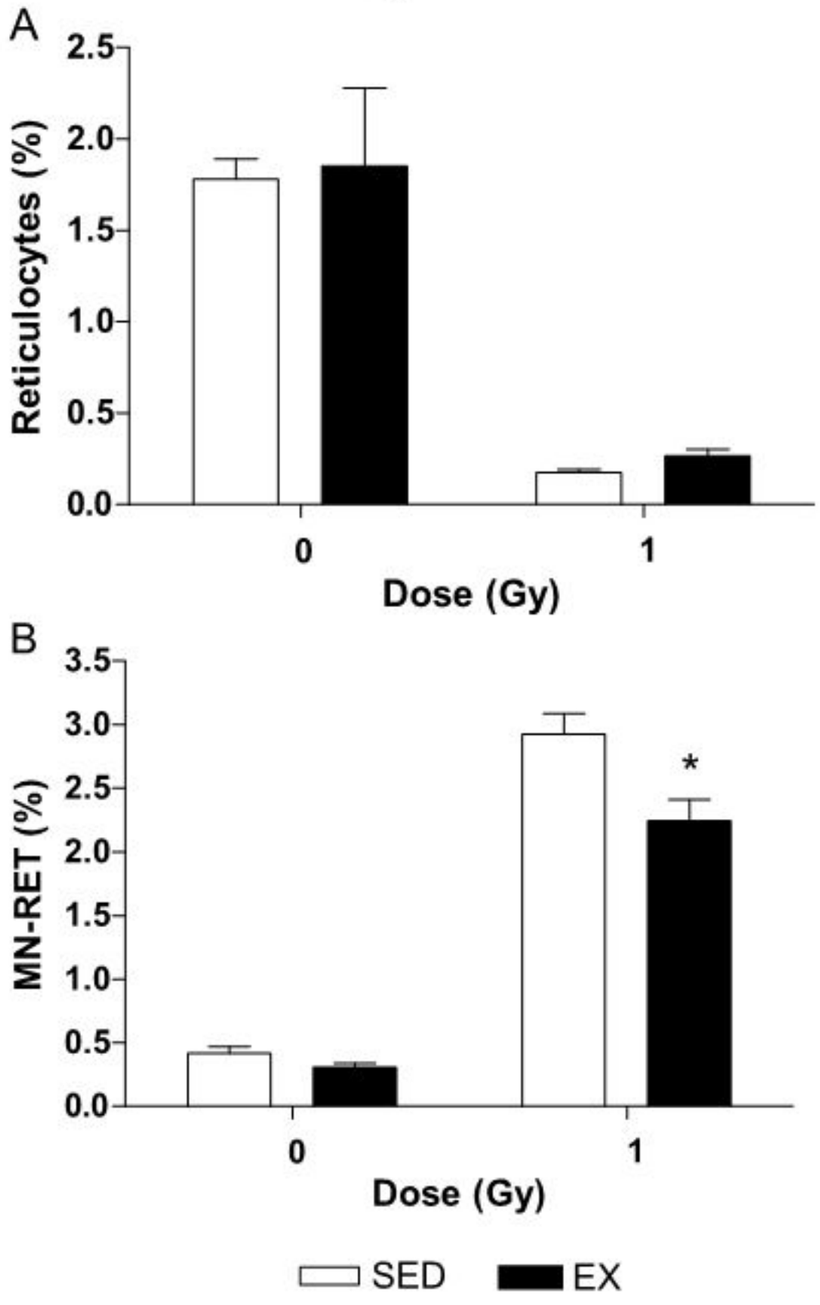
Reticulocytes were identified with an anti-CD71 antibody and analyzed using flow cytometry (Fig. S3[2]) (Dertinger et al. 2007). The percentage of reticulocytes did not differ between the exercise-trained mice and sedentary animals basally or in response to 1 Gy radiation (Fig. 3A). The basal percentage of MN-RET did not differ between exercise-trained and sedentary mice; however, in response to 1 Gy radiation, exercise-trained mice had significantly fewer MN-RET than sedentary mice (22%, $p < 0.05$, Fig. 3B).

Discussion

To our knowledge, the present report represents the first investigation into the effects of acute exercise and exercise training in the bone marrow compartment. Results from the present investigation suggest that exercise induces acute oxidative stress in bone marrow cells, resulting in adaptations that confer protection against future oxidative challenges. Acute exercise sensitizes bone marrow cells to oxidative stress, resulting in increased DNA damage in response to a radiation challenge and elevation in the percentage of cells expressing activated caspase-3 and -7. These perturbations in redox homeostasis from acute exercise result in an adaptive response to training either through increased apoptosis of susceptible cells or an up-regulation of protective mechanisms. These adaptations following training were manifested as an attenuation of DNA damage, MN-RET formation, and reduced apoptosis in response to high challenge doses of radiation.

Each acute exercise bout in a progressive exercise training

Fig. 3. The percentage of reticulocytes from sedentary (SED) and exercise-trained (EX) mice basally (0 Gy, SED $n = 5$, EX $n = 3$) and in response to 1 Gy, whole-body, in vivo radiation (1 Gy, SED $n = 3$, EX $n = 3$) (A). The percentage of micronucleated reticulocytes (MN-RET) from sedentary (SED) and exercise-trained (EX) mice basally (0 Gy, SED $n = 5$, EX $n = 3$) and in response to 1 Gy, whole-body, in vivo radiation (1 Gy, SED $n = 3$, EX $n = 3$) (B). Values are expressed as the percentage of total cells analyzed and are presented as means ± SE. *, $p < 0.05$ vs. SED.



program induces oxidative stress (Venditti and Di Meo 1996) that may result in DNA damage. DNA double-strand breaks are considered the most detrimental form of DNA damage, because the potential loss of genetic information and lack of repair or disrepair can lead to cell death or genetic damage. In the present investigation, basal levels of DNA double-strand breaks, as determined by γH2AX phosphorylation, were not different between acutely exercised and nonexercised control mice 6 h following exercise. We can conclude, however, that acute exercise sensitizes the bone marrow compartment to radiation, as levels of γH2AX foci were significantly elevated in acutely exercised mice in response to 2 Gy of radiation. These data are in agreement with the data of Umegaki and colleagues, who demonstrated increased DNA damage in peripheral lymphocytes challenged with 1.5 Gy of X-rays following an acute exercise bout (Umegaki et al. 1998). Together, these data suggest that acute, exhaustive exercise sensitizes lymphocytes and lymphocyte progenitors to oxidative stress. This sensitization, however, does not cause DNA damage to accumulate over the training period, as basal levels of DNA damage were unchanged between trained and sedentary mice. This agrees with previous studies indicating that the oxidative stress caused by each exercise bout in a training program does not result in increased oxidative damage to DNA with training (Radak et al. 2002, 2007). Rather, the stress induced by exercise training resulted in protective adaptations such that, following exposure to 1 Gy radiation, the increase in DNA damage from baseline levels was attenuated in exercise-trained mice as compared with sedentary mice. These data are in accordance with previous studies demonstrating unchanged levels of DNA damage in peripheral lymphocytes from trained subjects following an acute oxidative stress in the form of 1.5 Gy of X-rays (Umegaki et al. 1998) or an acute exercise bout (Peters et al. 2006). Taken together, our data suggest that acute exercise sensitizes the bone marrow compartment to oxidative challenges, such as a high dose of radiation. With training, these repeated acute exercise bouts cause an adaptive response that confers protection against future oxidative challenges.

Apoptosis is the primary means of death in hematopoietic cells exposed to high-dose radiation exposure (Lemon et al. 2008*b*). In the present investigation, acute exercise significantly increased the percentage of cells with activated caspase-3 and -7 in bone marrow cells, an indication that the cellular apoptotic process had been initiated. Conversely, exercise training significantly decreased the percentage of bone marrow cells with activated caspase-3 and -7. The training effects were due primarily to the decrease in cells undergoing the early stages of apoptosis defined as cells positive for active caspase-3 and -7 but not positive for 7AAD. Caspase-3 and -7 activation indicates that the apoptotic machinery within the cell has been activated; however, membrane permeabilization has not occurred, as 7AAD has not yet entered the cells. These results are in agreement with previous data suggesting that peak levels of apoptosis are first seen 8 h following an acute dose of radiation (Lemon et al. 2008*b*). Previous studies have also shown increased apoptosis in lymphocytes derived from the intestines (Hoffman-Goetz and Quadrilatero 2003) and peripheral blood (Mooren et al. 2004) from untrained or poorly trained mice and humans, respectively. In further agreement with the present findings, however, peripheral lymphocytes from well-trained individuals are protected from an apoptotic response following acute oxidative stress (Mooren et al. 2004; Peters et al. 2006). These data mirror our DNA damage results, in that acute exercise induced oxidative stress to bone marrow lymphocytes, and support our hypothesis that oxidative stress induced by acute exercise is responsible for an adaptive response that increases protection against future oxidative stress. Indeed, previous studies have reported increased gene expression of the anti-apoptotic genes BCL2A, Casp8, and FADD-like apoptosis regulator (CFLAR) following exercise in mature white blood cells (Büttner et al. 2007). Results from the present study suggest that similar adaptations may be occurring in more primitive cells of the hematopoietic hierarchy. Furthermore, these data suggest that a possible mechanism for the protective effects of exercise training may be the enhanced removal of highly susceptible cells and retention of cells with more robust defenses.

Quantification of reticulocytes and micronucleated reticulocytes (MN-RET) following a high-dose radiation exposure are established measures of cytotoxicity and genotoxicity, respectively (Dertinger et al. 2007). The percentage of

reticulocytes remains unchanged in athletes (Banfi and Del Fabbro 2007), a finding that is in agreement with data from the present study showing no effects of exercise training on reticulocyte number. Additionally, basal levels of MN-RET were not different between sedentary and exercise-trained mice. These data, along with results from our γH2AX experiments, support the notion that training does not cause DNA damage in cells from the bone marrow compartment. Conversely, exercise training conferred protection to reticulocytes, as was demonstrated by a significant reduction (22% relative to SED) in the percentage of MN-RET following 1 Gy of in vivo radiation. These data agree with the results of Umegaki and colleagues (1998), who demonstrated an increase in MN-RET formation in peripheral lymphocytes from untrained subjects exposed to 1.5 Gy of X-rays, while peripheral lymphocytes from trained subjects showed no such increase. Although the 22% reduction in MN-RET relative to that in sedentary mice represents a difference of <1% in absolute terms, we believe that the biological relevance of this difference is signficant. DNA damage and proliferation and differentiation of cells containing DNA damage are critical steps in cancer initiation and progression. This type of transformation in a single cell could lead to cancer development. Therefore, an absolute difference <1%, which represents thousands of cells, is very significant biologically, as it would translate into a large decrease in the risk of cancer development. Together, these data suggest that exercise training, either through enhanced apoptosis of damaged cells or enhanced protection against oxidative damage, induce protective adaptations against the cytotoxic and genotoxic effects of high-dose radiation exposure.

Overall, the ability of cells to adapt and become resistant to high-dose radiation through exercise training could be a potentially useful strategy to protect people from the detrimental effects of radiation. This could have applications in radiation therapy for protecting normal tissues, including bone marrow, during therapy, and also reducing the risks from diagnostic overexposure or overuse.

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Supplemental Figure Legends

Figure S1. Flow cytometry gating strategy for γH2A.X analysis. (a) Isolation of population enriched for lymphocytes and lymphocyte progenitor cells. (b) Determination of γH2A.X positivity. The area above the horizontal line on the plot represents cells positive for gamma H2AX foci as determined by FITC fluorescence. The black shading represents unchallenged cells, the gray shading represents cells challenged with 1 Gy and the white shading represents cells challenged with 2 Gy. Shifts to the right indicate increasing FITC fluorescence which represents increasing gamma H2AX foci.

Figure S2. Flow cytometry gating strategy for apoptosis analysis. (a) Gate for cells positive for FLICA and/or 7AAD, and removal of debris. (b) Gating strategy to determine cells that are FLICA positive (early apoptotic; D4), 7AAD positive (necrotic; D1), FLICA and 7AAD double positive (late apoptotic; D2) and events negative for both 7AAD and FLICA.

Figure S3. Flow cytometry gating strategy for reticulocyte and micronucleated reticulocyte (MN-RET) analysis. (a) Gate in low forward scatter region enriched for red blood cells. (b) Elimination of cells with high PI fluorescence (white blood cells) from previous gate. (c) Gate for elimination of platelets. (d) Gate for determination of percentage of reticulocytes and MN-RET. Gates A1 and A2 with high CD71 fluorescence represent reticulocytes. Gate A2 are cells positive for CD71 and PI. These represent the MN-RET. Gate A3 are the double negative events that do not express CD71 or PI. Gate A4 are events positive for PI only.

Figure S1

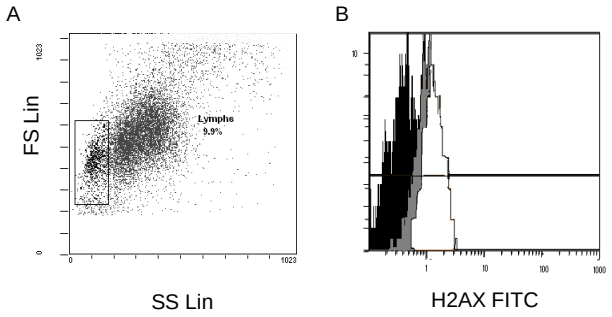


Figure S2

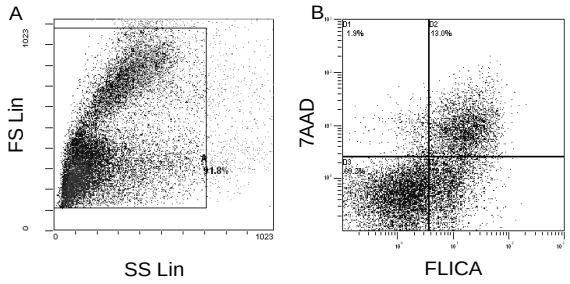
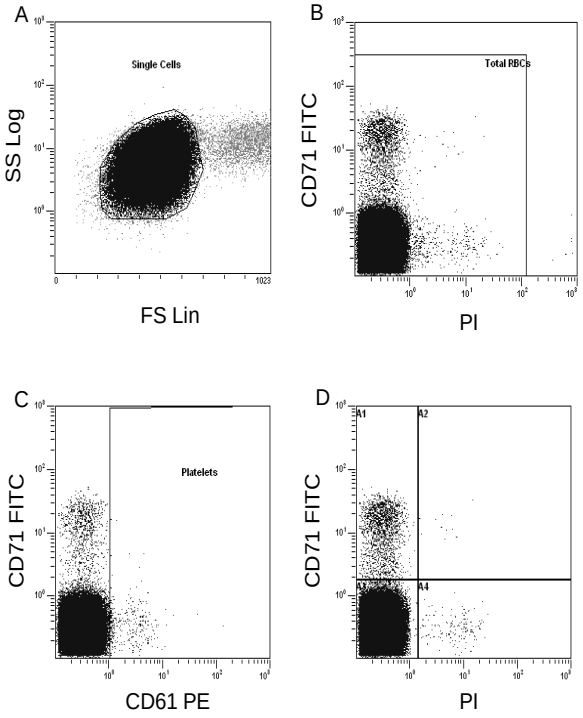


Figure S3


A
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CD71 FITC
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D
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A2
A4
CD71 FITC
PI

4. Chapter 4

Characterization of the Effects of Exercise Training on Hematopoietic Stem Cell Quantity and Function

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Abstract

The effect of exercise training on hematopoietic stem cells (HSC) is largely unknown. The aim of the present investigation was to determine whether exercise training could expand the bone marrow HSC pool, and influence various aspects of HSC function. Mice were either exercise-trained (EX; 1 hour/day, 3 days/week for 8 weeks) or remained sedentary (SED). Bone marrow (BM) from SED or EX mice was extracted from different HSC niches for cell cycle analysis, HSC (lineage$^-$, Sca-1$^+$, c-Kit$^+$; LSK) quantification, and differentiation along various hematopoietic lineages via flow cytometry. Serum was collected for evaluation of cytokines known to regulate HSC. To determine HSC function, BM from EX and SED mice was transplanted into primary and secondary recipients in a BM transplant (BMT) assay. EX increased HSC quantity in the vascular BM niche 20% versus SED ($p<0.05$), and increased the proportion of whole BM cells in G_2/M phase of cell cycle ($p<0.05$). The number of spleen colonies was 48% greater ($p<0.05$) in recipients transplanted with BM from EX. Serum IL-6 levels were decreased 38% in EX, and differentiation along the granulocyte and macrophage lineage trended to increase (16%, $p=0.053$ and 16%, $p=0.061$, respectively). Short- or long-term engraftment and homing in primary recipients were not altered in EX. HSC self-renewal as analyzed by hematopoietic regeneration in secondary recipients was also unaffected by EX. Here we demonstrate that HSC quantity is increased in the BM vascular niche associated with more activated, differentiated HSC, and that this expansion does not

improve or impair HSC function. **Keywords:** Interleukin-6, niche, bone marrow transplant, cell cycle, engraftment

Introduction

Hematopoietic stem cells (HSC) are currently the only cells regularly used in clinical stem cell therapy, and their remarkable clinical potential is evidenced by their use in the treatment of a variety of disease ranging from various forms of hematological cancers to congenital immunodeficiencies. HSC are the most primitive cells in the hematopoietic lineage, and act as a reserve cell population responsible for maintenance and production of circulating blood cells (18). HSC were first discovered in 1961 as the donor cell population responsible for survival and regeneration of all blood cell lineages in myeloablated hosts (33). HSC reside in two distinct but related niches within the bone marrow: the endosteal and vascular niche. HSC in the endosteal niche lining the bone in the bone marrow cavity are maintained in a state of quiescence via their association with osteoblasts (19), and have been shown to have greater long-term repopulating potential (13). HSC from the endosteal niche can migrate to the vascular niche where they associate with endothelial cells of the bone marrow sinusoids (19), become more mitotically active, and are primed for release into circulation or differentiation (15). Phenotypically, HSC are identified by their lack of expression of mature hematopoietic lineage markers, and positive expression of c-Kit and Sca-1, collectively the so-called LSK population (11, 24). Identification of cell

surface markers have been useful for the isolation and quantification of HSC, but the BMT assay persists as the gold standard assay for HSC characterization.

The BMT assay involves the ablation of recipient hematopoietic cells by radiation or chemotherapeutic agents, followed by transfusion of sufficient numbers of donor cells to allow for hematopoietic regeneration and recipient survival (25). For successful hematopoietic regeneration to occur, HSC must be in sufficient quantity, be able to successfully home to their niche in the bone marrow, be able to proliferate and differentiate to reconstitute all blood cell lineages, while maintaining self-renewal to ensure long-term engraftment (3, 19). Regulation of these cell fate decisions by *ex vivo* manipulation of HSC in culture has been the focus of numerous investigations in order to optimize the donor cell source for BMT. For example, treatment with cytokine cocktails *in vitro* has revealed key paracrine mediators of HSC survival, self-renewal and proliferation (12). Conversely, data from other studies has demonstrated that HSC expanded *in vitro* may result in impaired HSC function manifesting as decreased levels of engraftment upon transplantation (12, 17, 34). These data suggest that *ex vivo* expansion and manipulation of HSC may not be the optimal stimulus. It remains unknown if physiological stress *in vivo*, such as exercise, may provide the appropriate stimulus to induce HSC expansion and maintenance of function.

Exercise is a potent physiological stress with systemic health benefits related to changes in the hematopoietic system. For example, exercise is associated with increased red blood cell content (9), and enhanced disease

resistance via improved immune function (26). Whether these beneficial effects of exercise in the most mature cells of the hematopoietic lineage extend to more primitive hematopoietic cells, like the HSC, is unknown. The majority of studies in this field have focused on the mobilization of HSC into peripheral blood following an acute exercise stimulus in humans using flow cytometry and cell surface staining to identify HSC. These studies have demonstrated that acute exercise is a potent stimulus for the mobilization of the general HSC population into peripheral blood (20, 21, 32, 35, 37); however, the most primitive HSC population appears unresponsive to an acute exercise stimulus (4, 21). These acute changes are thought to be mediated by alterations in cytokines and growth factors involved in HSC mobilization or in proliferation with acute exercise (31, 35, 37). Even fewer studies have examined the effects of exercise training on HSC. Some investigations demonstrating an increase in peripheral blood HSC in trained individuals (4), while others fail to show a difference in peripheral blood HSC quantity (32, 35). The paucity of data regarding the effects of exercise training on HSC highlights the need for more research in this area.

Previous work from our laboratory (16) and others (5, 8, 38) has demonstrated an increase in protective mechanisms in the heterogeneous population of bone marrow cells in response to exercise training. Furthermore, we have demonstrated that exercise training promoted medullary hematopoiesis and that these effects may have been mediated through an improved cytokine milieu in the serum of exercise-trained mice, or beneficial niche adaptations (1).

The aim of the present investigation was to quantify HSC in their different niches within the marrow (i.e., endosteal and vascular) and examine their function using the BMT assay where marrow from exercise-trained or sedentary donors was used to reconstitute myeloablated recipients. In addition to allowing us to examine basic aspects of HSC function, the BMT assay also has the advantage of being clinically relevant as it is directly related to bone marrow transplantation in humans and could help determine whether using exercise-trained donors can improve recipient hematopoietic regeneration. We hypothesized that exercise training would increase HSC quantity and improve their function.

Materials and Methods

Mice

Animal protocols were approved by the McMaster Animal Research Ethics Board and conformed to Canadian Council for Animal Care Guidelines. Mice, housed at no more than five/cage, were maintained on a 12:12 hour light:dark schedule and provided food and water *ad libitum*. Male C57Bl/6-eGFP (23) (kind gift from Dr. B. Trigatti, McMaster University) or C57Bl/6 were bred in-house. Female C57Bl/6 mice (Jackson Labs, Bar Harbor, Maine) eight to ten weeks of age when used as recipient or six weeks of age when used for training studies were allowed to acclimatize to their new surroundings for one week prior to use in the experiments.

Exercise Protocol

At six weeks of age, male or female C57Bl/6 mice were randomly assigned to either an endurance exercise-trained (EX) or sedentary (SED) group. EX mice followed a standard training protocol, with minor modifications, previously shown to induce adaptations in the bone marrow compartment (1, 16). Mice were trained three days per week (Monday/Wednesday/Friday), 1 hour per day for 8 weeks. The exercise protocol consisted of a 10 minute warm-up at 12 m/min followed by a 45 minute training period that began at 14 m/min (week 1) and progressed to a max speed of 22 m/min (week 8), and concluded with a 5 minute cool-down period at 10 m/min. Speed during the training portion was adjusted to allow all mice to complete the full duration of exercise. EX mice were trained at approximately the same time each day to avoid any diurnal variation and were encouraged to run by mild electric shock. SED mice were place on the treadmill at the end of each training session to control for the stress of mouse handling and treadmill exposure without running.

LSK Quantification in Central vs. Endosteal Niche and Cell Cycle Analysis

Female mice were euthanized via cervical dislocation and their femurs and tibiae were quickly removed and cleaned of surrounding muscle, fat and connective tissue. Marrow from the vascular niche in the central marrow region was harvested as previously described (1). Flushed bones were then used to harvest HSCs from the endosteal region as previously described (1) with minor modifications. Bones were digested mechanically with scissors and enzymatically with collagenase (0.25% collagenase/20% FBS/PBS) for 10

minutes at 37^{o}C with shaking. The collagenase solution was diluted with buffer (2%FBS/1mM EDTA/PBS) and cells were filtered twice through a 70 µm filter to remove bone fragments. Cell suspensions were immediately processed for quantification of the LSK population via flow cytometry as previously described (1, 11). The following antibodies were used: Biotinylated Lineage panel (BD Biosciences, Mississauga, Canada), anti-mouse Sca-1 (1:10; BDBiosciences, Mississauga, Canada), anti-mouse c-Kit (1:10; eBiosciences, San Diego, California) and FITC anti-streptavidin (1:800; Biosource, Camarillo, California). Cells were analyzed immediately using the Epics XL flow cytometer (Beckman Coulter, Mississauga, Canada) with unstained and single-stained samples used as controls for compensation and gating. Data were analyzed and expressed as the percentage of c-Kit and Sca-1 positive cells from the lineage negative population.

A subset of cells from the vascular niche was used for cell cycle analysis. After counting, cells were washed, fixed in 70% ethanol and stored at -20^{o}C until analysis. For flow cytometric analysis of cell cycle status, fixed cells were centrifuged at 2000 rpm for 5 minutes, washed with PBS, resuspended in DAPI (Sigma, 1 µg/ml) and incubated on ice for 30 minutes. Single cell suspensions were immediately analyzed via the Partec Cyflow Space flow cytometer (Partec, Swedesboro, New Jersey). Debris was excluded and doublet discrimination was applied. Cell cycle analysis was based on samples with at least 2500 cells contributing to the main cell cycle display.

Lineage Panel Analysis

Previously frozen EX and SED samples were divided into 5 separate aliquots for staining with each individual antibody in the lineage panel (BD Biosciences, Mississauga, Canada) consisting of the following mature blood cell markers: Mac-1, TER-119, Gr-1, B220, and CD3ε. After incubation with biotinylated primary antibodies, cells were treated with FITC anti-streptavidin secondary antibody (1:800, Biosource, Camarillo, California), washed and immediately analyzed by flow cytometry. Unstained and secondary only samples were used to establish gates.

Serum Cytokine Analysis

Blood was collected from the submandibular vein. Serum was allowed to separate and samples were centrifuged at 4,500g for 10 minutes. Serum was removed and stored at -80^{o}C until analysis. Levels of the various cytokines were determined using the Bio-Plex Pro Assay (Bio-rad, Mississauga, Canada) according to manufacturer's instructions. If a sample had undetectable levels of a cytokine it was given a value of zero. This occurred in a single sample for a single measured cytokine.

Bone Marrow Transplants

Bone marrow transplantation was conducted as previously described (10). Briefly, 3 days after the final exercise session male donor mice were sacrificed by cervical dislocation. Female wild-type recipient mice, myeloablated with a fractionated dose of ~9 Gy irradiation (^{137}Cs; GammaCell 3000, Ottawa, Canada)

were reconstituted with 1 x 10^6 whole marrow cells isolated from both femurs and tibias of male C57Bl/6-eGFP or male C57Bl/6 donors immediately following the second irradiation dose via retro-orbital injection. Marrow from a single donor mouse was used to reconstitute 2-4 recipients. Females were chosen as recipients in accordance with common practices for the BMT assay (10), and males were used as donors for the potential to evaluate engraftment via Y chromosome expression if necessary. For secondary BMT, one primary recipient that originally received marrow from a sedentary mouse and two primary recipients who originally received marrow from exercise-trained mice were euthanized at least seven months following the initial BMT, and their marrow was used as the source of donor cells for transplantation into secondary recipients.

Colony Forming Unit Spleen (CFU-S)

Seven days following BMT, the CFU-S assay was conducted as described (22, 33). Briefly, mice were euthanized via cervical dislocation; spleens were quickly excised and fixed in Bouin's solution for 24 hours. Spleens were weighed and splenic colonies visualized as raised lumps with yellowish tinge, were counted by an investigator who was blinded to the conditions. Overlapping colonies were defined by a central nodular region of origin.

Recipient Reconstitution and Donor-Derived Engraftment

Blood was collected into heparinized tubes via facial bleed, and red blood cells were lysed with Tris-NH_4Cl (17mM Tris/0.75% NH_4Cl/ddH_20) lysis buffer Blood samples were then incubated with CD45 antibody (1:80, Invitrogen,

Burlington, Canada) and 7AAD (Beckman Coulter, Mississauga, Canada). Samples were analyzed on an Epics XL flow cytometer (Beckman Coulter, Mississauga, Canada). Single stained and unstained controls were used to establish gates and for compensation. The percentage of $CD45^+$ from the $7AAD^-$ population were identified as living leukocytes to evaluate total hematopoietic reconstitution, and the percentage of GFP^+ cells from this population were identified as donor-derived living leukocytes to evaluate donor-derived engraftment (Figure S2). Flow plots were analyzed with Expo32 analysis software (Beckman Coulter, Mississauga, Canada). Analysis was conducted in a blinded fashion with at least 3-6 different donor mice used to reconstitute multiple recipients at each time point for the short- and long-term repopulating assays. To allow for combination of data from separate days and experiments, peripheral blood from a non-transplanted wild type (non-GFP) mouse and non-transplanted GFP mouse were harvested and prepared in parallel with experimental samples on each experimental day for analysis of the percentage of CD45 cells and construction of a GFP standard curve in unmanipulated mice (Figure S3). Recipient reconstitution was expressed relative to the average percentage of CD45 cells from one non-transplanted wild-type mouse and one non-transplanted GFP mouse, and engraftment was normalized to the GFP standard curve. Any visibly ill mice, or with skin wounds from excessive grooming were excluded from analysis due to the potential for an inflammatory response to the wound, and potential activation of hematopoiesis for reasons unrelated to the study.

Blood from recipients reconstituted with wild type (non-GFP) marrow was used for complete blood count analysis at one month post-BMT. Approximately 300 µl of blood was collected, via facial bleed, into EDTA coated microtainer tubes for analysis by the Core Facility at McMaster University Hospital.

Acute homing to bone marrow

Bone marrow was collected as described above from both femurs and tibias of recipient mice five days following the BMT. An aliquot of 5 x10^6 cells was used for homing analysis, and remaining unused marrow cells were frozen in 10% DMSO/20% FBS/PBS followed by long-term storage at -80^{o}C. Whole marrow was tagged with 7AAD and CD45 as described above and analyzed via flow cytometry (Epics XL, Beckman Coulter, Mississauga, Canada). Flow cytometry gates and compensation were established based on unstained and single stained controls (Figure S4). Dead cells staining positively for 7AAD were excluded and analysis of CD45 cells expressing GFP, representing donor-derived blood cells, was conducted. Data is based on a number of recipients reconstituted with at least two donor mice. Data are normalized to standard curves developed from marrow harvested from non-manipulated wild type (non-GFP) and GFP mice harvested and processed in parallel with experimental samples on different analysis days. Analysis using the Expo32 analysis software (Beckman Coulter, Mississauga, Canada) was conducted in a blinded fashion.

Statistical Analysis

LSK, cell cycle, cytokine, lineage panel, CFU-spleen, and homing data were analyzed using an unpaired t-test in Excel. Since we were interested in the differences between groups and not the changes over time, early, late and secondary reconstitution and engraftment were also analyzed with an unpaired t-test in Excel. Data are presented as mean ± SEM with $p \leq 0.05$ considered significant. For experiments involving the BMT assay, statistical analysis was based on the number of recipients.

Results

Exercise increases the percentage of HSC as well as bone marrow cell proliferation in the vascular niche.

Previous literature identified two primary locations for HSC within the bone marrow cavity. The most primitive, quiescent HSC, responsible for long-term reconstitution of recipients are located along the endosteal lining of bones, while active, proliferating HSC, responsible for short-term reconstitution of recipients, are associated with the vasculature in the central region of the bone marrow cavity (3, 19). Exercise training significantly increased the percentage of HSC, defined as c-kit and Sca-1 positive within the lineage negative population (6) (LSK), in the vascular niche by 20% (Figure 1a-c, $p<0.05$). The percentage of HSC in the endosteal niche was not affected by exercise training (Figure 1d-f). To determine whether exercise training was causing a shift in the HSC population from the endosteal niche to the vascular niche, or increasing proliferation in the vascular niche, the ratio of HSC was compared between the two regions. The

ratio of vascular versus endosteal HSC was not changed with exercise (Figure 1g), supporting the notion that exercise increased proliferation of HSC specifically within the vascular niche without loss of more quiescent cells in the endosteal niche.

The vascular niche is home to a population of more proliferative HSC (15). Therefore, to confirm the observed increase in HSC quantity in the vascular niche with exercise training, we first analyzed the cell cycle status of bone marrow cells from the vascular region. The percentage of bone marrow cells in the vascular niche in the G_2/M phase of the cell cycle was significantly increased with exercise training as EX mice had 6.09 ± 0.44% cells in G_2/M while SED had 3.53 ± 1.03 cells in G_2/M (Figure 2a, $p<0.05$). It has been shown that colony forming cells in the spleens of irradiated mice seven days post-transplant represent a proliferative progenitor population from the transplanted cell source (22); therefore, we quantified spleen colonies seven days post-BMT. Mice transplanted with bone marrow from exercise-trained donors had significantly more spleen colonies than did mice transplanted with bone marrow from sedentary donors (Figure 2b, 48%, $p<0.05$).

The vascular niche is also associated with increased HSC differentiation to more mature hematopoietic cell populations (15); therefore, we quantified the percentage of bone marrow cells from the vascular niche expressing various hematopoietic lineage markers in sedentary and exercise-trained mice. No significant differences were observed between exercise-trained and sedentary

mice in any of the blood cell lineages analyzed; however, strong trends were observed in the Mac-1 lineage (Table 1; p=0.053), and Gr-1 lineage (Table 1; p=0.061) both increasing by approximately 15%.

A number of cytokines have been implicated in HSC regulation; therefore, we sought to determine the effects of exercise training on various cytokines involved in HSC fate decisions. All cytokines related to HSC regulation were down regulated (Table 2), with a significant 38% decrease in IL-6 (p=0.01), and a trend for a decrease observed in G-CSF (16%. p=0.08).

Increased HSC in the vascular niche does not result in increased recipient reconstitution or engraftment.

Given the observed increase in HSC quantity and bone marrow proliferation, we sought to determine if these adaptations would enhance reconstitution and engraftment in recipient mice using the BMT assay. We first analyzed recipients at early time-points post-BMT. We hypothesized that given the increases in the percentage of HSC in the proliferative niche; we would likely see the effect of exercise training in the week's post-BMT. Furthermore, if the effects of exercise training were transient or only isolated to more differentiated progenitors, we did not want to miss the optimal window of opportunity to observe the hypothesized effects. We first conducted the BMT assay in which recipient mice either received marrow from exercise-trained donors or sedentary donors. No differences in leukocyte reconstitution (Figure 3a) and donor-derived

engraftment (Figure 3b) were observed between recipient mice who received bone marrow from exercise-trained or sedentary donors.

Survival and reconstitution of recipients in the early week's post-BMT is primarily achieved through multi-potent progenitors and short-term repopulating HSC (ST-HSC) (6). Therefore, in order to determine the effects of exercise training on the more primitive self-renewing HSC population, we analyzed recipient mice at later time point's post-BMT. Marrow from exercise-trained donors did not enhance total leukocyte reconstitution (Figure 4a) or engraftment in recipients (Figure 4b) at any time point analyzed up to six months post-BMT. Furthermore, whole blood counts determined by complete blood cell count one month post-BMT did not differ between recipients who received marrow from sedentary or exercise-trained mice (Table 3). Similarly, recipient recovery as determined by functional test of treadmill endurance did not differ (Figure S1).

Exercise training does not impair donor cell homing or affect self-renewal capacity.

HSC expansion *in vitro* has been limited by defects in homing of cultured cells upon transplantation (17). To determine if similar functional defects were present with increased HSC quantity in exercise-trained mice, we examined donor cell homing in recipient marrow at five days post-BMT (7). Exercise training had no effect on the donor cells homing (Figure 5a-d) five days following BMT.

Proliferation of HSC in culture is associated with decreased engraftment potential due to a lack of self-renewal *in vitro* (12, 34). To determine if HSC self-renewal was maintained with the observed expansion in HSC in the vascular niche, we undertook a secondary reconstitution assay in which marrow from exercise-trained or sedentary donors reconstituted primary recipients and then marrow from the primary recipients was used to reconstitute secondary recipients. Levels of reconstitution and engraftment were determined in secondary recipients' one, three and six months post-BMT. In the secondary reconstitution assay, recipient reconstitution (Figure 6a), and donor-derived engraftment (Figure 6b) were not different between secondary recipients who received bone marrow from primary recipients previously transplanted with bone marrow from sedentary or exercise-trained mice.

Discussion

Previous studies have focused on the quantification of hematopoietic stem cells (HSC) in peripheral blood with training or acute exercise (4, 20, 21, 29, 32, 35, 37), or evaluation of HSC quantity and proliferation using *in vitro* assays (1); however, the effects of exercise training on HSC in bone marrow in their natural environment, *in vivo,* has never been examined. The present investigation evaluated both the quantity of HSC in two distinct niches within the bone marrow (i.e. endosteal and vascular), as well as HSC function using the bone marrow transplantation (BMT) model (25, 28). The primary findings from this study were that exercise training increases the quantity of HSCs in the vascular niche,

without altering the functional properties of HSC, such as homing or long-term engraftment.

HSC Quantity

It has been established that HSC residing in the endosteal niche are a quiescent reserve population of HSC, while those in the vascular niche are a more activated population primed for release into peripheral blood (19, 36). In the present study, we observed a significant increase in HSC quantity only in the vascular niche, which was supported by our cell cycle, colony forming unit-spleen (CFU-S) and lineage marker analysis. Exercise training increased the percentage of whole bone marrow cells in G_2/M phase of the cell cycle in the vascular niche. Although these data collected in whole bone marrow cells may not be directly applicable to HSC in the vascular niche, they do indicate that exercise training is a proliferative stimulus for bone marrow cells. At seven days post-BMT, recipient spleens are transiently colonized with donor-derived mature progenitor cells that are replaced later by more immature progenitor cells (22). The increase in spleen colonies at seven days post-BMT in mice transplanted with bone marrow from exercise-trained donors provides further support for their increased HSC quantity in the vascular niche with exercise training. Additionally, we observed a strong trend for an increase myeloid lineage positive markers, Mac-1 (15%, p=0.053) and Gr-1 (15%, p=0.061) in exercise-trained mice. Since the vascular niche is associated with myeloid differentiation of HSC (15), the trend for an increase in cells positive for myeloid markers provides further support

for an increase in HSC quantity in the vascular niche. Taken together, these data provide strong evidence that exercise training increases the quantity of HSC in the vascular niche of the bone marrow. It is believed that HSC mature and migrate from the endosteal niche to the vascular niche to prepare for release into circulation in response to hematological stress (19, 36). Since acute exercise has repeatedly been shown to mobilize more mature HSC into peripheral blood (21, 32, 35, 37), we speculate that with training, HSC are increased in the vascular niche in preparation for release in response to exercise.

Studies quantifying HSC with training are few, and have mostly focused on HSC circulating in human peripheral blood. Most studies demonstrate that basal levels of circulating HSC are not altered with training (32, 35); however, Bonsignore and colleagues did detect a significant increase in a specific population of more differentiated peripheral blood HSC in trained individuals (4). In a recent study from our lab (1), exercise training increased the number of hematopoietic progenitors of various lineages and of different stages of differentiation identified by a variety of colony forming cell assays *in vitro*. In our previous investigation (1), we also quantified the HSC population, identified by the LSK panel of markers, but failed to see a significant increase with exercise training. Importantly, the magnitude of increase, 20%, was identical between our previous (1) and the present investigation suggesting that increased statistical variability in our previous study (1), perhaps due to variability in the animal's response to training, can account for the differences in statistical outcomes.

Taken together, these data suggest that exercise training increases HSC quantity in the marrow cavity and may increase certain more mature populations of HSC in peripheral blood.

We considered the possibility that the increased HSC quantity in the vascular niche was due to a redistribution of HSC from the endosteal to the vascular niche. The quantity of HSC in the endosteal niche was unchanged, and the ratio of vascular to endosteal HSC was not affected by exercise training suggesting that redistribution was not occurring. In further support of this notion, we did not detect any defects in long-term engraftment potential of marrow from exercise-trained donors, which would have been expected if HSC quantity in the endosteal niche was reduced. Therefore, we speculate that exercise training expands a specific sub-population of HSC that is activated and primed for release into circulation but does not influence more primitive cells responsible for long-term and short-term repopulation. This hypothesis is supported by data from previous studies where exercise increased the quantity of circulating hematopoietic stem and progenitor cells but not those identified by their cell surface phenotype to be the most primitive population (4, 35).

Exercise likely induces its effects on HSC through the modulation of circulating levels of growth factors and cytokines. HSC in the vascular niche are highly sensitive and responsive to systemic factors due to their close proximity to the vasculature (25). In the present study, IL-6 , involved in HSC self-renewal (12), and G-CSF , involved in HSC mobilization (30), were down-regulated with

exercise training, although the decrease in G-CSF levels did not reach statistical significance (16%, p=0.08). Indeed, previous studies have demonstrated acute increases in IL-6 and G-CSF following a single exercise bout (4, 31, 37); however, the regulation of these factors with exercise training is less well known. Cross-sectional data of trained versus sedentary individuals suggests that circulating levels of IL-6 and G-CSF are not affected by training status (4); and that these factors are elevated following acute exercise (4, 27, 31, 35). We speculate that the observed down-regulation of these cytokines in the present study may represent a compensatory response to maintain homeostasis in response to the acute increases in these factors with exercise. Perhaps the short-term, pulsed increases in IL-6 with each exercise bout stimulate HSC proliferation, especially in the cells of the vascular niche. The basal decrease in IL-6 levels with exercise prevents a hyper-proliferative stimulus to HSC thereby maintaining their long-term reconstituting potential. In support of this hypothesis, HSC grown on stromal feeder layers in culture were maximally expanded when media changes occurred every other day (14). This alternating day media change schedule would be similar conditions in the present study where pulsed increases in hematopoietic growth factors may have occurred in mice exercised every other day. While interesting, further research will be required to test this theory.

HSC Function

HSC expansion via *ex vivo* manipulation has long been attempted to overcome the small quantity of HSC available in most tissue samples and increase their clinical applicability. HSC expansion *in vitro* has been limited by defects in HSC function such as impaired homing (17) and differentiation without self-renewal (34) upon transplantation. Unlike *in vitro* models, exercise training would maintain HSC in their natural microenvironment under physiological conditions. Given the importance of the niche in regulating HSC function (36), this approach may prevent functional defects often seen with *ex vivo* expanded HSC. Furthermore, the comprehensive time course of our analysis allowed us to evaluate the contribution of different sub-populations of HSC to recipient reconstitution. Short-term repopulating HSC (ST-HSC) are responsible for reconstitution of recipients in the early weeks and up to 3-4 months post-BMT, after which, long-term repopulating HSC (LT-HSC) with a higher capacity for self-renewal become more relevant in governing reconstitution (2, 6). Overall, recipient reconstitution was not improved by exercise training with no differences at any time point indicating no effect of exercise training on the function of ST- or LT-HSC. Unlike HSC expanded *in vitro* (12, 17, 34), pretreatment of donors with exercise did not impair donor cell hematopoietic reconstitution suggesting a maintenance of HSC function with the observed HSC expansion.

We considered a number of possibilities for the lack of improvement in hematopoietic regeneration when marrow from exercise-trained donors was used. We speculated that defects in HSC homing to the bone marrow niche, as

has been reported upon transplantation of culture-expanded HSC (17), might also be occurring with marrow from exercise-trained donors. In the present study; however, HSC homing to the bone marrow upon transplantation was preserved with exercise training. We next considered that HSC expansion with exercise training may result in a decrease in LT-HSC or impaired self-renewal as has been demonstrated with *in vitro* expanded HSC (12, 34). Results from our secondary transplantation assay where HSC were forced to undergo multiple rounds of proliferative stress to reconstitute multiple hosts indicate that a decrease in LT-HSC quantity was not occurring with exercise training and self-renewal capacity was maintained. These conclusions are further supported by our HSC quantification analysis where we did not observe any differences between exercise-trained and sedentary mice in HSC quantity in the endosteal niche, which is home to LT-HSC (13). These data lend further support to our suggestion that exercise training effects a population of more differentiated HSC not necessary for long-term engraftment and has no positive or negative effects on HSC function.

Data presented herein represent the first characterization of the effects of exercise training on HSC in their natural *in vivo* environment. Here we show that exercise training increases HSC quantity in the vascular niche while maintaining the population of HSC in the endosteal niche. We speculate that the mechanisms responsible for the observed increase may have been pulsed increases in hematopoietic growth factors associated with each exercise bout

with maintained HSC function by basal down-regulation of these factors. Increased HSC quantity was not associated with improved or impaired functional characteristics such as regeneration of the hematopoietic system in myeloablated recipients, homing to the bone marrow cavity or self-renewal suggesting that the effects of exercise training may be specific to a sub-population of HSC not directly responsible for recipient reconstitution. Whether these adaptations to exercise training induced in HSC are clinically relevant specifically to BMT will need to be determined by future studies perhaps examining transplantation in old or unhealthy mice.

Disclosures

The authors declare no conflict of interest, financial or otherwise.

Author Contributions

GP was the principal investigator of the study. MD and GP conceived and designed the experiments. GP contributed new reagents/materials/analytical tools. MD collected tissue, conducted the experiments, analyzed the data and wrote the manuscript. GP edited the manuscript. GP supervised the experimental work, interpretation of the data and edited the manuscript. All authors approved the manuscript prior to final submission.

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Figure Legends

Figure 1. HSC Quantification. HSCs were isolated from the vascular and endosteal niche from sedentary (SED) and exercise-trained (EX) mice, and quantified using the LSK markers via flow cytometry. Representative flow plots of the LSK population isolated from the vascular niche from SED (A) and EX (B) mice. The percentage of Sca-1 and c-Kit positive cells in the lineage negative population (LSK cells) isolated from the vascular niche (C). Representative flow plots of the LSK population isolated from the endosteal niche from SED (D) and EX (E) mice. The percentage of Sca-1 and c-Kit positive cells in the lineage negative population (LSK cells) isolated from the endosteal niche (F). The ratio of LSK cells in the vascular versus the endosteal niche (G). Data presented and analyzed as the percentage of Sca-1 and c-Kit positive cells in the lineage negative population. Graphs represent mean ± SEM (n=6 mice/group), and * denotes $p<0.05$ with t-test.

Figure 2. Cell Cycle Analysis and CFU-Spleen. Analysis of cell cycle status of whole marrow cells isolated three days following the final exercise bout from the vascular niche from sedentary (SED) and exercise-trained (EX) mice (A). Data are presented as mean ± SEM of the percentage of cells in each phase of the cell cycle (C) (n=6 mice/group), and * denotes $p<0.05$ with t-test. Spleen colonies were quantified in recipients transplanted with bone marrow from sedentary (SED) or exercise-trained (EX) mice 7 days following BMT. Data are presented

as mean ± SEM of spleen colonies normalized to total spleen weight of n=7-8 recipients per group, and * denotes $p<0.05$ with t-test.

Figure 3. Early Recipient Reconstitution Post-BMT. Peripheral blood was harvested from recipient mice in the BMT assay two and three weeks following transplantation. Analysis of peripheral blood reconstitution (donor and recipient derived); (A) and donor-derived engraftment (B) in peripheral blood of recipients at each time point following BMT. Reconstitution levels are presented relative to non-transplanted mice in arbitrary units (AU), while engraftment levels are normalized to a GFP standard curve, and data are presented as mean ± SEM (n=7-12 recipients/group/time point).

Figure 4. Long-term Recipient Reconstitution Post-BMT. Peripheral blood was harvested from recipient mice in the BMT assay monthly between one and six months following transplantation. Analysis of peripheral blood reconstitution (donor and recipient derived) (A) and donor derived engraftment (B) in peripheral blood of recipients at each time point following BMT. Reconstitution levels are presented relative to non-transplanted mice, while engraftment levels are normalized to a GFP standard curve, and data are presented as mean ± SEM (n=9-17 recipients/group/time point).

Figure 5. Donor Cell Homing. Recipient marrow was harvested five days following BMT for analysis of donor cell homing. Representative flow plots of non-GFP marrow (WT; A), and marrow from recipients transplanted with marrow from sedentary (SED; B) or exercise-trained (EX; C) mice. Homing of donor

hematopoietic cells to recipient bone marrow are normalized to a GFP standard curve, and presented as mean ± SEM (n=4-6 recipients/group) (D).

Figure 6. Secondary Recipient Reconstitution. Marrow from primary recipients reconstituted with whole marrow cells from sedentary (SED; n=3) or exercise-trained (EX; n=6-8) was harvested at least seven months following the initial transplant and was used as the donor cell population for transplantation into secondary recipients. Analysis of peripheral blood reconstitution (donor and recipient derived) (A) and donor derived engraftment (B) in peripheral blood of secondary recipients at each time point following BMT. Reconstitution levels are presented relative to non-transplanted mice, while engraftment levels are normalized to a GFP standard curve, and data are presented as mean ± SEM.

Tables

Table 1. Lineage Panel. Bone marrow isolated from the central marrow region from sedentary (SED) and exercise-trained (EX) mice was evaluated for the expression of various mature hematopoietic lineage panel markers. Values are presented as the mean (SEM) with units of percentage (n=5 mice/group).

Lineage Marker	SED	EX	p value
Mac-1	54.58 (3.39)	63.12 (1.62)	0.053
TER-119	9.11 (1.66)	7.37 (0.81)	0.374
Gr-1	53.28 (3.48)	61.53 (1.49)	0.061
B220	44.17 (3.84)	42.01 (1.77)	0.623
CD3ε	3.54 (0.26)	3.85 (0.21)	0.388

Table 2. Serum Cytokine Levels. Cytokine levels in sedentary (SED, n=12) and exercise-trained (EX, n=12) mice. Values are presented as mean (SEM) with units of pg/ml.

Cytokine	SED	EX	p value
IL-3	18.67 (1.9)	15.47 (1.7)	0.231
IL-6	24.86 (2.3)	15.33 (2.4)	**0.01**
G-CSF	165.6 (11.2)	138.33 (9.8)	0.08
GM-CSF	270.26 (20.5)	223.44 (19.7)	0.113

Table 3. Complete Blood Counts. Peripheral blood was harvested from primary recipients who received marrow from sedentary (SED) or exercise-trained (EX) mice one month following BMT and complete blood count analysis was performed. Data is presented as mean ± SEM based on n=9-10 SED and Ex mice per measure. MCV = mean corpuscular volume, MCH = Mean Corpuscular Hemoglobin, and MCHC = Mean Corpuscular Hemoglobin Concentration.

Parameter	SED	EX	p Value
Leukocytes (x 10^9/L)	5.32±0.79	4.73±0.79	0.606
Platelets (g/L)	622.80±57.9	627.00±66.43	0.963
Erythrocytes (x1012/L)	9.10±0.13	8.92±0.11	0.305
Hemoglobin (g/L)	144.70±1.98	141.90±1.77	0.307
Hematocrit (AU)	0.43±0.01	0.43±0.01	0.654
MCV (fL)	47.14±0.14	47.77±0.26	0.054
MCH (pg)	15.91±0.12	15.92±0.04	0.938
MCHC (g/L)	336.90±2.56	333.60±1.11	0.252

Figures

Figure 1

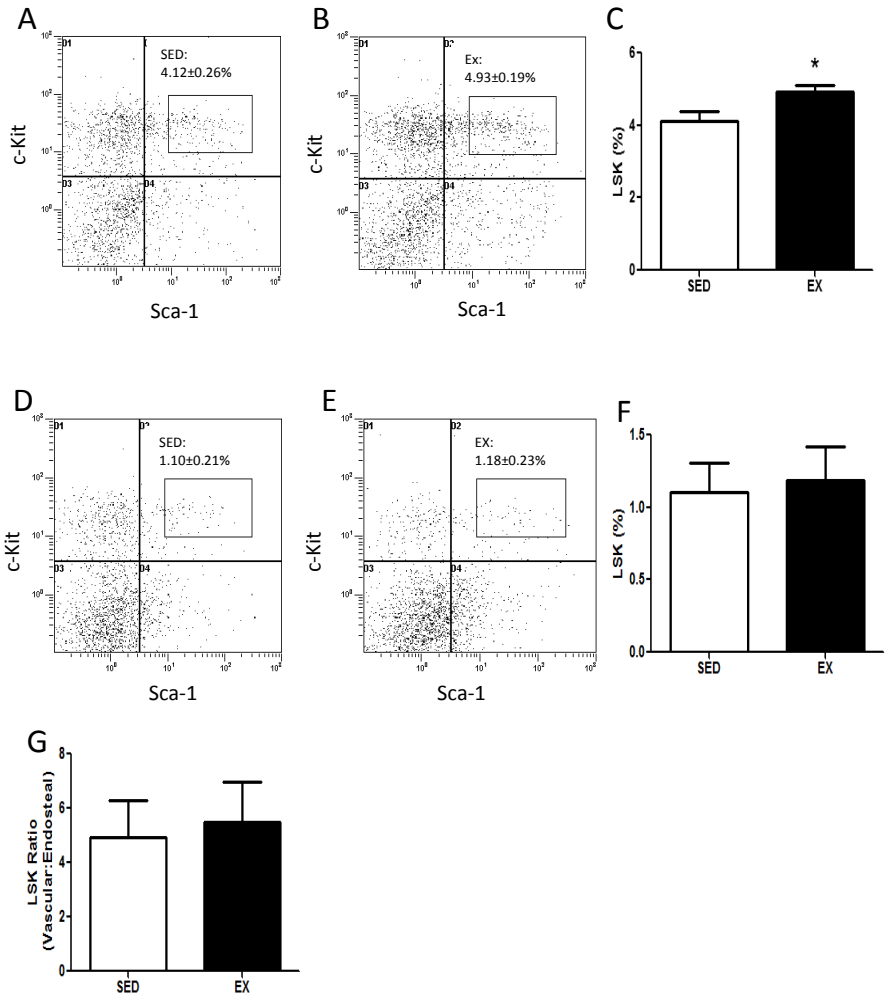


Figure 2

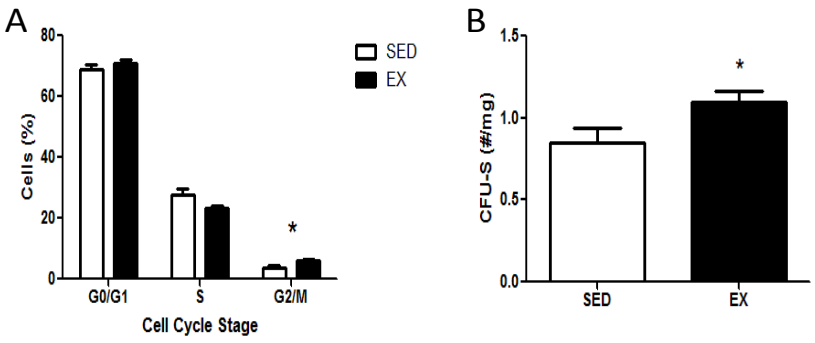

A
80
60
40
20
0
Cells (%)
SED
EX
*
G0/G1
S
G2/M
Cell Cycle Stage
B
1.5
1.0
0.5
0.0
CFU-S (#/mg)
*
SED
EX

Figure 3

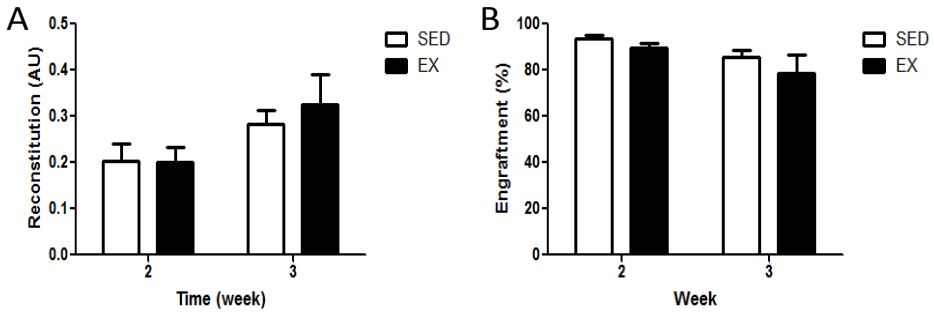

A
Reconstitution (AU)
0.5
0.4
0.3
0.2
0.1
0.0
2
3
Time (week)
SED
EX
B
Engraftment (%)
100
80
60
40
20
0
2
3
Week
SED
EX

Figure 4

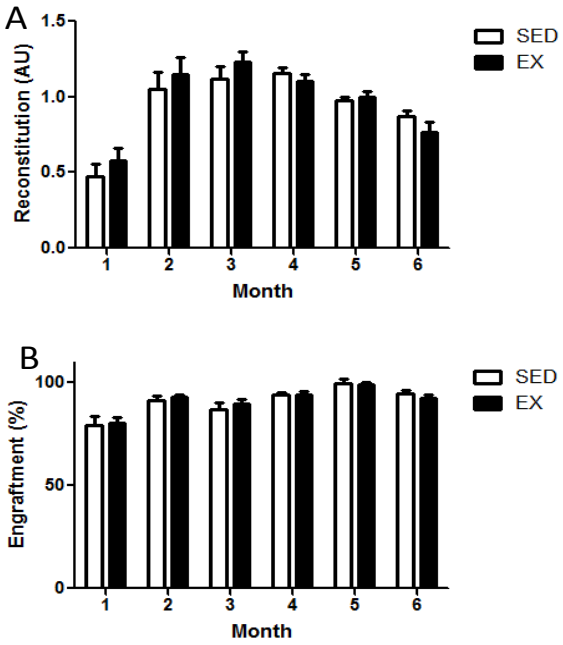

A
Reconstitution (AU)
1.5
1.0
0.5
0.0
1
2
3
4
5
6
Month
SED
EX
B
Engraftment (%)
100
50
0
1
2
3
4
5
6
Month
SED
EX

Figure 5

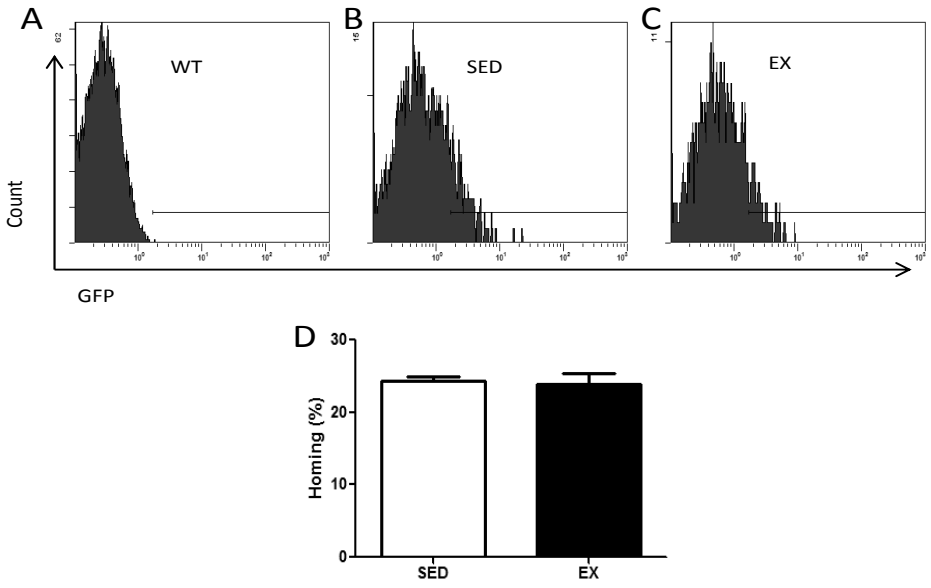
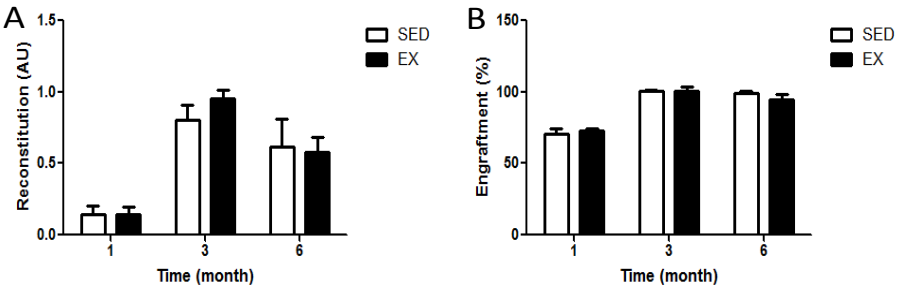


Figure 6


A
Reconstitution (AU)
1.5
1.0
0.5
0.0
1
3
6
Time (month)
SED
EX
B
Engraftment (%)
150
100
50
0
1
3
6
Time (month)
SED
EX

Supplemental Methods

Treadmill Test

One month following the BMT, return to function was analyzed in recipient mice via a treadmill test to exhaustion. Mice were placed on a motorized treadmill (Exer6, Columbus Instruments, Columbus, Ohio) beginning at 11 m/min. Speed was increased 1 m/min every 2 min and exhaustion was defined as when the mice did not respond to the shocker at the end of the treadmill for greater than 5 seconds continuously and were not responsive to manual encouragement. Exhaustion was evaluated by a researcher blinded to the group of each mouse.

Supplemental Figure Legends

Figure S1. Functional Recovery post-BMT. Functional recovery post-BMT, evaluated by a treadmill test to exhaustion, was evaluated one month post-BMT. Data are presented as mean ± SEM of total time (minutes) to exhaustion (n=18-19 recipients/group).

Figure S2. GFP Detection in Peripheral Blood. Representative gating strategy for analysis of donor-cell contribution to recipient hematopoiesis. Live cells were selected for based on negative staining for the viability dye 7AAD (A). Next, CD45 cells were selected for to exclude all non-hematopoietic cells (B). Gates to select for GFP positive cells were based on blood collected from wild type (non-GFP) mice (C) and maintained constant for analysis of short- and long-term donor-derived engraftment in primary and secondary recipients (D).

Figure S3. Standard Curve for GFP Detection. Standard curves were created on each analysis day for peripheral blood reconstitution analysis as well as marrow homing analysis and used for normalization of GFP signal detection. Curves were created by mixing blood from a wild type (WT; non-GFP) mouse with blood from a GFP mouse in percentages shown (A) collected on each analysis day. A representative standard curve is shown comparing the actual percentage mixture of GFP:WT blood to what was detected by the cytometer along with r^2 value (B).

Figure S4. Representative Gating for Donor Cell Homing. Gating was conducted similar to peripheral blood analysis where live cells were selected for based on their lack of expression of 7AAD. Next, hematopoietic cells were selected for based on their positivity for CD45, and the percentage of GFP^+ cells making up the $CD45^+$ population were quantified. Unstained controls used to establish gates are shown in (A) and representative histograms of sample analysis are shown in (B).

Supplemental Figures

Figure S1

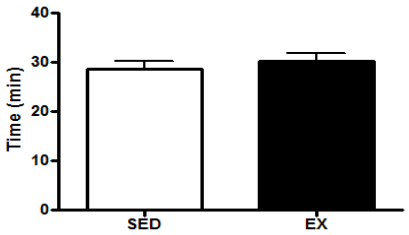


Figure S2

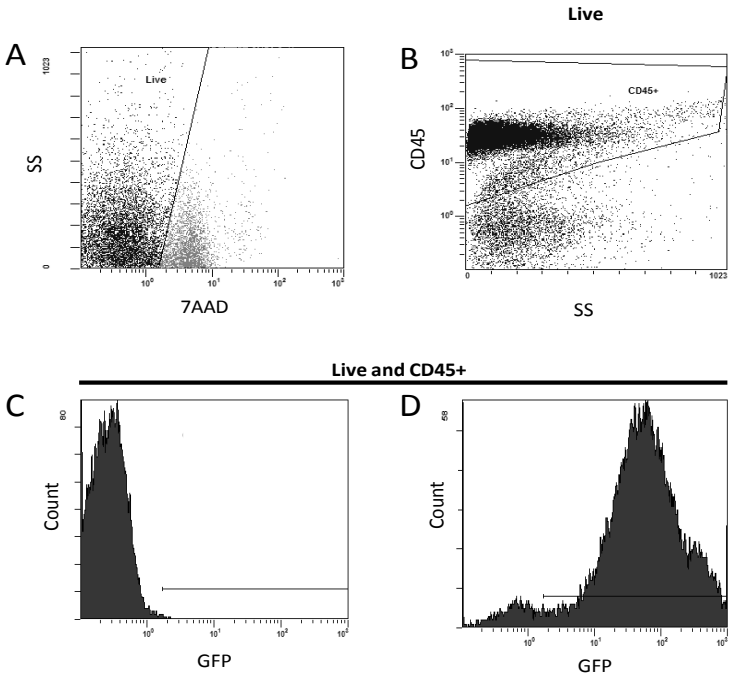

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Figure S3

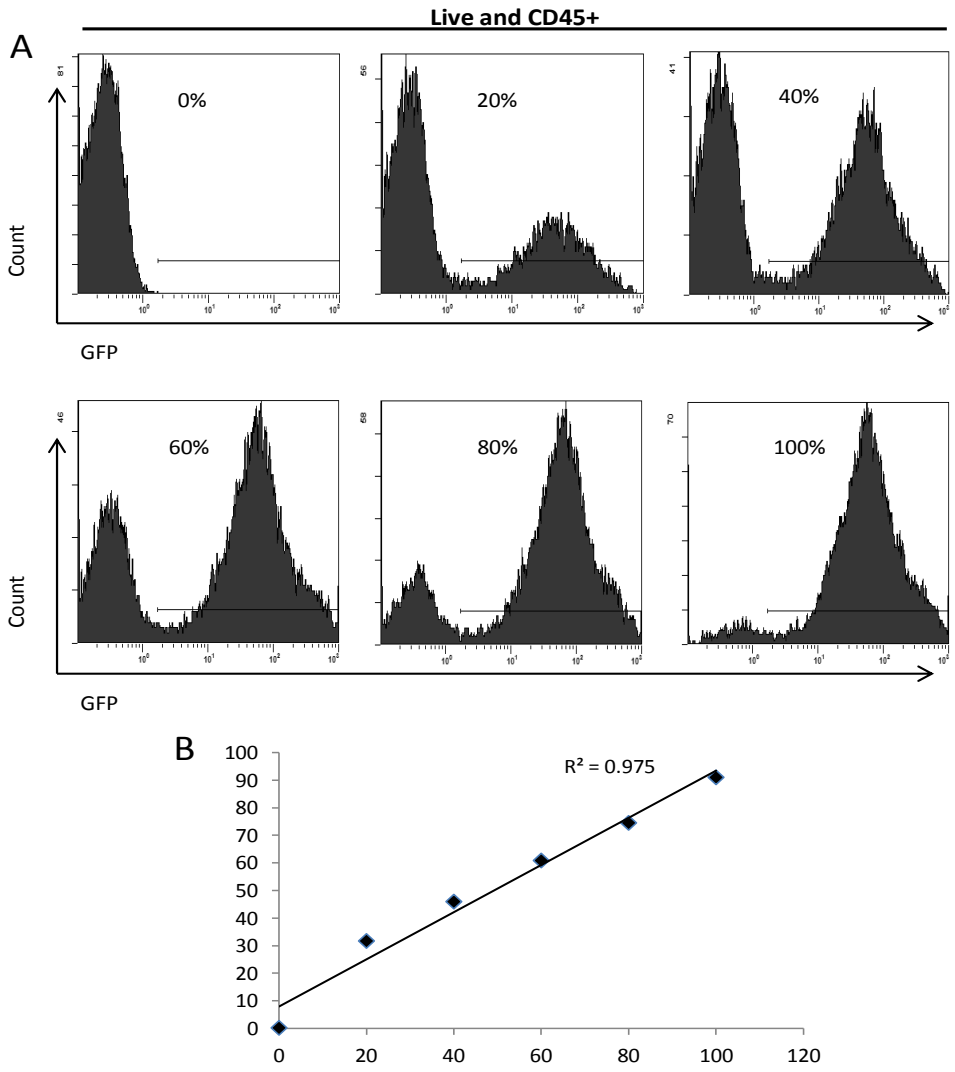
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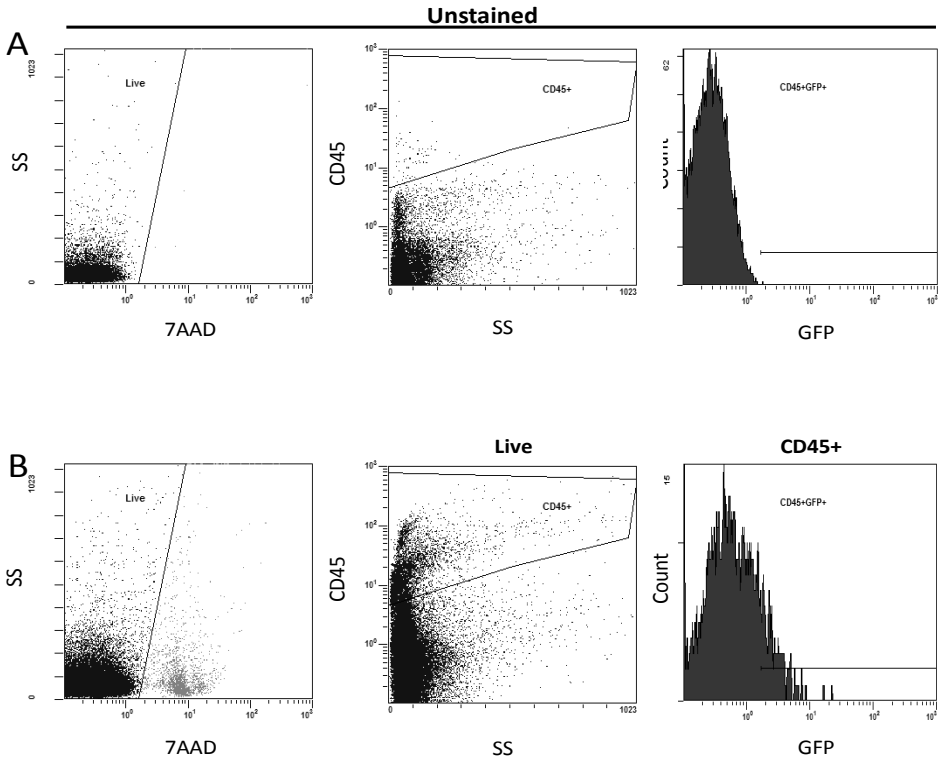
Figure S4


Unstained
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5. Chapter 5

Exercise training promotes recipient survival without impairing donor-derived engraftment following bone marrow transplantation

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Abstract

Bone marrow transplantation (BMT) is associated with a high risk of mortality partially due to the harmful effects of the preconditioning myeloablative regimens. We have recently demonstrated increased bone marrow cell survival and proliferation in response to exercise training, which may be attributable to increased quality of the niche. **PURPOSE.** The purpose of the present study was to determine the extent to which exercise preconditioning of recipients could increase the success of BMT. **METHODS.** We utilized a BMT assay where recipient C57Bl/6 mice remained sedentary (SED) or were exercise trained on a treadmill (EX; 3d/wk, 8 wks). Both groups of mice had their native marrow ablated prior to receiving GFP-labeled donor marrow. Successful BMT was established by recipient survival. Both donor-derived blood reconstitution and total (donor- and recipient-derived) blood reconstitution were measured by flow cytometry. One and four days post-BMT apoptosis, cellularity and donor cell homing were determined in the recipients' bone marrow cavity by flow cytometry. **RESULTS.** Whereas only 25% of SED survived, 82% of EX recipients survived the BMT. Homing of donor-derived marrow cells to the recipients' marrow cavity acutely post-BMT was not altered in EX, but EX mice displayed decreased levels (10%, $p<0.05$) of activated caspase-3/-7 one day following BMT leading to a maintenance of marrow cellularity in mice preconditioned with exercise. The acute inhibition of marrow cell apoptosis in EX resulted in increased total blood cell reconstitution at one and three and a half months post-BMT in EX (42% and

43% respectively, both $p<0.05$). Short- and long-term donor-derived engraftment was not different between exercise preconditioned and sedentary recipients. **CONCLUSION.** Exercise training increases recipient survival post-BMT with increased total blood cell reconstitution. Donor-derived reconstitution was not improved, possibly due to enhanced competition for niche availability created by the inhibition of apoptosis with exercise.

Introduction

Bone marrow transplantation (BMT) is used to treat a variety of diseases, and success depends on donor HSC engraftment and expansion in the recipient's marrow cavity. Despite their widespread use, mortality associated with BMT remains quite high *(1)*. BMT is preceded by conditioning regimens, such as irradiation, to remove native hematopoietic cells to allow for engraftment of donor cells *(2)*. Although radiation is an effective means of ablating native marrow cells, the effects of radiation can often be toxic to the recipient and the bone marrow niche *(3)*. Systemically, radiation induces a significant inflammatory response, which may result in organ failure *(4–6)*. Furthermore, the rapid loss of hematopoietic cells can result in hematopoietic failure and death *(5)*. Within the bone marrow microenvironment, radiation induces DNA damage not only in hematopoietic cells but also in osteoblasts *(3)* and endothelial cells *(7–9)* that comprise the HSC niche. In order for donor HSC to engraft, recipients must have sufficient niche capacity *(10, 11)*, and the niche must be healthy enough to

support hematopoiesis *(12)*. This is evidenced by the rapid regeneration of the niche post-BMT *(12, 13)*, where osteoblasts proliferate to repair the endosteal niche first followed soon after by the vascular niche *(12)*. Therefore, minimizing damage to the niche may promote donor cell engraftment and recipient survival. For these reasons, milder preconditioning protocols have been attempted that are less harmful to the recipients' hematopoietic system to increase BMT survival rates *(14)*. These approaches enhance recipient survival but impair donor-derived engraftment due to increased competition for niche positions from surviving native marrow cells within the niche *(14)*. Therefore, interventions designed to promote recipient survival from the preconditioning regime must also facilitate successful engraftment of donor cells.

Therapeutic interventions designed to target the HSC niche, and promote survival post-irradiation have recently been explored. These studies have demonstrated that directly targeting the cells that comprise the HSC niche can increase the capacity of the niche with concomitant increases in HSC number, and protect HSC during multiple rounds of chemotherapeutic insult *(15, 16)*. Furthermore, protection from radiation preconditioning can be achieved with a cocktail of hematopoietic promoting and anti-inflammatory cytokines *(17, 18)*. These data suggest that the control of inflammation, promotion of donor cell proliferation, and availability of niche spots for donor cell engraftment are important requirements for successful transplantation. Few studies have examined the effects of exercise training on hematopoietic parameters following

BMT. The available data suggests that beginning an exercise training program prior to BMT and continuing after the procedure attenuates hematopoietic cell loss *(19–21)*. Conversely, when an exercise program was started shortly after the BMT, no effects were observed in markers of hematopoiesis *(22)*.

We have previously demonstrated the beneficial effects of exercise training on hematopoietic cells and their microenvironment. Exercise training protects hematopoietic cells from radiation *(23)* and increases hematopoietic progenitor cell quantity (*(24)*,*De Lisio submitted*). We have also reported that these adaptations occur, at least in part, via alterations in the differentiation of the mesenchymal stem cells (MSC) that contribute to the bone marrow microenvironment and enhanced production of hematopoietic growth factors following exercise *(24)*. These adaptations may promote survival following radiation as a myeloablative strategy, as well as maintain a favourable microenvironment allowing donor cells to engraft and repopulate the new host more rapidly. Therefore, in the present study, we preconditioned recipients in the BMT assay with exercise training to determine the effects of preconditioning the HSC niche with exercise on recipient survival and donor-derived engraftment. Given the previous literature describing the beneficial effects of exercise training on the HSC niche, we hypothesized that exercise training would improve recipient survival and enhance donor cell engraftment.

Methods

Experimental animals

Mice were ordered from Jackson Labs, and allowed to acclimatize for at least one week prior to being used in any experiments. Mice were maintained on a 12 hour light cycle in temperature and humidity controlled room, and allowed food and water *ad libitum*. All protocols were approved by the McMaster Animal Research Ethics Board and conformed to guidelines established by the Canadian Council for Animal Care. Male mice expressing enhanced GFP driven by the actin promoter*(25)* (C57BL/6-Tg(CAG-EGFP)1Osb/J; Jackson Labs, Bar Harbor, Maine) 8-10 weeks old were used as donors for the BMT assay, and were housed in a clean room with non-ventilated racks for at least one week prior to BMT.

Exercise Training

Sedentary (SED) and exercise-trained (EX) female mice, aged six weeks, were housed three per cage in an ultraclean facility on ventilated racks during the exercise training protocol. Mice were exercise trained 1 hour/day, 3 days/week for 8 weeks on a motorized treadmill (Exer6, Columbus Instruments, Columbus, Ohio) as previously described *(23, 24)*. Each exercise session consisted of a 10 min warm up followed by 45 min of training beginning at 14 m/min in week 1 and increasing 1 m/min every week up to a final speed of 18 m/min, which was maintained for the remainder of the training period. Each training session was followed by a 5 min cool down. At the end of each training session, SED mice

were placed on the treadmill but were not required to run in order to control for the stress of handling and treadmill exposure.

Bone Marrow Transplantation Assay

Immediately following the final exercise training session SED and EX mice were transferred to a clean room with non-ventilated racks where radiation and transplants took place. Three days following the final exercise bout, female recipients were exposed to ~9 Gy radiation administered in fractionated doses of ~6 Gy and ~3 Gy separated by three hours. Donor marrow cells were harvested from male GFP^+ donors as previously described (*(26)*,*De Lisio submitted*). Immediately following the final radiation dose, recipient mice were reconstituted with 1×10^6 donor cells. Cells harvested from at least two male donors were mixed to ensure that the cell population the recipients received was uniform, and injected into multiple recipients. Daily health checks were performed and mice were defined as being at endpoint when they were deemed to be dehydrated, lethargic, non-responsive and had ruffled, unkempt coats.

Bone Marrow Analysis: Homing and Apoptosis

One and four days post-BMT a subset of mice were euthanized via cervical dislocation. Bone marrow was harvested as described above for analysis of donor cell homing to, and apoptosis in the bone marrow compartment. For homing analysis, 5×10^6 freshly isolated bone marrow cells were stained with CD45 antibody (1:80, Invitrogen, Burlington, Canada) to identify leukocytes and 7AAD (Beckman Coulter, Mississauga, Canada) to determine cell viability. Cells

were run immediately on Epics XL flow cytometer (Beckman Coulter, Mississauga, Canada) and single-stained controls were used to set up appropriate gates (Figure S1). For flow cytometry analysis, dead cells ($7AAD^+$) were excluded and the percentage of $CD45^+$ cells (donor and recipient derived leukocytes) as well as the percentage of GFP^+ cells from the $CD45^+$ population (donor-derived leukocytes) were analyzed. The percentage of $CD45^+$ cells was normalized to unmanipulated control mice.

Marrow not immediately used for homing analysis was frozen in freeze media (10% DMSO/20% FBS/PBS) and stored at -80^{o}C until further analysis. For analysis of apoptosis, frozen marrow was thawed and prepared for detection of activated caspase-3/-7 using the FLICA kit (ImmunoChemistry Technologies, Bloomington, Minnesota) according to manufacturer's instructions and as previously described *(23)*. Samples were analyzed immediately on Epics XL flow cytometer (Beckman Coulter, Mississauga, Canada) with appropriate single-stained controls to establish gates (Figure S2).

Blood Analysis: Reconstitution, Engraftment, Serum Cytokine Array

In another subset of mice, blood was collected via facial bleed at one, three and a half and six months post-BMT. These time points were chosen to evaluate the various populations of HSC that are responsible for recipient reconstitution. Short-term HSC with higher capacity for proliferation and lower capacity for self-renewal are the primary cell population for recipient reconstitution up to 3-4 months post-BMT, while long-term HSC having a higher

capacity for self-renewal reconstitute recipients beyond four months and are assumed to have undergone at least one round of proliferation and self-renewal, indicating the presence of truly engrafted stem cells by 6 months post-BMT *(27)*. For reconstitution and engraftment analysis, approximately 100 µL of blood was collected into a heparinised tube and mixed to prevent clotting. Red blood cells (RBC) were lysed with ammonium chloride lysis buffer (17mM Tris/0.75% NH_4Cl/ddH_20), washed, then stained with a CD45 antibody (1:80, Invitrogen, Burlington, Canada) as well as 7AAD in order to detect viable leukocytes.. Samples were run immediately on an Epics XL flow cytometer (Beckman Coulter, Mississauga, Canada). Non-viable cells were excluded and the percentage of $CD45^+$ cells was used as a measure of whole blood reconstitution, while the percentage of GFP^+ cells from the $CD45^+$ gate was used as a measure of donor-derived engraftment. Single-stained controls were used to establish appropriate gates (Figure S3). Data were normalized to blood from unmanipulated mice for CD45 analysis, and to standard curves established from a GFP^+ and a non-GFP mouse blood run on each experimental day (Figure S4).

For analysis of serum cytokine levels, blood was collected from the submandibular vein from female exercise trained and sedentary mice. Blood was collected two days following the final exercise bout in the exercise training protocol and these mice were never used in the BMT assay. Blood samples rested at room temperature for 30 min to allow serum to separate. The serum fraction was removed and stored at -80^{o}C until further analysis. A panel of

cytokines and growth factors was analyzed using the Bio-Plex Pro Assay (Bio-rad, Mississauga, Canada) according to manufacturer's instructions.

Bone Analysis

At one and four days post-BMT, a sub-set of recipient mice were euthanized via cervical dislocation and their left femur was quickly isolated. Femurs were immediately fixed in 10% neutral buffered formalin for three days then decalcified in 10%EDTA/PBS, pH 7.5 for 14 days as previously described *(24)*. Decalcified bones were stained with haematoxylin and eosin and longitudinally sectioned at a thickness of 4 µm by the Core Histology Facility at McMaster University. Using Nikon NIS Elements AR 3.2, regions of interest (ROI) were drawn around all non-bone containing areas within the femur marrow cavities of sedentary and exercise-trained donors. Using the automated measurement features within Elements, section density per ROI area was measured, totaled, and expressed relative to total ROI area for each sample. In separate analyses, we have found that the automated density measurement correlates with manual measurement of marrow cellularity. Analyses were performed by an experimenter blinded to experimental conditions.

Statistical Analysis

Data, presented as mean ± SEM, were analyzed using two-tailed t-test or two-way ANOVA (group x time) in either Excel or SigmaPlot. Probability of survival was analyzed via chi-squared test. A p value of ≤ 0.05 was considered to be statistically significant.

Results

Exercise training suppresses inflammation and enhances likelihood of survival following BMT

Radiation is associated with increased inflammation leading to organ failure and death *(4–6)*; therefore, we sought to determine if exercise training improved the systemic inflammatory environment. Using a serum multiplex cytokine array we examined a number of different inflammatory cytokines associated with hematopoietic regulation in exercise trained and sedentary mice prior to BMT. Collectively, exercise training decreased levels of circulating inflammatory cytokines (Table 1) with the following being significantly lower with exercise: IFN-γ (28%, $p<0.05$), TNF-α (24%, $p<0.05$). Additionally, the anti-inflammatory cytokine, IL-10 was also significantly reduced (34%, $p<0.05$). To determine if decreased systemic inflammation induced by exercise training contributed to improved survival from BMT, health checks were performed daily for at least 14 days following the BMT procedure. While 82% of the exercise-trained recipients survived, only 25% of sedentary recipients survived (Figure 1, $p<0.05$). All mice that died did so in the first week post-BMT.

Exercise training does not improve donor cell homing to the bone marrow but inhibits apoptosis

We next sought to determine the mechanisms responsible for increased survival associated with exercise training. To assess whether an exercise-conditioned niche was more amenable to receiving donor hematopoietic cells,

acute homing to the bone marrow compartment was evaluated in recipients one and four days following BMT. No effect was observed in the homing ability of donor hematopoietic cells in exercise or sedentary niches; however, a general increase in donor hematopoietic cell homing was observed from one to four days post-BMT ($p<0.05$; Figure 2a-e).

Since exercise training did not enhance donor hematopoietic cell homing to the bone marrow niche, we hypothesized that early inhibition of native cell apoptosis may be an important factor in the increased survival of exercise trained recipients. Apoptosis was determined by measuring the levels of activated caspase-3/-7 in recipient marrow one and four days post-BMT. Caspase-3 and -7 are the two main effector caspases responsible for the execution of apoptosis *(28)*. The level of activated caspase-3 and -7 in bone marrow cells of exercise trained recipients was significantly reduced by ~10%, one day post-BMT ($p=0.01$), with no differences observed four days post (Figure 3a-e). To confirm these findings, we evaluated bone marrow cellularity and observed a significant decreased from one to four days post-BMT in both groups (Figure 4a-e; 2-way ANOVA). Upon further analysis, the decreased cellularity was primarily due to a significant ~32% decrease in SED ($p<0.01$) but not EX (3% decrease, $p=0.77$) mice (Figure 4e). Together, these data suggest that exercise training promotes survival of native marrow cells in response to BMT.

To identify if exercise training was specifically preserving hematopoietic cells in the bone marrow in response to radiation, we evaluated the percentage of

$CD45^+$ cells in the marrow one and four days post-BMT. The percentage of leukocytes was significantly decreased in both SED and EX recipients between one and four days post-BMT with no differences between groups (Figure 5a-e). These data suggest that exercise training was preserving non-hematopoietic cells comprising the HSC niche acutely post-BMT.

Exercise training increases early reconstitution without impairing donor-derived engraftment.

To determine if inhibition of apoptosis and a maintenance of cellularity translated to enhanced blood reconstitution in EX recipients, we analyzed the percentage of leukocytes and the percentage of donor derived leukocytes with flow cytometry. Exercise-trained recipients had higher percentages of blood leukocytes by 42% ($p<0.05$) and 43% ($p<0.05$) at one and three months respectively (Figure 6a) with no differences in donor-derived engraftment between groups at any time points measured (Figure 6b). Together, these data suggest that exercise training enhances early leukocyte reconstitution without impairing long-term engraftment of donor cells.

Discussion

In the present study, we determined the effects of exercise training on the HSC microenvironment by preconditioning BMT recipients with exercise. We report that recipients preconditioned with exercise had an increased likelihood of survival following BMT. This phenotype may be due to a decreased inflammatory environment prior to transplant, and decreased native cell apoptosis immediately

post transplant which maintained bone marrow cellularity. These adaptations promoted recipient survival and early hematopoietic reconstitution, without diminishing donor-derived engraftment.

In the present study, 82% of the mice preconditioned with exercise training survived the BMT process, whereas only 25% of the sedentary mice survived. These mortality rates were higher than expected and indeed higher than we have observed in our previous studies (*De Lisio, submitted*). In the present study, mice were moved from the room where they were housed during the training program to a new room where the BMT took place. Both rooms were sterile environments and SED and EX mice were moved simultaneously. The stress associated with changing rooms prior to the BMT procedure, although inadvertent, may have contributed to the increased mortality observed in the present study. Regardless, both SED and EX recipients were housed and handled in exactly the same manner, including regular exposure to the treadmill; therefore, the observed effects can solely be attributed to the adaptations to exercise training. These experimental conditions may more closely resemble conditions patients experience prior to BMT, as patients undergoing BMT experience a great deal of psychological stress *(29)*.

Radiation induces systemic inflammation that can damage hematopoietic cells in the bone marrow and other organs *(4–6)*. Reducing the inflammatory effects associated with radiation is therefore an important factor in reducing radiation-

induced mortality *(30)*. In agreement with previous studies *(31–34)*, we observed a significant decrease in systemic levels of pro-inflammatory cytokines IFN-γ and TNF-α. . IFN-γ and TNF-α, have been shown to work synergistically to promote apoptosis induction *(35)*. Additionally, an acute increases in systemic levels of TNF-α was correlated with a decrease in levels of circulating HSC 24 hours later *(36)*. Therefore, decreased circulating levels of these pro-inflammatory cytokines with exercise may promote hematopoiesis. Although we were unable to measure cytokine levels immediately post-BMT, it has previously been demonstrated that the inflammatory response to an acute inflammatory stimulus in exercise-trained subjects is blunted *(31, 37)*. Therefore, similar mechanisms may have been activated in the exercise-trained mice in response to the radiation preconditioning. We speculate that the decreased basal levels of circulating inflammatory cytokines, prior to the myeloablation strategy, may have diminished the inflammatory response immediately post-BMT and promoted survival in exercise-trained mice.

Interestingly, we also observed a significant decrease in the anti-inflammatory cytokine, IL-10, with exercise training. This observation is in agreement with previous exercise training studies where the level of IL-10 was decreased *(33, 34)*. IL-10 has been shown to directly inhibit TNF-α production *(31)*, and elevated levels of both cytokines, contribute to mortality in heart failure patients *(38)* suggesting that the mechanisms regulating the levels of these cytokines in

circulation may be related. Similar to previous studies *(34)*, we speculate that the decreased levels of TNF-α with exercise training necessitated lower levels of its inhibitor IL-10, accounting for the decrease in both cytokines.

To further examine the potential mechanisms underlying improved survival in exercise-trained recipients, we investigated levels of apoptosis, cellularity and donor cell homing in recipient marrow at early time points following BMT. Homing of donor cells to the bone marrow did not differ between groups and increased equally in both groups across time post-BMT. These data are in agreement with previous studies reporting minimal donor cell homing to the bone marrow immediately post-BMT, which increases in the first week post-transplant *(12, 39)*. Therefore, preconditioning with exercise does not promote donor cell migration and engraftment in the niche.

Myeloablation by radiation results in the removal of hematopoietic cells, primarily via apoptosis *(40)*; therefore, we identified the level of activation of two primary effector caspases, caspase-3/-7 *(28)*, in whole bone marrow isolated from recipients one and four days post-BMT. One day following BMT, levels of activated capsase-3/-7 in recipient marrow was significantly decreased in mice preconditioned with exercise training suggesting that exercise increased survival of recipient cells in response to the myeloablative strategy. In support of our findings demonstrating a decrease in apoptosis following exercise training, the overall decrease in marrow cellularity from one to four days post-BMT was

primarily due to a decrease in cellularity in sedentary mice. Cellularity in sedentary recipients decreased 32% from one to four days post-BMT, while only a 3% decrease was observed in exercise trained recipients. Following radiation, hematopoietic cells, lost through apoptosis, are replaced by adipocytes *(41)* which are known to negatively regulate bone marrow hematopoiesis *(41, 42)* . Indeed, inhibition of adipogenesis either genetically or pharmacologically increases peripheral blood cell recovery in transplanted mice *(41)* . In the present study we observed increased hematopoietic reconstitution at one and three and a half months post-BMT in exercise-trained recipients. We speculate that inhibition of apoptosis and maintenance of bone marrow cellularity in exercise trained recipients prevented adipocyte infiltration in the bone marrow cavity post-irradiation thereby promoting hematopoietic recovery.

The specific cell population preserved by exercise training remains unknown. Our data suggests that exercise did not specifically preserve hematopoietic cells in the bone marrow as the percentage of $CD45^+$ cells decreased similarly in both groups from one to four days post-BMT. These data suggest that the effects of exercise were specific to non-hematopoietic cells. It has been demonstrated that pre-treatment with radiation for myeloablation destroys the vascular HSC niche *(43)*. It is possible that the reduced caspase-3/-7 activation was localized to the endothelial cells of the bone marrow sinusoids and by preservation of these cells; exercise training maintained the vascular niche. Since the vascular niche is associated with increased HSC proliferation *(44)*, this may explain the increased

early hematopoietic reconstitution in exercise trained recipients at early time points post-BMT. Alternatively, exercise training may have preserved bone marrow MSC. MSC do not express CD45 *(45)*; therefore, they would not have been detected by staining for CD45 but could have contributed to the decrease in caspase-3/-7 activation as that analysis was performed in whole bone marrow. Increased survival of MSC could have promoted early hematopoietic regeneration as MSC secrete growth factors and cytokines that promote HSC survival and growth *(46)*, and MSC transplantation without HSC has been shown to rescue mice from lethal doses of radiation *(4)*. It is possible that exercise training induced adaptations in bone marrow endothelial cells or MSC that supported their survival post-BMT and contributed to enhanced early hematopoietic reconstitution.

In the present study, hematopoietic reconstitution was increased at one and three and a half months post BMT in exercise trained mice. These data are in agreement with previous studies that have shown an attenuation in hematopoietic cell loss with in-patient exercise programs for BMT patients *(19–21)*. Since donor-derived hematopoietic reconstitution was not enhanced or impaired, these data suggest that the increase in overall (donor- and recipient-derived) hematopoietic reconstitution in exercise-trained mice resulted from proliferation and differentiation of recipient HSC that persisted post-BMT. Donor-derived engraftment post-BMT is accomplished by donor cells homing and engrafting in their niche in the bone marrow. In response to radiation, the vascular niche

within the bone marrow compartment breaks down as marrow cells supporting the niche are destroyed causing HSC to migrate to the endosteal niche *(13)*, the location of LT-HSC *(47)*. It is well known that donor derived engraftment is directly related to the number of available niche spots in the recipient marrow *(10, 11, 48)*. If these niche spots are maintained with exercise training then any surviving recipient HSC as well as donor-derived HSC have equal opportunity to fill these niches. Indeed, as donor cell homing takes several days *(39)*, and is not improved with exercise, surviving recipient HSC would have the advantage of being present in the niche immediately post-BMT and would be able to take up residence in any remaining niches prior to donor HSC which must first home back to the bone marrow. This may account for the increased total hematopoietic cell reconstitution in exercise trained mice at early time points.

Taken together, the present data can be summarized using the following model (Figure 7). Pre-treatment with exercise training decreases inflammation which enhances likelihood of survival. At the cellular level, exercise training recipients does not improve donor cell homing to the bone marrow; however, whole bone marrow cell apoptosis is acutely inhibited post-BMT. The acute inhibition of apoptosis does not preserve native hematopoietic cells but may increase survival of endothelial cells of the bone marrow sinusoids or bone marrow MSC. Preservation of these cell types would promote hematopoietic reconstitution, as observed in exercise trained animals, by whichever cells could rapidly populate the niche. This niche preservation would result in more rapid

hematopoietic reconstitution by both recipient- and donor-derived HSC. This model may explain the improved indices of hematopoiesis in patients who participate in an in-patient exercise training program that begins prior to BMT *(19–21)*, and suggests that exercise may be an effective adjuvant therapy to promote successful BMT.

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Tables

Table 1. Serum Cytokine Levels. Serum levels of various inflammatory cytokines in non-transplanted sedentary (SED, n=12) and exercise-trained (EX, n=12) mice. Data are presented as mean (SEM) with units of pg/ml.

Cytokine	SED	EX	p value
IL-1α	321.47 (62.70)	221.68 (64.17)	0.278
IL-10	132.8 (14.4)	87.6 (14.3)	**0.036**
IFN-γ	69.17 (5.26)	51.94 (5.11)	**0.028**
TNF-α	1007.39 (53.90)	790.24 (72.80)	**0.025**

Figure Legends

Figure 1. Recipient Survival Post-BMT. Recipient mice were exercise-trained (EX, n=11) or remained sedentary (SED, n=12) for 8 weeks prior to transplantation, and following BMT, SED and EX recipients were health-checked daily. The graph represents the percentage of recipients that survived beyond the first week post-transplant. Data are combined from two separate sets of experiments. Combined data were analyzed via x^2-test, and * denotes $p<0.05$.

Figure 2 Donor Cell Homing in Recipient Bone Marrow. One and four days post-BMT, recipient marrow was harvested and analyzed for donor cell homing to the bone marrow cavity in previously sedentary (SED, n=6) or exercise-trained (EX, n=6) recipients. Representative flow cytometry scatter plots from SED one (A) and four (C), and EX one (B) and four (D) days post-BMT. (E) Combined data from two separate experiments are expressed as mean ± SEM, * denotes a main effect for time ($p<0.001$; 2-way ANOVA).

Figure 3. Bone Marrow Apoptosis. One and four days post-BMT, recipient marrow was harvested and analyzed for levels of caspase-3/-7 activation, an indicator of apoptosis, in previously sedentary (SED; n=6) or exercise-trained (EX; n=6) recipients. Representative flow cytometry scatter plots from SED one (A) and four (C), and EX one (B) and four (D) days post-BMT. Caspase-3/-7 activation is expressed as mean ± SEM of mean fluorescence intensity (MFI) (E). A significant group x day interaction was observed with * denoting a significant

difference ($p<0.001$) within each group from one day post-BMT, and ** denoting a significant difference ($p=0.01$) between SED and EX at one day post-BMT via 2-way ANOVA.

Figure 4. Bone Marrow Cellularity. One and four days post-BMT, previously sedentary (SED, n=5-6) or exercise-trained (EX, n=6) recipients were sacrificed and bone marrow cellularity was determined. Representative images of SED one and four days post-BMT (A and C respectively) and EX one and four days post-BMT (B and D respectively) are shown. Quantification of the total density per region of interest area (Sum Density/ROI Area), an indication of bone marrow cellularity is shown in (E). Data are presented of mean ± SEM of arbitrary units (AU) for marrow cellularity, and * denotes a main effect for time ($p<0.05$, 2-way ANOVA). If the difference within each group from one to four days post-BMT was analyzed via t-test, a significant decrease in marrow cellularity in SED (32% decrease, $p<0.01$, t-test) with no change (3% decrease, $p=0.77$, t-test).

Figure 5. Leukocyte Content in Recipient Bone Marrow. One and four days post-BMT, recipient marrow was harvested and analyzed for total (recipient and donor-derived) leukocyte in previously sedentary (SED, n=6) or exercise-trained (EX, n=6) recipients. Representative flow cytometry scatter plots from SED one (A) and four (C), and EX one (B) and four (D) days post-BMT. Leukocyte content is expressed relative to non-transplanted controls (E). Combined data from two

separate experiments are expressed as mean ± SEM, * denotes a main effect for time ($p<0.001$; 2-way ANOVA).

Figure 6. Recipient Reconstitution and Donor-Derived Engraftment. Reconstitution of total (recipient and donor-derived) peripheral blood leukocytes (A) and donor-derived leukocytes (B) were evaluated 1, 3.5 and 6 months post-BMT in previously sedentary (SED, n=3) or exercise-trained (EX, n=9) recipients. Recipient reconstitution is expressed relative to untreated control mice (A) and expressed as arbitrary units (AU), and donor-derived engraftment (B) is expressed relative to a GFP standard curve created on each analysis day. Combined data from two separate experiments are presented as mean ± SEM, and * denotes $p<0.05$ versus SED.

Figure 7: Working Model. EX recipients have increased likelihood of survival post-BMT likely mediated by the anti-inflammatory effects of exercise. In the bone marrow cavity, exercise training inhibits native BM apoptosis and maintenance of BM cellularity in EX recipients. These effects are not localized to hematopoietic cells; therefore, exercise must be preserving non-hematopoietic cells in the niche. Niche preservation promotes earlier hematopoietic reconstitution in EX recipients without impairing donor-derived engraftment. Please refer to text for more detailed explanation. Green circles represent donor and donor-derived cells, blue circles represent recipient and recipient-derived cells, ⊤ represents enhancement, and ⊥ represents inhibition.

Figures

Figure 1

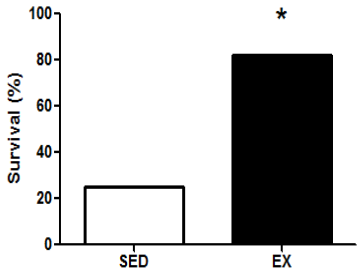
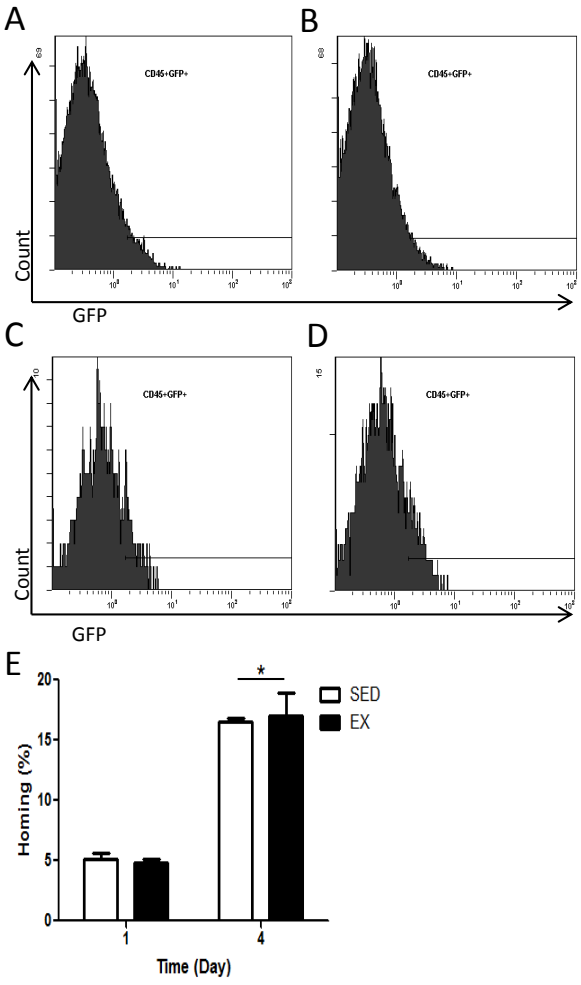


Figure 2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CD45+GFP+
CD45+GFP+
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GFP
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D
CD45+GFP+
CD45+GFP+
Count
GFP
E
*
SED
EX
Homing (%)
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15
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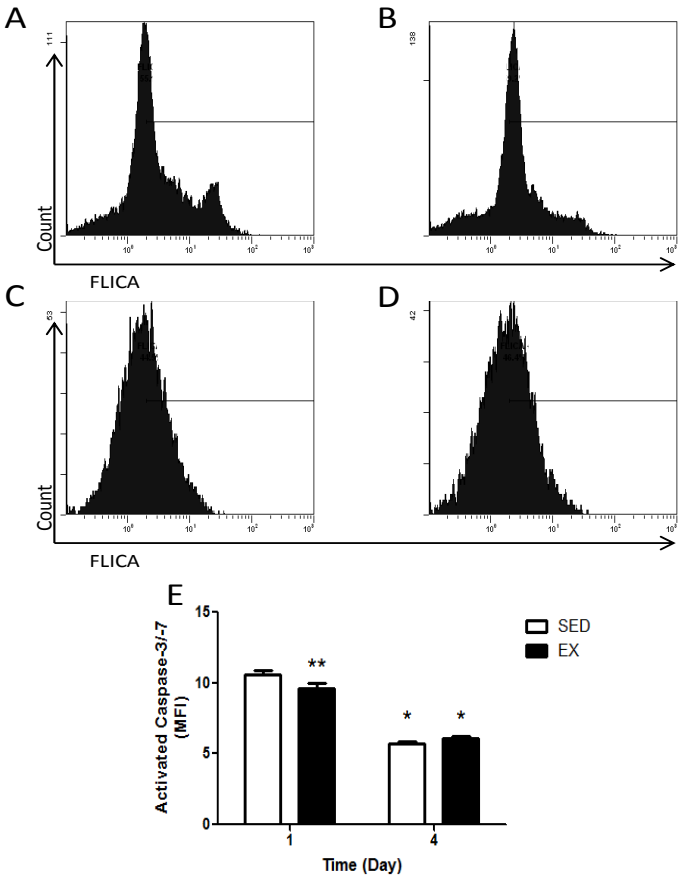
1
4
Time (Day)

Figure 3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*
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1
4
Time (Day)

Figure 4

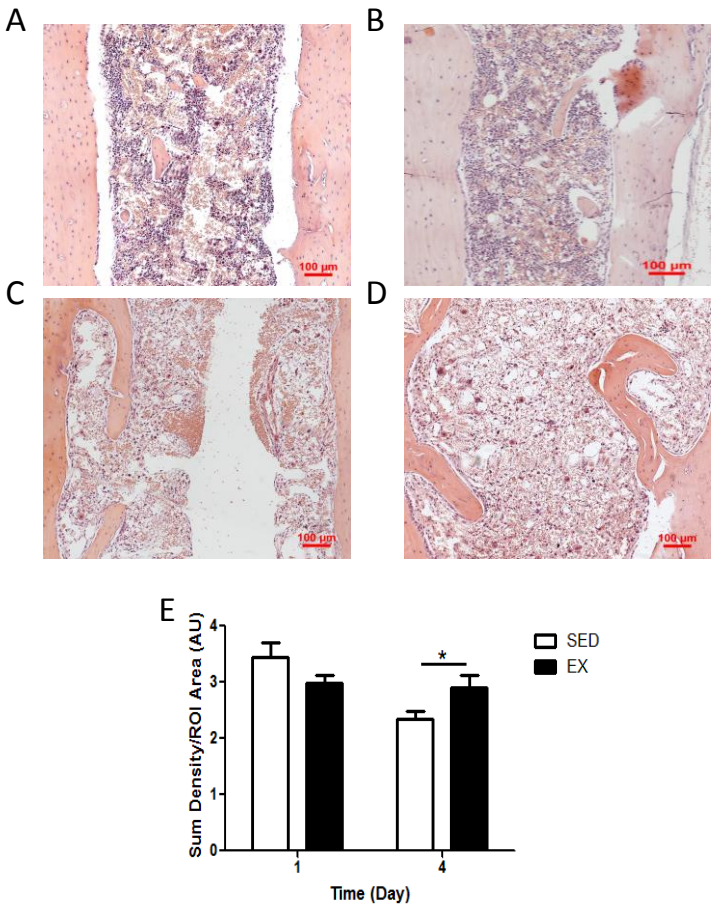
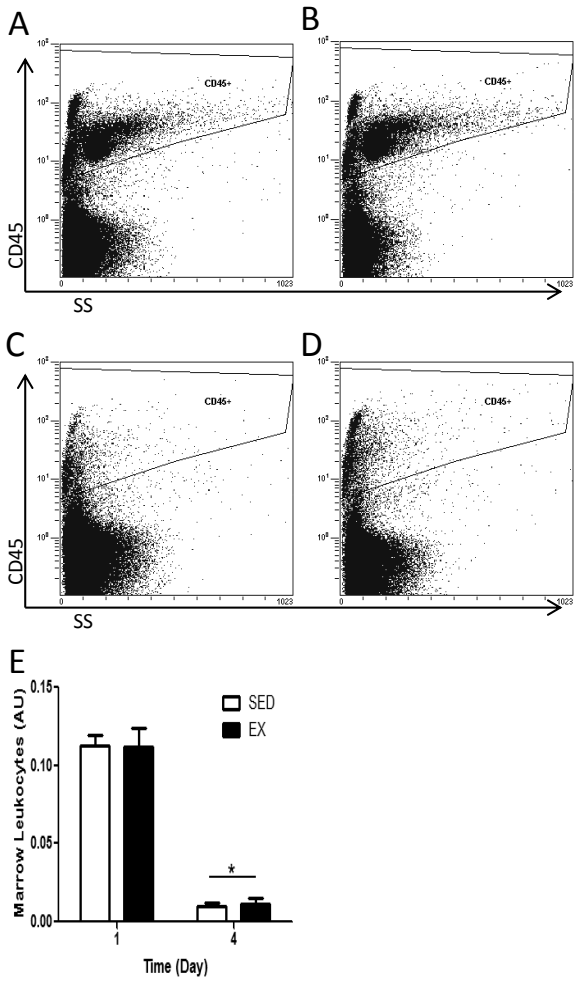


Figure 5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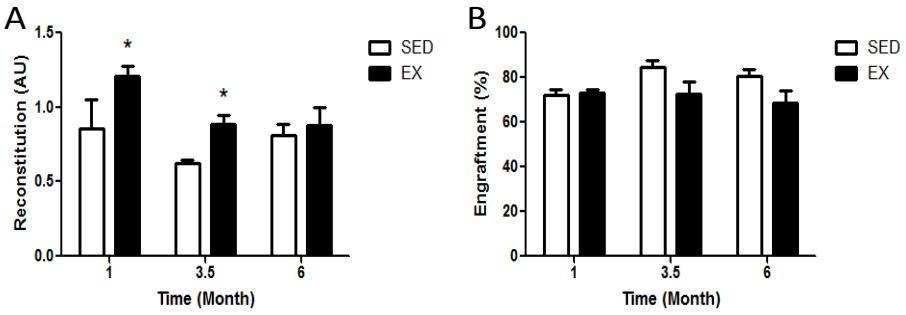
Time (Day)

Figure 6


A
Reconstitution (AU)
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1.0
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0.0
*
*
1
3.5
6
Time (Month)
SED
EX
B
Engraftment (%)
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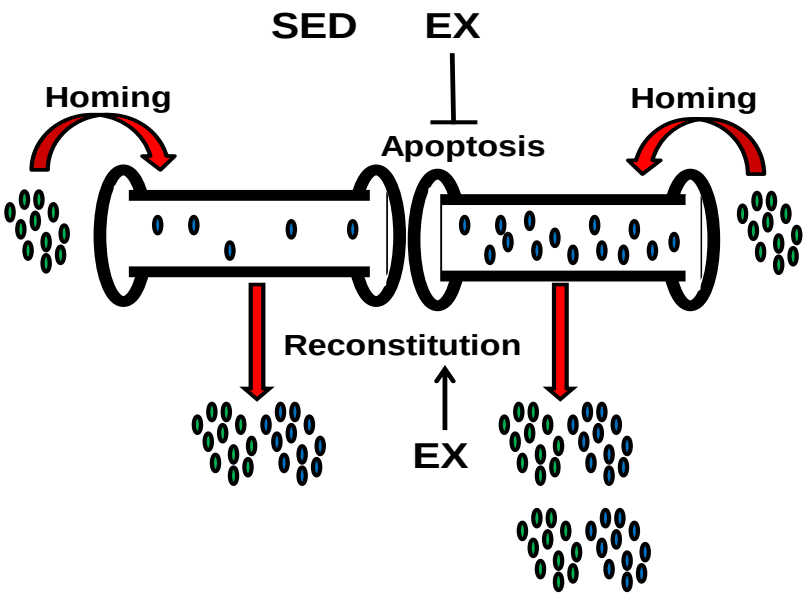
80
60
40
20
0
1
3.5
6
Time (Month)
SED
EX

Figure 7



Supplemental Figure Legends

Figure S1. Negatives for Bone Marrow Homing Analysis. Representative flow cytometry gates for homing analysis were based on unstained bone marrow from unmanipulated mice to determine CD45 gates (A) and from unmanipulated wild-type (non-GFP) mice to determine GFP gates (B).

Figure S2. Negatives for FLICA Analysis. Representative flow cytometry gates for FLICA analysis was based on unstained bone marrow from unmanipulated mice.

Figure S3. Gating Strategy for Reconstitution and Engraftment Analysis. Representative flow cytometry histograms depicting the analysis of total leukocyte reconstitution and donor-derived leukocyte reconstitution. Live ($7AAD^-$) cells were selected for (A), then $CD45^+$ cells from the live gate were analyzed for total reconstitution (B). GFP^+ cells from the $CD45^+$ gate were analyzed for donor-derived engraftment (D). A representative histogram from an unmanipulated wild-type (non-GFP) mouse is shown in (C).

Figure S4. GFP Standard Curve. A representative GFP standard curve is shown. GFP standard curves were established each analysis day using blood from unmanipulated wild-type (non-GFP) and GFP mice.

Figure S1

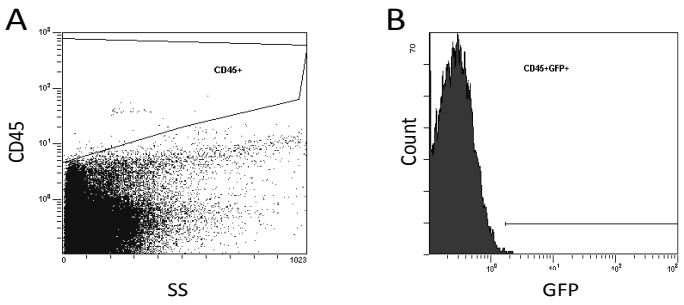


Figure S2

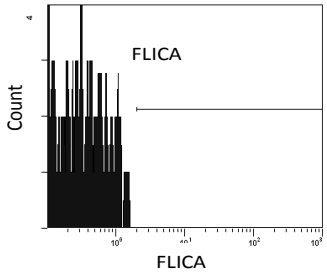
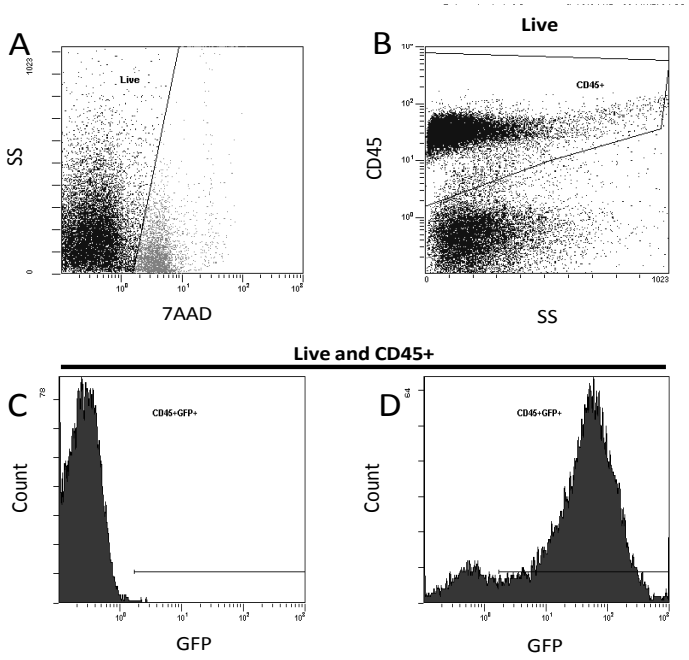


Figure S3


A
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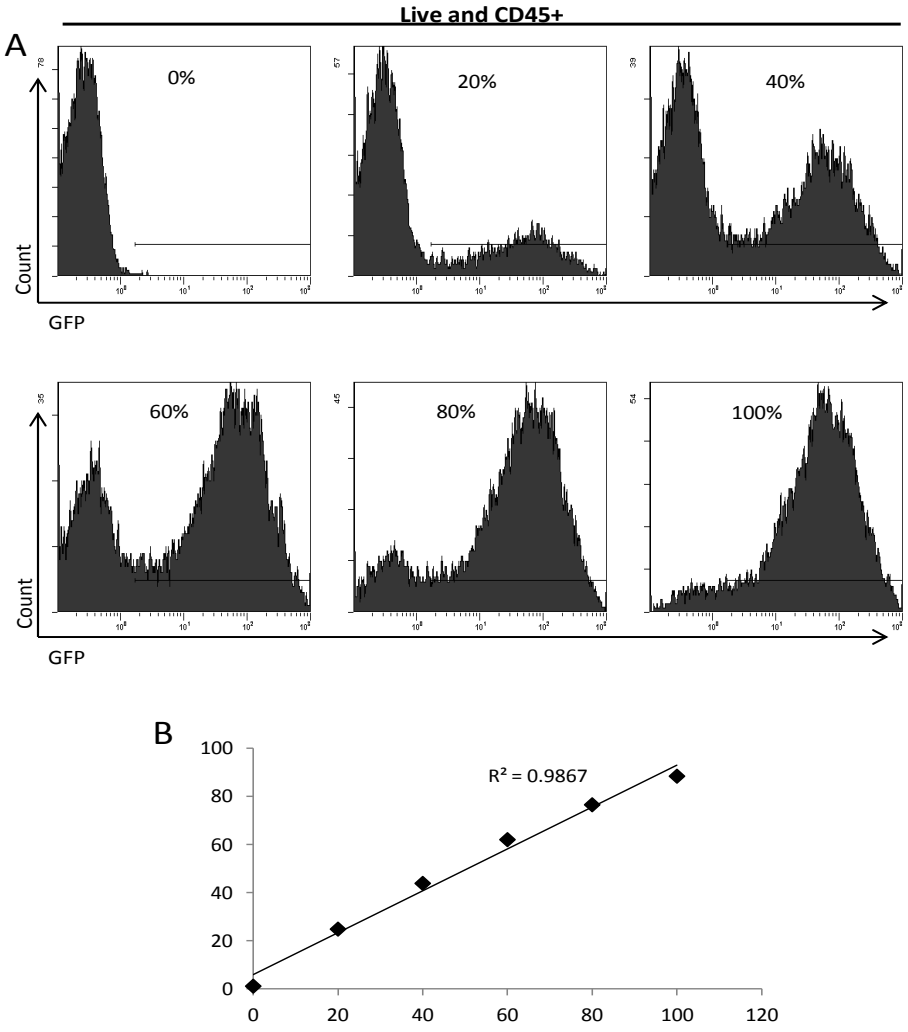
CD45+GFP+
Count
GFP

Figure S4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R² = 0.9867
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120

Chapter 6: General Discussion

6.1 INTRODUCTION

The overall aim of the present thesis was to determine if exercise training could protect against radiation damage and improve outcomes in bone marrow transplantation (BMT). We began by examining the effects of radiation in skeletal muscle and bone marrow of exercise-trained mice. Having shown an enhanced response to irradiation in skeletal muscle [1] (Chapter 2) and an attenuation of radiation-induced damage in bone marrow cells [2] (Chapter 3), we turned our attention to the most primitive hematopoietic cells located in the bone marrow, the hematopoietic stem cells (HSC). We demonstrated that exercise training increased a more mature, proliferative population of HSC and did not decrease overall HSC function (Chapter 4). To functionally characterize HSC we employed the BMT assay commonly used in HSC biology but never previously applied in exercise physiology. This assay has direct clinical relevance to BMT outcomes and allowed us to examine the effects of preconditioning donors and recipients with exercise prior to BMT. We did not demonstrate any improvement in BMT success when donors were exercise-trained (Chapter 4); however, when we examined the effects of exercise training recipients prior to BMT we observed increases in survival and hematopoietic recovery (Chapter 5). The following section will discuss the significance of the studies conducted in the present thesis both from a methodological and clinical perspective, summarize and integrate

data from the studies while discussing their limitations and offering suggestions for future experiments.

6.2 EXERCISE TRAINING: A RADIOPROTECTANT

6.2.1 Significance of Studies

Previous studies have demonstrated that exercise training inhibits damage caused by oxidative stress induced by intense exercise [3–5] and ischemia-reperfusion injury [6]. In both of these models, the source of reactive oxygen species (ROS) production is endogenous to the organism. These models may be problematic, especially when applied to exercise training, as adaptations to exercise may prevent the production of equivalent levels of oxidative stress in sedentary or exercise-trained mice. For example, exercise training has been shown to improve the efficiency of mitochondria [7] and increase capilarization of skeletal muscle [8]; therefore, induction of oxidative stress by intense exercise or reperfusion injury will likely not create the same level of ROS stimulus due to adaptations in the exercise group with training. As a result, it is difficult to conclude whether the results are due to alterations in ROS production or in the response to ROS. Another disadvantage of previous models is that the response to ROS cannot be deemed specific. For example, damage induced by intense exercise will increase ROS production in the muscle but it will also cause metabolic and mechanical stress in skeletal muscle which will induce its own set of responses.

In Chapter 2 we improve upon these established methods by employing radiation as a novel model of inducing oxidative stress in skeletal muscle [1]. Radiation-induced oxidative stress is advantageous to previous models because all mice can be treated with an equivalent ROS-inducing stimulus that is independent of any adaptations that may have occurred with exercise. This approach enabled us to determine that the effects of exercise were in response to ROS, and not in the production of ROS. Another advantage of our radiation model is that radiation is a "clean" stressor in that the cellular effects of radiation are primarily mediated via the production of ROS [9,10]. This allowed us to study the response to ROS specifically without any mechanical or metabolic changes. Although not examined in the present thesis, the purely ROS-mediate effects of radiation could be applied to examining the mechanisms responsible for the adaptive response to exercise. Using radiation, one could isolate adaptations to ROS independent of the mechanical and metabolic component of exercise. Together, the application of radiation to the study of exercise physiology could lead to a deeper understanding of the ROS-mediated effects of exercise.

From a clinical perspective, minimizing radiation-induced damage is an important issue as the use of imaging modalities (common source of radiation) for disease diagnosis has recently increased dramatically [11] and will continue to increase as our population ages [12]. Additionally, radiation therapy for cancer treatment is employed in fractionated doses similar to those used in the studies in Chapters 2 and 3 [13]. Non-specific effects of radiation therapy can be

deleterious to tissues adjacent to the tumor, such as skeletal muscle [13,14], and healthy cells particularly susceptible to radiation, such as bone marrow progenitor cells [15]. In Chapters 2 and 3 we examined the response of skeletal muscle and bone marrow, to radiation with the aim of developing exercise-based interventions to prevent the negative consequences of radiation exposure.

6.2.2 Potential Mechanisms of Protection

In Chapter 2, we demonstrated that adaptations to exercise training were primarily localized to mitochondrial enzymes. Specifically, we observed significant increases in the mitochondrial isoform of superoxide dismutase, manganese superoxide dismutase (MnSOD; 51%) as well as the mitochondrial metabolic enzymes, citrate synthase (CS) and cytochrome c oxidase (COX) (42% and 38% respectively) [1] (Chapter 2) only in exercise-trained muscle exposed to radiation. Mitochondria are a major source of ROS production in muscle [16], and are also highly sensitive to radiation treatment, especially the mitochondrial membranes [17]. Mitochondrial membranes are important for cellular function in that they are the location of the electron transport chain (ETC) involved in oxidative metabolism [18], and they regulate the release of proteins involved in the apoptosis cascade [19]. Therefore, increased MnSOD activity following radiation in exercise-trained mice may help prevent mitochondrial membrane damage induced by ROS which may prevent ETC dysfunction and prevent initiation of apoptosis by preventing leakage of cytochrome c from the mitochondrial matrix. In support of this notion, specific SOD2 over expression in

mitochondria of HeLa cells increased their survival and decreased levels of DNA damage relative to controls when exposed to radiation [20]. It is intriguing to speculate that a similar mechanism is occurring with exercise training in skeletal muscle, and the observed increase in the activity of COX, the final complex in the ETC, suggests that this may have been occurring in our mice. Therefore, increased ROS scavenging by MnSOD following radiation may prevent apoptosis and damage to vital organelles having an overall protective effect.

The mechanisms responsible for the increased mitochondrial enzyme activity remain unknown but should be a focus of future studies. Exercise training is associated with an increased mitochondrial density in skeletal muscle [21] mediated by increased nuclear localization of PGC-1α [22,23]. Additionally, exercise training resulted in increased PGC-1α mRNA expression and activation [24]. Furthermore, photo-stimulation of cells has been shown to promote mitochondrial biogenesis [25] and increase mitochondrial electron transport chain component activity [26]. Therefore, increased PGC-1α activation by exercise training may prime cells for mitochondrial biogenesis induced by radiation so that mitochondrial number could increase quickly following exposure. Another possible explanation for the increased mitochondrial enzyme activity is via post-translational modification. Indeed, phosphorylation of MnSOD, CS, COX has been demonstrated in porcine heart [27]. However, given the timing of evaluation (2 days post-radiation), it is likely that the results observed are due to increased protein or mitochondrial content as phosphorylation and dephosphorylation

events tend to occur rapidly to maintain tight regulation of intracellular processes. Furthermore, acute exercise studies show rapid up regulation of mitochondrial mRNA [22,28] with increases in enzyme activities not occurring until days later [29]. Taken together, these data suggest that the adaptations observed in exercise-trained mice exposed to radiation are due to increased mitochondrial protein content and not due to post-translational modifications induced by oxidative stress. Future studies should examine the mechanisms responsible for the synergistic effects of exercise and radiation on skeletal muscle mitochondria.

An important question that remains unresolved is whether the protective effects of exercise training on the bone marrow are due to an enhanced response of antioxidant enzymes to radiation, similar to that observed in skeletal muscle? In Chapter 3 we demonstrated that a single exercise bout acutely sensitized bone marrow cells to radiation as evidenced by increase DNA damage and apoptosis [2]. These data suggest that similar to the acute effects of exercise in skeletal muscle [3–5], acute exercise also induced oxidative stress in bone marrow cells. Therefore, the proposed model of exercise hormesis demonstrated in skeletal muscle and the brain [30–32] may also apply to bone marrow. Indeed, previous studies have demonstrated that exercise training increased antioxidant enzyme activity in mature blood cells [33], as well as circulating hematopoietic/endothelial stem/progenitor cells [34], and was associated with lower levels of reactive oxygen and nitrogen species [34]. These effects; however, have not been extended to progenitor cells in the bone marrow. We speculate that similar to

skeletal muscle, the acute oxidative stress induced by each exercise bout in a training program induces an up-regulation of cellular protective mechanisms (i.e., antioxidant enzymes) in bone marrow cells resulting in an adaptive response that diminishes damage induced by future oxidative insults (Figure 1).

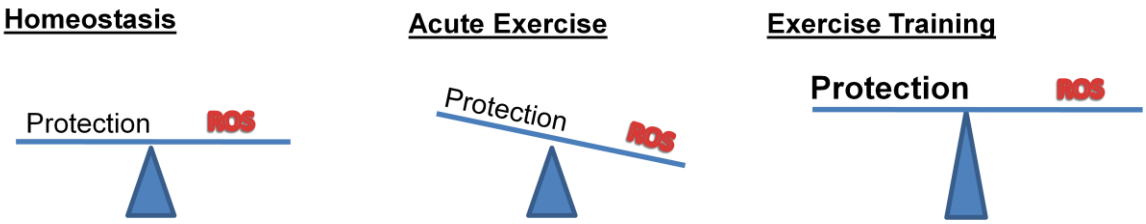

Figure 1. Exercise Hormesis. Under basal conditions, oxidative homeostasis is maintained with cellular protective mechanisms balancing both endogenous and exogenous ROS production. During an acute exercise bout, endogenous mitochondrial ROS production is increased leading to cellular oxidative stress that sensitizes cells to oxidative damage in the short-term but leads to an adaptive response that increases inherent levels of protection with training. These protective adaptations result in an enhanced response to (Chapter 2) or increased protection from (Chapter 3) a severe oxidative stress in the form of a high dose of radiation.

6.2.3 Implications and Limitations

In Chapter 3, we demonstrated that exercise training decreased apoptosis, and attenuated DNA damage in response to radiation [2]. From these data we concluded that exercise training may be an effective means of protecting radio-sensitive tissues, such as bone marrow cells, from radiation induced damage. An important question for future study will be to determine if exercise training induces similar protective adaptations in unwanted cells (i.e. cancer cells) that may also protect them from treatment. Epidemiological evidence suggests that physical activity levels are inversely related to cancer relapse [35], and cancer

society's worldwide promote exercise as both a preventative intervention and a recovery intervention [36]. These recommendations suggest that exercise is beneficial in cancer patients. Mechanistically, most types of cancer cells exhibit increased oxidative stress, despite elevated levels of some antioxidant systems, and cancers cells are increasingly susceptible to oxidative stress as compared to non-malignant cells [37]. It is possible that the antioxidant defense mechanisms in cancer cells may be at maximal capacity to combat the constantly elevated levels of ROS thus minimizing their ability to adapt with exercise. If this is true, then enhanced protective effects of exercise in non-malignant cells may attenuate the non-specific effects of cancer treatment while maintaining its efficacy in removing malignant cells.

The response of different tissues to oxidative stress is variable with oxidative damage manifesting at time points that are tissue- and dose-dependent. This was evidenced by results from Chapter 2 and 3 where markers of oxidative damage were not elevated in skeletal muscle but were elevated in bone marrow following radiation exposure. The radiation doses for the present studies were determined based on previous literature where a similar dose was established to induce oxidative damage in a variety of tissues [38,39]. A limitation of the studies in both Chapter 2 and 3, which likely contributed to the lack of observable difference in markers of oxidative damage in Chapter 2, was that only one time point following radiation treatment was evaluated for each indicator of damage. Previous studies examining the effects of radiation exposure in the bone marrow

informed our decisions for the timing of these analyses. It has previously been shown that in bone marrow cells exposed to radiation, γH2AX foci peaks at 30 minutes following exposure [40], apoptosis peaks 8 hours following exposure [41], and micronucleated reticulocyte levels peak two days following exposure [42]. No previous studies had examined the effects of radiation treatment on skeletal muscle *in vivo*. Since our primary interest in this study (Chapter 2) was examining the antioxidant enzyme response to exercise and radiation we based our time point for analysis on previous literature exploring the effects of antioxidant enzymes in skeletal muscle to an acute oxidative stress. Previous studies have demonstrated that increased antioxidant protein content, and not post-translational modifications are primarily responsible for increased antioxidant activity in response to oxidative stress [3], and that antioxidant protein content was elevated 1-3 days following a 1 Gy exposure in various tissues [43]. Based on these data, we selected 2 days post-irradiation as our time point for analysis. Indeed, future studies should be undertaken to evaluate the time course of radiation-induced damage in skeletal muscle, and if exercise training prior to radiation exposure alters this time course.

6.3 EXERCISE TRAINING AND HSC

6.3.1 Cell Intrinsic vs. Niche Effects

Having established an enhanced antioxidant response in skeletal muscle from exercise-trained animals exposed to radiation (Chapter 2), and an attenuation of radiation-induced damage in bone marrow cells from exercise-

trained mice (Chapter 3) we were interested in determining the effects of exercise training on the most primitive cells of the hematopoietic system, the HSC. We used multicolour immunophenotyping via flow cytometry to quantify HSC and characterize bone marrow cells directly from their *in vivo* environment. We then extended these findings by evaluating various functional characteristics of HSC via the BMT assay. The BMT assay is ideal for studying the function of HSC *in vivo* as successful BMT is dependent upon: (i) adequate HSC quantity in the donor graft, (ii) donor HSC homing to the recipient's bone marrow niche, (iii) survival and engraftment of HSC within the recipient's bone marrow niche, (iv) proliferation with self-renewal to regenerate the HSC compartment and reconstitution of all blood cell lineages in the recipient [44]. Given the importance of the niche for HSC regulation [45], conditions in the niche likely also contribute to BMT success. The experiments conducted in Chapters 4 and 5 allowed us to directly characterize the effects of exercise training on each of these important factors related to BMT success.

The studies outlined herein are the first to evaluate the quantity and function of HSC in their natural environment *in vivo*, in response to exercise training. Furthermore, we quantified HSC in two distinct bone marrow niches, the endosteal and vascular niche, home to populations of HSC with different characteristics. To date, the majority of exercise studies have quantified HSC in circulation removed from their important regulatory cues within the bone marrow [46–51]. Recent work from our lab evaluated hematopoietic stem and progenitor

cell quantity using various colony forming unit assays *in vitro* [52]. *In vitro* models will allow for specific study of cell intrinsic effects of exercise but will not account for the complexities of the microenvironment *in vivo*. Quantifying HSC directly from their various bone marrow niches allowed us to understand the effects of exercise directly on HSC quantity while maintaining all interactions with their physiological niches. We determined that exercise training significantly increased the quantity of HSC specifically in the vascular niche by 20% (Chapter 4) with no effects on HSC quantity in the endosteal niche. The vascular niche is home to a less primitive, more proliferative population of HSC and is associated with increased differentiation, especially along the myeloid lineage [53]. Therefore, the increased HSC quantity in the vascular niche was confirmed by significant increases in cycling whole marrow cells and spleen colony forming capacity (Chapter 4). We also observed strong trends for increased differentiation along the myeloid lineage (Chapter 4). Exercise training did not alter the quantity of HSC in the endosteal niche, nor was there a shift in the ratio of vascular to endosteal HSC with exercise. These data are confirmed by the observed maintenance in donor-derived hematopoietic reconstitution with exercise-trained marrow in the long-term engraftment (out to six months) and secondary transplantation assays. HSC from the endosteal niche have greater long-term repopulating potential than do HSC from the vascular niche [54]; therefore, any changes in the endosteal HSC population would have manifested in these assays. From these data we concluded that exercise training increases a

specific population of more differentiated HSC with higher proliferative potential, perhaps not vital to BMT success. This conclusion is in agreement with studies from other groups demonstrating that exercise increased the quantity of circulating HSC but not the most primitive HSC population [46,47].

In Chapter 4, we speculated that the mechanism responsible for increased HSC quantity with exercise training was through alterations in the serum cytokine milieu. Although we observed a significant decrease in IL-6 and a trend for a decrease in G-CSF, hematopoietic growth factors involved in self-renewal, proliferation and mobilization of HSC [55,56], we speculated that these basal decreases were an adaptation to the pulsed increases known to occur in these factor in response to acute exercise [46,49,57]. Regulation of HSC quantity with exercise training by paracrine factors would also explain the lack of effect on HSC in the endosteal niche. The endosteal niche is poorly vascularised with minimal blood supply and oxygen content [58]. This is believed to be a protective mechanism for long-term HSC (LT-HSC) preventing damage from exposure to inflammatory cytokines and ROS [58]. This protective mechanism aimed towards preserving HSC, may also prevent exercise-mediated signals from reaching the most primitive HSC population.

Expansion of niche capacity, or lack thereof, may also explain the effects of exercise training on HSC quantity. HSC quantity is directly related to niche capacity as increased osteoblast quantity, the primary cells of the endosteal niche [59], resulted in a parallel increase in HSC quantity [59,60]. It has been

shown that exercise increases osteoblasts activity [61], while immobilization prevents it [62], and exercise promotes the differentiation of MSC along the osteogenic lineage while restricting adipogenic differentiation [52]. Conversely, other studies have demonstrated that mechanical stress applied to bones, similar to the forces experienced by bone during running, only induces bone remodeling in specific areas where the bone is most stressed [63]. Together, these data suggest that exercise training may only affect osteoblasts in a specific and isolated region of the marrow compartment. Therefore, only a small percentage of osteoblasts in the endosteal niche may be responsive to exercise having minimal effects on the overall HSC population in that niche. This mechanism may explain the lack of effects observed in HSC in the endosteal niche (Chapter 4). Conversely, the effects of exercise in the bone marrow vascular niche may be more pronounced accounting for the increased quantity in HSC from this niche with exercise training (Chapter 4). Exercise training increases blood flow in the bone marrow in dogs [64]. The mechanisms responsible for increased blood flow in the marrow with exercise training were not determined [64]; however, it would be interesting to examine if the increased blood flow was due to increased marrow vasculature. If this is the case, then more vascular niche spots would be created with exercise training allowing for a concomitant increase in HSC quantity. Future work will be necessary to confirm these speculations by perhaps co-localizing specific areas of new bone and capillary formation and HSC quantity in response to exercise training.

Although quantification of HSC is informative, true characterization of HSC must be conducted via the BMT assay. HSC were first defined by their function as cells that can regenerate the hematopoietic system of myeloablated recipients using the BMT assay [65]. Using the BMT assay we were able to separate the cell intrinsic effects of exercise training from niche. In exercise-trained donors, HSC were removed from their exercise-conditioned environment and placed in a new, unconditioned environment, allowing specific evaluation of cellular adaptations to exercise. The function of exercise-conditioned HSC was not improved relative to non-conditioned HSC from sedentary mice (Chapter 4). To facilitate donor cell engraftment and evaluate donor cell hematopoietic repopulating activity in the BMT assay, recipients are often preconditioned with radiation or chemotherapeutic agents to ablate the recipients' hematopoietic system. A limitation of these myeloablative regimens is that they are not specific to hematopoietic cells but also negatively affect supporting cells in their environment. For example, radiation destroys the HSC vascular niche [66,67], and induces prolonged oxidative stress in the bone marrow [68]. Our BMT model, like many others [69] employed a lethal dose of radiation as a myeloablative strategy, which is known to exhibit increased and prolonged oxidative stress in the bone marrow [68]. Perhaps signals received by HSC once removed from their exercised environment and placed in a novel, damaged environment, override any cell intrinsic adaptations in HSC function associated with exercise.

Since exercise appeared not to impart cell intrinsic benefits leading to improved HSC function we chose to examine whether preconditioning of the niche could lead to improved outcomes following BMT. To do this we reversed our exercise condition, preconditioning recipients with exercise prior to BMT. When recipient hematopoietic cells were ablated in the BMT assay, only exercise-induced alterations in the niche remained. We were confident that this approach may be beneficial as exercise is pleiotropic and may influence multiple aspects of HSC-niche interaction. For example, recent data from our lab indicated that exercise training enhanced hematopoietic cytokine production in skeletal muscle, decreased adiposity in the bone marrow cavity and enhanced the differentiation of bone marrow derived mesenchymal stem cells along the osteogenic as opposed to the adipogenic lineage [52]. Additionally, we previously demonstrated the protective effects of exercise on total bone marrow cells [2] (Chapter 3) and altered hematopoietic cytokine levels with training (Chapter 4). Together, these data suggest that exercise training induces adaptations in the niche that promote hematopoiesis and hematopoietic cell survival.

Directly assessing HSC-niche interactions are difficult for a number of reasons. First, HSC identification requires the use of multiple markers making the evaluation of HSC and the niche, *in situ*, extremely difficult. Second, the marrow environment is complex and is composed of a myriad of cell types. These cell types include osteoblasts, endothelial cells, mesenchymal stem cells

(MSC), hematopoietic cells and adipocytes, which regulate HSC function via paracrine mechanisms or via direct cell to cell interactions [45]. Therefore, we chose to evaluate the niche indirectly by exercise training recipients prior to BMT and evaluating survival and engraftment as surrogate markers of niche health (Chapter 5). Since both sedentary and exercise-trained recipients received the same donor cell source, differences could be directly attributed to alterations in the niche with exercise. In Chapter 5 we demonstrated that exercise training recipients prior to BMT significantly increased their likelihood of survival by approximately 3 fold. The mechanisms responsible for the effects on survival were decreased pro-inflammatory cytokine levels with exercise and an acute inhibition of native bone marrow cell loss by apoptosis. These effects of exercise immediately following BMT resulted in increased hematopoietic regeneration in exercise-trained recipients one and three and a half months post-BMT. These data are in agreement with previous studies that demonstrated an attenuation [70,71] and decreased time [72] of hematopoietic cell loss in patients who exercised prior to BMT. The effects of exercise training neither improved nor inhibited donor-derived hematopoietic regeneration suggesting that acute survival of recipient hematopoietic cells promotes recipient survival in the short-term following BMT but does not impair successful regeneration of their hematopoietic system by donor cells. We conclude that the beneficial effects of exercise are primarily mediated by alterations in the HSC niche.

6.3.2 Integrating the Effects of Exercise Training on the Bone Marrow

The effects of exercise training on cells in the bone marrow compartment, including HSC are summarized in Figure 2. Taken together, the studies presented in this thesis demonstrate an attenuation of the effects of radiation in the bone marrow (Chapter 3 and 5) as well as HSC expansion with exercise training (Chapter 4). These adaptations in bone marrow cells with exercise training are mediated by the acute stress of each exercise session (Chapter 3) which may result in up-regulation of antioxidant defense mechanisms similar to those observed in skeletal muscle (Chapter 2). In BMT, the radioprotective effects of exercise contribute to survival of recipient cells in the bone marrow that promote hematopoietic reconstitution (Chapter 5). The anti-inflammatory effects of exercise training in the serum cytokine milieu (Chapter 5) potentially contributed to these protective effects; however, future studies should also include measurements of cytokine levels acutely post-radiation in exercise-trained versus sedentary mice. These acute protective effects contributed to the enhanced likelihood of survival in exercise-trained mice following BMT (Chapter 5). Although blood reconstitution was enhanced at the earliest time points, donor-derived engraftment was not (Chapter 5). Collectively, we demonstrated that exercise training increased HSC quantity (Chapter 4) as well as protected HSC from radiation (Chapter 3 and Chapter 5).

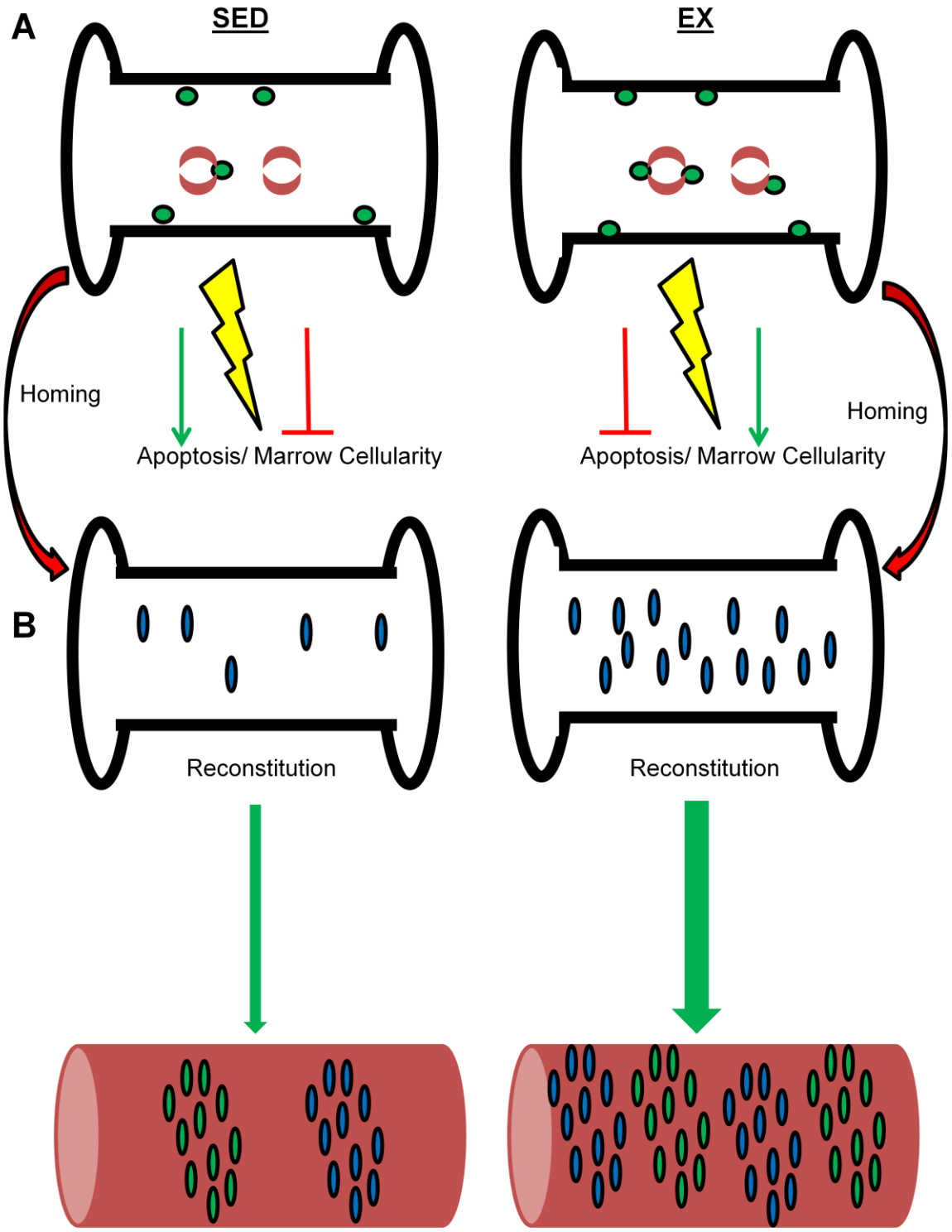

Figure 2. Theoretical Model of the Effects of Exercise Training on Hematopoietic Stem Cells and their Niche. (A) Exercise training increases

HSC quantity in the vascular niche with no decrease in HSC quantity in the endosteal niche, and increases the percentage of whole bone marrow cells undergoing mitosis. With exercise preconditioning prior to transplantation, no enhancement was observed in any parameters of HSC function such as homing, donor-derived engraftment or recipient reconstitution (Chapter 4). (B) Preconditioning BMT recipients with exercise enhanced their probability of survival by decreasing levels of apoptosis and maintaining bone marrow cellularity. These protective effects on native marrow cells resulted in enhanced recipient reconstitution at early time points following BMT with donor-derived engraftment not differing early but continuing to increase with time post-BMT (Chapter 5). Red lines indicate inhibition or decrease, green arrow indicate increase or maintenance, yellow lightning bolt represents irradiation, blue circles are recipient marrow/hematopoietic cells, green circles are donor marrow/hematopoietic cells.

6.3.3 Limitations and Future Studies

As outlined extensively in Chapter 1, the hematopoietic system is complex consisting of a variety of cells at various levels of maturity and stages of differentiation. In the present work, we were unable to identify specific cell types affected by exercise. For quantification, HSC were identified via the LSK panel (Chapter 4). The LSK population can only be considered as enriched for HSC as it is itself heterogeneous containing multipotent progenitors (MPP), and both short-term (ST-) and long-term (LT-) HSC [73]. To specifically quantify and identify the different cell populations within the LSK fraction of the bone marrow, sophisticated multilabel-immunophenotyping techniques are required. For example, dormant HSC (LSK $CD34^-$ $CD48^-$ $CD150^+$ $CD135^-$) with long-term repopulating potential can be differentiated from activated HSC (LSK $CD34^+$ $CD48^-$ $CD150^+$ $CD135^-$) and multi-potent progenitors (LSK $CD34^+$ $CD48^+$ $CD150^-$ $CD135^+$) [74]. Furthermore, we were unable to directly determine the cell cycle

status of HSC, or the exact populations homing to the bone marrow (Chapter 4). Technical limitations of our flow cytometer, or cell quantity limitations associated with transplantation prevented us from conducting such an analysis.

Our approach to circumvent these issues was to analyze marrow harvested from two different locations within the bone marrow cavity, and to evaluate donor-derived engraftment in recipients at multiple time points post-BMT. It has been suggested that progression from LT-HSC to MPP is regulated, at least in part, by the HSC niche with a higher proportion of LT-HSC found in the endosteal niche [54]. Therefore, by quantifying HSC in both niches we were able to more specifically determine if exercise training was affecting the HSC population enriched for LT-HSC in the endosteal niche, or the population enriched for more differentiated ST-HSC and MPP in the vascular niche. Furthermore, it has been established that different HSC populations are responsible for short-term engraftment (up to 3-4 months post-BMT) of recipients and long-term engraftment (6 months and secondary recipients) [73]. Therefore, by evaluating multiple time points post-BMT we were able to examine the effects of exercise on various sub-populations of HSC.

It will be important for future studies to better clarify the specific relationship between HSC and niche cells with exercise. These analyses are extremely difficult given the complexity of the HSC niche and the dynamic phenotype of cells in the niche. It would be interesting to determine the exact location of homing of HSC following exercise preconditioning. It has been

suggested that LT-HSC are maintained in the endosteal niche via interactions with N-cadherin osteoblasts [60]. Although overall homing to the marrow cavity was not altered with exercise training (Chapters 4 and 5), it would be interesting to determine if exercise promoted or impaired homing specifically to the endosteal niche (N-cadherin expressing osteoblasts) or vascular niche (CD31 positive endothelial cells). This analysis would determine if exercise training promoted HSC homing to the niche associated with the most primitive HSC (endosteal niche) or more differentiated HSC (vascular niche).

A limitation of Chapter 5 is that we were unable to determine the specific bone marrow cell population preserved in recipients by exercise that contributed to their increased likelihood of survival. The quantity of hematopoietic cells (identified by their positivity for the hematopoietic marker CD45) was not different between sedentary and exercise-trained recipients and decreased equally from day-one to day-four post-BMT in both groups (Chapter 5). These data suggest that maintenance of recipient hematopoietic cells with exercise training was not the mechanism for increased probability of survival. We considered three other cell populations in the marrow: endothelial cells, MSC and adipocytes that may have contributed to our findings. Preconditioning with radiation destroys the vascular niche by ablating the hematopoietic cells in the marrow that support the structure of the sinusoids [75] and also by inducing damage in sinusoidal endothelial cells [67]. Since the vascular niche is home to a more proliferative population of HSC [53], maintenance of the vascular niche with exercise

preconditioning may have contributed to the enhanced early reconstitution of hematopoietic cells in exercise-trained recipients. MSC support HSC growth and survival by paracrine mechanisms [76] and support regeneration of the hematopoietic system independent of transplanted HSC [77]. Therefore, maintenance of recipient MSC may be a second mechanism responsible for improved hematopoietic regeneration in exercise-trained recipients. Finally, adipocytes negatively regulate HSC [78,79] and populate the marrow following hematopoietic ablation by radiation [78]. We have previously shown that exercise that exercise training inhibits MSC differentiation into adipocytes [52]; therefore, a third mechanism responsible for the improved hematopoietic reconstitution in exercise-trained recipients may be through inhibition of the adipocyte population in the bone marrow cavity following BMT. Future studies should explore these mechanisms.

6.4 DONOR VS. RECIPIENT PRECONDITIONING AND BMT SUCCESS

The BMT assay not only allows evaluation of various HSC and niche characteristics, it also has direct clinical implications. Although BMT have commonly been used for decades to treat a number of diseases, survival outcomes are poor [80]. Developing interventions to improve BMT survival would have significant implications for public health. A number of factors have been identified that are related to recipient survival following BMT. Most of these are focused on the immune response between donor cells and recipient tissue [80]. Physiological characteristics of the donor also impact BMT success. For

example increasing donor age is associated with decreased recipient survival [81]. In cases where young donors cannot be found, it would be extremely beneficial if simple preconditioning programs, such as exercise, could be implemented to improve donor cell characteristics and increase BMT success. In Chapter 4, we observed nearly 100% survival of all mice given BMT, preventing us from seeing any potential improvements with exercise training. In these experiments, conditions were optimized for success such that recipients were given a large cell dose, both donors and recipients were young and healthy, and recipients were exposed to minimal stress. Perhaps the benefits of exercise in BMT would be realized in non-ideal conditions, such as with elderly donors. Future experiments should evaluate this hypothesis.

An alternative approach to improving BMT success has been to diminish the harmful effects of recipient preconditioning (myeloablation) prior to transplantation by using lower doses of radiation [82]. While this strategy increases recipient survival, donor-cell engraftment is impaired, decreasing the efficacy of this approach. In Chapter 5 overall survival rates were lower than in Chapter 4, and we were able to observe an approximately 3 fold increase in survival in exercise-trained recipients without any impairment in donor-derived engraftment. We are confident that the decreased overall survival rates observed in Chapter 5 were not due to technical errors during the transplantation process for a number of reasons. First, the transplants conducted in Chapter 5 were completed after the transplantation technique had been optimized in our lab and

after we had successfully completed a large quantity of transplants with very low mortality rates. Second, successful transplants were completed after those conducted for the survival data in Chapter 5 with no mortality. Third, every precaution was taken to eliminate any potential sources of bias. During the transplantation procedure cages of sedentary and exercise-trained recipients were alternated to account for any improvements during the course of the transplants on each day. Both sedentary and exercise-trained recipients were given donor cells from the same vial to ensure the source of donor cells was not different. Mice were deemed to be at end-point in consultation with animal facility staff that was blinded to the experimental conditions. Finally, the experiments were completed twice with increased survival in exercise-trained recipients in both sets of experiments.

We suspect that the increased mortality rates observed in Chapter 5 were related to stress associated with moving the mice three days prior to transplantation. This increased stress was inadvertent as moving the mice was mandated by the animal facility in order to maintain the mice in a sterile environment during the exercise training protocol prior to transplantation. In Chapter 4 recipient mice did not need to be moved and they were housed in the same room as the transplantation procedure for at least one week prior to BMT. In Chapter 5, recipients only spent three days in their new room prior to BMT to maintain consistency between Chapters 4 and 5 in the timing of BMT in relation to the final exercise session. BMT was conducted in both Chapters 4 and 5 three

days following the final exercise session in the training protocol based on the increased hematopoiesis and beneficial adaptations observed in cells of the HSC niche from our previous study [52]. In Chapter 5, both sedentary and exercise-trained recipients were housed, moved and handled in exactly the same manner. This included placing sedentary mice on the treadmill without running during each exercise session; therefore, the increased survival in exercise-trained mice cannot be attributed to an increased resistance to stress due to increased handling. The experimental conditions in Chapter 5 are likely more translatable to clinical BMT as BMT is associated with a great deal of psychological stress [83]. Taken together, these data suggest that exercise training may improve BMT outcomes when conditions may not be optimal and future studies should explore this possibility.

A criticism of Chapter 5 may be that patients preparing for BMT may not be in any condition to exercise. The majority of studies examining the effects of exercise on BMT recovery have focused on exercise training patients' post-BMT. These studies have been conducted both in in-patient and out-patient settings and have demonstrated improvements in strength, endurance, fatigue levels, quality of life and overall happiness when exercise was used in rehabilitation [83]. The in-patient studies began the exercise strategy shortly following the transplantation procedure when patients were presumably at their weakest having undergone the myeloablative protocol and the transplantation procedure. Few studies have examined the role of exercise in the peri-operative phase of the

BMT. In these studies, patients began an exercise protocol when they checked into the hospital prior to the transplant and continued exercising throughout the transplantation process. Adherence was high in these subjects, and all studies report similar psychological and physiological improvements as those that examine exercise in the post-transplantation phase [83]. Only three studies evaluated hematological parameters when exercise training was conducted in the peri-operative phase of transplantations with all three demonstrating improvements in various indices of hematopoietic cell survival [71,72,84]. These data are generally supported by results from Chapter 5, and indicate decreased inflammation and inhibition of bone marrow cell apoptosis as a potential mechanism (Chapter 5). In general, these studies suggest that a well-designed, moderate intensity exercise program can be effectively implemented in patients preparing for BMT and may support recovery of hematological parameters.

6.5 CONCLUSION

The present thesis incorporated novel approaches such as radiation-induced oxidative stress and BMT to study the potential use of exercise training as a radioprotectant and an adjuvant therapy to BMT. The data presented herein has significantly contributed to the literature by demonstrating that adaptations to exercise training extend their protective effects to bone marrow and that exercise may enhance the systemic response to bone marrow transplant (BMT) which is primarily mediated by protective adaptations in the niche. These studies may lead to new investigations examining the underlying mechanisms of exercise-

induced benefits in bone marrow and the potential clinical relevance of exercise-training for populations requiring BMT.

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