Effects of Exercise on Myeloid-Derived Suppressor Cell-Related Tumor Progression and Metastasis

Jacob Garritson

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EFFECTS OF EXERCISE ON MYELOID-DERIVED SUPPRESSOR CELL-RELATED TUMOR PROGRESSION AND METASTASIS

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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Entitled: Effects of Exercise on Myeloid-Derived Suppressor Cell-Related Tumor Progression and Metastasis

has been approved as meeting the requirement for the Degree of Doctor of Philosophy in College of Natural and Health Sciences in School or Department of Sport and Exercise Science, Program of Exercise Physiology

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ABSTRACT


Myeloid-derived suppressor cells (MDSCs) are potent suppressors of immune function and may play a key role in the development and progression of metastatic cancers. Aerobic exercise has been shown to have anticancer effects, yet the mechanisms behind this protection are largely unknown. **Purpose:** to determine the effects of physical activity on MDSC accumulation and function. **Methods:** Female BALB/c mice were assigned to one of two primary groups: sedentary tumor (SED+TUM) or wheel run tumor (WR+TUM). After 6 weeks of voluntary wheel running, all animals were randomly subdivided into 4 different timepoint groups; 16, 20, 24, and 28 days post-tumor cell injection. All mice were inoculated with 4T1 mammary carcinoma cells in the mammary fat pad and WR groups continued to run for the specified time post-injection. Spleen, blood, and tumor samples were analyzed using flow cytometry to assess proportions of MDSCs (CD11b/Ly6G/Ly6C). MDSCs were purified from the spleen to assess T-cell suppressive capacity and metastatic lesions were quantified in the lung. **Results**

Compared to SED+TUM, levels of MDSCs in the spleen were significantly lower ($p < 0.05$) in WR+TUM at day 16 (33 ± 5%; 23 ± 10% of total cells, respectively) and day 20 (34 ± 8%; 24 ± 5% of total cells, respectively). Additionally, there were significantly fewer circulating MDSCs ($p < 0.05$) in WR+TUM at day 16 and there was a non-
significant ($p = 0.08$) trend toward lower MDSCs at day 28 in the tumors of WR+TUM. While this delay in MDSC accumulation did not translate to a decrease in tumor growth, physical activity led to 62% and 26% fewer metastatic lung nodules at days 24 and 28, respectively. At day 28, MDSCs harvested from SED+TUM significantly suppressed CD3$^+$CD4$^+$ T-cell proliferation (3.2 ± 1.3 proliferation index) while T-cell proliferation in WR+TUM MDSC co-cultures (5.1 ± 1.7 proliferation index) was not different from controls. **Conclusions:** These findings suggest that physical activity may delay the accumulation of immunosuppressive MDSCs providing a broader window of opportunity for early interventions with increasingly common immunotherapies.
ACKNOWLEDGEMENTS

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CHAPTER I
INTRODUCTION

Recent reports from the American Cancer Society project that more than 1.7 million new cancer cases will occur in 2019, and over 600,000 people will die from cancer this year. In general, incidence rates for all types of cancer have decreased from 2009-2015. This is potentially credited to a sharp decline in colorectal cancers (CRC) in men over the past 5 years as overall cancer incidence rates in women have remained relatively stable for the past few decades (Siegel, Miller, & Jemal, 2019). For example, female breast cancer incidence increased by 0.4% from 2004-2015, which has been partly attributed to the obesity epidemic (Cronin et al., 2018). Consequently, a significant number of people are living with cancer, which is the second leading cause of death in the United States (Heron & Anderson, 2016).

Epidemiological data support an association between physical activity and a decrease in cancer risk and mortality. This is important to consider due to the worldwide increase in physically inactive individuals. It is estimated that 31% of adults are physically inactive worldwide and this number continues to rise each year (Hallal et al., 2012). Fortunately, regular physical activity (PA) can reduce the risk for developing cancer. Specifically, meeting the current recommendation of 150 min/week of moderate-vigorous PA (MVPA) may result in a 12% reduction in the risk for developing breast cancer (Liu et al., 2016). Several mechanisms behind the protective effect of PA on
cancer risk and mortality have been proposed, including a reduction in sex hormones, insulin levels, and chronic low grade inflammation (Dethlefsen, Pedersen, & Hojman, 2017). Additionally, exercise has the potential to influence the balance between pro-inflammatory and anti-inflammatory signals, as well as the recruitment and function of myeloid and lymphoid lineage immune cells. This relationship is of particular interest, especially in the context of cancer and cancer therapy.

Myeloid-derived suppressor cells (MDSCs) have garnered significant interest among cancer researchers in recent years. As the name suggests, MDSCs are immune cells of myeloid origin with the ability to suppress both innate and adaptive immune responses. Also, MDSCs have been implicated as contributors in the development of metastatic disease. MDSC induction is a result of overproduction of tumor-produced growth factors and pro-inflammatory signals. For example, granulocyte-macrophage colony-stimulating factor (GM-CSF) has been shown to generate MDSCs with potent immune suppressive function (Morales, Kmieciak, Knutson, Bear, & Manjili, 2010; Serafini et al., 2004). Additionally, interleukin-6 (IL-6) and interleukin-1β (IL-1β) are two pro-inflammatory cytokines that are important for the accumulation and suppressive function of MDSCs (Bunt et al., 2007; Zhang et al., 2018). Chronic inflammation is one of the hallmarks of cancer and the resulting generation of immune suppressive MDSCs supports the hypothesis that inflammation drives tumor progression and metastasis by suppressing antitumor immune responses. MDSCs also play a role in the failure of modern immunotherapies. For instance, melanoma patients with low levels of circulating MDSCs had an improved response to ipilimumab treatment (Schilling et al., 2016).
Therefore, targeting MDSCs is a logical approach to improve the outcomes of patients receiving immunotherapy.

Exercise has been shown to have antitumor effects and one potential mechanism explaining this is a reduction in chronic low-grade inflammation, or perhaps even more important, is an acute increase in anti-inflammatory cytokines. IL-6 has been studied extensively and has traditionally been thought of as a pro-inflammatory cytokine. However, depending on the context, IL-6 may also stimulate anti-inflammatory signals. Specifically, increases in muscle-derived IL-6 during exercise leads to an increase in the anti-inflammatory cytokines interleukin 10 (IL-10) and interleukin 1 receptor antagonist (IL-1ra) (Steensberg et al., 2003). It has been suggested that chronic exercise may decrease systemic levels of pro-inflammatory mediators such as c-reactive protein (CRP), tumor necrosis factor α (TNF-α), and IL-6, but the reductions are modest at best (Shaw et al., 2015; Thomas et al., 2012). As such, a regular accumulation of acute bouts of aerobic exercise that result in consistent increases in systemic anti-inflammatory cytokines may be more important when it comes to the antitumor effects of exercise. Further, acute exercise can improve antitumor immunity by mobilizing highly cytotoxic natural killer (NK) cells to the tumor microenvironment in an epinephrine and IL-6 dependent manner (Pedersen et al., 2016). In order to facilitate antitumor immunity by cytotoxic immune cells, it will also be important to consider how exercise affects intratumoral immune suppression.

For cancer patients receiving immunotherapy, it is imperative to elucidate methods that may reduce MDSC-related immune suppression. To date, no studies have investigated the effects of exercise on the accumulation and function of MDSCs.
Considering recent increases in the use of immunotherapies and a growing population of individuals living with cancer, it is imperative to answer questions surrounding the role exercise may play in modulating MDSCs.

**Statement of Purpose**

The purpose of this study was threefold: (a) to examine the effects of endurance exercise training on the time course of MDSC accumulation in a mouse tumor-bearing model, (b) to examine the effects of exercise on the suppressive quality of MDSCs in a mouse tumor-bearing model, and (c) examine the effects of exercise on lung metastasis in a mouse tumor-bearing model.

**Research Hypotheses**

**H1**  Endurance exercise training will delay the onset of MDSC accumulation in the spleen, blood, and tumor of tumor-bearing mice.

**H2**  MDSCs harvested from exercised mice will have reduced suppressive quality.

**H3**  Endurance exercise training will decrease the number of metastatic lesions in the lungs of tumor-bearing mice.
<table>
<thead>
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<th>Abbreviation</th>
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<td>Akt</td>
<td>protein kinase B</td>
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<td>Arg1</td>
<td>arginase 1</td>
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<td>CRC</td>
<td>colorectal cancer</td>
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<td>CRP</td>
<td>c-reactive protein</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<td>EGF</td>
<td>epidermal growth factor</td>
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<td>EMT</td>
<td>epithelial mesenchymal transition</td>
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<td>FACS</td>
<td>fluorescence activated cell sorting</td>
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<td>GM-CSF</td>
<td>granulocyte-macrophage colony-stimulating factor</td>
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<tr>
<td>HGF</td>
<td>hepatocyte growth factor</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>hypoxia inducible factor-1α</td>
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<td>HMGB-1</td>
<td>high mobility group protein 1</td>
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<td>IL-10</td>
<td>interleukin 10</td>
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<tr>
<td>IL-1β</td>
<td>interleukin-1β</td>
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<td>IL-1R</td>
<td>IL-1 receptor</td>
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<td>IL-1ra</td>
<td>interleukin 1 receptor antagonist</td>
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<td>IL-6</td>
<td>interleukin-6</td>
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<td>MDSC</td>
<td>Myeloid-Derived Suppressor Cell</td>
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<td>MVPA</td>
<td>moderate-vigorous physical activity</td>
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<td>NK</td>
<td>natural killer cell</td>
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<td>NS</td>
<td>natural suppressor</td>
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<td>PA</td>
<td>physical activity</td>
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<td>TCR</td>
<td>T-cell receptor</td>
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<td>TGF-β</td>
<td>transforming growth factor β</td>
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<td>TME</td>
<td>tumor microenvironment</td>
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<td>TNF-α</td>
<td>tumor necrosis factor α</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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CHAPTER II

REVIEW OF LITERATURE

Recent reports from the American Cancer Society project that more than 1.7 million new cancer cases will occur in 2019 and over 600,000 people will die from cancer this year. Breast cancer incidence has remained relatively unchanged over the last decade and the obesity epidemic is one explanation for this observation (Cronin et al., 2018; Heron & Anderson, 2016). Significant epidemiological data suggest there is a relationship between physical activity and reductions in cancer risk and mortality. For example, meeting the recommended 150 min/week of MVPA may reduce the risk for developing breast cancer by as much as 12% suggesting that breast cancer can be preventable with a change in lifestyle (Liu et al., 2016). MDSCs are a population of immune cells that expand in cancer patients and promote tumor progression through the suppression of antitumor immunity. Exercise has been shown to slow the progression of solid tumors in various pre-clinical animal models (Betof et al., 2015; Goh, Endicott, & Ladiges, 2014; Pedersen et al., 2016). Additionally, exercise can modulate the tumor microenvironment in a manner that facilitates invasion of cytotoxic immune cells and cancer therapies (Betof et al., 2015; Pedersen et al., 2016), however, its effects on MDSC-related intratumoral immune suppression have not been investigated.
**Tumor Model**

The 4T1 mammary carcinoma is a transplantable tumor cell line that was originally isolated in 1978. Four morphologically and karyotypically distinct sublines were isolated from a spontaneously arising mammary tumor in BALB/c mice (Dexter et al., 1978). All four sublines were shown to be tumorigenic *in vivo* and the 4T1 cell line was chosen for its selective resistance to thioguanine (Aslakson & Miller, 1992). Human breast cancers frequently present with mutations to the p53 tumor suppressor gene (Bertheau et al., 2013). The p53 protein is a potent regulator of the cell cycle and its modification and/or deletion results in uncontrolled cell growth. 4T1 cells have been described as p53 null (Yang et al., 2017; Yerlikaya, Okur, & Ulukaya, 2012) and also have constitutively active protein kinase B (Akt) (Xue et al., 2012), another common mutation frequently observed in human breast cancer.

Regarding the experimental advantages of the 4T1 cell line, this cell line closely resembles the progression of human breast cancer as it is one of the few animal models of breast cancer that can spontaneously metastasize from the mammary fat pad (Aslakson & Miller, 1992; Pulaski & Ostrand-Rosenberg, 1998). The progression and metastatic pattern of 4T1 tumors closely resembles that of human breast cancer with a particular tropism for the lungs and liver (Pulaski & Ostrand-Rosenberg, 1998). Also, primary 4T1 tumors can be easily established in the mammary gland of female BALB/c mice. Further, primary tumors can be surgically removed in order to study metastasis in an animal model that is similar to clinical models where primary tumors have been surgically removed (Pulaski & Ostrand-Rosenberg, 2001). Thioguanine resistance allows for the quantification of metastatic nodules in distant organs, which has traditionally been
difficult experimentally. Organs from 4T1 tumor bearing mice can be harvested and cultured at specific time points, then treated with thioguanine, leaving only the metastatic drug resistant 4T1 colonies which can be fixed and quantified (Aslakson & Miller, 1992; Pulaski & Ostrand-Rosenberg, 1998; Pulaski & Ostrand-Rosenberg, 2001).

The 4T1 tumor model has been used extensively to study the response of triple negative breast cancers to chemotherapy (Dalezis et al., 2017; Hu et al., 2017; Liu et al., 2018) and immunotherapy (Du et al., 2017; Finke et al., 2011; Gebremeskel et al., 2017; Le et al., 2009; Ling et al., 2017; Liu et al., 2018; Noman et al., 2014). Moreover, 4T1 tumors are known to stimulate production of MDSCs, which has made it a popular pre-clinical model to study the role of intratumoral immune suppression on tumor progression and metastasis (Bunt, Sinha, Clements, Leips, & Ostrand-Rosenberg, 2006; Bunt et al., 2007; Finke et al., 2011; Noman et al., 2014; Zhang et al., 2018).

4T1 Tumors and Myeloid-Derived Suppressor Cells

4T1 tumors secrete many pro-inflammatory signals and chemokines that result in emergency myelopoiesis and the generation of MDSCs. In recent years the 4T1 tumor model has been one of the most common animal models used to study MDSC biology. Bunt and colleagues (2007) investigated the effects of chronic inflammation on MDSC accumulation and tumor progression (Bunt et al., 2007). 4T1 tumors were implanted into IL-1 receptor (IL-1R)-deficient mice, which have lower potential for inflammation, and IL-1ra-deficient mice, which have increased potential for inflammation. MDSC accumulation was significantly delayed in IL-1R-deficient mice which resulted in reduced tumor progression compared to IL-1ra-deficient mice. Additionally, IL-6 partially restored MDSCs and tumor progression in IL-1R-deficient mice. These data
suggest that chronic inflammatory signaling from IL-1β and IL-6 is one of the driving forces behind the accumulation of MDSCs.

Chronic inflammation, which is common among many cancers, has also been shown to promote survival of MDSCs. One study used proteomic analysis to identify proteins expressed in the highly inflammatory 4T1 tumor environment (Chornoguz et al., 2011). They found that the Fas pathway and caspase proteins were likely candidates that could explain resistance to apoptosis in MDSCs. Inflammation-induced MDSCs showed significantly lower levels of activated caspases following treatment with a Fas agonist suggesting that an inflammatory environment protects MDSCs from extrinsic apoptosis signals and results in increased half-life of MDSCs. Taken together, these results support the hypothesis that inflammation is a driving force for the accumulation and survival of MDSCs, which promote 4T1 tumor progression through the suppression of antitumor immunity.

**Myeloid-Derived Suppressor Cells**

MDSCs are most frequently described as a heterogeneous population of immature immune cells that suppress innate and adaptive immune responses. These cells were originally discovered in the 1970’s and described as natural suppressor (NS) cells (Bennett, Srinivasa Rao, & Mitchell, 1978). In mice, two subtypes of MDSCs have been identified; monocytic MDSCs (M-MDSC) and polymorphonuclear MDSCs (PMN-MDSC). M-MDSCs are defined as CD11b+Ly6G−Ly6C<sup>hi</sup> and PMN-MDSCs are defined as CD11b+Ly6G+Ly6C<sup>lo</sup> (Youn et al., 2008). The phenotypic profile of these cells was, and still is, contentious due to the heterogeneity of these cells and the common markers shared among other immune cell types such as neutrophils (Talmadge & Gabrilovich,
2013). Nonetheless, there has been increasing interest in immunosuppressive cells of myeloid origin due to their role in tumor progression. Due to the lack of well-defined nomenclature, Gabrilovich and colleagues (2007) suggested that these cells be called “myeloid-derived suppressor cells” to reflect their origin and function and facilitate further scientific discussion and progress (Gabrilovich et al., 2007). Although some still debate the phenotype of these cells, it is now accepted that suppressive cells of myeloid origin play a critical role in the progression of cancer.

**Myeloid-Derived Suppressor Cells: Generation and Accumulation**

Chronic inflammation is one of the hallmarks of cancer and it is now well established that tumor-produced inflammatory signals promote the accumulation of MDSCs with potent suppressive activity. For example, high levels of GM-CSF, which is a common chemokine expressed in 4T1 tumors (Finke et al., 2011), has been shown to stimulate production of MDSCs that inhibit antigen-specific T-cell responses (Morales et al., 2010; Serafini et al., 2004). Additionally, Sceneay and colleagues were able to demonstrate that bone marrow cells cultured with IL-6 and GM-CSF results in the generation of MDSCs that are phenotypically and functionally similar to MDSCs from tumor bearing mice (Sceneay et al., 2018). Furthermore, vascular endothelial growth factor (VEGF) expression is frequently increased in hypoxic tumor environments and VEGF has been shown to be a chemoattractant for MDSCs (Gabrilovich et al., 1996; Kusmartsev et al., 2014). Pre-clinical studies using mice have demonstrated that a variety of pro-inflammatory signals drive MDSCs accumulation.

IL-6 and IL-1β are two pro-inflammatory cytokines that have frequently been implicated in MDSC generation. MDSCs may not express the IL-1R, however,
overexpression of IL-1β in 4T1 tumors decreased survival time and elevated levels of MDSCs in tumor bearing mice (Bunt et al., 2006). This suggests there is a relationship between IL-1β and IL-6 signaling in the generation of MDSCs. For instance, administering IL-6 in IL-1R-deficient mice harboring 4T1 tumors restores MDSCs and hastens tumor progression (Bunt et al., 2007) suggesting that IL-6 is downstream of IL-1β. Additionally, TNF-α, another proinflammatory cytokine commonly found in the tumor microenvironment, also promotes the accumulation of MDSCs. Pharmacological inhibition of TNF-α has been shown to facilitate the differentiation of MDSCs into dendritic cells and macrophages (Sade-Feldman et al., 2013). Collectively, the results of these studies suggest that chronic inflammatory signals such as IL-1β and TNF-α may stimulate the release of IL-6 and GM-CSF from other cell types such as macrophages, which in turn promotes MDSC generation in the bone marrow.

**Myeloid-Derived Suppressor Cells: Mechanisms of Immune Suppression**

Multiple mechanisms are involved in immune suppression by MDSCs. The primary mechanisms that have been described involve the depletion of key nutrients involved in T-cell proliferation and the generation of oxidative stress that affects T-cell trafficking and activation. Overexpression and/or production of arginase 1 (Arg1) (Highfill et al., 2010), nitric oxide synthase (Arina & Bronte, 2015), hydrogen peroxide, superoxide (Corzo et al., 2009), and peroxynitrite (Nagaraj et al., 2007) are some of the key phenotypical and functional characteristics of MDSCs. The mechanisms listed above are most frequently studied in peripheral lymphoid organs, as there are technical
challenges with isolating MDSCs from tumors at a high enough frequency that allows for functional assessment (Kumar, Patel, Tcyganov, & Gabrilovich, 2016).

Arg1 catalyzes the following reaction: \( \text{L-arginine} + \text{H}_2\text{O} \rightarrow \text{ornithine} + \text{urea} \) and the overexpression of Arg1 in MDSCs leads to significant depletion of L-arginine (Atkins et al., 2009; Raber, Ochoa, & Rodríguez, 2012; Rodriguez et al., 2005). L-arginine is an amino acid used in the biosynthesis of proteins and has been shown to be important for T-cell proliferation and function. For example, Zea et al. demonstrated that T-cells activated \textit{in vitro} in the absence of L-arginine had decreased expression of CD3\(\zeta\), an important co-receptor in the T-cell receptor (TCR) complex that generates activation signals in T-cells (Zea et al., 2004). Additionally, T-cells cultured in the absence of L-arginine are arrested in the G0-G1 phase of the cell cycle, while cells cultured with L-arginine progress through to the M phase (Rodriguez, Quiceno, & Ochoa, 2007). Therefore, the overexpression of Arg1 in MDSCs and depletion of L-arginine presents a major barrier to T-cell proliferation and activation in tumor bearing individuals.

MDSCs generate a significant amount of oxidative stress through their production of reactive oxygen species (ROS). This is accomplished primarily by an increased expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase which catalyzes the production of a superoxide free radical by transferring an electron to oxygen from NADPH. Increased expression of NADPH oxidase in MDSCs causes large amounts of ROS to be released, which inhibits activation of T-cells (Corzo et al., 2009; Huang et al., 2013). Specifically, when MDSCs are in close proximity to T-cells, nitration of the TCR and/or CD8 receptor alters the conformation of the protein resulting in significantly reduced antigen recognition (Nagaraj et al., 2007). In summary, depletion of key nutrients
and nitration of the TCR represent two of the primary mechanisms by which MDSCs exert their immunosuppressive effects on adaptive immune cells.

**Myeloid-Derived Suppressor Cells and Metastasis**

Evidence exists for a role of MDSCs in the majority, if not all, steps that lead to the development of metastatic disease. MDSCs employ numerous mechanisms that facilitate invasion of tumor cells into surrounding tissue and seeding in distant organs. Clinically, high levels of MDSCs have been correlated with an increase in metastasis in the lymph nodes of breast cancer patients (Yu, Wang, Yan, Li, & Ren, 2013). Additionally, MDSCs are correlated with increased metastasis in several other cancer types including non-small cell lung cancer (Huang et al., 2013) and melanoma (Weide et al., 2014). Consequently, there has been increasing interest in uncovering the mechanisms utilized by MDSCs that promote metastasis.

The current research suggests that MDSCs can be recruited to the pre-metastatic site before tumor cells and facilitate tumor seeding by creating an immunosuppressive environment. Additionally, MDSCs contribute to tumor invasion by promoting neovascularization, degradation of the extracellular matrix (ECM), and may even be involved in the epithelial-mesenchymal transition (EMT) (Condamine, Ramachandran, Youn, & Gabrilovich, 2014; Safarzadeh, Orangi, Mohammadi, Babaie, & Baradaran, 2018). For example, the chemokines CXCL1, CXCL2, and CXCL5 recruit MDSCs to the pre-metastatic site and enhance the survival of tumor cells when they arrive by creating an immunosuppressive environment (Acharyya et al., 2012; Connolly et al., 2009; Toh et al., 2011). This process represents a paracrine signaling loop between tumor cells and
MDSCs that enhances tumor seeding in distant organs through the formation of a pre-metastatic niche.

Neovascularization is important for tumor progression and metastatic dissemination. Naturally, MDSCs appear to be involved in this process. For instance, rapid tumor growth results in hypoxia and up-regulation of proangiogenic factors that enhance MDSC recruitment to the tumor microenvironment via hypoxia inducible factor-1α (HIF-1α) (Noman et al., 2014; Qian et al., 2019). Also, MDSCs secrete matrix metalloproteinases (MMP) that degrade the ECM and increase bioavailability of VEGF to stimulate blood vessel formation in the tumor (Safarzadeh et al., 2018). Further, Shen and colleagues (2014) showed that activated MDSCs secrete VEGF which contributes to the formation of new blood vessels (Shen, Wang, He, Wang, & Zheng, 2014). In general, these data suggest that MDSCs play a role in the neovascularization of solid tumors through the production of various pro-angiogenic factors and degradation of the ECM.

Finally, it has been shown that MDSCs play a role in EMT, a critical step in tumor invasion and metastasis. EMT represents a loss of cell-cell adhesion and downregulation of E-cadherin which allows for invasion into the basement membrane and intravasation into the blood stream. In some cases, tumor cells appear to acquire this motile phenotype through various chemical signals rather than selective, random mutations. For example, Toh et al. (2011) demonstrated that purified MDSCs induce EMT in cancer cells through the secretion of transforming growth factor β (TGF-β), epidermal growth factor (EGF), and hepatocyte growth factor (HGF) (Toh et al., 2011). Additionally, recruitment of MDSCs can lead to the activation of EMT-related
transcription factors such as nuclear factor-κB (NF-κB) and Snail through the secretion of high mobility group protein 1 (HMGB-1) (Simpson, Templeton, & Cross, 2012).

**Aerobic Exercise and Cancer**

Significant epidemiological data exists suggesting that physical activity reduces the risk for developing cancer. The overall risk of cancer may be reduced by 7% in individuals who meet the current WHO recommendations for physical activity, which is 150 min/week of MVPA or 10 MET-hours/week. Breast cancer seems to be particularly responsive to physical activity as both premenopausal and postmenopausal women who are physically active have a relative risk (RR) of 0.79 and 0.89 respectively (Liu et al., 2016). The benefits of regular physical activity also show a dose-dependent response. That is, increased amounts of exercise above the minimum recommended amount by the WHO show increased protection against the development of cancer (Liu et al., 2011; Ruiz-Casado et al., 2017). Cancer risk decreases by 1% for every additional 20 MET-hours/week above 20 MET-hours/week (Liu et al., 2016). Additionally, risk of mortality due to breast cancer is significantly lower in physically active individuals. When comparing the highest vs. lowest levels of PA, RR of breast cancer mortality is 0.77 for pre-diagnosis PA and 0.72 for post-diagnosis PA (Schmid & Leitzmann, 2014). Current mechanisms that may explain the protection of exercise on cancer development and mortality include a combination of reduced systemic cancer risk factors and accumulation of anticancer components following acute bouts of exercise.

**Effects of Exercise on Tumorigenesis**

It is now well established that physical inactivity is a major risk factor for the development of cancer and mortality due to cancer. As such, several studies have
investigated the effects of exercise on tumorigenesis in pre-clinical animal models. Although differences exist between tumor types depending on the tissue of origin, most solid tumors utilize oncogenic pathways that allow for overproduction of inflammatory signals, immune system evasion, promotion of angiogenesis, and apoptosis resistance. Exercise has the potential to affect the variables listed above and has consequently become the primary focus of current research.

Recently, Pedersen and colleagues showed that voluntary wheel running in mice reduced tumor growth by as much as 60% across five different tumor models (Pedersen et al., 2016). This reduction in tumor growth was associated with an epinephrine and IL-6-dependent mobilization of NK cells to the tumor microenvironment as blockade of these signaling pathways negated the effects of exercise on tumor growth. In agreement with these results, Betof et al. demonstrated that wheel running in Balb/c mice bearing 4T1 tumors significantly reduced tumor growth (Betof et al., 2015). Exercise led to an increase in tumor cell apoptosis, improved blood vessel maturity, and reduced hypoxia in these animals. Additionally, Buss and Dachs (2018) showed that voluntary exercise can decrease tumor hypoxia, which led to a decrease in tumor growth (Buss & Dachs, 2018). Further, wheel running and treadmill training in mice can reduce intratumoral IL-6 and VEGF (Amani Shalamzari et al., 2014), increase TNF-α in tumor tissue (Molanouri Shamsi et al., 2019), and slow tumor growth in a distance-dependent manner (Goh et al., 2014). In summary, exercise in tumor bearing mice can promote blood vessel maturation that leads to reductions in intratumoral hypoxia and facilitates infiltration of cytotoxic immune cells. Additionally, proinflammatory cytokines that have been implicated in the
recruitment of pro-tumor immune cells may be reduced in the tumor which would improve immune detection by cytotoxic immune cells.

**Effects of Exercise on Inflammation**

As described above, tumor-associated inflammation drives cancer progression through the recruitment of immunosuppressive MDSCs that allow for evasion of immune surveillance. Pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α have been shown to regulate MDSC recruitment from the bone marrow. Additionally, CRP is an acute phase protein released in response to IL-6 and TNF-α that is associated with poorer prognosis in breast cancer patients (Allin, Nordestgaard, Flyger, & Bojesen, 2011). Exercise has shown to affect these signaling pathways, and thus has the potential to temper tumor-induced immune suppression. Various studies have examined the effects of exercise training on systemic inflammatory signals with both chronic and acute exercise.

Systemic CRP is one of the most frequently measured inflammatory markers following exercise training. Chronic exercise interventions have been shown to significantly decrease systemic CRP levels following training (Fairey et al., 2005; Friedenreich et al., 2012). Additionally, highly trained individuals present with low levels of circulating CRP compared to sedentary controls. For example, Tomaszewski and colleagues (2003) showed that CRP concentrations in male ultramarathon runners were less than half that of controls (Tomaszewski et al., 2003). Systemic decreases in CRP levels appear to be the most prominent when the exercise intervention exceeds 4 months (Campbell et al., 2009; Dethlefsen et al., 2017), providing evidence for the importance of lifelong physical activity for cancer prevention. There is limited data examining CRP levels in breast cancer patients and the majority of studies show no effect on systemic
CRP following exercise intervention (Gómez et al., 2011; Guinan et al., 2013; Rogers et al., 2013). It should be noted that many of these studies involved only 8-12 weeks of exercise. However, a recent study demonstrated that in a population of cancer survivors engaged in exercise-based rehabilitation, those with low plasma CRP concentrations had a significantly higher levels of cardiorespiratory fitness compared to those with moderate/high concentrations of plasma CRP (Christensen et al., 2019).

The effects of chronic exercise on levels of IL-6 and TNF-α are much more varied. Some studies have shown a significant decrease in IL-6 and TNF-α (Balducci et al., 2010; Kullo, Khaleghi, & Hensrud, 2006; Shanely et al., 2013) while others show no effect of exercise (Friedenreich et al., 2012; Libardi, De Souza, Cavaglieri, Madruga, & Chacon-Mikahil, 2012). Further, a meta-analysis of over 150 studies showed that IL-6 and TNF-α concentrations were unchanged with exercise in individuals without a cancer diagnosis (Lin et al., 2015). Similarly, a meta-analysis of exercise interventions in breast cancer patients showed no effect of training on IL-6 and TNF-α levels (Kang et al., 2017). Again, most of these interventions were of a relatively short duration suggesting that the most pronounced effects of chronic exercise on systemic inflammatory markers require longer periods of training.

Many studies have investigated the chronic effects of exercise on systemic inflammation and cancer risk factors, yet the acute effects of exercise on tumor biology remain a somewhat neglected area of research. However, several preclinical studies have shown that exercise-conditioned serum following acute exercise can directly inhibit the growth of cancer cells in vitro (Dethlefsen et al., 2016; Gannon, Vaughan, Garcia-Smith, Bisoffi, & Trujillo, 2015; Hojman, Dethlefsen, & Pedersen, 2010). Also, It is now well
established that IL-6 significantly increases following acute bouts of exercise evident by a >10 fold increase following prolonged bouts of PA (Pedersen & Febbraio, 2012). Unlike IL-6 secreted from immune cells in response to pro-inflammatory signals such as TNF-α, this is generally considered an anti-inflammatory response due to the acute induction of anti-inflammatory cytokines. For example, infusion of IL-6 does not increase levels of the pro-inflammatory cytokine TNF-α while enhancing the plasma levels of the anti-inflammatory cytokines IL-1ra and IL-10 (Steensberg et al., 2003). Additionally, Nieman et al. (2006) reported that there is a 2.7-fold increase in blood leukocyte IL-10 mRNA and a 2.2-fold increase in blood leukocyte IL-1ra mRNA 1-hour post-exercise (Nieman et al., 2006). Further, this anti-inflammatory milieu is maintained for several hours following exercise (Mendham, Duffield, Marino, & Coutts, 2015). These data suggest that both long-term training and the accumulation of repeated bouts of acute exercise are important for the anti-inflammatory effects of exercise. The accumulated surge of anti-inflammatory cytokines following acute exercise may also directly counteract pro-inflammatory signals that are frequently associated with MDSC induction and tumor progression such as IL-1β and pro-inflammatory IL-6.

Effects of Exercise on Metastasis

Metastatic disease is the primary cause of cancer-related deaths. As described above, regular physical activity can reduce the risk for mortality due to cancer by as much as 20% (Schmid & Leitzmann, 2014). Therefore, it has been hypothesized that exercise may decrease the dissemination of tumor cells to distant organs. Indeed, there have been several studies investigating the effects of exercise on metastasis, however, methodological differences make it difficult to definitively answer this question. For
example, many studies use intravenous (tail-vein) injection of tumor cells (Hoffmann-Goetz, MacNeil, & Arumugam, 1994; Jadeski & Hoffman-Goetz, 1996; MacNeil & Hoffman-Goetz, 1993; Pedersen et al., 2016), while others investigate metastasis arising from the primary tumor (Dethlefsen et al., 2016; Gershbein, Benuck, & Shurrager, 1974). Intravenous injection of tumor cells allows for evaluation of the ability of circulating tumor cells to survive and colonize in an organ, however it does not allow for investigation of the early steps in the metastatic cascade. Thus, the most appropriate model of metastasis is orthotopic primary tumors that spontaneously metastasize to distant organs.

Concerning the effects of exercise on metastasis, it is difficult to make meaningful comparisons between studies due to differences in tumor models and exercise protocols. In models of metastasis that arise from primary tumors, most studies show no effect of exercise. For instance, Jones et al. (2012) used voluntary wheel running in mice orthotopically injected with C-1 prostate cancer cells and showed a non-significant decrease (34%) in the number of metastatic lesions. In support of these results, Yan and colleagues (2011) demonstrated that there was a non-significant trend toward a decrease in tumor metastasis with voluntary wheel running after surgical resection of Lewis lung carcinoma tumors (Yan & Demars, 2011). Conversely, a recent study showed that pulmonary metastasis was increased with voluntary wheel running in the 4T1 tumor model (Smeda et al., 2017). Further, studies using intravenous injection of tumor cells have shown that exercise can decrease radioactivity in the lungs from radiolabeled tumor cells (Hoffmann-Goetz, MacNeil, & Arumugam, 1994), decrease tumor cell retention in the lungs (Jadeski & Hoffman-Goetz, 1996), and lower the number of B16 tumors in the
lungs (Pedersen et al., 2016). These data suggest that exercise has minimal effects on tumor metastasis, however differences in training modality, tumor model, and quantification of metastases make it difficult to make definitive conclusions. Therefore, more studies using orthotopic tumor models are needed.

Summary

Breast cancer incidence remains relatively unchanged and is largely attributed to worldwide increases in lifespan, physical inactivity and obesity. Consequently, a significant number of people are living with breast cancer. While a large body of evidence suggests that regular PA can reduce cancer risk and mortality, the biological mechanisms explaining this relationship remain unclear. Tumor progression is driven in part by chronic inflammation that results in the generation of pro-tumor MDSCs. MDSCs inhibit antitumor immunity, promote metastasis, and reduce the efficacy of immunotherapy. Pre-clinical studies show that exercise has the potential to slow tumor growth by normalizing tumor vasculature, which reduces intratumoral hypoxia and facilitates infiltration of cytotoxic immune cells. Additionally, exercise can reduce systemic inflammation and promote an anti-inflammatory phenotype following acute exercise. The anti-inflammatory effects of exercise have the potential to directly oppose signals that have been implicated in MDSC generation, yet no studies have investigated the effects of exercise on MDSC-related tumor progression.
CHAPTER III

METHODS

Experimental Design

This study utilized endurance training to examine the effects of exercise on MDSC-related tumor progression and metastasis in an orthotopic mouse model of breast cancer. The primary purpose of this study was to determine if exercise temporally delays the onset of MDSC accumulation in the blood, spleen, and tumor of tumor-bearing mice. A secondary purpose of this study was to examine the effects of exercise on MDSC function by measuring the suppressive quality of these cells in vitro. The final purpose of this study was to determine the effects of exercise on lung metastasis. These exercise effects were evaluated using a 10-week endurance exercise voluntary wheel running protocol. On the 6th week of the training protocol, animals were inoculated with $1 \times 10^4$ 4T1 tumor cells in the dorsal mammary fat pad and then continued to train for an additional 4 weeks. Subgroups were sacrificed at varying time points following tumor inoculation to examine the time course of MDSC accumulation. Figure 1 outlines the experimental design of this study.
**Figure 1.** Experimental Design. WR, wheel run; SED, sedentary; TUM, tumor.

WR+TUM groups, \( n = 10 \) per time point. SED+TUM groups, \( n = 10 \) per time point.

**Animals and Animal Care**

Female Balb/c mice (\( n = 80 \)) were housed individually in a temperature-controlled facility with a 12:12 hour light-dark schedule. Additionally, female age-matched non-tumor control mice (\( n = 20 \)) were used to harvest naïve splenocytes for T-cell suppression assays. Mice were given standard chow and distilled water *ad libitum*. All procedures were approved by the University of Northern Colorado’s Institutional Animal Care and Use Committee (IACUC) and were in compliance with the Animal Welfare Act guidelines.

**Exercise Training Protocol**

Mice, 8 weeks of age, were randomly assigned to either a wheel run (WR+TUM) or sedentary (SED+TUM) group on day one of the exercise intervention. SED+TUM was restricted to normal cage activity for the duration of the study. WR+TUM animals were given 24-hour access to commercially available running wheels (11.5 cm by diameter) (MiniMitter; Bend, OR) for the duration of the study. Running distance was monitored using a Vital View data acquisition system (MiniMitter; Bend, OR).
Cell Culture, Tumor Inoculation, and Measurement

The 4T1 mouse mammary carcinoma (ATCC, Manassas, VA) cell line was used to establish orthotopic tumors in the mammary gland. Cells were grown in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal bovine serum (FBS) and maintained in a humidified incubator with 5% CO₂. Cell growth medium was replaced every two to three days.

On week 6 of the study, animals were sedated with isoflurane and inoculated subcutaneously with $1 \times 10^4$ 4T1 cells suspended in 100 µl of PBS in the fourth mammary gland on the right side using a 25-gauge syringe needle. Mice were the most active from 8:00 PM – 8:00 AM during this study. Therefore, to ensure that injections were performed during a period of reduced activity, all tumor inoculations occurred between 11:00 AM and 1:00 PM. Following tumor inoculation, SED+TUM and WR+TUM were randomly subdivided into different time point groups: 16 days (n = 10), 20 days (n = 10), 24 days (n = 10), or 28 days (n = 10). Previous unpublished data in our lab demonstrated that mice injected with $1 \times 10^4$ 4T1 cells develop palpable tumors after 8 days on average. Also, these tumors become measurable with digital calipers after 14 days on average. Although tumors are present well before the 16-day time point, it is unlikely that enough cells could be harvested for flow cytometric analysis. Thus, the earliest time point of 16 days was chosen. Additionally, pilot data from our lab indicate that MDSCs are not present in the tumor or peripheral lymphoid organs by 12 days post-tumor injection, making functional analysis of these cells impossible. For additional time-points of sacrifice, 4-day intervals were chosen because previous data have shown that 4T1 tumors grow at a relatively linear rate when measured every 3-5 days (Bunt et al., 2007).
Subgroups of mice were sacrificed at the specified time points post-injection. Body condition, and tumor volume was monitored three times per week for the duration of the study. Tumor length and width was measured using digital calipers. These measurements were used to estimate tumor volume using the following formula: \( \pi/6 \times \text{width} \times \text{length}^2 \), where length equals the largest diameter, and width was measured 90° from length.

**Flow Cytometry**

**Tissue Preparation and Analysis**

Spleen, blood, and tumor samples were harvested and prepared for flow cytometry. Spleens were mechanically dissociated using the frosted end of two microscope slides and filtered through a 100 µm cell strainer. Blood samples were collected via cardiac puncture into a syringe containing 0.1 mL heparin. Tumors were excised and mechanically homogenized using a cell dissociation sieve tissue grinder (Sigma Aldrich), then enzymatically digested in type IV collagenase (2 mg/mL) and DNase (100 µg/mL) for 45 minutes at 37°C on a platform rocker. Following digestion, tumor samples were filtered through a 100 µm cell strainer. Single cell suspensions of splenocytes, tumor, and peripheral blood were cleared of red blood cells (RBC) by incubating in ammonium-chloride-potassium lysis buffer for 3 minutes at room temperature. Fc receptors were blocked by incubating samples on ice for 10 minutes in anti-mouse CD16/32 antibody (BioLegend). Cells were then resuspended in antibody solution containing fluorochrome-conjugated monoclonal antibody against CD11b, Ly-6G, Ly-6C, or the appropriate isotype control (BioLegend). Samples were incubated on ice, in the dark, for 35 minutes and analyzed on an Attune NxT flow cytometer. Initial forward scatter/side scatter gating was used to exclude debris and doublets from analysis.
Unstained and isotype controls were used to exclude background fluorescence and ensure there is no evidence of non-specific binding. Proportions of MDSCs were quantified in the blood, spleen, and tumor and were reported as total MDSCs as a percentage of the total cells in the sample. A representative example of the gating strategy used to identify MDSCs is provided in Appendix B.

**Myeloid-Derived Suppressor Cell Sorting Using Fluorescence Activated Cell Sorting**

PMN-MDSCs and M-MDSCs were purified from the spleens of tumor bearing mice for functional assessment using fluorescence activated cell sorting (FACS). Single cell suspensions of splenocytes were cleared of red blood cells and stained with zombie violet viability dye (BioLegend) for 15 min according to manufacturer recommendations. Next, cells were stained for CD11b, Ly-6G, and Ly-6C as described above. Gates were established to identify zombie violet negative (viable) cells, then CD11b^+Ly6G^+Ly6C^{lo} and CD11b^+Ly6G^−Ly6C^{hi} cells for sorting. These subpopulations of MDSCs were pooled and sorted into the same 15 mL conical containing complete RPMI (cRPMI) supplemented with 20% FBS using a SH800S cell sorter. After sorting, cells were centrifuged for 7 minutes at 300g. The cell pellet was resuspended in cRPMI growth medium at a concentration of 1x10^6/mL and set aside.

**T-cell Suppression Assay**

**Antibody Coating of Microwell Plates**

Anti-CD3ε antibody was prepared in sterile PBS (5 µg/mL) and 50 µL was dispensed into each experimental well of a 96-well flat-bottom tissue culture plate. Fifty µL of sterile PBS was added to unstimulated control wells. The plate was tightly covered
with Parafilm and incubated for 2 hours at 37°C. Before adding cells, antibody solution was removed, and each well was washed 2 times with sterile PBS.

**Addition of Cells**

All samples used for the T-cell suppression assay were handled under sterile conditions. Splenocytes were harvested from a naïve female Balb/c mouse, 12-15 weeks of age, and cleared of red blood cells as described above. The cell suspension was resuspended in cRPMI at a concentration of 2x10^6/mL and centrifuged for 7 minutes at 300g. The cell pellet was then resuspended in CellTrace Violet (1:1000) and incubated at 37°C for 20 minutes protected from light. CellTrace Violet was quenched by adding cRPMI and incubating cells for an additional 5 minutes at 37°C. Cells were pelleted and resuspended in cRPMI. Next, 100 µL of the splenocyte cell suspension (2x10^5 total cells) was added to each well. One hundred µL of the MDSC suspension (1x10^5 total cells) was added to each experimental well for a 2:1 splenocyte to suppressor ratio. To account for higher cell numbers and added competition for resources in the experimental wells, 3x10^5 splenocytes were added to control wells to equal the total number of cells in experimental wells. One hundred µL of cRPMI was added to unstimulated control and stimulated control wells for a final volume of 200 µL/well. Next, soluble anti-CD28 (2 µg/mL) antibody was added to each stimulated well. Finally, samples were incubated at 37°C for 72 hours and analyzed on an Attune NxT flow cytometer.

Figure 2 outlines an example of the setup of control and experimental wells. Representative results from dye dilution analysis are presented in Figure 3. The peak farthest to the right, which has the highest fluorescence of CellTrace Violet, represents cells that have not divided. Peaks to the left represent T-cells that have divided one or
more times. CellTrace fluorescence decreases (becomes more dilute) with multiple cell divisions. Using the FCS express software, a proliferation index can be calculated from this histogram which is the average number of divisions of responding cells. The proliferation index should be lower in splenocytes co-cultured with MDSCs.

Figure 2. T-cell suppression assay design. Theoretical setup of a culture dish for functional assessment of MDSCs from an individual animal run in duplicate. All splenocytes were labeled with CellTrace Violet for detection of proliferation via dye dilution. Stimulated wells were coated with anti-CD3ε antibody and soluble anti-CD28 antibody for activation of CD3+CD28+ T-cells. MDSCs were isolated from individual tumor-bearing mice and added to experimental wells at a ratio of 1:0.5 splenocyte to MDSC.
Figure 3. Detection of cell division by dye dilution. Cells were stained with CellTrace Violet and stimulated with CD3 antibody. The peak outlined in black are cells that have not divided (unstimulated controls). Violet peaks represent successive generations of cells.

**Lung Metastasis**

An incision was made along the midline of the abdomen through the ribcage and up toward the salivary glands. The trachea was identified and elevated by threading a pipette tip underneath. Using a 27-G syringe needle, India Ink (10% India Ink and 0.1% ammonium hydroxide) was injected into the lungs via the trachea until resistance was felt. The trachea was cut, and the lungs were removed. Lungs were rinsed briefly with deionized water and transferred to a glass vial containing 3 mL of Fekete’s solution (50% ethanol, 6% formaldehyde, 3% glacial acetic acid). After at least 10 minutes, tumor nodules were visualized as white dots on black lungs (Figure 8B). Lungs were transferred to a dish and individual white dots were counted as a single metastatic tumor nodule (Paschall & Liu, 2016). Metastatic nodules were manually counted on the surface of both the right and left lung. Each animal has a total surface metastatic nodule count, and an
average surface nodule count was obtained for each group. Group averages were
compared to determine the effects of exercise on the number of metastatic lung nodules.

**Statistical Analysis**

All data are presented as means ± standard error of the mean. For tissue-resident
MDSC proportions, a student’s t test was used to identify group differences for the
number of MDSCs in spleen, blood or tumor samples. For tumor volume, tumor mass,
and metastatic lung nodules, student’s t tests were performed for the specified time points
to determine group differences. If variances were significantly different, a t test with
Welch’s correction was used. For T-cell proliferation, repeated measures ANOVA was
used to identify differences between stimulated controls, and co-cultures containing
MDSCs harvested from SED+TUM or EX+TUM. P-values with p < 0.05 were
considered significant.
CHAPTER IV

RESULTS

General Observations

A total of 7 animals (SED+TUM day 16, 20, 24, and 28, n = 1/timepoint; WR+TUM day 16, 24, and 28, n = 1/timepoint) were euthanized early and excluded from analysis due to poor body condition as a result of tumor growth during this study. No differences were observed between groups for initial body mass or final body mass over the course of the study (Figure 4). Additionally, no differences were observed in running distance between different timepoint groups (Figure 5). This is expected as mice tend to run more during the first weeks of being housed in a wheel running cage and progressively decrease their running volume each subsequent week, resulting in only slightly higher post-injection distances in the 24 and 28 day groups (Turner, Kleeberger, & Lightfoot, 2005).
Figure 4. Body Mass. Day 0 represents initial body mass for all animals included in the study (n = 40/group). Day 16-28 points represents final body mass at the corresponding timepoint at the time of sacrifice (n = 9-10/group).
Figure 5. Running distance in WR+TUM animals. (A) Pre-tumor running distance, (B) post-tumor cell injection, and (C) total running distance at the specified timepoints.
Myeloid-Derived Suppressor Cell Accumulation and Tumor Progression

Spleen, blood, and tumor samples were analyzed in tumor-bearing mice at the indicated time points to determine the effects of physical activity on MDSC accumulation. At day 16, a marked increase in the number of these cells was observed in tumor bearing animals. In WR+TUM animals, MDSCs in the spleen were significantly ($p < 0.05$) lower than SED+TUM at day 16 (23.0 ± 10.0% vs. 33.0 ± 5.2%) and day 20 (24.3 ± 5.1% vs. 33.9 ± 8.0%) (Figure 6A). The effect of exercise on MDSC accumulation in the spleen was lost by day 24 and 28 as no differences were observed between groups. Additionally, at day 16, circulating MDSCs in the blood were significantly lower ($p < 0.05$) in WR+TUM (14.7 ± 8.5%) when compared to SED+TUM (23.2 ± 7.1%) (Figure 6B). Interestingly, it appears that MDSCs do not begin to infiltrate 4T1 tumors until the later stages of progression. While MDSC populations increased in the spleen and blood by day 16, MDSC levels in the tumor remained relatively low until day 28. A recent study showed that a combination of physical activity and energy restriction reduced total MDSCs in the tumor microenvironment (TME) 35 days post-tumor implantation by about 15% (Turbitt et al., 2019). In comparison, we observed a non-significant ($p = 0.08$) 24% decrease in MDSCs in the TME of WR+TUM (17.2 ± 5.3%) when compared to SED+TUM (23.2 ± 8.7%) at day 28 independent of energy restriction (Figure 6C). While the delay in accumulation of MDSCs did not translate to smaller tumors at any timepoint (Figure 7), there was a non-significant 62% and 26% reduction in metastatic lung nodules in WR+TUM at days 24 and 28, respectively (Figure 8A). Spleen mass progressively increased as tumor size increased, and no differences were observed with physical activity (Figure 9).
Figure 6. MDSC accumulation. (A) Total accumulation of MDSCs in the spleen (B) blood, and (C) tumor at the specified time-points post-4T1 cell injection. Means ± SEM. *p < 0.05 vs. WR+TUM. For tissue MDSCs, a student’s t test was used to compare groups.
Figure 7. Tumor mass. Progression of tumor mass over the course of the study. Means ± SEM. A student’s t test was used to compare groups at each respective timepoint post-4T1 cell injection.

Figure 8. Metastatic lung nodules. (A) Progression of the absolute number of metastatic lung nodules over the course of the study. (B) Representative lung samples from day 28 animals showing visualization of white nodules on black lungs. Means ± SEM. A student’s t test was used to compare groups at each respective timepoint post-4T1 cell injection.
Figure 9. Spleen mass. Progression of spleen mass over the course of the study. Means ± SEM. A student’s t test was used to compare groups at each respective timepoint post-4T1 cell injection.

T-cell Suppression Assay

MDSCs were identified and purified from the spleen using FACS. Representative samples from the spleen of an individual WR+TUM animal at day 28 before sorting and after sorting are presented in Figure 10. Average purity of all sorted samples was >80%, which is within the normal expected range when using this method (Solito et al., 2019). Pathological activation of MDSCs has been described previously as a gradual process that is related to tumor progression (Gabrilovich, 2018). The results presented here support this idea as MDSCs purified from tumor bearing mice did not become significantly suppressive toward T-cell proliferation until day 28. CD3+CD4+ T-cell proliferation index in SED+TUM MDSC co-cultures (3.19 ± 0.47) was significantly
lower \( (p < 0.05) \) than stimulated control samples \( (5.30 \pm 0.53) \) (Figure 11A). In comparison, CD3\(^+\)CD4\(^+\) cell proliferation index in WR+TUM MDSC co-cultures \( (5.05 \pm 0.59) \) was not significantly suppressed suggesting that endurance exercise delayed the acquisition of suppressive function by MDSCs. At all timepoints, CD3\(^+\)CD8\(^+\) T-cell proliferation was not significantly suppressed in either group (Figure 11B). However, CD3\(^+\)CD8\(^+\) T-cell proliferation in WR+TUM co-cultures \( (5.54 \pm 0.53) \) at day 24 was significantly higher \( (p < 0.05) \) than stimulated controls \( (4.04 \pm 0.69) \) suggesting that these cells were potentially promoting CD3\(^+\)CD8\(^+\) T-cell proliferation.

**Figure 10.** Gating strategy for MDSC purification using FACS. (A) Spleen sample from individual day 28 WR+TUM animal before sorting. (B) Post-sort analysis of purified MDSCs.
**Figure 11.** T-cell suppression assay in MDSC co-cultures. (A) CD3^+^CD4^+^ T-cell proliferation in stimulated controls and MDSC co-cultures at the specified timepoint post-4T1 cell injection. (B) CD3^+^CD8^+^ T-cell proliferation in stimulated controls and MDSC co-cultures at the specified timepoint post-4T1 cell injection. (C) Representative CD3^+^CD4^+^ T-cell proliferation plots in day 28 samples. Median CellTrace violet fluorescence in unstimulated samples was used to set the starting undivided population (vertical black line). Splenocytes were stimulated to proliferate using anti-CD3ε and anti-CD28 antibody. *p < 0.05 vs. stimulated control. For statistical analysis a repeated-measures ANOVA with Tukey’s multiple comparison was used.
CHAPTER V

DISCUSSION AND CONCLUSIONS

This was one of the first studies to examine the effects of exercise on the time-course of MDSC accumulation and function in a murine model of metastatic breast cancer. The hypothesis in response to our first specific aim was partially supported, as the numbers of MDSCs in WR+TUM were significantly lower in the spleen and blood when compared to SED+TUM. However, while MDSCs in the tumor of WR+TUM were trending lower compared to SED+TUM, this difference was not significant (p = 0.08). The second hypothesis of this study stated that MDSCs from exercising mice would have a reduced suppressive capacity. This hypothesis was also supported, evidenced by reduced suppression of T-cells in MDSC co-cultures from WR+TUM. Although there was a trend toward lower metastatic lung nodules in WR+TUM at day 24 and 28, this difference was not significant, which resulted in rejection of our third hypothesis.

A growing body of literature suggests that physical activity, both acute and chronic, can favorably modulate immune function, with beneficial effects that potentially extend into the prevention and treatment of cancer. Physical activity and exercise is known to modulate inflammatory signaling, as well as the expansion and recruitment of both myeloid and lymphoid lineage immune cells (Hojman, 2017). Nearly all leukocyte populations are mobilized to the blood during acute exercise, and moderate intensity exercise is known to improve chemotaxis, phagocytosis, and oxidative burst activity in
neutrophils (Pedersen & Hoffman-Goetz, 2017). MDSCs are immature myeloid cells routinely associated with a poorer prognosis in several cancer types (Veglia, Perego, & Gabrilovich, 2018) and pharmacological blockade of MDSCs can slow the growth of transplantable tumors (Davis et al., 2017; Kumar, Cheng et al., 2016). To date, only one study has investigated the effects of voluntary wheel running on the accumulation of MDSCs in a pre-clinical animal model and the present study aimed to determine if physical activity could improve antitumor immunity by reducing the accumulation of MDSCs in an orthotopic mouse model of breast cancer. Our data show that voluntary wheel running significantly delayed the accumulation of MDSCs in the spleen and blood. Additionally, there was a modest reduction in intratumoral MDSCs, however, these effects did not translate to smaller tumors by size and weight. Few groups have investigated the effects of physical activity on metastasis in 4T1 tumor-bearing mice and here we observed a non-significant 62% and 26% reduction in the number of metastatic lung nodules at days 24 and 28, respectively. In comparison, a recent study showed that wheel running in 4T1 tumor-bearing mice increased metastatic burden in the lungs when mice began running concurrently with tumor injection (Smeda et al., 2017). Turbitt et al. recently reported that energy restriction preventing weight gain in combination with voluntary wheel running significantly reduced M- and PMN-MDSCs in the spleen and tumor which led to a decrease in tumor growth (Turbitt et al., 2019). They also observed a non-significant decrease in metastatic burden in the lungs of wheel running mice fed ad libitum. By comparison, we report that physical activity can reduce the numbers of these cell populations independent of energy restriction. Taken together, these results suggest that weight management may be important to maximize the beneficial
immunomodulatory effects of physical activity in tumor-bearing mice. One potential mechanism explaining these findings is the normalization of tumor vasculature with physical activity. For example, others have shown that 18 days of wheel running following implantation of 4T1 tumor cells resulted in improved vessel maturity, perfusion, and reduced hypoxia in the tumor microenvironment (Betof et al., 2015).

Reductions in hypoxia have been shown to reduce the accumulation of MDSCs in 4T1 tumors, decrease the expression of programmed cell death receptor ligand 1 (PD-L1), and increase the recruitment of T-cells to the tumor microenvironment (Qian et al., 2019).

A hallmark of MDSCs is the ability to suppress T-cell proliferation. MDSCs purified from SED+TUM mice appeared to preferentially suppress CD3⁺CD4⁺ T-cell proliferation while MDSCs from WR+TUM mice did not significantly suppress T-cell proliferation at 28 days, suggesting that physical activity may help normalize myelopoiesis and consequently the function of MDSCs. Compared to the findings of Turbitt et al. where they observed an increased proliferation of CD4⁺ T-cells harvested from physically active tumor-bearing mice (Turbitt et al., 2019), we showed that physical activity may directly alter the ability of MDSCs to suppress naïve T-cells evidenced by reduced T-cell suppression in MDSC co-cultures at day 28. Additionally, CD3⁺CD8⁺ T-cell proliferation was significantly higher than controls in WR+TUM MDSC co-cultures at day 24 suggesting that these cells were promoting cytotoxic T-cell proliferation.

MDSCs are often described as immature immune cells that are highly plastic depending on the context of the microenvironment. It is possible that the anti-inflammatory effects of physical activity may help normalize the aberrant myelopoiesis associated with accumulation and activation of MDSCs.
Exercise had no effect on the rate of tumor progression in our study. Mixed results have been reported in the literature, with some studies in 4T1 tumor bearing mice showing that voluntary wheel running can slow the growth of 4T1 tumors (Betof et al., 2015; Goh et al., 2014; Hagar et al., 2019), while other recent reports show that wheel running alone had no effect on tumor growth in 4T1 tumor-bearing mice (Smeda et al., 2017; Turbitt et al., 2019). We propose that pre-training is important for the observed antitumor effects of physical activity which may be related to the running habits of rodents. Mice run the highest volumes the first several weeks after being housed with a running wheel and progressively decrease their running volume during subsequent weeks (Turner et al., 2005). Adaptations that promote an anti-inflammatory environment and progression toward more low-moderate running intensity may be important for the anticancer effects observed in pre-training models. In comparison, animals that begin running on the day of tumor inoculation may run at a volume and intensity that is ultimately immunosuppressive, negating any potential anticancer effects.

Immune checkpoints regulate the immune system by promoting self-tolerance and preventing autoimmunity. Tumors are known to co-opt these immune checkpoint pathways to evade immune surveillance by T-cells specific for tumor antigens. Checkpoint inhibitor immunotherapy targets these immune checkpoints by blocking the interaction between checkpoint proteins that inactivate tumor-specific T-cells (Pardoll, 2012). High levels of circulating MDSCs have been used as a predictor of the clinical response to immunotherapy with higher MDSCs being associated with treatment failure (De Henau et al., 2016; Ostrand-Rosenberg & Fenselau, 2018). In patients treated with the checkpoint inhibitor ipilimumab, low levels of circulating MDSCs were associated
with increased survival and improved treatment outcomes (Schilling et al., 2016). Additionally, depletion of MDSCs in pre-clinical mouse models significantly improves the outcomes of immunotherapy (Iida et al., 2017) and exercise has been shown to regulate inflammatory signals such as IL-6 and IL-1β that are implicated in MDSC accumulation (Bunt et al., 2007; Murphy et al., 2011; Pedersen & Hoffman-Goetz, 2017). Also, human breast cancer survivors who participated in a six month endurance training intervention had significant reductions in serum levels of systemic inflammatory cytokines such as TNF-α and IL-6 (Christensen et al., 2019). Further, an acute bout of exercise has been shown to increase serum concentrations of the anti-inflammatory cytokines interleukin 10 (IL-10) and interleukin-1 receptor antagonist (IL-1ra) (Petersen & Pedersen, 2004). Taken together, the anti-inflammatory effects of exercise described above in both animal models and human subjects have the potential to regulate several of the mechanisms involved in the accumulation and maintenance of MDSCs, specifically the IL-1β and IL-6 signaling pathways. However, future work is necessary to determine whether these factors are underlying mechanisms behind the beneficial effects of physical activity observed in this study.

**Conclusions**

The present study investigated the effects of voluntary wheel running on the temporal accumulation of pro-tumor MDSCs and metastatic burden in a murine model of breast cancer. Despite a profound uptick in MDSC production in tumor-bearing animals, voluntary wheel running was able to delay the accumulation of immunosuppressive MDSCs. Furthermore, MDSCs harvested from physically active mice were less suppressive toward T-cell proliferation *in vitro* and enhanced cytotoxic T-cell
proliferation at day 24 post-tumor injection. Thus, findings from the present study suggest that physical activity may be a cost-effective way to reduce the severity of aberrant myelopoiesis that results in the accumulation of pathologically activated MDSCs, which could potentially improve the efficacy of now common immunotherapies such as programmed cell death 1 receptor (PD-1)/PD-L1 checkpoint inhibitors.

**Limitations**

While the 4T1 tumor model has been used extensively to study MDSC biology, there are some limitations to our design. It is possible that with a higher ratio of MDSCs, some suppressive effects may be observed at earlier timepoints. However, due to low numbers of MDSCs at the early timepoints, only a 2:1 splenocyte-to-suppressor ratio was used. Additionally, different subpopulations of MDSCs exert their immune suppressive effects using different mechanisms. Again, due to low cell numbers at early timepoints, especially in the M-MDSC population, both populations were pooled together in this study making it difficult to determine where the observed effects of physical activity are occurring. These findings were limited to the 4T1 breast cancer tumor model and future research into the effects of physical activity on MDSC accumulation in additional tumor models will help to confirm the results presented here. Experiments using S100A9 knockout mice, which have a reduced capacity to generate MDSCs, would help confirm that the observed effects in this study were physical activity-dependent. Additional assays further characterizing the phenotype and function of T-cells are necessary to determine the effects of physical activity on antitumor immunity. Finally, mice were individually housed to accurately measure running activity. This may potentially affect immune function, as mice experience less stress in social groups and have an increased metabolic
rate to maintain core temperature when housed individually (Krohn, Sørensen, Ottesen, & Hansen, 2006). All animals in the present study were housed individually to account for these effects.
References


https://doi.org/10.1097/CAD.0000000000000484


https://doi.org/10.1038/nature20554


*Cancer Research, 38*(10), 3174–3181.

[https://doi.org/10.2147/IJN.S149235](https://doi.org/10.2147/IJN.S149235)


[https://doi.org/10.1016/j.intimp.2011.01.030](https://doi.org/10.1016/j.intimp.2011.01.030)

*Cancer Prevention Research, 5*(1), 98–108. [https://doi.org/10.1158/1940-6207.CAPR-11-0369](https://doi.org/10.1158/1940-6207.CAPR-11-0369)


Molanouri Shamsi, M., Chekachak, S., Soudi, S., Gharakhanlou, R., Quinn, L. B. S., Ranjbar, K., … Voltarelli, F. A. (2019). Effects of exercise training and supplementation with selenium nanoparticle on T-helper 1 and 2 and cytokine levels in tumor tissue of mice bearing the 4 T1 mammary carcinoma. *Nutrition, 57*, 141–147. https://doi.org/10.1016/j.nut.2018.05.022


Confirming 4T1 Tumors Stimulate MDSC Production

Early experiments were conducted to confirm that the 4T1 model stimulates aberrant myelopoiesis and production of MDSCs in our hands. To confirm this, animals were assigned to one of four groups (n = 3-10/group); sedentary control (SED), wheel run control (WR), sedentary tumor (SED+TUM), or wheel run tumor (WR+TUM). Both WR groups ran for 6 weeks and SED groups were restricted to normal cage activity. After the 6-week pre-training period, both TUM groups were inoculated with $1 \times 10^4$ 4T1 cells and tumors were allowed to grow for 28 days. After 28 days, animals were sacrificed and MDSCs were analyzed in the spleen and blood. As expected, there was a significant increase in these cells in the spleen and blood of tumor-bearing animals when compared to non-tumor controls (Figure 12). Additionally, no differences were observed between SED and WR controls indicating that physical activity does not alter myelopoiesis in non-tumor animals, which is why this study excluded non-tumor control animals.

*Figure 12.* 4T1-dependent MDSC production. MDSCs were analyzed in the spleen and blood after 28 days of tumor growth. Means ± SEM. **$p < 0.01$ vs. non-tumor controls.
Determining Earliest Timepoint for MDSC
Functional Analysis

To determine the earliest timepoint to begin purification and functional analysis of MDSCs, pilot experiments were performed to determine when an uptick in MDSCs is observed in tumor-bearing animals. Animals were assigned to either a sedentary tumor (SED+TUM) or wheel run tumor (WR+TUM) group. After 6 weeks of wheel running, all animals were inoculated with 4T1 tumor cells and sacrificed 12 days later. While palpable tumors were present in these animals, MDSCs were relatively undetectable. MDSCs in the spleen (Figure 13A), blood (Figure 13B), and tumor (Figure 13C) were less than 5% of the total cells, which is similar to what is observed in non-tumor control animals (Figure 12). It is likely that these are normal myeloid cells that have not yet been pathologically activated, thus a later timepoint of 16 days was chosen as the earliest timepoint for this study.
Figure 13. Early MDSCs. MDSCs in the (A) spleen, (B) blood, and (C) tumor of tumor-bearing animals after 12 days of tumor growth. Means ± SEM.
APPENDIX B

MYELOID-DERIVED SUPPRESSOR CELL GATING STRATEGY
Gating Strategy for Identifying Myeloid-Derived Suppressor Cells

The cell surface markers CD11b, Ly6C, and Ly6G are the most common markers used to identify MDSCs in mice. PMN-MDSC are CD11b\(^+\)Ly6C\(^{lo}\)Ly6G\(^+\) and M-MDSC are CD11b\(^+\)Ly6C\(^{hi}\)Ly6G\(^-\). As described in the methodology, samples were stained with fluorescent monoclonal antibodies for these markers and run on an Attune NxT flow cytometer. Data from the flow cytometer were exported and analyzed using FCS express (De Novo software). The gating strategy to identify and quantify these cells in a homogenous tissue sample is presented in Figure 14 below. Initial gating is used to identify cells and exclude debris with a low forward scatter (FSC-A) and low side scatter (SSC-A). Next, to exclude doublets, or cells that pass through the flow cytometer stuck together, the singlets gate was included. Single cells appear at a 45-degree angle on a FSC-A by forward scatter height (FSC-H) plot. After excluding doublets, primary gates around CD11b positive cells were established and then secondary gates were established around Ly6C positive or Ly6G positive cells. As described previously, both populations of MDSCs were combined and reported as total MDSCs in the sample.
Figure 14. Myeloid-derived suppressor cell gating strategy.
APPENDIX C

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE PROTOCOL APPROVAL
IACUC Memorandum

To:    Reid Hayward
From:  Laura Martin, Director of Compliance and Operations, ARF
CC:    IACUC Files
Date:  June 11, 2019
Re:    IACUC Protocol 1906CE-RH-RM-22 Approval

The UNC IACUC has completed a final review of your protocol "Strategies to Alleviate the Negative Effects of Cancer and Cancer Treatments". The protocol review was based on the requirements of Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training; the Public Health Policy on Humane Care and Use of Laboratory Animals; and the USDA Animal Welfare Act and Regulations. Based on the review, the IACUC has determined that all review criteria have been adequately addressed. The PEPD is approved to perform the experiments or procedures as described in the identified protocol as submitted to the Committee. This protocol has been assigned the following number 1906CE-RH-RM-22.

The next annual review will be due before June 11, 2020.

Sincerely,

Laura Martin, Director of Compliance and Operations