THE EFFECTS OF CHANGES IN ENERGY BALANCE ON IMMUNE REGULATION AND TUMOR PROGRESSION IN THE 4T1.2 MAMMARY TUMOR MODEL

A Dissertation in
Integrative and Biomedical Physiology

by
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ABSTRACT

One significant challenge in the field of breast cancer (BC) research is to determine how to reduce and/or eliminate the mortality associated with metastatic BC. Novel therapies, especially non-pharmacological, lifestyle-based interventions that prevent or slow metastatic disease with less severe side effects are greatly needed. Numerous lifestyle factors (including dietary components, body weight, and physical activity patterns) significantly impact BC risk and survival. Emerging population data suggests an inverse relation between physical activity and BC incidence, as well as an important role for exercise in the prevention of cancer recurrence and mortality. The observational nature of these studies limit the ability to determine biological mechanisms and the extent to which exercise, as opposed to changes in body weight, drive beneficial effects. Additionally, very little is known about the mechanisms contributing to the relation between physical activity and survival. Given the importance of metastases in the mortality of women with BC, understanding the role of exercise on metastatic burden may reveal important new targets for secondary and tertiary cancer prevention.

The aim of study one was to control for weight and examine the effects of exercise, mild dietary restriction, or the combination of diet and exercise on the inflammation-immune axis and tumor progression in a preclinical metastatic BC model to determine the extent to which exercise or body weight contribute to cancer prevention. Dietary energy restriction-induced weight control (i.e., SED+ER mice) was effective at altering host splenic immunity and the expression of key genes in the tumor microenvironment (TME) related to immunosuppression and metastatic progression; however, this intervention failed to induce changes in
primary tumor growth or spontaneous metastases. Moderate exercise in weight stable mice (EX+ER) resulted in a similar reduction in immunosuppressive and metastatic genes in the TME compared with the SED+ER mice; however, in addition, EX+ER mice had the greatest reduction in splenic immunosuppressive cells and plasma insulin-like growth factor-1 (IGF-1). The effects of moderate exercise in weight stable mice culminated in a significant delay in primary tumor growth and spontaneous metastases, suggesting that exercise-induced alterations in metabolic drivers of tumorigenesis, not simply a change in body weight, underlie the protective effects in the dual intervention group. Interestingly the exercise-induced protective effect on the emergence of immunosuppressive factors and reduced tumor burden was lost when mice continued to gain weight over the course of the study, suggesting that weight gain-induced disturbances on hormonal, inflammatory, and/or immunological function can override the exercise-induced benefits. Collectively, study one provided a deeper understanding of the extent to which exercise, and changes in body weight, underlies cancer protection.

Few researchers have examined the effect of energy balance interventions on the efficacy of immunotherapeutic strategies. Two subsequent studies were designed to investigate the response to emerging cancer therapeutics in mice randomized to an energy balance paradigm (i.e., sedentary, ad libitum, weight gain [WG] group vs. exercising, mild dietary restriction, weight maintenance [WM] group) to identify potential mechanisms and provide translational support.

Study two aimed to determine if there were any additive effects of moderate exercise in weight stable mice and the therapeutic administration of a broad-based, allogeneic, whole tumor cell cancer vaccine (VAX). There was a significant effect
of both WM and VAX alone on primary tumor growth; and an additive effect of WM+VAX on primary tumor growth, lung and heart spontaneous metastases, splenocyte count at sacrifice, the number of total splenic myeloid-derived suppressor cells (MDSCs) and granulocytic subset of MDSCs, and plasma levels of IGF-1. Splenic interferon gamma (IFNγ) secretion in response to re-stimulation with tumor antigens was significantly elevated in response to VAX and WM; however, there was no additive effect of WM+VAX. These results suggested that our whole tumor cell cancer vaccine augmented the weight maintenance (via diet and exercise) effects on primary tumor growth and spontaneous metastasis; and suggested that vaccination may provide an immune stimulus to further promote the protective effects of moderate exercise alone in the metastatic 4T1.2 mammary tumor model.

Study three aimed to determine if there were any additive effects of moderate exercise in weight stable mice and the dual therapeutic administration of a whole tumor cell cancer vaccine and programmed cell death protein-1 (PD-1) checkpoint blockade. We observed a cancer prevention effect of PD-1 checkpoint blockade in WG mice on primary tumor growth and spontaneous lung metastasis. However, moderate activity in weight stable mice, independent of PD-1 checkpoint blockade, was effective in reducing primary tumor growth and metastatic burden. The WM+PD-1 group displayed the lowest number of splenic MDSCs and granulocytic MDSCs and maintained its splenic lymphoid populations. Neither the number of tumor-infiltrating immune cells, the effector or activation status of tumor-infiltrating CD4+ helper and CD8+ cytotoxic T cells, nor functional outcomes were significantly different between groups. PD-1 checkpoint blockade in WG mice,
moderate exercise in weight stable mice, and PD-1 checkpoint blockade in
moderately exercising, weight stable mice showed comparable, albeit subtle
differences, in tumor-immune crosstalk gene expression markers that drive the
expansion of immunosuppressive cell types and impact metastatic progression.
The lack of responsiveness to VAX+PD-1 checkpoint blockade in WM mice
suggests that moderate exercise in weight stable mice may be enhancing
antitumor immunity and/or reducing protumorigenic factors (i.e., similar
mechanisms mediated by VAX+PD-1 checkpoint blockade).

Results from the current studies provided insight into the extent to which
exercise in weight stable mice underlie cancer protection. Also, results provided
insight into potential mechanisms by which exercise can act via the inflammation-
immune axis to attenuate the generation of a protumorigenic and
immunosuppressive TME. These data demonstrated that preventing weight gain
through diet and exercise may be an important recommendation to maintain
prolonged antitumor effector responses and improve clinical outcomes. Results
from the current studies provided insight into potential mechanisms by which
physical activity exerts primary and secondary cancer prevention effects and
provides a biological rationale for randomized clinical trials to investigate physical
activity strategies to prevent metastatic progression in BC survivors and ultimately
improve survival outcomes.
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>4T1</td>
<td>Murine metastatic breast cancer model</td>
</tr>
<tr>
<td>Akt</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>AL</td>
<td>Ad libitum</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
</tr>
<tr>
<td>Arg-1</td>
<td>Arginase-1</td>
</tr>
<tr>
<td>BC</td>
<td>Breast cancer</td>
</tr>
<tr>
<td>CCL</td>
<td>CC-chemokine ligand</td>
</tr>
<tr>
<td>CCR</td>
<td>CC-chemokine receptor</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>Cox-2</td>
<td>Cyclooxygenase-2</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T-lymphocyte-associated protein 4</td>
</tr>
<tr>
<td>CXCL</td>
<td>CXC-chemokine ligand</td>
</tr>
<tr>
<td>CXCR</td>
<td>CXC-chemokine receptor</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMT</td>
<td>Endothelial to mesenchymal transformation</td>
</tr>
<tr>
<td>ER</td>
<td>Dietary energy restriction</td>
</tr>
<tr>
<td>EX</td>
<td>Exercise</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FoxP3</td>
<td>Forkhead box O3</td>
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<tr>
<td>G-CSF</td>
<td>Granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>gMDSC</td>
<td>Granulocytic myeloid-derived suppressor cell</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epidermal growth factor 2</td>
</tr>
<tr>
<td>IDO</td>
<td>Indoleamine 2,3-dioxygenase</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
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<tr>
<td>IGFBP</td>
<td>Insulin-like growth factor binding protein</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G, isotype control</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iIMC</td>
<td>Immature myeloid-lineage cell</td>
</tr>
<tr>
<td>iNOS</td>
<td>Nitric oxide synthase 2</td>
</tr>
<tr>
<td>IP</td>
<td>Intrapertitoneal</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motifs</td>
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<tr>
<td>ITIM</td>
<td>Immunoreceptor tyrosine-based inhibitory motifs</td>
</tr>
<tr>
<td>I.V.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>KLRG1</td>
<td>Killer cell lectin-like receptor 1</td>
</tr>
<tr>
<td>LUC</td>
<td>Luciferase</td>
</tr>
<tr>
<td>Ly6C</td>
<td>Lymphocyte antigen 6C</td>
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<tr>
<td>Ly6G</td>
<td>Lymphocyte antigen 6G</td>
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MAPK  Mitogen-activated protein kinase
MCP-1  Monocyte chemotactic activating protein
M-CSF  Macrophage colony-stimulating factor
MDSC  Myeloid-derived suppressor cell
MH  Major histocompatibility complex
mMDSC  monocytic myeloid-derived suppressor cell
mTOR  The mechanistic target of rapamycin
nDLN  Non-draining lymph node
NFAT  Nuclear factor of activated T cell
NK  Natural killer cell
NKCC  Natural killer cell cytotoxicity
NO  Nitric oxide
NF-κB  Nuclear factor kappa B
PBS  Phosphate-buffered saline
PD-1  Programmed cell death protein-1
PD-L1  Programmed death-ligand 1
PD-L2  Programmed death-ligand 2
PI3K  Phosphoinositide 3-kinase
PLC  phospholipase-C
PPARα  Peroxisome proliferator-activated receptor alpha
RIN  RNA integrity number
ROS  Reactive oxygen species
RPMI  Roswell Park Memorial Institute
SED  Sedentary
STAT  Signal Transducer and Activator of Transcription
TAA  Tumor-associated antigens
TAM  Tumor-associated macrophage
T<sub>CM</sub>  Central memory T cell
TCR  T cell receptor
T<sub>EM</sub>  Effector memory T cell
TERT  Telomerase reverse transcriptase
TGFβ  Transforming growth factor beta
TH  Helper T cell
TME  Tumor microenvironment
TNBC  Triple-negative breast cancer
TNFα  Tumor necrosis factor alpha
Treg  Regulatory T cell (CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> TH cells)
VAX  Vaccine
VEGF  Vascular endothelial growth factor
VEH  Vehicle
WG  Weight gain
WM  Weight maintenance
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CHAPTER 1: INTRODUCTION

Breast cancer (BC) is the most commonly diagnosed cancer and the second leading cause of cancer-related deaths among women in the United States (1, 2). Approximately 60% of BCs are diagnosed at the local stage and the subsequent 5-year survival is 98.5%. Unfortunately, 5% of BCs are diagnosed at the distant stage, meaning the cancer cells have metastasized. Metastatic disease remains incurable and is the underlying cause of death in the majority of BC patients who die of the disease (3). Median survival with metastatic BC is three years, and there is no statistically significant change in median survival from metastatic BC cancer in over 20 years. One significant challenge in the field of BC research is to determine how to reduce and/or eliminate the mortality associated with metastatic BC. Currently, treatments are available that slow metastatic tumor growth (e.g. estrogen blockers, chemotherapy, radiation); however, these treatments carry their own risks of morbidity and mortality. Thus, novel therapies, especially non-pharmacological, lifestyle-based interventions that may prevent or slow metastatic disease, with less severe side effects, are greatly needed.

Emerging population data suggests an inverse relation between physical activity and BC incidence (4, 5), as well as an important role for exercise in the prevention of cancer recurrence and mortality. The observational nature of these studies limit the ability to determine biological mechanisms. Several exercise-induced mechanisms are proposed, including exercise-induced improvements in the inflammation-immune axis that favors sustained immunosurveillance mechanisms. An additional limitation of the epidemiologic data is that it remains
uncertain the extent to which exercise, as opposed to changes in body weight, account for the reduction in BC risk and improvements in survival outcomes. Well-designed preclinical studies are needed to investigate mechanisms, as well as determine if direct exercise effects or exercise-induced changes in body weight drive protection.

Based on the recent success of immunotherapy in multiple solid tumors, this personalized treatment option is under active investigation in BC, especially in metastatic BC patients who have failed to respond to other treatment modalities. Two immunotherapy treatment strategies are therapeutic cancer vaccines and immune checkpoint blockade. Cancer vaccines provide tumor-associated antigen(s) to stimulate an effector T cell response (6); whereas, immune checkpoint blockade uses specific antibodies to maintain T cell activation by either binding and agonizing co-stimulatory signals or binding and antagonizing co-inhibitory signals. Cancer vaccines and checkpoint blockade are both designed to stimulate a robust antitumor effector response to eliminate tumor cells through two separate mechanisms. The release of tumor-derived factors and the generation of an immunosuppressive tumor microenvironment reduces efficacy of immunotherapy. Therefore, understanding the mechanisms by which exercise can enhance immunosurveillance and determine if exercise-induced effects can blunt the emergence or function of immunosuppressive cell populations could be a non-pharmacological approach to improve therapeutic outcomes. It is of utmost importance to delineate new, previously unidentified strategies to attenuate tumor-induced inflammation and enhance immunotherapeutic efficacy to improve treatment responses and clinical outcomes in BC patients.
CHAPTER 2: LITERATURE REVIEW

2.1. INTRODUCTION

Cancer encompasses a broad family of complex diseases that involve abnormal and unregulated cell proliferation. A group of transformed cells often form a neoplasm, or tumor; however, cells may be distributed diffusely. Cancer is the second leading cause of death globally and the number of new cases is expected to increase by approximately 70% over the next two decades (7, 8). The cause of cancer is diverse and mutations can be driven by chemical (9) or physical (10) carcinogenesis or exposure to cancer causing pathogens (11). Despite the complex nature of cancer, Hanahan and Wineberg (12, 13) detail similar characteristics that all cancers possess, allowing for unchecked proliferation and survival. These characteristics include: sustainment in proliferative signals, avoidance of programmed cell death, induction of angiogenesis, and activation of invasion and metastasis. Updated hallmarks include emerging and enabling characteristics of cancer cells, specifically cancer cell’s unique ability to drive genomic instability, generate tumor-promoting inflammation, deregulate cellular energetics, and ultimately avoid immune destruction (13).

2.2. BREAST CANCER

2.2.1. Overview of the breast

The breast is a secretory organ composed of different cell types, including mammary stem cells, epithelial cells, adipocytes, vascular endothelial cells, fibroblasts, and immune cells (14). Mammary epithelial cells are organized into two layers, the basal myoepithelial layer and the luminal
epithelial layer (15). The basal myoepithelial cell layer is composed of specialized contractile cells that provide structural support to the luminal epithelial layer and play a role in milk ejection during lactation (16). The luminal epithelial layer can be subdivided into ductal cells, which form a single layer of polarized epithelial cells around the ductal lumen, or alveolar luminal cells, which constitute the alveolar units that arise during pregnancy and lactation (17). The breast is made up of 15-20 sections called lobes. Each lobe contains smaller lobules formed by ductal and alveolar luminal epithelial cells. Lobules are the functional unit of the breast and site of milk production in lactating women (18). The development and growth of the breast is highly regulated and involves a complex interaction of hormones (e.g., estrogen, progesterone, androgens), cytokines, and growth factors (e.g., fibroblast growth factor, epidermal growth factor) that bind to specific membrane receptors to induce a cascade of intracellular events that promote cell activation, proliferation, and survival (19). However, intrinsic mutations or extrinsic stressors can result in the dysregulation of signaling cascades and the promotion of a cancer initiating event (17, 20).

2.2.2. Breast cancer

Breast cancer (BC) is the most commonly diagnosed cancer in United States with current estimates predicting 252,710 new cases in 2017 (1). BC the second leading cause of cancer-related deaths among women in the United States (1, 2) with current estimates predicting that 40,610 patients (or 6.8% of all cancer-related deaths) will succumb to the disease this calendar year.
Female BC is most commonly diagnosed in middle-aged and older women with a median age at diagnosis of 62 years (1). Overall, the five-year survival for all BC is 89.7%. Despite significant advances in detection, diagnosis, and treatment of BC, several unresolved questions remain. Polyak, et al. (21) postulates that progress must be made related to the prevention of BC, enhancements in diagnostic sensitivity, a better understanding of tumor progression and the causes of recurrence, improvements in treatment modalities, greater access to high-quality medical care, and overcoming therapeutic resistance.

BC is a heterogeneous disease characterized by a diverse spectrum of molecular phenotypes that are associated with different disease presentation events, treatment modalities, response to treatment, and clinical outcomes (21-23). Tumors can arise from the basal myoepithelial layer or the luminal epithelial layer of mammary epithelial cells and are associated with diverse molecular phenotypes. Through histological and comprehensive gene expression profiling, BC can be distinguished by up to 21 distinct histological subtypes (24) and four different molecular subtypes (25, 26). The main molecular subtypes, categorized based on epithelial cell of origin and the presence or absence of hormone receptors (estrogen or progesterone) and levels of human epidermal growth factor 2 (HER2), are luminal A, luminal B, HER2+, and basal-like (27). The majority of BCs are luminal A and B subtypes, which are typically positive for estrogen and progesterone hormone receptors. Luminal B is further defined as HER2+ and tends to be a higher grade and more
aggressive than luminal A (28, 29). The HER2\(^+\) subtype lacks hormone receptors, but overexpresses HER2, an epidermal growth factor receptor that possesses oncogenic properties (i.e., stimulates an increase in proliferation, angiogenesis, and invasiveness). Basal-like tumors lack the hormone and HER2 receptors and are often called triple-negative BC (TNBC). Although BC classification aids in determining treatment strategies and predicting prognosis, a more sophisticated classification system is needed to address the heterogeneous nature of BC and improve predictive power and prognosis (23, 28, 30, 31).

Women with operable, early, localized BC patients typically undergo surgery; either breast-conserving surgery (32) or mastectomy, coupled with radiotherapy, chemotherapy, endocrine therapy, and/or targeted therapy depending on molecular subtype (33, 34). Luminal A-type tumors are associated with the best prognosis due to the expression of hormone receptors and favorable response rates to hormonal therapy. HER2\(^+\) tumors can be effectively controlled with a diverse array of anti-HER2 therapies (e.g., Trastuzumab). Whereas, basal-like, TNBC tumors lack a molecular target, respond poorly to standard chemotherapy (approximately a 20% response rate), and are associated with the worst prognosis due to limited treatment options (30). Additionally, TNBC are often detected as grade III tumors, resulting in poor prognosis (35). A high priority for BC research is to identify better detection methods and develop more favorable treatment options to
improve TNBC outcomes. The emergence of personalized medicine and targeted immunotherapy is a promising treatment approach for TNBC.

Due to increased awareness and enhanced detection methods, many BCs are diagnosed in early stages (36). Approximately 60% of BCs are diagnosed at the local stage and the subsequent five-year survival is 98.9% (1). Regional detection (i.e., the cancerous cells have spread to a regional lymph node) accounts for approximately 30% of diagnoses with a subsequent 5-year survival of 85.2% (1). Unfortunately, 6% of BCs are diagnosed at the distant stage, meaning the cancer cells have metastasized. Metastatic disease remains incurable, has a 5-year survival of 26.9%, and is the underlying cause of death in the majority of BC patients who die of the disease (3). Although death rates are falling on average 1.8% each year over 2005-2014, likely due to enhanced treatment strategies, one significant challenge in the field of BC research is to determine how to reduce and/or eliminate the mortality associated with metastatic BC (37, 38).

Researchers have been studying metastasis for over 100 years, dating back to Stephen Paget’s 1889 ‘seed-soil’ hypothesis detailing the crosstalk between cancer cells (i.e., the seeds) and specific organ microenvironments (i.e., the soil) (39). Metastatic progression is a multi-step process that involves the interaction between tumor cells, stromal cells, and tumor-infiltrating immune cells to alter tumor angiogenesis, cell invasiveness, and cell migratory pathways to promote the dissemination of tumor cells to distant sites (39, 40). However, this dynamic process remains poorly understood (41). Currently,
treatments are available that slow metastatic progression (e.g., estrogen blockers, chemotherapy, radiation); however, these treatments carry their own risks of morbidity and mortality (42, 43). Median survival with metastatic BC is three years, and there has been no statistically significant change in median survival from metastatic BC in over 20 years (44, 45). All molecular BC subtypes are capable of progressing to metastatic disease and the molecular subtype can predict metastatic progression patterns (46, 47). For example, TNBC is characterized by an increase rate of metastatic spread to the brain and lung and a decrease in overall survival compared with patients with other metastatic molecular subtypes. TNBCs are more often diagnosed at a later stage and therefore associated with a more advanced disease. Thus, novel therapies that prevent (48) or slow metastatic disease with less severe side effects, especially within the metastatic TNBC population, are greatly needed (45).

Based on recent advancements in the fields of immunology and molecular biology, immunotherapy has become a promising emerging treatment for cancer (49-54), including TNBC and advanced metastatic disease (55-63). Immunotherapy treatments include therapeutic cancer vaccines, monoclonal antibodies, adoptive cell therapies, and the administration of immunostimulatory cytokines, all of which are designed to stimulate a robust antitumor effector response to eliminate tumor cells through several techniques (51, 64). The identification of numerous tumor-associated antigens (TAAs) (65) and the use of tumor-infiltrating immune cells as a prognostic indicator (66-76)
provides compelling evidence that BC is immunogenic. Therefore, a brief overview of the immune system is warranted.

2.3. THE IMMUNE SYSTEM IN CANCER

2.3.1. Overview of the immune system

The immune system is a complex network of cells, tissues, and organs that protect the body from infection and identify and eliminate cancerous cells (77). Immune cells originate from hematopoietic precursor cells within the bone marrow microenvironment. Diverse and multifunctional immune cell arise through an intricate series of highly regulated signaling events. Broadly, the immune system can be classified into two arms: innate (or humoral) or adaptive (or cell mediated) immunity.

2.3.2. Innate vs. adaptive immunity

The innate immune response to an invading pathogen is rapid and non-specific. Innate immune cells include dendritic cells (DCs), natural killer (NK) cells, granulocytes, myeloid-derived suppressor cells (MDSC), monocytes (macrophage precursors), and macrophages. Collectively, under normal conditions, innate immune cells play a pivotal role in first-line defense mechanisms (e.g., phagocytosis) and in the communication with and activation of the adaptive immune response to assist in pathogen clearance, targeting infected or transformed cells for death, and tissue remodeling (77, 78). DCs are antigen-presenting cells (APCs) and act as messengers between the innate and adaptive immune response by processing antigen material and presenting this information to cells of the adaptive immune system (77, 79). NK cells are
critical for innate immunity and can recognize stressed cells in the absence of antibodies or major histocompatibility complex (MHC) and lyse cells directly through the release of intracellular granules (e.g., perforin, granzymes) (80, 81). Granulocytes and macrophages are capable of phagocytosis and involved in tissue remodeling (77, 78).

The adaptive immune system is composed of highly specialized cells with specificity for an individual pathogen, toxin, or allergen (77). The adaptive response occurs secondary to the innate response because cells must encounter the antigen and clonally proliferate in sufficient numbers to mount an attack. Additionally, a key feature of the adaptive response is its ability to generate long-lived memory cells, which confer lasting protection (82). Adaptive immunity is carried out by lymphocytes. Lymphocytes can be categorized as B cells, which perform antibody-specific responses, or T cells, which perform cell-mediated responses (83). B cells are central in antibody-mediated responses, but also have an important role in processing and presenting antigens and secreting cytokines to refine the immune response (84). T cells represent a heterogeneous population that include cluster of differentiation (CD)4+ helper T cells and CD8+ cytotoxic T cells. Broadly, CD4+ helper T cells play a major role in mediating immune responses through the secretion of specific cytokines. Various activation signals and differentiation pathways generate a diverse population of CD4+ helper T cell (T_H) subsets with distinct functions (classical T helper 1 [T_H1], T helper 2 [T_H2], T helper 9 [T_H9], T helper 17 [T_H17], T helper 22 [T_H22], follicular helper T cell [T_FB], and
regulatory T cells [Tregs]) (85). Lastly, activated CD8⁺ cytotoxic T cells can recognize infected, stressed, or transformed cells through recognition of peptides presented on MHC class I molecules and induce apoptosis via the release of cytotoxins, perforins, and granzymes or through the expression of surface proteins (e.g., Fas ligand) (86).

The innate immune system is capable of phagocytoses and aids in the clearance of extracellular pathogens. Both the innate system, specifically NK cells, and the adaptive system, most notably CD8⁺ cytotoxic T cells, are instrumental in the detection and clearance of intracellularly infected or transformed cell populations (77). They do so through the detection and interaction with MHC molecules present on target cells. Two classes of MHC molecules exist, MHC class I and MHC class II. MHC class I molecules are found on all nucleated cells and platelets. NK cells are primed to induce programmed cell death; however, extracellular inhibitory receptors on NK cells can bind to MHC class I and prohibit cell killing (80, 81). Pathogenic signaling and tumorigenic factors can induce the downregulation of MHC class I molecule as a mechanism to avoid immune detection. The removal of MHC class-I mediated inhibition allows NK cells to release factors to promote the programmed cell death of the target cell (81). Infected and transformed cells can load and present endogenous antigens via MHC class I molecules. CD8⁺ cytotoxic T cells recognize their specific antigen in the context of MHC class I, triggering programmed cell death mechanisms.
APCs, like DCs, can phagocytose exogenous (i.e., viral or bacterial peptides) or endogenous (i.e., self-peptides) antigens and process them by proteases to peptide fragments (77). Activated DCs migrate to proximal draining lymph nodes where they can present antigens (79). The activation of a naïve T cell occurs through the simultaneous engagement of the T cell receptor (TCR) and co-stimulatory molecules (e.g., CD28 or inducible T cell co-stimulator [ICOS]) on the T cell surface by MHC (CD4 in the context of MHC class II and CD8 in the context of MHC class I) and co-stimulatory molecules (e.g., CD80 [B7.1] and CD86 [B7.2]) on APCs (87). TCR stimulation alone results in anergy (or T cell tolerance); whereas, co-stimulation alone has no effect on T cell activation. TCR engagement orchestrates essential changes in the gene expression profile to transition a naïve cell to an actively expanding immune effector cell. Intracellular signaling involves a myriad of molecules, with the most common being Src family kinases (e.g., Fyn, Lck), which phosphorylate tyrosines on intracellular immunoreceptor tyrosine-based activation motifs (ITAMs) on the intracellular domain of CD3 zeta (ζ)-chain (88). Phosphorylation events result in downstream signaling mediated by phospholipase-C (PLC)γ, mitogen-activated protein kinase (MAPK), nuclear factor kappa B (NF-κB), and nuclear factor of activated T cell (NFAT), which results in gene transcription in the nucleus of factors that promote cell survival and proliferation (87). Co-stimulatory and cytokine signaling, especially signaling via interleukin (IL)-2 (89), activate phosphoinositide-3 kinase (PI3K) and its downstream target, protein kinase B (Akt), which increases expression
of glucose transporters on the cellular membrane and upregulates glycolytic enzyme activity to meet the metabolic needs of the highly proliferative effector cells (90). Cytokine signaling also activates specific transcriptional factors to induce CD4+ helper T cell polarization (i.e., TH1, TH2, TH9, TH17, TH22, TFH, and Tregs).

2.3.3. Immunosurveillance

The role of the immune system in controlling primary tumor growth is well studied (91-93) and involves anti-tumor responses by NK cells and CD8+ cytotoxic T cells, which recognize transformed cells and prevent their expansion in a process called immunosurveillance. In humans, NK cells comprise approximately 5-15% of all circulating lymphocytes (94) and can infiltrate a pathogen-infected site or a malignant tumor microenvironment (TME) upon activation (95, 96). The number of NK cells within the tumor and in the stroma near the cancer nests significantly correlates with a prolonged survival time in patients with various types of cancer, including BC (74). Within the TME, NK cells can detect malignant cells lacking MHC class I or via the identification of “stress” ligands on targets through NK cell receptors (e.g., NKG2D), which can render tumor cells susceptible to NK cell-mediated lysis (81). Activated NK cells eliminate targets through the release of cytotoxic enzymes (e.g., perforins, granzymes, granulysin) and/or soluble factors (chemokines and inflammatory cytokines [e.g., interferon-gamma (IFNγ)] (97-99)) which, in turn, can recruit and/or activate other immune effectors cells. CD8+ cytotoxic T cells recognize antigens in the context of MHC class I on the
surface of virally infected or malignant cells and rapidly proliferate in response to activation (100). Activated CD8+ cytotoxic T cells lyse target cells through perforin/granzyme and Fas/FasL mediated mechanisms, as well as the production of IFNγ, an immunoregulatory and antitumor effector molecule (101, 102). IFNγ plays a role in promoting protective host responses against tumors by up-regulating tumor cell MHC class I antigen processing and presentation (100, 103). There is compelling evidence for an improved prognosis for BC patients that have increased intratumoral CD8+ cytotoxic T cell infiltration (66-73). Additionally, type I CD4+ helper T cells (Th1) produce cytokines (e.g., IFNγ) and chemokines to recruit and activate NK cells and CD8+ cytotoxic T cells (104) to further promote anti-tumor immunity. When immunosurveillance is successful, immune-mediated cell death of transformed cells can result in complete elimination or pressure the growing tumor into a state of equilibrium (105). Accumulating clinical (106) and experimental evidence (107) strongly suggests similar immunosurveillance mechanisms are involved with the recognition of disseminated tumor cells. Memory T cells and NK cells in metastatic niches (including the bone (108-111), lung (112-115), and liver (116) microenvironment) can promote immune-mediated tumor dormancy (117). Thus, BC progression is highly linked to immunosurveillance mechanisms.

2.3.4. The tumor microenvironment

As a transformed, cancerous cell progresses to a neoplasm, or tumor, it generates a complex TME that is characterized by increased intrinsic inflammatory gene transcription factors, inflammatory cytokines, and
proinflammatory signaling molecules (118, 119). These factors deregulate hematopoietic pathways resulting in the expansion and accumulation of immunosuppressive cell types of both lymphoid (e.g., Tregs) (120, 121) and myeloid lineage (e.g., tumor-associated macrophages [TAMs] (122, 123) and MDSCs) (124-128). The abundance of both lymphoid and myeloid immunosuppressive cells is positively correlated with tumor burden (129-133). Other immune cell types (e.g., regulatory B cells (134), neutrophils (135, 136)) or supporting cells within the TME (e.g., mesenchymal stromal cells (137)) are intricately involved in the generation of a proinflammatory, immunosuppressive TME. Crosstalk exists between these cell types to promote a feed-forward loop favoring a proinflammatory immune evading microenvironment (131, 138-140). The balance between anti-tumor immunosurveillance mechanisms and the accumulation of immunosuppressive cells within the TME is critical for regulating primary tumor growth (141) and metastatic progression (142).

2.3.5. Protumor immunosuppressive cells

Tumors can commandeer numerous cell types to promote sustained proliferation and aid in cancer progression. The main immunosuppressive cells, Tregs, TAMs, and MDSCs, are summarized within this literature review.

2.3.5.1. Regulatory T cells

Tregs are a subset of CD4\(^+\) helper T cells that co-express CD4, CD25, and the gene transcription factor FoxP3 (143). Tregs can develop in the thymus (i.e., natural Tregs), which comprise 5-10% of total peripheral CD4\(^+\) helper T cells, or be generated in the periphery from
conventional CD4+ helper T cells (i.e., inducible Tregs) (144). Under normal conditions Tregs are essential in the homeostasis and modulation of the immune response by resolving inflammation and promoting peripheral immune tolerance (143). Dysregulated Treg expansion and function can play a role in autoimmune diseases, transplantation tolerance, infectious diseases, allergic disease, and tumor immunity.

Tumor-infiltrating Tregs are observed in BC (145-148) and the Treg subset can comprise up to 45% of total CD4+ helper T cell populations within the TME (149). Increased intratumoral Tregs is correlated with BC progression and is an indicator of poor prognosis (150). Recent findings support the investigation of Treg-targeted therapies to attenuate the Treg-induced suppression of antitumor effector responses and improve therapeutic outcomes.

2.3.5.2. Tumor-associated macrophages

Monocytes are recruited to the TME by secreted factors (e.g., vascular endothelial growth factor [VEGF], macrophage colony-stimulating factor [M-CSF]) and differentiate into macrophages. Based on the cytokine milieu generated by tumor cells, stromal cells, and other tumor-infiltrating immune cells, macrophages within the TME can polarize into cells with antitumor or protumor function, and are broadly categorized as M1 (classical) or M2 (alternative) macrophages, respectively (122). M1 macrophages inhibit tumor growth via phagocytosis and the secretion of proinflammatory cytokines (e.g., tumor-necrosis factor alpha [TNFα], IL-
1, IL-6, IL-12, and IFN$\gamma$) (135). Also, M1 cytokines can recruit both CD4$^+$ helper and CD8$^+$ cytotoxic T cells to the TME and promote the polarization of CD4$^+$ helper T cells to the antitumor, IFN$\gamma$-producing T$_{H1}$ phenotype. M2 macrophages play an important role in wound healing and tissue repair through the secretion of growth factors (e.g., VEGF, epidermal growth factor, and platelet-derived growth factor) and anti-inflammatory cytokines (e.g., IL-10, transforming growth factor beta [TGF$\beta$], IL-4, and IL-13); however, in the context of cancer, these mechanisms can promote tumor growth (122). TAMs closely resemble M2-polarized macrophages and aid in cancer progression by promoting tumor cell proliferation, angiogenesis, immunosuppression, and extracellular matrix turnover (123).

TAM density within the TME is correlated with angiogenesis and tumor cell invasion and migration (151-153). The presence of TAMs as a prognostic indicator in BC is emerging. Two recent meta-analyses collected data from five (154) and sixteen (155) studies and evaluated the correlation between TAMs (detected via immunohistochemistry) and clinical staging, overall survival, and disease-free survival in BC patients. Results indicate that high intensity TAM expression was correlated with poor disease-free and overall survival, and suggest that TAM infiltration can serve as a novel prognostic factor in BC patients. Two active areas of research are identifying the negative impact of TAM on BC therapies or directly targeting TAMs to enhance current and emerging therapies.
2.3.5.3. Myeloid-derived suppressor cells

MDSCs represent a heterogeneous population of immature myeloid-lineage cells that significantly expand in both animal models and human BC patients and possess immunosuppressive and proangiogenic properties, which facilitate tumor growth and promote metastases (125, 127, 156). In healthy individuals and mice, MDSCs are present in low numbers in the circulation and regulate immune responses and promote tissue repair. Under normal conditions, immature myeloid-lineage cells (iMCs) quickly differentiate into mature granulocytes, macrophages, or DCs (125, 127). However, in pathological inflammatory conditions such as infectious diseases (157, 158), autoimmunity (159), and cancer (160, 161), this heterogeneous family of iMCs is halted in an immature state and expands from the BM into the circulation and peripheral organs. Clinical studies reveal that BC stage and metastatic tumor burden is positively correlated with the level of circulating MDSCs (162) and elevated circulating MDSCs is indicative of a poor prognosis (163, 164).

The intricate network regulating the expansion, trafficking, and activation of MDSCs is reviewed elsewhere (124, 125, 127) and not only involves crosstalk between MDSCs and tumor cells, but also communication between MDSCs and other tumor-infiltrating cells (e.g., TAMs and Tregs) (139, 165-167). Briefly, key cytokines and soluble factors that block myeloid maturation and promote MDSC expansion include granulocyte-colony stimulating factor (G-CSF) (168, 169),
granulocyte macrophage-colony stimulating factor (GM-CSF) (170), M-CSF (171), stem cell factor (172), VEGF (173), IL-6 (171, 174), and prostaglandin E2 (PGE₂) (175). Collectively, these factors trigger a signaling cascade in iMCs, mainly through Janus tyrosine kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3), resulting in alterations in downstream targets that regulate cell differentiation, proliferation, survival, and apoptosis and block normal physiological differentiation of iMCs; thus, driving the expansion of pathological MDSCs (125).

Numerous factors are involved in MDSC trafficking to the tumor site and/or premetastatic niche (124, 156, 176). Chemokine ligands of the C-C (CCL2 and CCL12) (177-179) and C-X-C motif (CXCL1, CXCL5, and CXCL12) (178, 180) are released from the TME and interact with related chemokine receptors on MDSCs (CCR2, CXCR2, and CXCR4) (177-180) to promote mobilization. Additional studies detail the essential roles of integrin and selectin adhesion molecules (181) and tumor cell Fas-ligation induced PGE₂ production (182) in promoting MDSC mobilization. Expansion-inducing factors can also induce mobilization, most notably tumor-derived G-CSF, GM-CSF, and S100A8/A9 proteins (125, 183). Tumor-derived S100A8/A9, in addition to STAT3-induced production of S100A8/A9 from MDSCs, promote accumulation via an autocrine feedback loop. S100A8/A9 bind to surface receptors for advanced glycation end products on MDSCs and inhibit DC differentiation pathways.
and promote the mobilization of MDSCs through NF-κB signaling (183).

Thus, MDSC recruitment to the TME represents a vicious cycle, with tumor-infiltrating MDSCs augmenting the initial tumor-driven recruitment.

2.3.6. Immunosuppressive mechanisms

The mechanism(s) of Treg-mediated (184-186), TAM-mediated (187), and MDSC-mediated (125, 127, 139, 167) immunosuppression of antitumor effector cells is thoroughly reviewed and likely function through cell-surface receptors and/or through the release of short-lived soluble mediators. In the context of the TME, Tregs exert immunosuppressive effects by direct interaction with cells through cell surface expression of inhibitory molecules (e.g., cytotoxic T-lymphocyte-associated protein 4 [CTLA-4] (188)) and the production of immunosuppressive cytokines, such as IL-10, IL-35, and TGFβ (184, 185). In general, TAMs and MDSCs can mediate suppressive functions through multiple players, such as Arg-1 and nitric oxide synthase 2 (iNOS), the hyperproduction of NO and reactive oxygen species (ROS), induction of Tregs, as well as deregulation of cyclooxygenase-2 (Cox-2)/PGE₂, TGFβ, and IL-10 pathways.

ROS can catalyze the nitration of the T cell receptor (TCR) (189), downregulate the ζ-chain of the TCR complex (190), and interfere with IL-2 signaling (a signal essential to T cell proliferation) (191) resulting in the desensitization of the TCR response and T cell unresponsiveness. Furthermore, enzyme metabolites from iNOS, specifically NO, can interfere with T cell JAK/STAT signaling proteins required for numerous T cell functions,
inhibit MHC class II expression, and induce T cell apoptosis (125). These enzymatic pathways also produce superoxide, which combines rapidly with NO to form peroxynitrite, a powerful oxidant (192). Increased peroxynitrite induces post-translational modifications, such as nitration of the TCR and CD8 molecules, resulting in antigen-specific T cell unresponsiveness (193).

Tumor cell and tumor-infiltrating immunosuppressive cells deplete nutrients (such as L-arginine, L-cysteine, and L-tryptophan) from the environment via consumption and sequestration to dysregulate APC function and the activation and proliferation of antitumor effector cells (194, 195). Depletion of L-arginine downregulates the ζ-chain of the TCR complex and results in the proliferative arrest of antigen-activated T cells (194-196). Tumor cells, TAMs, and MDSCs express Ido1, which encodes for indoleamine 2,3-dioxygenase (IDO), the first and rate-limiting enzyme of tryptophan catabolism through the kynurenine pathway (197). Depletion of tryptophan, an amino acid essential for T cell activation, results in blunted T cell proliferation, differentiation, effector functions, and viability (198). Additionally, bioactive kynurenine pathway compounds can activate the aryl hydrocarbon receptor on CD4+ helper T cells, promoting Treg polarization (199). Lastly, T cells are unable to synthesize cysteine and rely on APCs to convert methionine or cystine to cysteine and export surplus cysteine to T cells during antigen presentation (195). However, MDSCs also import cystine for intracellular conversion to cysteine, but lack the ability to export cysteine back into the extracellular space; thus, limiting cystine pools for APC conversion (195). As a
result, APCs cannot export cysteine, which deprives T cells of an amino acid essential for activation and proliferation. Tumor-derived S100A8/A9, in addition to STAT3-induced production of S100A8/A9 from MDSCs, inhibit the maturation of DCs (200). The culmination of these suppressive mechanisms results in less functional DCs and less activated effector T cells.

2.4. IMMUNOTHERAPY

2.4.1. Overview of immunotherapeutic strategies

Cancer immunotherapy, deemed by Science magazine (49) as the 2013 “Breakthrough of the Year” and the 2016 and 2017 ASCO “Advance of the Year” (201, 202), has demonstrated success in treating cancer, including very advanced, metastatic diseases. Immunotherapy treatments include therapeutic cancer vaccines, monoclonal antibodies, adoptive cell therapies, and the administration of immunostimulatory cytokines, all of which are designed to stimulate a robust antitumor effector response to eliminate tumor cells through several techniques (51, 64). Therapeutic cancer vaccines provide TAAs to stimulate an effector T cell response (6); whereas, immune checkpoint blockade, like anti-CTLA-4 and anti-programmed cell death protein-1 (PD-1), use specific antibodies to target inhibitory cell surface molecules to promote sustained effector cell activation (203). Emerging immunotherapy strategies are currently being investigated in advanced-stage cancer patients who have failed to respond to other treatments (49-54), including TNBC and advanced metastatic disease (55-63).
Despite the recent successes of immunotherapy in advanced-stage cancer patients, less than half of patients who receive immunotherapy experience an objective, durable response (204). Thus, there is great interest in understanding the factors that contribute to the heterogeneity in response rates to immunotherapy. Areas of research include the identification of clinically meaningful biomarkers to direct treatment choices (i.e., personalized medicine) and/or through the selection of appropriate combinatorial strategies (aimed to enhance antigen presentation, reverse T cell dysfunction, and/or target immune inhibitory mechanisms) without inducing adverse effects (56-58, 205). The release of tumor-derived factors and the generation of an immunosuppressive TME reduces immunotherapeutic efficacy. No study, to our knowledge, has retrospectively or prospectively assessed the impact of host factors known to modulate immune function (e.g., physical activity, body mass index, or dietary and sleep patterns) on the efficacy of immunotherapeutic outcomes. Therefore, understanding the mechanisms by which lifestyle factors can enhance antitumor immune mechanisms or blunt the emergence of immunosuppressive cell populations could be a non-pharmacological approach to improve therapeutic outcomes. It is of utmost importance to delineate new, previously unidentified strategies to attenuate tumor-induced inflammation and enhance immunotherapeutic efficacy to improve treatment responses and clinical outcomes in cancer patients.
2.4.2. Cancer vaccines

Cancer vaccines can be prophylactic (i.e., preventative) (206) or therapeutic (51). Therapeutic cancer vaccines, categorized as DC or whole tumor cell based, provide APCs, like DCs, with TAAs to stimulate a durable antitumor effector CD8+ cytotoxic T cell response (51, 52, 207-209). Providing tumor-derived peptides (210) or full-length recombinant tumor proteins are two strategies for DC-based therapeutic cancer vaccines; however, the development of in vivo tumor-derived peptide vaccinations has proven problematic as free peptides can be rapidly cleared prior to uptake by APCs (51). The co-administration of immune-stimulant adjuvants (e.g., IL-2 or GM-CSF) with tumor-derived peptides to further promote the activation of either DCs or CD8+ cytotoxic T cells has increased vaccine efficacy, generating a more robust antitumor effector CD8+ cytotoxic T cell response (209, 211). An alternative strategy is to generate DCs ex vivo from peripheral blood mononuclear cells, pulse with TAAs, and inject the activated DCs back into the host for subsequent activation of a CD8+ cytotoxic T cell response (208, 212, 213). Two limitations in DC-based vaccines is that T cell epitopes against TAAs are largely undefined, making it difficult to screen and select tumor antigens (214, 215), and ex vivo generation of DCs is laborious and costly (216).

Whole tumor cell based cancer vaccines represent an attractive alternative source of TAAs (sourced from whole tumor cells, tumor cell lysates, tumor oncolytes, apoptotic bodies, or transduced tumor cells), as this type of vaccine typically displays some intrinsic adjuvant activity and allows DCs to
process and present numerous TAAs to induce a polyclonal effector CD8⁺ cytotoxic T cell response against numerous tumor cell subpopulations (207, 217). Through epitope spreading, or the process in which T cells respond to peptides not present in the whole tumor cell cancer vaccine, tumor cells that are not originally targeted through TAAs within the whole tumor cell cancer vaccine can become targets through secondary priming (218-220). Autologous (i.e., sourced from the same host receiving the vaccination) and allogeneic (i.e., sourced from a different host or tumor cell line) whole tumor cell cancer vaccines are under preclinical (221-226) and clinical (227-229) development.

Several host-related factors (e.g., tumor-induced inflammation and/or emergence of immunosuppressive cell types) can influence the efficacy of whole tumor cell based cancer vaccines (208, 230), therefore combinatorial strategies are investigating if current (e.g., chemotherapy, radiation) and emerging (e.g., monoclonal antibodies, tyrosine kinase inhibitors) therapies can reduce tumor-induced inflammation to enhance therapeutic efficacy (224, 230-234).

2.4.2.1. Clinical studies

Cancer vaccine therapies, both tumor-derived peptide based and whole tumor cell based are under investigation in BC patients. The majority of clinical trials assessing peptide-based strategies target HER2-derived peptides alone, or in combination with additional immunostimulatory agents (235). For example, a well-studied (236) tumor-derived peptide-based vaccine targeting HER-2 (E75, also known
as NeuVAX) is now being investigated clinically. NeuVAX combined with GM-CSF administration prolonged disease-free survival in a subset of BC patients who express low levels of HER2 with a high risk of relapse (237). A phase II study (NCT01570036) is underway to investigate the combination of NeuVAX and targeted HER2 therapy in node positive (or node-negative if estrogen and progesterone receptor negative) BC patients who are disease-free after standard-of-care therapy. However, a study investigating the administration of NeuVAX+GM-CSF in early-stage, node-positive BC patients with low-to-intermediate HER2 expression was halted due to ineffectiveness (NCT01479244). New targets including peptides against Wilms tumor protein antigen (238) and immunization with vaccinia virus modified to express mucin-1 and IL-2 (239) can induce tumor regression in BC patients. The identification of tumor-specific antigens in which to target remains an active area of research.

Clinically, whole tumor cell cancer vaccines are rarely investigated as a monotherapy in patients with advanced stage disease. These therapies are more typically genetically modified to secrete various cytokines, such as GM-CSF, administered in combination with an immune adjuvant (214, 229), or administered in combinatorial treatment strategies coupled with chemotherapy, endocrine therapy, and/or targeted therapy (240-242). In a phase I clinical trial, the safety of a plasmid transfected/allogeneic HER2-positive cell-based GM-CSF-secreting vaccine was confirmed in metastatic BC patients. The vaccine alone or in
sequence with low-dose chemotherapy induces HER2-specific T cell-mediated immunity; however, survival data is forthcoming (240). A separate Phase I trial modified MDA-MB-231 cells to express B7-1 (CD80), a costimulatory molecule required for antigen-presentation to T cells. Vaccinated stage IV metastatic BC patients had an increase in tumor-specific immune responses, but failed to show differences in tumor progression (243). The administration of a mixed vaccine composed of autologous breast tumor cells supplemented with allogeneic breast tumor cells, 3 additional TAAs antigens combined with IL-2 and GM-CSF to BC patients in a phase II study increases antigen-specific lymphocyte response and improves clinical response (244). The administration of this mixed vaccine to BC patients with depressed immunity improves ten-year survival compared with historical data (228). Two active clinical trials are investigating the safety and efficacy of modified autologous vaccinations engineered to express GM-CSF in metastatic BC patients (NCT00317603 and NCT00880464). Future studies are required to identify the optimal combinatorial or multi-antigen approach to improve survival outcomes.

2.4.2.2. Preclinical studies

Few researchers have designed preclinical studies to investigate cancer vaccine monotherapy in murine models of mammary carcinogenesis. Cell lysate from 4T1 encapsulated in poly(lactic-co-glycolic acid) acid (PLGA) microparticles reduces spontaneous lung metastasis, but has no effect on primary tumor growth (222). A placental
endothelial cell vaccine (ValloVAX) administered SQ for four weekly vaccinations after tumor implantation inhibits 4T1 tumor growth, however immune effector function was not characterized (245). Combinatorial strategies are being investigated in both peptide-based and whole tumor cell based vaccines. Combinatorial immunostimulatory strategies include genetically modifying cells to express GM-CSF, CD40L, OX-40, and B7-1 or the co-administration of IL-12 in the vaccination protocol (225, 226, 234, 246). Soliman, et al. (225) vaccinated 4T1 tumor-bearing mice with irradiated B78H1 bystander cell lines engineered to secrete GM-CSF and CD40 ligand, a costimulatory ligand important for T cell activation. Vaccinated mice display a reduction in tumor growth, concurrently with an increase in the secretion of IL-2 and IFNγ from splenic T cells and an increase in the number of tumor-infiltrating T cells. The anchoring of IL-12 and the B7-1 costimulatory molecules to 4TO7 cells results in significantly lower tumor burden compared with controls after one treatment (246). Vaccinated mice also display a reduction in tumor angiogenesis and an increase in tumor-infiltrating CD8⁺ cytotoxic T cells. Data from Yan, et al. (226) demonstrates that the administration of irradiated, transfected 4T1 cells that express VEGFR2 inhibits subsequent tumor growth and lung metastasis. Lastly, the intranodal administration of autologous tumor-derived autophagosome-based therapeutic vaccine (DRibbles) with an anti-OX40 co-stimulatory antibody enhances T cell priming and antitumor efficacy in 4T1 tumor-bearing mice (234). The findings from these results
indicate that anchored cytokines or the addition of co-stimulatory molecules may improve therapeutic potency.

The investigation of combinatorial strategies that prevent the emergence or function of immunosuppressive factors is underway. To minimize the induction of Tregs, Monzavi-Karbassi, et al. (247) developed a vaccine composed of cell lysate lacking lectin-reactive glycoconjugates. The vaccination of 4T1 tumor-bearing mice reduces tumor growth and spontaneous lung metastasis. In contrast, the crude lysate activates Tregs and induces their suppressive function measured via a mixed suppression assay. This suggests that minor refinements in TAAs can be performed to weaken immune suppression and improve antitumor immune responses.

Cyclooxygenase-2 (Cox-2), a rate-limiting enzyme in the synthesis of prostaglandins from arachidonic acid, is highly upregulated in mammary cancers (248) and plays a key role in stimulating epithelial cell proliferation, inhibiting apoptosis, stimulating angiogenesis, and mediating immune suppression (248, 249). The short-term administration of celecoxib, a selective Cox-2 inhibitor, as a monotherapy can reduce 4T1 tumor growth (250-252). Celecoxib administered in combination with a tumor-lysate pulsed DC vaccine plus GM-CSF adjuvant therapy, results in the greatest reduction in 4T1 tumor growth and spontaneous lung metastasis, an increase in the secretion of IFN\(\gamma\) and IL-4 from re-stimulated CD4\(^+\) helper T cells, and an increase in abundance of tumor-infiltrating CD4\(^+\) helper and CD8\(^+\) cytotoxic T cells compared to
unvaccinated tumor-bearing controls (251). These data suggest that inhibiting the expansion of Tregs or blocking Cox-2 via short-term celecoxib therapy may be safely used to increase vaccine efficacy for treating metastatic BC.

2.4.3. Immune checkpoint blockade

In normal physiology, inhibitory immune checkpoints are important to protect against activation events targeted toward self-antigens (i.e., autoimmunity) and to return the adaptive response to a basal level and function following the clearance of a foreign agent in a process called homeostatic control (50). However, inhibitory immune checkpoints may act as a barrier to the successful identification, activation, and eradication of malignant self-cells by effector immune cell populations. Following T cell activation by APCs, T cells migrate to specific sites and perform their effector function. Upon activation, T cells upregulate inhibitory cell surface markers, including OX-40, lymphocyte-activation gene 3 (Lag-3), T cell immunoglobulin and mucin-domain containing-3 (TIM-3), PD-1, and an increasingly expanding list of other inhibitory molecules (53, 203, 253). The immune checkpoint blockade approach to immunotherapy involves using antibodies to maintain T cell activation by either binding and agonizing co-stimulatory signals or binding and antagonizing co-inhibitory signals (53, 57). The mechanism of PD-1 inhibition and the use of PD-1 checkpoint blockade in clinical and preclinical models are reviewed.

Upon activation, TCR-mediated calcium influx can induce Pdcd1 transcription (the gene encoding for PD-1) via NFATc1 (254). In tumor-bearing
hosts, proinflammatory signals (e.g., IFNγ) produced in the TME, signal to tumor cells, stromal cells, and tumor infiltrating immune cells (e.g., MDSCs) to increase the expression of programmed death-ligand 1 (PD-L1 or CD274) and PD-L2 (CD273), which can bind to PD-1 on T cells (203). When PD-1 is engaged by its ligand, the PD-1 intracellular domain, composed of an immunoreceptor tyrosine-based inhibition motif (ITIM), inhibits kinases involved in T cell activation through the recruitment of Src homology 2-containing tyrosine phosphatase (SHP2) (53). PD-1 induced SHP2 can directly inhibit TCR transduction by dephosphorylating and inactivating Zap70, a major integrator of TCR-mediated signaling (254). Additionally, SHP2 can block the induction of PI3K activity and downstream Akt signaling, resulting in an inhibition in the ability of T cells to import glucose, rendering T cells more susceptible to apoptosis (255, 256); thus, shutting down the antitumor response.

PD-1 is widely expressed on several cells important in antitumor immunity, including B cells, NK cells, and CD4+ helper and CD8+ cytotoxic T cells (257). Therefore, PD-1 checkpoint blockade has broad action on a number of antitumor effector responses. Although there is excitement in the field and clinical successes are reported, the response rate to PD-1 blockade is under 50%. Thus, there is a growing interest in improving response rates to immune checkpoint blockade through the identification of the phenotype of responders with the long-term goal of administering targeted, personalized therapy and/or combinatorial treatment strategies (e.g., chemotherapy, cancer vaccines).
2.4.3.1. Clinical studies

Recent clinical BC studies target PD-1 in TNBC because it is associated with limited treatment options (55, 258), high expression of PD-L1 in the TME, and an increase in tumor-infiltrating immune cells (72, 259-261); thus, making immunotherapy an attractive treatment option. Clinically, PD-L1 protein expression is detected in 20-30% of BC patients and single-agent PD-1 blockade can result in objective and durable clinical responses in BC patients, with responses more favorable for patients with tumors positive for PD-L1 (257). Currently, there are close to 50 ongoing, or soon to open, clinical trials evaluating the efficacy of immunotherapy strategies alone or in combination with current and emerging therapies in the neoadjuvant and metastatic setting in BC patients (55-58).

2.4.3.2. Preclinical studies

In the preclinical setting, the 4T1 series of BC cells are largely considered poor responders to single-agent PD-1 blockade due to the expansion of immunosuppressive MDSCs (262, 263). Data from Hirano, et al. (264) confirm that 4T1 tumors upregulate PD-L1 in vivo and blockade of PD-L1 (100 µg) at day 7 and 10 post-tumor implantation partially inhibits primary tumor growth. Beavis, et al. (265) demonstrates that single agent PD-1 checkpoint blockade (200 µg, administered at day 7, 11, and 15 post-tumor implantation) is not effective at reducing 4T1.2 primary tumor growth or spontaneous lung metastasis when mice are removed from
study at day 18 post-tumor implantation. This lack of effect was attributed to low levels of PD-1 cell surface expression on T cell subsets during 4T1.2 tumor progression.

Dual interventions that target the proinflammatory TME concurrently with PD-1 or CTLA-4 checkpoint blockade result in regression of 4T1 tumors, even when disease burden is advanced and metastatic (266). The administration of PD-1 checkpoint blockade in combination with therapies that target a reduction in systemic inflammation (e.g., celecoxib [selective Cox-2 inhibition], azacitine or entiostat [epigenetic modulating drug], and J32 [phosphoinositide 3-kinase inhibitor]) are the most effective at driving an enhancement in antitumor effector function, a reduction in established tumor growth, and a reduction in spontaneous metastasis (266-270). However, no preclinical study has tested if lifestyle-based interventions, i.e., physical activity and the prevention of weight gain, which are low-cost and have known benefits across the cancer continuum (271), can augment T cell responses (272, 273) and can enhance the efficacy of immunotherapeutic strategies.

2.5. RISK FACTORS FOR BREAST CANCER

Although the etiology of BC remains unclear (274), several non-modifiable and modifiable risk factors are implicated in the risk of developing BC. While it is nearly impossible to determine the cause of a cancer in an individual, population-based statistics can draw broad conclusions on factors that increase or decrease the risk of developing certain cancers. Cancer is likely caused by a combination of
non-modifiable and modifiable risk factors (275-280). Due to the complexity of events that drive cancer initiation, it is unlikely that one factor is responsible for cancer initiation. An appreciation of one’s risk factors can guide decision making over one’s lifetime to reduce the risk of developing cancer.

Non-modifiable, or fixed, risk factors of BC include age, sex, race and ethnicity (White > African > Asian), family history of BC, personal history of BC, menstrual history (early menarche, late menopause), dense breast tissue, previous ionizing radiation exposure to the chest, and genetic predisposition (e.g., mutations in BRCA1 or BRCA2 genes) (281-285). Increased age (greater than 45 years-old) is the largest non-modifiable risk factor for BC (1). Compared with white women, African American women present with BC at a younger age, are less likely to be diagnosed with local stage cancer, and have worse disease-free and overall survival rates within each stage (1, 286, 287). The reason for race/ethnicity differences in BC risk and prognosis is difficult to disentangle.

Modifiable risk factors that decrease the risk of BC include age at first pregnancy (35 or younger is protective) and physical activity (4, 288-292). Emerging research suggests that non-starchy vegetables, foods containing carotenoids, dairy products, and diets high in calcium may lower the risk of BC; however, additional studies are needed confirm this relation (293-297). Data from clinical (298-300) and epidemiologic (4, 5, 301) studies support the existence of an inverse relation between moderate-to-vigorous physical activity and both pre- and postmenopausal BC incidence, although the evidence is stronger for postmenopausal women (275). Modifiable risk factors that increase the risk of BC
include smoking, the use of hormone replacement therapy, nulliparity, age at first pregnancy (35 or older increases risk), alcohol consumption, and physical inactivity (302-307). Elevated weight has contrasting effects on the risk of premenopausal and postmenopausal BC risk (308, 309); however, being overweight or obese serves as a predictor of poor prognosis once BC is diagnosed regardless of menopausal status (310). The combination of increased body weight and physical inactivity are estimated to account for 30% of the BC risk in industrialized societies (311, 312) and are highly linked to the etiology of BC. Therefore, since an increase in body weight represents a known, modifiable risk factor for postmenopausal BC, general weight control during adulthood may be an effective strategy for BC prevention.

The current nutrition and physical activity guidelines proposed by the American Cancer Society (ACS), as well as the World Cancer Research Fund (WCRF)/American Institute for Cancer Research (AICR) recommend that individuals maintain a healthy weight throughout life by balancing caloric intake, engaging in physical activity, and losing weight if overweight or obese (275, 313). Dietary recommendations include consuming a healthy diet with an emphasis on plant foods and a reduction in processed meat and red meat. Physical activity recommendations for cancer prevention include engaging in at least 150 minutes of moderate intensity or 75 minutes of vigorous intensity activity each week. Several cohort studies have evaluated adherence to the ACS and WCRF/AIC guidelines and assessed BC prevention (314-317). Collectively, these studies report 16% to 60% risk reductions in postmenopausal women who adhere to the
guidelines, with the association mainly linked to reduced body fatness and alcohol intake rather than specific differences in dietary patterns. BC survivorship populations often face lasting psychological, physical, and social side effects; therefore, similar guidelines are recommended for BC survivors to improve quality of life measurements.

2.5.1. Diet and breast cancer incidence and mortality

2.5.1.1. Clinical and epidemiologic studies

The role of diet, a modifiable risk factor in BC development, is studied extensively; however, there is a lack of consensus on several key dietary issues (318). The relation between dietary components (e.g., dietary fat intake or micronutrient content), whole-foods (e.g., fruits, vegetables, red meat), or dietary patterns (e.g., calorie restriction) and BC risk are currently under clinical and epidemiologic investigation.

Recent research suggests that non-starchy vegetables, foods containing carotenoids, dairy products, and diets high in calcium may lower the risk of BC; however, more studies are needed confirm these relations (293-297). Conversely, it is hypothesized that high dietary fat intake is associated with an increased risk of BC. The daily intake of dietary fat in industrialized countries, including the United States, is approximately 30-40% of total energy intake, much higher than the recommended 15-30% of total energy intake (275). A high-fat diet is associated with an increase in several inflammatory biomarkers; however, the mechanisms underlying the link between a high-fat diet and cancer risk
and/or progression are poorly understood. A recent meta-analysis was completed assessing dietary total fat and fatty acids intake from 24 independent prospective cohort studies involving over 38,000 BC patients (319). Dietary intake information was acquired via self- or interviewer-administered food frequency questionnaires or 24-hour recall methods. Results indicate that the total dietary fat and specific fatty acids are not associated with increased risk of BC. Brennan, et al. (320) performed a meta-analysis assessing dietary fat and BC mortality from 15 prospective cohort studies. Total fat intake was not associated with BC-specific or all-cause death; however, women in the highest vs. the lowest category of total fat intake had an increased risk of all-cause death in studies that assessed diet pre-diagnosis. The association between dietary fat and BC risk and survival are inconsistent and require further investigation (321, 322). Although there is limited or inconclusive evidence for several key dietary factors, strong evidence links alcohol intake to BC risk (293, 303, 323). Consuming alcoholic drinks increases the risk for both pre- and postmenopausal BC; however, the mechanisms whereby alcohol influences BC risk remain uncertain.

Evidence from observational, randomized controlled, and bariatric surgery studies suggest that dietary energy restriction reduces the risk of BC in women (324-331). Dietary energy restriction in premenopausal and postmenopausal years is most effective at reducing the risk of postmenopausal primary BC. Studies investigating the influence of war-
related famine (332-336) on BC risk later in life have provided conflicting results. Famine exposure prior to the age of ten is protective against BC later in life; whereas, famine exposure after 18 years of age increases BC risk later in life (337). Additionally, Michaels, et al. (338) prospectively followed women diagnosed with anorexia nervosa prior to age 40, reporting that subjects had nearly a 50% risk reduction in BC compared with age-matched controls. It is well-established that prolonged voluntary dietary energy restriction is difficult to maintain. Further complicating the clinical dietary energy restriction field is the debate over the best approach to restricting diet: continuous dietary energy restriction, intermittent dietary energy restriction, or intermittent fasting (339). Despite convincing preclinical evidence (340-344), there is a shortage of epidemiologic and mechanistic human data demonstrating benefits of dietary energy restriction on delaying cancer progression or improving survival outcomes (275).

2.5.1.2. Preclinical studies

Several preclinical studies have been carried out to analyze the effects of dietary components (both micro- and macronutrients) on primary prevention (345-349) and a reduction in metastatic progression (350, 351) in murine models of BC. Numerous inconsistent results exist within the preclinical literature. Preclinical studies poorly model the complex gene-environment-diet interaction and clinical translation is often difficult due to supraphysiological doses (352). However, preclinical studies on diet and
cancer prevention provide important insight into potential mechanisms of action and targets for future pharmacological drug development.

Dietary energy restriction has proven benefits in terms of delaying primary (341, 342, 353-355) and metastatic (343, 356) BC progression in mice. Preclinical studies demonstrate that moderate to severe dietary energy restriction (20%-40% reduction in energy intake) delays primary tumor growth in orthotopic (356-358) and carcinogen-induced (341, 359, 360) mammary tumor models. The protective effect of dietary energy restriction on carcinogenesis is proportional to the degree of restriction within the range of 20-40% (341, 361, 362). However, 10% dietary energy restriction does not alter the percent of carcinogen-induced mammary lesions, or the proportion of pre-malignant to malignant lesions as compared with \textit{ad libitum}-fed rats (341) suggesting that restriction alone may need to exceed 10% to exert a cancer prevention effect. Furthermore, a 30% reduction in calories resulted in a delay in both 4T1 and 67NR tumor growth compared with control feeding or alternate-day feeding (363). Additional studies are needed to assess continuous dietary energy restriction, intermittent dietary energy restriction, or intermittent fasting in preclinical models. A recent meta-analysis by Lv, et al. (340) summarized data from 44 preclinical studies (including models of mammary, prostate, brain, pancreatic, and hepatic cancers) from 1994 to 2014 exploring the role of dietary energy restriction (20-50%) on cancer initiation and progression. Approximately 90% of the studies showed a
dietary energy restriction effect on primary cancer growth. An assessment of the 16 preclinical studies using BC models from 1994 to 2014 showed that dietary energy restricted animals developed approximately 50% less BC than controls, similar to a meta-analysis summarizing dietary energy restriction in preclinical BC models from 1942 to 1994 (364). Few preclinical studies have assessed dietary energy restriction and metastatic progression. De Lorenzo, et al. (356) reports that a 40% restriction in dietary intake reduces 4T1 primary tumor growth and both spontaneous and I.V.-induced metastatic outgrowth. Collectively, the preclinical data on dietary energy restriction and cancer prevention is promising. Future clinical trials are needed to investigate the effectiveness and safety of these dietary regimens in human populations.

2.5.2. Physical activity and breast cancer incidence and mortality

2.5.2.1. Clinical and epidemiologic studies

Data from clinical (298-300) and epidemiologic (4, 5, 301) studies support the existence of an inverse relation between moderate-to-vigorous physical activity and both pre- and postmenopausal BC incidence, although the evidence is stronger for postmenopausal women (275). Wu, et al. (4) conducted a recent meta-analysis using data collected from 31 prospective studies involving over 63,000 subjects. Physical activity was assessed using validated self-reported questionnaires and expressed as times per week, hours per week (h/week), metabolic equivalent of task (MET)-h/week, or energy expenditure in calories per
week. Dose-response analysis suggests that the risk of BC decreases by 3% for every 10 MET h/week increment in recreational activity and 5% for every 3 h/week increment in moderate plus vigorous recreational activity. Additionally, women who did the most activity (recreational, occupational, or household activities characterized as greater than 2 h/week) had a 12% lower risk of developing BC compared with the least active women. This effect is observed when controlling for body mass index, suggesting that exercise may be protective independent of weight status. Assessing the dose and intensity of physical activity in subset analysis in Wu, et al. and in the American Cancer Society Cancer Prevention study II Nutrition cohort (365) suggests that moderate activity, such as brisk walking provides a benefit; however, vigorous physical activity or walking greater than seven h/week provides the greatest reduction in BC risk. Studies also suggest that sustained activity over each stage of life (from adolescence onward) provides the greatest risk reduction (366). The primary mode for capturing physical activity data in retrospective studies is survey questionnaires of self-reported physical activity. Self-reported data may be unreliable due to inaccuracies in recall and is difficult to determine the intensity of activity performed, therefore prospective studies are important to continue to refine the relation between physical activity and BC prevention (367).

Evidence from epidemiologic studies suggest that regular physical activity decreases the risk of recurrence and improves BC-specific and
overall survival in BC patients (292, 368-378). Two recent meta-analyses were completed using data from 16 cohort studies involving over 40,000 BC patients (292, 377). Results of both analyses indicate that patients who participated in physical activity prior to a BC diagnosis had approximately a 20% risk reduction in BC-specific mortality when comparing the most active to the least active women. Additionally, patients who participated in physical activity after a cancer diagnosis had a 40-50% risk reduction in BC-specific mortality (379, 380). The beneficial effects of physical activity on BC-specific mortality remain significant after stratifying by body mass index or menopausal status, suggesting the effects of physical activity on cancer survival may be independent of metabolic or reproductive hormone status (379-381).

A similar association between baseline cardiorespiratory fitness and reduced BC-specific mortality is demonstrated in the Aerobics Center Longitudinal Study, in which 14,811 women were followed for 31 years (382). It is unknown if the protective effect of exercise on cancer outcomes (primary or secondary prevention) is due, in part, to the prevention of weight gain (i.e., obesity prevention) or to a direct effect of exercise on cancer risk and progression. Also, it remains unclear if the physical activity improvements in BC survival are due to a reduction in metastatic disease occurrence and/or progression (373-375, 378).
2.5.2.2. Preclinical studies

Numerous preclinical studies demonstrate that exercise, achieved via access to motorized treadmill or voluntary activity wheels, reduces primary tumor growth in orthotopic (383-386), carcinogen-induced (387-393), and transgenic (394-396) mammary tumor models. For example, exercise reduces 4T1 primary tumor growth when the intervention is started concomitantly with the injection of 4T1 tumor cells into the mammary gland (385). Few preclinical studies exist studying the effect of exercise on metastatic progression. Interestingly, exercise has no effect on primary tumor growth when MDA-MB-231 cells are subcutaneously (397) or orthotopically (398) injected into athymic mice. These data suggest that the beneficial effects of exercise on primary tumor growth may be mediated via an immune-dependent mechanism.

2.5.3. Weight and breast cancer incidence and mortality

Strong evidence exists for an association between obesity and postmenopausal BC development (275, 293, 308, 309). Conversely, substantial clinical (308, 325, 399-406) and epidemiologic (407) evidence exists suggesting that weight control is associated with decreased risk of primary BC (309, 408, 409). A meta-analysis was completed using data from seven studies involving 4,570 women with no history or low use of hormone-replacement therapy and an adult weight gain range from 0-35 kg. Results indicate that every 5 kg increase in adult weight gain is associated with approximately an 11% increase in the risk of postmenopausal BC (407). The
study found no association between adult weight gain and premenopausal BC risk. The observational nature of retrospective studies makes it difficult to determine if the protective effects of weight control are attributable to certain lifestyle interventions (e.g., diet or physical activity). Studies in overweight and obese postmenopausal women are beginning to separate the effects of diet alone, aerobic exercise alone, and the combination of diet and exercise on inflammatory, metabolic, and sex hormone mediators (330, 410-413). Data from these studies show that sustained weight loss can be achieved via moderate dietary energy restriction alone (restriction to achieve a 10% weight loss); however, diet plus aerobic exercise results in the greatest reduction in chronic inflammation (e.g., reduced C-reactive protein, IL-6, and leptin) in overweight and obese postmenopausal women. The reduction in chronic inflammation may help explain how weight control achieved through diet and exercise reduces the risk of postmenopausal BC.

Being overweight or obese serves as a predictor of poor prognosis once BC is diagnosed regardless of menopausal status (310). Weight gain often occurs in BC patients undergoing treatments; however, few studies have been done to investigate the effects of weight control in BC survivorship populations. The limited studies report generally consistent findings that weight control can reduce mortality and improve quality of life measurements in BC survivors (414-417). However, inconsistencies in the literature exist with Makari-Judson, et al. (416) suggesting that weight gain after a BC diagnosis does not impact BC prognosis, but does have a negative impact on quality of life measurements.
Future clinical studies are needed to assess changes in body composition and BC outcomes.

2.6. MECHANISMS BY WHICH MODIFIABLE RISK FACTORS INFLUENCE CANCER PROGRESSION

The extent to which dietary energy restriction or physical activity, as opposed to changes in body weight, protect against primary tumor growth and metastatic progression remains unclear in epidemiologic, clinical, and preclinical studies. One common hypothesis explaining the cancer protective effect of diet and physical activity on BC risk is a mediation of the effect through body weight (366). The observational nature of the epidemiologic studies limit the ability to determine biological mechanisms and the extent to which diet or exercise, as opposed to changes in body weight, account for the reduction in BC risk and improvements in survival outcomes. Diet and exercise can directly impact oncogenic mutation rates and the proliferative capacity of malignant cells. Additionally, diet and exercise may mediate beneficial changes in several physiological systems, including skeletal muscle, bone marrow, adipose tissue, and liver, and impact tumor progression through changes in adiposity, sex hormone levels, metabolic signals (e.g., insulin and IGF-1), inflammatory status, and/or the immune response. It is likely that the cumulative action of these mechanisms contributes to the effects of diet and exercise on primary cancer prevention and improvement in survival (298, 299, 418, 419).

2.6.1. Genetic and epigenetic alterations

Genetic and epigenetic alterations can induce changes in oncogenic gene expression resulting in an expression profile that promotes tumor
initiation, progression, and recurrence (420, 421). Epigenetic modulation is a stable and heritable molecular alteration in the gene expression profile of a cell during somatic cell division without changing the DNA sequence. These changes include histone variants, posttranslational modifications of amino acids on the amino-terminal tail of histones, and covalent modifications of DNA bases (422). A recent meta-analysis reports an association between gene-specific DNA methylation and prognosis, as well as distinct DNA methylation levels within specific BC subtypes (423). Lifestyle-based interventions (e.g., diet and exercise) could induce alterations in genetic and epigenetic events, resulting in an expression profile that prohibits or reduces the development, progression, and/or recurrence of BC (424), yet few clinical or preclinical studies have examined these relationships. Physical activity-induced improvements in survival (425) and obesity-induced impairments in prognosis (426) after a BC diagnosis may be mediated through changes in methylation profiles of BC-related genes, with physical activity favoring the expression of genes involved in tumor suppression and a decrease in the expression levels of oncogenes. Zeng, et al. (427) reports that a six-month moderate-intensity aerobic exercise program in BC survivors improves overall survival and results in a reduction in the hypermethylation of L3BTL1, a tumor suppressor gene, suggesting that aerobic exercise may maintain proper transcription and translation of important tumor suppressing molecules. Calorie restriction in the preclinical MMTV-HER2/neu murine model of BC reduces primary tumor growth, spontaneous metastasis, and hinders aberrant epigenetic alterations
in genes related to BC prognosis, including estrogen receptor alpha and beta, (428). Future studies are needed to identify specific BC-related genes of interest and the extent to which diet and exercise can modulate epigenetic control.

2.6.2. Tumor cell survival

Cancer cells sustain proliferative capacity through numerous mechanisms, including an evasion in growth suppressor signaling, the upregulation of cell cycle control proteins (e.g., cyclins, cyclin-dependent kinases), and a resistance in apoptotic-induced cell death (13). The dysregulation in cell cycle progression has broad influences in cancer cell biology and induces alterations in cell metabolism, tumor-induced secretory factors, and metastatic potential that favor cell survival. Sustained proliferative capacity can be induced via intrinsic and autocrine signaling cascades or through the dysregulation of cells within the TME to provide additional growth factor signaling (13).

Experimental animal models suggest that dietary energy restriction can reduce cancer cell proliferation by altering the expression of cell cycle proteins (decrease cyclins, increase levels of cyclin-dependent kinase inhibitors, and decrease cyclin-dependent kinases) (275) and altering the phosphorylation status of tumor suppressor genes. Thompson, et al. (429) reports that dietary energy restriction can reduce retinoblastoma protein phosphorylation, a tumor suppressor that is dysfunctional in several cancers. Hypophosphorylation of retinoblastoma protein maintains tumor suppressor activity; thus, promoting the
inhibition of cell cycle progression. In addition, dietary energy restriction can promote the maintenance of cellular proapoptotic machinery through the induction of B-cell lymphoma 2 (Bcl-2) family of proteins (429). A review of preclinical in vivo studies investigating the effects of aerobic exercise on cancer initiation, progression, and metastasis by Ashcraft, et al. (430) suggests that physical activity can also modulate cell cycle proteins and the phosphorylation status of tumor suppressor genes to reduce proliferation and/or favor apoptosis in cancer cells. Future studies are required to determine the timing of diet- and exercise-induced alterations in cell proliferation and apoptotic signaling cascades across the cancer continuum.

2.6.3. Cellular energetics

It is well established that metabolic rewiring is orchestrated by oncogenes; however, emerging data suggest that dysregulated metabolism can play a primary role in oncogenetic and epigenetic events that promote cancer (431). The hyperproliferation associated with uncontrolled tumor growth requires an adjustment in cellular metabolism not only for fuel needs, but to provide biosynthetic intermediates necessary for assembling new cells (13). To meet the macromolecule demands, cancer cells reprogram glucose energetics from oxidative phosphorylation to oxidative glycolysis, even in conditions where oxygen is present, in a process termed aerobic glycolysis (i.e., the Warburg effect) (432). This shift is achieved through the upregulation of glucose transporters (GLUT), notably GLUT1, and glycolytic enzymes (13). However,
this shift results in substrate inflexibility and a reliance on an influx of glucose carbons through glycolysis (433).

It is therefore feasible that lifestyle-based interventions (e.g., dietary energy restriction (433, 434) and physical activity (435)) could induce alterations in glucose availability, resulting in a reduction in glucose to cancer cells; thus, limiting their ability to replicate or rendering them susceptible to cell death. Specifically, dietary energy restriction may have an anticancer effect through the activation of peroxisome proliferator-activated receptor α (PPARα), which simulates lipid oxidation and inhibits glycolysis (434). Physical activity can directly control whole-body metabolism through changes in glucose, lipid, and amino acid metabolism. An exercise-induced activation of AMP-activated protein kinase (AMPK), which activates energy-producing pathways and inhibits biosynthetic and anabolic pathways, could help to control cancer cell growth (435). An eight-week motorized wheel-running intervention increased AMPK signaling and reduced Akt and the mechanistic target of rapamycin (mTOR) signaling in the methylNitrosurea-induced mammary carcinomas model in rats, suggesting a shift in anabolic and protein synthesis pathways (436-438). Lastly, weight maintenance achieved through diet and exercise in BC survivors may reduce the rate of recurrence and improve BC-specific mortality via an inhibition in the dysregulation of cancer cell metabolism (439). Future studies are needed to investigate the pathways by which diet and exercise impact the metabolism of cancer cells, as well as the physiological
regulation of substrate availability of glucose, lactate, glutamine, and additional amino acids, and if these mechanisms can alter cancer progression.

2.6.4. Tumor vascularization

Solid tumors are highly metabolic and acquiesce tissue repair mechanisms to increase tumor vasculature; thus, increasing nutrient availability. Tumors and supporting cells (e.g., mesenchymal cells, MDSCs, TAMs) (131, 440, 441) can secrete proangiogenic factors (e.g., VEGF) to promote TME vascularization, and thus increase nutrient availability. However, tumor vascularization is highly unorganized. Tumor vascularization is correlated to the level of hypoxia within the TME. It is commonly accepted that angiogenesis and the expression of angiogenic factors, such as VEGF, is associated with the increased risk of metastasis and poor patient outcome (442). Emerging studies suggest that antiangiogenic therapy can normalize vasculature and hypoxia in the TME and improve the delivery of therapy (443). Few clinical studies have assessed the effects of diet and exercise on tumor vascularization or their combination with antiangiogenic therapy.

Emerging preclinical data suggests that diet and exercise can alter tumor vasculature. Betof, et al. reports that an exercise-induced reduction in primary tumor growth is accompanied by an increase in the density of apoptotic cells, and microvessel density and maturity in the tumor (385). These data suggest that exercise may alter the TME resulting in a reduction in tumor mass and tumor hypoxia (385). Running wheel activity induces a reduction in intratumoral VEGF and is associated with a reduction in primary tumor growth.
in the estrogen-dependent MC4L2 murine BC model (444). Long-term exercise exposure increases VEGF expression resulting in enhanced tumor vascularization and a reduction in tumor burden, multiplicity, and histological grade in the methyl nitrosurea-induced mammary carcinoma (445). Lastly, dietary energy restriction reduces blood vessel density in pre-malignant and malignant breast pathologies in rats (429). Thus, exercise and dietary energy restriction likely affect tumor vascularization; however, the relationships are likely complex.

2.6.5. Inflammation

It is well established that chronic inflammation can drive tumorigenesis (446). However, as cancerous cells progress to a tumor mass, intrinsic inflammatory gene transcription factors (e.g., NF-κB, STAT3, HIF1α), inflammatory cytokines (e.g., IL-1β, IL-6, IL-23, TNFα), and proinflammatory chemokine molecules (e.g., CXCR4, CXCL12) generate a highly inflammatory, non-resolving TME that promotes cell proliferation, survival, and invasiveness (13, 447-449). Also, tumor-secreted factor orchestrate extrinsic changes within the originating tissue (e.g., induction of cancer-associated-fibroblasts (450)) and promote the expansion and accumulation of immunosuppressive tumor-infiltrating cells (e.g., MDSCs, TAMs, Tregs (131)) to generate a feed-forward proinflammatory milieu. The mechanisms by which tumor intrinsic inflammatory signals promote the hallmarks of cancer (e.g., sustained cell proliferation, oncogenic and epigenetic mutations, alterations in cellular energetics, inhibition in apoptosis) is thoroughly reviewed (13, 447-449).
The clinical investigation of lifestyle-based interventions (e.g., dietary energy restriction and physical activity) in cancer patients typically characterize the systemic inflammatory milieu (e.g., plasma or serum C-reactive protein, IL-6, or TNFα), rather than directly assessing tumor-induced gene expression levels or secretion of proinflammatory factors within the TME. Recent advances in microarray technology have made the analysis of tumor biopsies more practical; however, deciphering between tumor-derived, stromal-derived, or tumor-infiltrating immune-cell derived factors remains a logistical problem. Preclinical studies analyzing the TME show an exercise-induced reduction in proinflammatory signals (e.g., IL-6, TNFα) within the TME (444). Diet- and exercise-induced pathways that reduce proliferative signal or inhibit the dysregulation of tumor cell metabolism could also play a role in the inhibition of tumor-secreted proinflammatory mediators. However, future studies are needed to determine diet- and exercise-induced effects directly on intrinsic inflammatory signaling cascades within tumor cells.

Acute inflammation is essential to initiate the immune response to a harmful stimulus. However, chronic systemic inflammation can dysregulate several pathways that promote tumor initiation and escape (449, 451). Several studies are investigating the use of inflammatory biomarkers (e.g., C-reactive protein, IL-6, TNFα) as predictive and prognostic indicators in BC (452-457). Elevated serum C-reactive protein, an acute phase protein and nonspecific marker of systemic inflammation often used in the clinical assessment of inflammatory status, as well as serum IL-6, is associated with BC stage and an
indicator of poor prognosis. The relation between diet and exercise, inflammatory mediators, and BC is emerging in the clinical and epidemiologic literature. Plasma TNFα and IL-6 is unchanged in response to a six-month aerobic exercise trial in BC survivors. Subset analysis reveals a significant reduction in plasma IL-6 in women who reached 80% of the intervention goal compared with those who did not (458). Studies in tumor-free overweight and obese postmenopausal women show that sustained weight loss is achieved via moderate dietary energy restriction alone (restriction to achieve a 10% weight loss). Diet plus aerobic exercise results in the greatest reduction in chronic inflammation (e.g., reduced plasma C-reactive protein and IL-6) (330, 410-413); however, the relation to postmenopausal BC risk is forthcoming. The reduction in chronic inflammation may help explain how weight control achieved through diet and exercise reduces the risk of postmenopausal BC.

Numerous preclinical studies suggest that proinflammatory cytokines can facilitate tumor growth and metastatic progression by directly impairing cells within the TME (mesenchymal cells, tumor-infiltrating immune cells), resulting in an increase in tumor vasculature and tumor cell invasiveness (394, 449, 459). Treadmill running in the C3(1)SV40Tag transgenic BC model (394) and voluntary wheel activity in the methylNitrosurea-induced mammary carcinoma model in rats (460) reduces plasma IL-6 and is associated with a reduction in primary tumor incidence. Both voluntary wheel activity and dietary energy restriction (15% reduction) interventions reduce plasma IL-6 and TNFα and reduce methylNitrosurea-induced mammary carcinoma incidence and
multiplicity (342). Therefore, targeting systemic cytokines and inflammatory signals may be a potential therapeutic target to enhance current and emerging therapies (461).

### 2.6.6. Sex hormones

Sex hormones, or steroids, are important in sexual development and other bodily functions. A complex relation exists between estrogen levels, adiposity, and menopausal status (462). Prior to menopause, estrogens are produced mainly in the ovaries of women and elevated adiposity results in the dysregulation of ovary-specific production of estrogen. After menopause, adipose tissue is the main source of estrogen and generally, elevated body weight means higher circulating estrogen levels. Epidemiologic studies show a strong association between elevated circulating estrogen levels and postmenopausal BC risk (308, 309, 463). However, being overweight or obese serves as a predictor of poor prognosis once BC is diagnosed regardless of menopausal status (310). In established BC, elevated plasma estrogens correlate with gene expression of estrogen-dependent genes with the expression varying across the menstrual cycle of premenopausal women (463). The role of diet, exercise, and weight control on estrogen levels and BC risk (291, 305, 306, 309, 339, 366, 419, 464, 465) and progression (312, 466, 467) is thoroughly reviewed and likely dependent on BC subtype classification. For example, Ibrahim, et al. (373) reports that post-diagnosis physical activity reduces BC deaths and all-cause mortality among patients with estrogen receptor-positive tumors; whereas, women with estrogen receptor-negative
disease show no gain. Therefore, the beneficial effects of diet, exercise, and weight control on BC risk and progression is intricately linked to menopausal status and BC subtype.

2.6.7. Metabolic signaling and growth factors

Numerous researchers have reported the role of inflammatory metabolic mediators (e.g., insulin, IGF-1, IGFBP-3) and growth factors (e.g., G-CSF, GM-CSF) in tumor growth (360). The effects of IGF-1 on tumor progression are discussed.

IGF-1 is a peptide hormone involved in modulating cell growth and survival by stimulating proliferation (468). In circulation, the action of IGF-1 is regulated by a group of six IGF-binding proteins (IGFBPs) (469, 470). IGFBP-3 is the primary binding protein and can exhibit IGF-1 independent actions, such as promoting growth inhibition and apoptosis (471). Elevated circulating concentrations of IGF-1 are firmly established as a risk factor for the development of BC, especially estrogen positive tumors (472-477). A recent meta-analysis was completed using data from 17 prospective studies quantifying pre-diagnosis circulating IGF-1 concentration and BC risk involving 4,790 BC patients (475). Results indicate that patients with the highest IGF-1 concentration had a 28% increase in BC risk compared with the lowest fifth. This association was not altered when adjusting for IGFBP3 and did not vary by menopausal status; however, it does seem to be confined to estrogen-receptor-positive tumors (475).
The role of IGF-1 in BC survivorship populations is emerging (478-481). Since IGF-1 is responsive to changes in energy balance, researchers are investigating the role of physical activity as a non-pharmacological intervention to reduce IGF-1 levels in BC survivors (433, 482, 483). A recent meta-analysis was completed using data from five randomized controlled trials involving 235 BC survivors (484). Aerobic exercise results in a significant reduction in circulating IGF-1; however, the effect on BC recurrence and survival has yet to be established.

Preclinical studies have focused on the impact of IGF-1 in cancer cell proliferation, migration, and metastatic potential using in vitro and in vivo models to identify the signaling pathways involved in these processes (485). It is well established that dietary energy restriction prevents mammary tumorigenesis in rodents and IGF-1 may play a key role in the reduction in tumor growth. IGF-1 signaling can activate PI3K/Akt and Raf-1/MEK/ERK pathways and downstream nuclear factors in cancerous cells to promote proliferation and inhibit apoptosis (486-488). Thus, a dietary energy restriction-induced reduction in IGF-1 could result in blunted tumor proliferation.

Emerging evidence suggests that IGF-1 may play a role in the epithelial-mesenchymal transition (EMT) process (343, 489). A number of studies show a strong correlation between EMT and high invasive and metastatic behavior of BC (490). Results from a recent study suggest that IGF-1 levels may regulate luminal tumor growth by modulating genes related to EMT and chemokine
signaling (358). Thus, alterations in energy balance achieved via dietary energy restriction and exercise are likely linked to the EMT process.

Additionally, the IGF family can also dysregulate immune compartments. For example, IGF-1 can activate Raf-1/MEK/ERK and decrease ERK1/2 phosphorylation and p38 dephosphorylation pathway in DCs resulting in a delay in maturation (or an induction in a tolerogenic DC phenotype) (491, 492). Reduced antigen uptake and presentation abilities in DCs will activate fewer antigen-specific CD8+ cytotoxic T cells (491, 492). IGF signaling in DCs can also induce the secretion of IL-6, TNFα, and IL-10, generating an immunosuppressive phenotype that promotes tumor escape (491, 492).

2.6.8. Myokines

Skeletal muscle is now recognized as an endocrine organ that can secrete peptides in response to exercise-induced skeletal muscle contraction (435) to promote an anti-inflammatory milieu (e.g., IL-6, IL-10, IL-Ira) and regulate physiological processes in distant organs (493). An exercise-induced shift in circulating concentrations of pro- and anti-inflammatory cytokines may have direct effects on tumor proliferation (494), or an indirect effect through the control of systemic levels of hormones (299) or altered immune responses (e.g., acute mobilization of NK and T cells, enhancements in NK cytotoxicity) (112, 495, 496). Regular physical activity may prevent cancer initiation and improve survival via exercise-induced changes in skeletal muscle-derived cytokines that reduce proinflammatory signaling.
2.6.9. Adipokines

Adipose tissue is now recognized as a major endocrine organ capable of secreting numerous cytokines, or adipokines (497). Over 40 adipokines have been identified; however, leptin and adiponectin are the most abundant in human serum. Circulating leptin is positively correlated with adiposity; whereas, circulating adiponectin is generally lower in obese compared with lean individuals (497). Leptin has broad effects on the immune system, cytokine production, angiogenesis, and carcinogenesis (498). Adiponectin has direct anti-diabetic, anti-atherogenic, and anti-inflammatory properties. Data on the association of adipokines and BC risk and prognosis are mixed (499); however, recent meta-analyses support a link. A recent meta-analysis reports that the concentration of plasma leptin levels increase with disease severity in BC patients (500). Recent meta-analyses summarizing both prospective cohort and case-control studies report an inverse relation between adiponectin levels and BC risk (501-503). Leptin and adiponectin are responsive to changes in energy balance and are being investigated as modulating factors mediating the effects of diet and exercise on cancer progression (339). Numerous preclinical studies report that dietary energy restriction and exercise can induce systemic changes in adipokine levels and alter primary tumor growth or metastatic progression (342, 356, 392, 504). Further studies are needed to characterize the link between adipokines and BC risk and progression.
2.6.10. The immune response

Immune cells have multiple flavors of cell surface receptors and can respond to numerous signaling molecules (e.g., inflammatory, sex hormone, metabolic, adipokine, myokine) that direct and refine the immune response (505). Immune cells are diverse and play a critical role in tumorigenesis (immunosurveillance reviewed in section 2.3.3. and immunosuppression reviewed in 2.3.5). Cancer related inflammation subverts antitumor immunity and promotes the emergence of immunosuppressive cell types. Therefore, weight gain-induced perturbations or diet and/or exercise-induced changes in inflammatory tone, sex hormones, metabolic signaling, adipokines, or myokines can not only directly affect tumor initiation and progression, but can also impact the immune response to cancer (506-508). Changes in the inflammation-immune axis may underlie the relation between physical activity and BC risk and progression. Identifying the specific molecules and mechanisms driving an exercise-induced benefit in the immune response to cancer is an active area of research (435, 509-511).

Physical training can improve quality of life in BC survivors (414-417). However, clinical data is emerging that physical training, both aerobic (512-515) and resistance (516, 517), can also increase immunological anti-cancer activity. Schmidt, et al. (518) recently reviewed relevant clinical trials and meta-analyses studying the effects of physical activity on the immune system of BC patients and summarized that exercise-induced benefits are likely mediated via an increase in the number and cytotoxicity of monocytes, natural killer cells,
and cytokines. Exercise-induced changes in immune compartments and its potential influence in cancer therapies is emerging (519).

Preclinical studies suggest exercise-induced benefits in T cell (273, 496) and NK cell (520, 521) compartments. Numerous preclinical studies demonstrate that activity (running wheel, treadmill, swimming) can improve immune effector responses and increase the number of tumor-infiltrating immune cells concurrently with a delay in primary tumor growth (342, 394, 520-523) and a reduction in metastasis (524). Few studies have investigated exercise-induced alterations in immunosuppressive cell types. Goh, et al. (395), reports that running wheel activity decreases intratumoral expression of Ccl22, a cytokine associated with Treg recruitment. Immunosuppressive cells can promote tumor survival and interfere with current and emerging therapies, therefore, additional studies on exercise-induced changes in immunosuppressive cell expansion and/or function are needed.

Severe caloric restriction or starvation impairs immunity (525, 526). However, preclinical studies suggest that fasting-mimicking diets increase circulating CD8+ cytotoxic T cells and reduce Treg cells, resulting in improvements in immunosurveillance mechanisms (527, 528). Dietary energy restriction changes in immune compartments and its potential influence in cancer therapies is emerging (529).

2.7. RATIONALE FOR CURRENT RESEARCH

Metastatic disease remains incurable and a significant challenge in the field of BC research is to determine how to reduce and/or eliminate the mortality
associated with metastatic BC (37, 38). Currently, treatments are available that slow metastatic progression (e.g., estrogen blockers, chemotherapy, radiation); however, these treatments carry their own risks of morbidity and mortality (42, 43). TNBC is characterized by an increase rate of metastatic spread to the brain and lung and a decrease in overall survival compared with other metastatic molecular subtypes. TNBCs are often diagnosed at a later stage and therefore associated with a more advanced disease. Thus, novel therapies that may prevent (48) or slow metastatic disease with less severe side effects, especially within the metastatic TNBC population, are greatly needed (45).

Emerging population data suggests an inverse relation between physical activity and BC incidence, as well as an important role for exercise in the prevention of cancer recurrence and mortality. The extent to which dietary energy restriction or physical activity, as opposed to changes in body weight, protect against primary tumor growth and metastatic progression remains unclear in epidemiologic, clinical, and preclinical studies. One common hypothesis explaining the cancer protective effect of diet and physical activity on BC risk is a mediation of the effect through body weight (366). The observational nature of the epidemiologic studies limit the ability to determine biological mechanisms and the extent to which diet or exercise, as opposed to changes in body weight, account for the reduction in BC risk and improvements in survival outcomes. Thus, well-designed preclinical studies are needed to investigate mechanisms, as well as determine if direct exercise effects or exercise-induced changes in body weight underlie protection.
Based on recent advancements in the fields of immunology and molecular biology, immunotherapy has become a promising emerging treatment for BC, especially metastatic disease (62, 63). Immunotherapy treatments include therapeutic cancer vaccines, monoclonal antibodies, adoptive cell therapies, and the administration of immunostimulatory cytokines, all of which are designed to stimulate a robust antitumor effector response to eliminate tumor cells through several techniques (51, 64). Therapeutic cancer vaccines provide TAAs to stimulate an effector T cell response (6); whereas, immune checkpoint blockade, like anti-PD-1, use specific antibodies to target inhibitory cell surface molecules to promote sustained effector cell activation (203). An active area of research attempts to identify ways in which to increase immunotherapy efficacy through the identification of clinically meaningful biomarkers to direct treatment choices (i.e., personalized medicine) and/or through the selection of appropriate combinatorial strategies (aimed to enhance antigen presentation, reverse T cell dysfunction, and/or target immune inhibitory mechanisms) without inducing adverse effects. The number of possible combinatorial treatment strategies grows exponentially. A deeper understanding of the mechanisms by which diet, exercise, or weight control can modulate the systemic host milieu or the TME resulting in a more favorable inflammation-immune axis would not only provide more support for public health interventions, but also identify novel mechanisms to target with future pharmacological therapies. Therefore, testing if lifestyle-based interventions, i.e., physical activity and the prevention of weight gain, which are low-cost, have known benefits across the cancer continuum (271), and can augment T cell responses
(272, 273), can enhance the efficacy of immunotherapeutic strategies represents an attractive option.

2.7.1. Model selection

Several murine models of human BC exist and can be broadly categorized as xenograft, inducible (chemically or virally), or genetically engineered. The advantages and disadvantages of these model systems is extensively reviewed (530, 531). Injectable tumors are inherently variable (e.g., induction rates, number of tumors induced, and metastatic progression) (532), which is further complicated by inconsistencies in the literature in terms of the number of cells injected or the method of injection (e.g., subcutaneous, orthotopic). Nonetheless, transplantable mouse models remain a valuable approach because an intact tumor-host environment is maintained, allowing for the evaluation of therapies that require an immune response (532).

The most widely studied syngeneic murine mammary model is the biomarker receptor triple-negative 4T1 series, consisting of several genetically related cell lines (533). The 4T1.2 variant has a basal-like phenotype (534) and shows characteristics of advanced human stage IV BC. 4T1.2 cells spontaneously metastasize to several organ systems when implanted orthotopically, including lung and bone (168, 535, 536), two common sites of metastasis in human BC patients (38). 4T1.2 cells lack expression of estrogen receptor, progesterone receptor, and HER2. Thus, 4T1.2 cells represent an aggressive preclinical stage IV basal-like TNBC model (536). The 4T1 (537, 538) and 4T1.2 (figure 2.1) mammary tumor model can release endocrine (e.g., G-CSF and GM-CSF) and proinflammatory (e.g., IL-6) signals that dysregulate
hematopoiesis, resulting in the expansion and accumulation of iMCs, like MDSCs, into the blood, spleen (e.g., splenomegal), TME, and metastatic niches. Additionally, 4T1 and 4T1.2 tumor growth is correlated with an increase in immunosuppressive Tregs (539), which can further promote tumor progression. As 4T1.2 tumors advance, there is a progressive decrease in lymphoid-lineage cells and an increase in myeloid-lineage cells within the tumor and secondary lymphoid organs. This dysregulated immune phenotype can generate an immunosuppressive environment that dampens antitumor effector functions; thus, promoting tumor survival and escape.

Historically, it is hypothesized that the 4T1 series is weakly immunogenic and poor responders to individual immune stimulatory therapies such as adenovirus expression of B7.1 or IL-12 (540) or cellular vaccines consisting of MHC class II, B7.1, or the staphylococcal enterotoxin B superantigen (541, 542) due to the emergence of the highly immunosuppressive effects of MDSC and Treg populations. Recent studies show that the immune system can recognize the 4T1 mammary tumor, but only through the combination of MDSC (126, 262, 266, 543-546) or Treg (539, 547) inhibition plus immune stimulatory treatments. Thus, studying if mild dietary energy restriction and moderate exercise can blunt tumor-induced inflammation, reduce the expansion of immunosuppressive cell types, and sustain antitumor immunity resulting in a reduction in primary tumor growth and metastatic disease in a clinically relevant, immunocompetent, orthotopic, mammary tumor model is highly innovative and readily translatable.
A. Splenomegaly

Non-tumor bearing

Tumor bearing

B. Tumor volume (cm³)

Days post tumor implantation

C. CD3 T cells (percent)

D. CD4⁺ helper T cells (percent)

E. CD8⁺ cytotoxic T cells (percent)

Days post tumor injection

F. Gr-1⁺CD11b⁺ MDSCs (percent)

G. Gr-1⁺CD11b⁺ mMDSCs (percent)

H. Gr-1⁺CD11b⁺ gMDSCs (percent)

Days post tumor injection

I. Spleenic MDSCs x 10⁶

End tumor wt (g)

J. G-CSF (pg/ml)

End tumor wt (g)

K. IL-6 (pg/ml)

End tumor wt (g)
Figure 2.1. Immune and plasma alterations over time in the 4T1.2 mammary tumor model. The 4T1.2 mammary tumor model is characterized by splenomegaly (A, B) with splenocyte cell counts increasing proportionately with tumor volume. Splenomegaly is characterized by a decrease in the percent of (C) CD3+ T cells, (D) CD4+ helper T cells, and (E) CD8+ cytotoxic T cells and an increase in the percent of (F) Gr-1+CD11b+ MDSCs, (G) Gr-1loCD11b+ mMDSCs, and (H) Gr-1hiCD11b+ gMDSCs as tumor progression occurs. Tumor weight at sacrifice is correlated with (I) splenic Gr-1+CD11b+ MDSCs (Spearmen correlation, $\rho=0.849$, $p<0.001$), (J) plasma G-CSF (Spearmen correlation, $\rho=0.459$, $p<0.001$), and (K) plasma IL-6 (Spearmen correlation, $\rho=0.499$, $p<0.001$).

2.7.2. Activity intervention selection

Based on the association between physical activity and a reduction in BC incidence, reduction in recurrence, and improvements in survival in observational studies, my dissertation studies were designed to model women who remain weight stable throughout adulthood via two lifestyle-based strategies, a modest reduction in calories (10% of caloric intake) and moderate physical activity to test the extent to which exercise as opposed to changes in body weight protect against tumor growth and metastasis. Rodent models of activity can utilize forced (i.e., treadmill running or swimming) or voluntary (i.e., wheel running) intervention paradigms with the advantages and disadvantages of each intervention thoroughly reviewed (430, 548). Briefly, forced exercise interventions can quantify intensity and duration, but are associated with an elevated stress response (i.e., sympathetic nervous system and/or hypothalamic-pituitary-adrenal axis activation). Conversely, mice can acclimate to voluntary running wheel activity over time; thus, reducing stress-related changes in the adrenal hormone corticosterone. To reduce stress-related detrimental effects on the immune response and tumorigenesis, voluntary wheel running was selected.
2.8. SPECIFIC AIMS AND HYPOTHESES

Overarching hypothesis: Moderate exercise achieved via access to voluntary activity wheels will alter the inflammation-immune axis resulting in a reduction in primary tumor growth and spontaneous metastasis; and an enhancement in therapeutic efficacy in a stage IV metastatic BC model.

Specific aim 1. Determine the extent to which exercise, as opposed to changes in body weight, protects against primary tumor growth and metastatic progression in a stage IV metastatic BC model.

Hypothesis 1.1. Changes in body weight via mild dietary energy restriction alone or moderate exercise in mice gaining weight over the course of the study will reduce primary tumor growth and metastatic burden in 4T1.2 tumor-bearing mice.

Hypothesis 1.2. Moderate exercise in weight stable mice (achieved via the combination of mild dietary energy restriction [10% dietary energy restriction based on control food intake] and access to an activity wheel) will prevent the emergence of immunosuppressive factors, concurrently with the greatest reduction in primary tumor growth and spontaneous metastases in 4T1.2 tumor-bearing mice.

Hypothesis 1.3. Moderate exercise in weight stable mice will reduce proinflammatory tumor-immune gene transcription factors within the TME.

Specific aim 2. Determine if moderate exercise in weight stable mice can enhance the efficacy of a broad-based, allogeneic whole tumor cell cancer vaccine in a stage IV metastatic BC model.
**Hypothesis 2.1.** Moderate exercise in weight stable mice will enhance the efficacy of the broad-based immunogenic stimulus resulting in the greatest reduction in primary tumor growth and spontaneous metastases, as well as enhancement in antitumor immune effector function in 4T1.2 tumor-bearing mice.

**Specific aim 3.** Determine if moderate exercise in weight stable mice can enhance the efficacy of the dual administration of a broad-based, allogeneic whole tumor cell cancer vaccine and PD-1 immune checkpoint blockade in a stage IV metastatic BC model.

**Hypothesis 3.1.** The beneficial effects of moderate exercise in weight stable mice are working through an enhancement in antitumor immune responses.

**Hypothesis 3.2.** Moderate exercise in weight stable mice will prevent the emergence of immunosuppressive factors.

**Hypothesis 3.3.** Antagonizing the co-inhibitory PD-1 immune checkpoint via antibody blockade will allow the immune system to completely eradicate the tumor and prevent metastases in moderately exercising, weight stable mice.
CHAPTER 3: WEIGHT MAINTENANCE ACHIEVED VIA MILD DIETARY ENERGY RESTRICTION AND MODERATE PHYSICAL ACTIVITY ON TUMOR PROGRESSION IN THE 4T1.2 MAMMARY TUMOR MODEL*

3.1. ABSTRACT

Regular physical activity and the prevention of weight gain significantly decreases breast cancer (BC) incidence and improves BC-specific and overall survival in BC patients. Possible mechanisms in which physical activity- and diet-induced weight control reduce the risk for primary BC include changes in metabolic, inflammatory, and immune mediators; however, the mechanisms underlying the improvement in survival are not well understood. How physical activity and diet-induced weight control improve survival is of utmost importance because metastatic disease remains incurable and is the underlying cause of death in the majority of BC patients who die of the disease. Thus, the discovery of any lifestyle intervention(s) that could prevent or delay metastatic disease could be transformative in terms of cancer therapy and survivorship. The goals of the current study were to determine if: 1. The protective effect of diet and exercise on primary tumor growth could prevent metastases; 2. This protective effect is due largely to a reduction in calories or to a direct effect of exercise on tumor outcomes and metastasis; and 3. A reduction in calories, exercise, or the combination alter the tumor microenvironment (TME) and reduce the expansion of protumor immunosuppressive cells in tumor-bearing mice. Female BALB/c mice

(n=15-20/group) were randomized to sedentary (SED) or activity wheel (EX) cages and fed *ad libitum* (AL) or 90% of control food intake (10% reduction in dietary energy intake compared with *ad libitum*-fed controls) to study the effects of EX alone, ER alone, and the combination of EX+ER on tumor and metastatic outcomes. Importantly, the *ad libitum* groups (SED+AL and EX+AL) and dietary energy restriction groups (SED+ER and EX+ER) were weight matched to allow the investigation of the effects of moderate exercise in weight gain vs. weight maintenance groups. After eight-weeks on the interventions, all mice were orthotopically injected with 5x10⁴ luciferase-transfected 4T1.2 cells into the fourth mammary fat pad and continued on their respective interventions. Mice were sacrificed at day 35 post tumor implantation. Weight maintenance mice, both SED+ER and EX+ER groups, weighed significantly less than weight gain mice, both SED+AL and EX+AL groups, throughout the study (p<0.001). Primary tumor growth (time x treatment, p<0.001) and tumor weight at sacrifice (p=0.021), as well as lung (p=0.054) and femur (p=0.018) spontaneous metastasis, were significantly different between groups, with EX+ER mice displaying the lowest tumor volume, tumor weight, and metastatic burden. Moderate exercise in weight stable mice reduced splenomegaly (p=0.002) and the abundance of splenic myeloid-derived suppressor cells (MDSCs; p=0.003) and MDSC subsets, reduced the concentration of plasma insulin-like growth factor-1 (IGF-1; p=0.020), and altered chemokine, proinflammatory, immunostimulatory, and immunosuppressive gene expression in the TME. To our knowledge, this is the first study to: 1. Control for body weight and mechanistically tease apart the contribution of diet vs. exercise.
on mammary tumor growth and metastatic progression; and 2. Show a cancer protective effect of moderate exercise in weight stable mice on metastatic progression to lung and bone, common sites of metastases in BC patients, concurrently with a shift in inflammatory status and immune responses in a highly aggressive, stage IV BC model.

3.2. INTRODUCTION

Breast cancer (BC) is the most commonly diagnosed cancer and the second leading cause of cancer-related deaths among women in the United States (1, 2). Approximately 60% of BCs are diagnosed at the local stage (i.e., cancer cells are confined to the breast tissue or auxiliary lymph node) with a subsequent 5-year survival rate of 98.8%. However, 6% of BCs are diagnosed at the distant stage (i.e., cancer cells have spread, or metastasized, outside of the breast tissue) with a subsequent 5-year survival rate of 26.9% (1, 2). Therefore, reducing the occurrence of metastatic disease could be transformative in terms of cancer therapy and survivorship.

Although the etiology of BC remains unclear (274), several non-modifiable and modifiable risk factors are implicated in the risk of developing BC. Non-modifiable risk factors include age, sex, race, ethnicity, family history, personal history, menstrual history, and genetics (e.g., BRCA1 or BRCA2). Modifiable risk factors that increase the risk of BC include smoking, the use of hormone replacement therapy, nulliparity, age at first pregnancy (35 or older increases risk), alcohol consumption, and physical inactivity; whereas, factors that decrease the risk include age at first pregnancy (35 or younger is protective) and physical activity.
Elevated weight has contrasting effects on the risk of premenopausal and postmenopausal BC risk (308, 309); however, being overweight or obese serves as a predictor of poor prognosis once BC is diagnosed regardless of menopausal status (310). The combination of increased body weight and physical inactivity are estimated to account for 30% of the BC risk in industrialized societies (311, 312) and are highly linked to the etiology of BC. Therefore, since an increase in body weight represents a known, modifiable risk factor for postmenopausal BC risk, general weight control during adulthood may be an effective strategy for BC prevention. In fact, the current nutrition and physical activity guidelines proposed by the American Cancer Society recommend that individuals maintain a healthy weight throughout life by balancing caloric intake, engaging in physical activity, and losing weight if overweight or obese (313). Similar guidelines are recommended for survivorship populations.

Data from epidemiologic studies suggest that regular physical activity significantly decreases BC incidence (4, 5) and improves BC-specific and overall survival in BC patients (375, 380, 549). This effect is observed when controlling for body mass index, suggesting that exercise may be protective independent of weight status. However, the observational nature of these studies limit the ability to determine biological mechanisms (e.g., improvements in inflammation-immune axis) and the extent to which exercise, as opposed to changes in body weight, account for the reduction in BC risk and improvements in survival outcomes. Also, it remains unclear if the physical activity improvements in BC survival are due to a reduction in metastatic disease occurrence and/or progression (373-375, 378).
Therefore, well-designed preclinical studies controlling for weight and examining the effects of exercise, mild dietary energy restriction, or the combination of diet and exercise on the inflammation-immune axis and tumor progression are essential to determine the extent to which exercise or body weight contribute to protective benefits.

Several broad categories of host factors are identified as possible mediators underlying the relation between exercise and BC risk and progression because they are modulated by changes in energy balance and are implicated in the process of carcinogenesis (299, 360, 366, 549-553). These include reproductive hormones (412, 464, 554-556), metabolic mediators (557-559), and adipokine and inflammatory mediators (413, 557, 560, 561). Emerging evidence suggests that exercise in weight stable animals may also modulate antitumor immune responses. In non-tumor-bearing, weight stable mice, moderate exercise (achieved via access to voluntary activity wheels) significantly enhances antigen-specific T cell responses and natural killer (NK) cell activity (562), two cell types important for mediating antitumor immunity. Thus, moderate physical activity may also reduce recurrence and increase survival in BC patients by preserving or restoring mechanisms (e.g., metabolic and inflammatory responses) that promote immunosurveillance.

The tumor microenvironment (TME) is characterized by dysregulated energy metabolism and by the production of intrinsic inflammatory gene transcription factors, inflammatory cytokines, and proinflammatory signaling molecules (118, 119). In addition to the transformed hyperproliferative cell mass,
the primary TME consists of components that can be broadly classified into three groups (563): a) non-cellular components, b) cells of mesenchymal origin, and c) cells of hematopoietic origin. Non-cellular components, like the extracellular matrix, provide structural and functional support to the expanding tumor mass. Cells of mesenchymal origin, like fibroblasts, adipocytes, or endothelial cells, can respond to tumor-derived factors to promote tumor vascularization, metastatic dissemination, and immune suppression (564). Cells of hematopoietic origin (e.g., myeloid- and lymphoid-lineage immune cells) have disparate function (565). The role of the immune system in controlling primary tumor growth is well studied (91-93) and involves antitumor responses by NK cells and CD8\(^+\) cytotoxic T cells. These cells can recognize transformed cells and prevent their expansion through the release of cytotoxic enzymes (perforin 1, granzyme A and B, granulysin) and/or soluble factors (chemokines and inflammatory cytokines [e.g., interferon (IFN)\(\gamma\)] (97-99)). Additionally, type I CD4\(^+\) helper T cells (T\(\text{H}1\)) produce cytokines (e.g., interleukin (IL)-2 and IFN\(\gamma\)) and chemokines to recruit and activate NK cells and CD8\(^+\) cytotoxic T cells (104) to further promote antitumor immunity. However, immunosuppressive cells of both lymphoid (e.g., Tregs (120)) and myeloid (e.g., tumor-associated macrophages [TAMs] (122, 123) and myeloid-derived suppressor cells [MDSCs] (124-128)) lineage are recruited to the TME by proinflammatory tumor-derived factors and their abundance is positively correlated with tumor burden (129-133). Immunosuppressive cells reduce antitumor immune mechanisms through the depletion of nutrients in the TME, inhibitory cell-surface receptors, and/or through the release of short-lived soluble mediators. The balance
between antitumor immunosurveillance mechanisms and the accumulation of tumor-driven immune suppressive cells is critical for regulating primary tumor growth (141) and metastatic progression (142). The complexity of the TME has become more appreciated in the past two decades (12, 13); however, the effect of changes in energy balance on the TME remains understudied.

The extent to which exercise, as opposed to changes in body weight, protect against primary tumor growth and metastatic progression remains unclear. Therefore, the goal of the current study was to determine if exercise or changes in body weight via mild dietary energy restriction drive protection in an orthotopic model of state IV metastatic BC. A 2x2 factorial design was used to test energy balance interventions: Female BALB/c mice were randomized to sedentary (SED) or activity wheel (EX) cages and fed ad libitum (AL) or 90% of control food intake (10% dietary energy restriction, ER) in a 2x2 factorial design to investigate the effects of EX alone, ER alone, and the combination of EX+ER on tumor, metastatic, and immune outcomes. Since metastatic disease remains incurable and is the underlying cause of death in the majority of BC patients who die of the disease (3), the discovery of any lifestyle intervention(s) that could prevent or delay metastatic disease could be transformative in terms of cancer therapy and survivorship. Additionally, a deeper understanding of the mechanisms by which exercise can modulate systemic host milieu or the TME resulting in a more favorable inflammation-immune axis would not only provide more support for public health interventions, but also identify novel mechanisms to target with future pharmacological therapies.
3.3. MATERIALS AND METHODS

3.3.1. Screening for intrinsic running behavior

Acclimated mice were screened for voluntary running behavior by placing mice into individual cages fitted with a running wheel apparatus (Starr Life Sciences Corporation; Oakmont, PA) for four days as described previously (562). Briefly, wheel revolutions of individual mice were recorded and analyzed using Vital View software (Starr Life Sciences Corporation). Wheel revolutions were converted to total kilometers run over the four-day test period and histogram of distance run was generated. Mice that ran above the 25th percentile of kilometers during the four-day test period were randomized to one of the four intervention groups.

3.3.2. Tumor cell line and cell culture

The 4T1.2 cell line is a murine metastatic BC line derived from a spontaneously arising mammary tumor in a BALB/cfC3H mouse (566). When implanted orthotopically, the 4T1.2 cell line mimics the metastatic progression of human BC with a tendency to metastasize to lung and bone (567). 4T1.2 cells stably expressing luciferase (4T1.2\textsuperscript{Luc}) were provided by Dr. Robin Anderson (Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia) and maintained in Dulbecco’s modified Eagle’s medium (Life Technologies; Grand Island, NY) containing 10% fetal bovine serum (Gemini Bio-Products, Sacramento, CA), 2 mM glutamine (Mediatech; Manassas, VA), 1X nonessential amino acid (Mediatech), and 8 μg/ml puromycin (Mediatech).
3.3.3. Animal model

Six-week-old female BALB/c mice were obtained from Jackson Laboratory (Bar Harbor, Maine). Mice selected for their intrinsic running potential were randomized to sedentary (SED) or activity wheel (EX) cages and fed *ad libitum* (AL) or 90% of control food intake (10% dietary energy restriction, ER) in a 2x2 factorial design to investigate the effects of EX alone, ER alone, and the combination of EX+ER on tumor and metastatic outcomes (Fig. 3.2A). After eight-weeks on the interventions, all mice were orthotopically injected with \(5 \times 10^4\) luciferase-transfected 4T1.2 cells into the fourth mammary fat pad and continued on their respective energy balance intervention. Primary tumor growth was measured 2-3x/week via caliper. Mice were sacrificed at day 35 post tumor implantation. An additional study randomized 38-week old, female BALB/c mice (n=5-6/group) to single-housed running wheel cages or standard cages and followed the same energy balance protocol as above to investigate the energy balance effects in middle-aged, adult tumor-bearing mice. All mice were fed AIN-76A diet (Research Diets, New Brunswick, NJ). Movement was not monitored in mice that did not have access to running wheels. Food intake, body weight, and tumor size \((v= (short^2 \times long)/2)\) were monitored as previously reported (562, 568), and mice were observed daily for signs of ill health. All mice were housed at the Pennsylvania State University and maintained on a 12-hour light/dark cycle with free access to water. The Institutional Animal Care and Use Committee of the Pennsylvania State University approved all animal experiments.
3.3.4. Metastatic burden

Lung, femur, liver, kidney, heart, tibia, brain, and spine were collected, flash frozen in liquid nitrogen, and stored at -80°C. Tissues were homogenized and genomic DNA was isolated using DNeasy Blood and Tissue Kit (Qiagen; Valencia, CA), per manufacturer’s instructions. DNA was quantified using a Nanodrop ND-1000 spectrophotometer (Nanodrop Products; Wilmington, DE). Metastatic tumor burden of the tissues was quantified using the Taqman™ system performed on a Step-One Plus (Life Technologies) real time PCR instrument to quantify luciferase. Assay sequences for luciferase were CAGCTGCACAAAGCCATGAA (forward primer), CTGAGGTAATGTCCACCTCGATATG (reverse primer) and TACGCCCTGGTGCCCGGC (probe with 5’ FAM reporter and 3’ BHQ quencher). The reference gene used for normalization was mouse telomerase reverse transcriptase (TERT) and was assayed using the Taqman Copy Number Reference Assay TERT (Life Technologies). The standard curve included 5-10-fold serial dilutions (200 ng to 20 pg) of DNA extracted from cultured 4T1.2luc cells. The standard curve and 200 ng of the tissue DNA samples were run in duplicate for luciferase and TERT on the Step-One Plus using the quantitative data analysis option and standard cycling parameters. Luciferase data was normalized to the quantitative values for TERT in each sample to correct for fluctuations in DNA amount, quality, and reaction efficiency.
3.3.5. Bioluminescent in vivo imaging

Mice were weighed and injected I.P. with XenoLight D-Luciferin-K⁺ Salt Bioluminescent Substrate (150 mg luciferin/kg body weight) (Perkin Elmer; Waltham, MA) 10 minutes prior to imaging. Anesthetized mice were imaged for a two-minute exposure in an IVIS 50 Imaging System (Perkin Elmer) and analyzed with Igor Pro - Scientific Imaging Analysis Software (Wavemetrics; Lake Oswego, OR).

3.3.6. Splenic and tumor-infiltrating immune cell assays

3.3.6.1. Isolation of splenic immune cells.

Spleens were harvested via gross dissection and splenocytes were prepared from individual mice by mechanical dispersion, as previously described (345). Briefly, spleens were mechanically disrupted with a syringe plunger and passed through a 70 μm nylon mesh strainer (BD Biosciences; Bedford, MA), erythrocytes were lysed with ACK lysing buffer (Lonza; Basel, Switzerland), and remaining cells washed twice in complete medium (RPMI 1640 (Mediatech) supplemented with 10% FBS (Gemini Bio-Products), 0.1 mM non-essential amino acids (Mediatech), 1 mM sodium pyruvate (Mediatech), 2 mM glutamine (Mediatech), 10 mM HEPES (Mediatech), 100 U/mL penicillin streptomycin (Mediatech). Cell counts and viability were determined via trypan blue exclusion (Mediatech).
3.3.6.2. Splenic immune cell proliferation and cytotoxicity assays

Splenic CD4⁺ helper T cells were isolated via Dynabeads Untouched Mouse CD4 Cells Kit following manufacturer's instructions (Life Technologies). Splenic CD4⁺ helper T cells (1x10⁵) plus 1.0 μg/ml unlabeled anti-CD28 (BD Biosciences) were incubated in flat-bottomed, 96-well plates (Greiner Bio-One; Monroe, NC) in the presence of increasing concentrations of anti-CD3 antibody (BD Biosciences) for 72 hours (562). Proliferation data was analyzed by tritiated (H³) thymidine (Perkin Elmer) incorporation and quantified on a Microbeta plate reader (Perkin Elmer). Each assay was performed in triplicate. CD4⁺ helper T cell supernatant was collected following 48-hour stimulation with 0.5 μg/ml anti-CD3 and 1.0 μg/ml anti-CD28 and stored at -80°C. IL-2 and IFNγ were quantified using Legend Max ELISA kits (Biolegend; San Diego, CA).

Natural killer cell cytotoxicity (NKCC) was assessed in a standard 4-hour chromium release assay, as previously described (569), using 100:1, 50:1, 25:1, 12.5:1, and 6.25:1 effector: target ratios. NKCC experiments were performed in triplicate using ⁵¹Cr-labeled (Perkin Elmer) YAC-1 target cells in log phase of growth. Gamma emission was detected using Packard Cobra II Auto-Gamma, model E5002 (Perkin Elmer). Yac-1 targets were plated alone (spontaneous release) or with 1% Triton-X (EMD Millipore) to induce total release and percent lysis was calculated by: [(observed-spontaneous) / (total-spontaneous)] * 100.
3.3.6.3 Splenic IFN$\gamma$ secretion post five-day bulk culture

Splenic immune cells, depleted of Gr-1$^+$ cells via magnetic bead isolation (Miltenyi, San Diego, CA), were co-cultured for five days with 1x10$^6$ irradiated 4T1.2$^{luc}$ cells to generate tumor antigen-specific T cells. Splenic effector cells were isolated and plated (0.5x10$^6$/well) in 24 well plates (Greiner Bio-One). Supernatants were harvested (48 hours post-incubation) and stored at -80$^\circ$C. IFN$\gamma$ was measured using Legend Max ELISA kits (Biolegend), per manufacturer instructions. Cytokines were quantified with an Epoch Microplate Spectrophotometer (Biotek; Winooski, VT). Each assay was performed in duplicate.

3.3.6.4. Splenic MDSC suppression assay

Granulocytic and monocytic MDSC subsets were isolated from a single cell suspension of splenocytes per manufacturer’s instructions (MSDC Isolation kit; Miltenyi). Post-isolation MDSC subsets were counted and viability determined via trypan blue exclusion. C57BL/6 T cells were isolated via negative selection using the Dynabeads® Untouched™ Mouse T Cell Kit (Life Technologies). BALB/c APCs were isolated using Dynabeads® Mouse Pan T (Thy1.2) Kit (Life Technologies). After isolation, T cells and APCs were counted and viability was determined via trypan blue exclusion. Isolated splenic T cells (1x10$^5$) from C57BL/6 animals were co-cultured in a mixed lymphocyte reaction with irradiated (2000 rads) APCs (5x10$^5$) isolated from 4T1.2$^{luc}$ tumor-bearing BALB/c mice and either (Gr-1$^{hi}$CD11b$^+$ granulocytic or Gr-1$^{lo}$CD11b$^+$ monocytic
MDSCs (5x10⁴)). Cells were incubated in flat-bottomed, 96-well plates and were pulsed with 1 μCi per well of tritiated thymidine (H³) at 72 hours. Proliferation was assessed by tritiated thymidine incorporation at 96 hours (Perkin Elmer) on a microbeta plate reader (Perkin Elmer). Each assay was performed in triplicate.

3.3.6.5. Isolation of tumor-infiltrating immune cells

Primary tumors were harvested during dissection, weighed, minced into fine pieces (<10mg), and incubated with 0.03 mg/ml Liberase (Roche; Indianapolis, IN) and 12.5 U/ml DNase I (Sigma-Aldrich; St. Louis, MI) for 45 minutes at 37°C on an orbital shaker. Following the digestion, remaining pieces were mechanically disrupted with a syringe plunger, passed through a 70 μm nylon mesh strainer (BD Biosciences), layered over Lympholyte-M cell separation media (Cedarlane; Burlington, NC), and centrifuged at room temperature for 20 minutes at 1200g. Isolated cells were washed twice in cold PBS (Mediatech) and cell counts and viability of tumor immune infiltrates were determined via trypan blue exclusion.

3.3.6.6. Flow cytometric analyses

Single cell suspensions of splenocytes, splenic effector cells (after five-day bulk culture with irradiated tumor cells), and tumor-infiltrating immune cells were washed twice in PBS containing 0.01% bovine serum albumin (flow buffer) at 4°C. Cells were incubated with Fc block (Biolegend) and 1x10⁶ cells were stained with saturating concentrations
of conjugated antibodies for 30 min at 4°C, as previously described (345). Fluorescently conjugated antibodies for flow cytometry included rat α-mouse CD19 (1D3), hamster α-mouse CD3 (145-2C11), rat α-mouse CD4 (RM4-5), rat α-mouse CD8 (53-6.7), rat α-mouse CD25 (PC61.5), mouse α-mouse NK1.1 (PK136), mouse α-mouse I-Ab (AF6-120.1), hamster α-mouse CD11c (HL3), rat α-mouse CD11b (M1/70), rat α-mouse F4/80 (T45-2342), rat α-mouse Ly6G and Ly6C [Gr-1] (RB6-8C5), rat α-mouse Ly6C (AL-21), and rat α-mouse Ly6G (1A8). Antibodies were obtained from BD Biosciences, Biolegend, and eBioscience; San Diego, CA. Following incubation with the conjugated antibodies, cells were washed twice in flow buffer and fixed in 1% paraformaldehyde (BD Biosciences) in flow buffer for flow cytometric analysis. Additionally, splenic regulatory T cells (Tregs) were quantified using the Mouse Regulatory T Cell Staining Kit #1 (eBioscience) per manufacturer’s instructions. Briefly, following the extracellular staining for CD4 and CD25, cells were permeabilized for 30 minutes, washed in a fixation/permeabilization buffer, and intracellularly stained for rat α-mouse FoxP3 (FJK-16s) for 30 minutes. Following incubation with the FoxP3 antibody, cells were washed twice in fixation/permeabilization buffer and read within 12 hours. Cells were gated on forward vs. side scatter, and a total of 50,000 events for extracellular staining of lymphoid and myeloid populations and a total of 100,000 events for intracellular staining for Treg populations were analyzed. Flow cytometric analyses were performed on
a Beckman Coulter FC500 (Beckman Coulter; Indianapolis, IN) or a BD LSR-Fortessa (BD Bioscience) flow cytometer. Flow cytometric analyses were plotted and analyzed using Flow Jo software (Tree Star; Ashland, OR).

3.3.7. Plasma mediators

Fasting blood was collected at sacrifice (day 35) via submandibular bleed in microtainer tubes (BD Biosciences), centrifuged, and plasma was stored at -80°C. Granulocyte colony-stimulating factor (G-CSF), Interleukin-6 (IL-6), Interleukin-1 alpha (IL-1α), monocyte chemoattractant protein-1 (MCP-1), leptin, and adiponectin were measured using a Milliplex MAP Multiplex or Singleplex Assay (EMD Millipore; Billerica, MA) and quantified on a Bio-plex 200 system (Bio-Rad; Hercules, CA) using Luminex-200 software (Luminex; Austin, TX), per manufacturer’s instructions. Insulin-like growth factor-1 (IGF-1) and insulin-like growth factor binding protein-3 (IGFBP-3) were measured using R&D Systems Quantikine ELISA kits (R&D Systems; Minneapolis, MN) and quantified with an Epoch Microplate Spectrophotometer (Biotek; Winooski, VT). Each assay was performed in duplicate.

3.3.8. Gene expression in the tumor microenvironment

At sacrifice, tumor samples were incubated overnight in RNAlater (Qiagen) followed by RNA isolation from individual mice using Qiashredder columns followed by RNeasy Mini Plus Kit (Qiagen). RNA quality was assessed via Bioanalyzer analysis (Agilent Technologies; Santa Clara, CA) with samples requiring an RNA Integrity Number (RIN) above 7 to pass quality control testing.
RNA sample was retrotranscribed using RT² First Strand Kit (Qiagen) according to manufacturer’s instructions and samples were loaded onto qPCR plates (Mouse Cancer Inflammation & Immunity Crosstalk PCR Array, Cat. # PAMM-181Z, Qiagen) and cycled according to the following conditions: 95°C for 10 minutes; 95°C for 15 seconds; 60°C for 1 minutes for 40 cycles on a StepOnePlus (Applied Biosystems; Foster City, CA). Qiagen’s online Data Analysis Center was used to normalize raw CT values to the Actb housekeeping gene and fold change ($2^{\Delta\Delta Ct}$) was calculated as previously described (572).

3.3.9. Statistical analyses

Tumor weight, metastatic burden, the distribution of cells in the spleen, splenic bulk culture cells, and tumor-infiltrating immune cells, cytokine secretion, and plasma mediators were assessed for normality and equal variances; and either parametric or nonparametric analyses were used based on sample distribution to detect differences between treatment groups. Metastatic burden, cytokine secretion, and metabolic mediators were skewed; thus, the data were transformed (log or square root) prior to statistical analysis. Differences in tumor weight, metastatic burden, the distribution of cells in the spleen, splenic bulk culture cells, and tumor-infiltrating immune cells, cytokine secretion, and plasma mediators were assessed between groups via a one-way analysis of variance (ANOVA), followed by a Bonferroni correction for multiple comparisons where appropriate, or Kruskal-Wallis test, depending on normality and variance. Additionally, if groups were collapsed based on body
weight (i.e., WG: [SED+AL and EX+AL] vs. WM: [SED+ER and EX+ER]), significance was assessed between groups via a Student’s t-test or Mann-Whitney test, depending on normality and variance. Body weight, primary tumor volume, CD4+ helper T cell proliferation, and NKCC were examined using a two-way ANOVA, followed by a Bonferroni correction for multiple comparisons where appropriate. All data are presented as the mean plus or minus the standard deviation of the mean. All analyses were conducted using GraphPad Prism 5 software (GraphPad Software; La Jolla, CA) and statistical significance was accepted at the p≤0.05 level.

3.4. RESULTS

3.4.1. Intrinsic running behavior

Following the four-day screening, mice were ordered from low to high intrinsic running behavior and broken into quartiles (Fig. 3.1A). The top 75% of intrinsic runners (average km/day > 3.5 km/day) were randomized to energy balance interventions (sedentary or activity wheel cages).
Figure 3.1. Screening BALB/c mice for intrinsic running activity. (A) Acclimated mice were screened for voluntary running behavior by being placed into individual cages fitted with a mouse running wheel apparatus for 4 days. Wheel revolutions of individual mice were recorded and analyzed using Vital View software (Starr Life Sciences Corporation) and the top 75% were randomized to energy balance interventions (sedentary or activity wheel cages). (B-E) Wheel revolutions (collected over 30 minute bins) for four representative mice from each quartile over a four-day period.
3.4.2. Body weight and wheel activity

The experimental design is displayed in Fig. 3.2A. Briefly, female BALB/c mice were randomized to sedentary (SED) or activity wheel (EX) cages and fed *ad libitum* (AL) or 90% of control food intake (10% dietary energy restriction, ER) in a 2x2 factorial design to investigate the effects of EX alone (in mice weight matched to the sedentary *ad libitum* group), ER alone (in mice weight matched to EX+ER group), and the combination of EX+ER on tumor and metastatic outcomes. After eight-weeks on the interventions, all mice were orthotopically injected with $5 \times 10^4$ luciferase-transfected 4T1.2 cells into the fourth mammary fat pad and continued on their respective energy balance intervention. Mice were sacrificed at day 35 post tumor implantation. Mice that maintained body weight, i.e., the SED+ER and EX+ER groups, weighed significantly less than mice that gained weight, i.e., SED+AL or EX+AL groups, over the course of the study (Fig. 3.2B; n=15-20/group; 2-way ANOVA, time x treatment, $F_{(39,897)}=12.76$, $p<0.001$). Wheel activity was maintained for 13 weeks over the course of the study, i.e., prior to and after tumor injection (Fig. 3.2C; n=40; average distance run per day=6.2±2.8 km).
3.4.3. Primary tumor growth

The middle-aged, adult mice displayed similar tumor growth, metastatic outcomes, and immune profiles compared with the 14-week mice (data not shown); therefore, these groups were combined for subsequent data analysis. Primary tumor growth was significantly reduced in EX+ER mice compared with
control (SED+AL) and single intervention (EX+AL and SED+ER) groups (Fig. 3.3A; n=15-20/group; 2-way ANOVA, time x treatment, $F_{(18,414)}=3.33$, $p<0.001$) compared with control (SED+AL) and single intervention (EX+AL or SED+ER) groups. Tumor weight at sacrifice was reduced in EX+ER mice compared with SED+AL mice (Fig. 3.3B; n=15-20/group; 1-way ANOVA, $F_{(3,68)}=3.492$, $p=0.021$).

### 3.4.4. Metastatic burden

Lung (Fig. 3.3C; 1-way ANOVA, $F_{(3,58)}=2.72$, $p=0.054$), femur (Fig. 3.3D; Kruskal-Wallis, KW=10.06, $p=0.018$), and liver (Fig. 3.3E; Kruskal-Wallis, KW=7.21, $p=0.065$) spontaneous metastasis were significantly different between groups with EX+ER mice displaying the lowest metastatic burden. Kidney (Fig. 3.3F; Kruskal-Wallis, KW=6.26, $p=0.100$), heart (Fig. 3.3G; Kruskal-Wallis, KW=5.97, $p=0.113$), and tibia (Fig. 3.3H; Kruskal-Wallis, KW=1.58, $p=0.664$) spontaneous metastasis were not significantly different between groups. Representative IVIS images (Fig. 3.3I). Samples were collapsed into weight gain (composed of SED+AL and EX+AL mice) and or weight maintenance (composed of SED+ER and EX+ER) to further investigate metastatic burden. Brain (Fig. 3.3J; Mann-Whitney test, $p=0.293$) and spine (Fig. 3.3K; Mann-Whitney test, $p=0.275$) spontaneous metastasis were not significantly different between WG (i.e., SED+AL and EX+AL) and WM (i.e., SED+ER and EX+ER) groups.
Figure 3.3. Moderate exercise in weight stable mice reduced primary tumor growth and metastatic outcomes. (A) Primary tumor growth (measured 2-3x/week via caliper) was significantly reduced in EX+ER mice compared with control (SED+AL) and single intervention (EX+AL and SED+ER) groups (n=15-20/group; 2-way ANOVA, time x treatment, $F_{(18,414)}=3.33$, $p<0.001$) compared with control (SED+AL) and single intervention (EX+AL or SED+ER) groups. (B) Tumor weight at sacrifice was reduced in EX+ER mice compared with SED+AL mice (n=15-20/group; 1-way ANOVA, $F_{(3,68)}=3.49$, $p=0.012$).
p=0.021). (C-J) At sacrifice, organs (n=15-20/group) were flash frozen, homogenized, and DNA was extracted. Quantitative PCR was used to detect luciferase expression as a marker of metastatic burden reported as ng of luciferase DNA normalized to mouse TERT DNA in 200 ng of sample. (C) Lung (1-way ANOVA, $F_{(3,58)}=2.72$, p=0.054), (D) femur (Kruskal-Wallis, KW=10.06, p=0.018), and (E) liver (Kruskal-Wallis, KW=7.21, p=0.065) spontaneous metastasis were significantly different between groups with EX+ER mice displaying the lowest metastatic burden. (F) Kidney (Kruskal-Wallis, KW=6.26, p=0.100), (G) heart (Kruskal-Wallis, KW=5.97, p=0.113), and (H) tibia (Kruskal-Wallis, KW=1.58, p=0.664) spontaneous metastasis were not significantly different between groups. (I) Representative IVIS images. (J) Brain (Mann-Whitney test, p=0.293) and (K) spine (Mann-Whitney test, p=0.275) spontaneous metastasis were not significantly different between WG (i.e., SED+AL and EX+AL) and WM (i.e., SED+ER and EX+ER) groups. Significantly different from SED+AL (*) and SED+ER (‡).

### 3.4.5. Splenic immunity

Splenocyte count was significantly reduced in EX+ER mice compared with both SED+AL and EX+AL mice (Fig. 3.4A; 1-way ANOVA, $F_{(3,72)}=5.60$, p=0.002). CD4$^+$ helper T cells from EX+ER mice proliferated more than CD4$^+$ helper T cells from EX+AL (Fig. 3.4B; n=15-20/group; 2-way ANOVA, $F_{(3,315)}=2.99$, p=0.038); however, IL-2 (Fig. 3.4C; Student’s t-test, p=0.754) and IFN$\gamma$ (Fig. 3.4D; Student’s t-test, p=0.354) secretion were not significantly different between WG (i.e., SED+AL and EX+AL) and WM (i.e., SED+ER and EX+ER) groups. NK cell cytotoxicity was not significantly different between WG and WM groups (Fig. 3.4E; n=7-8/group; 2-way ANOVA, $F_{(1,65)}=0.06$, p=0.812). No differences in the percentage of CD3$^+$ T cells, CD3$^+$CD4$^+$ helper T cells, CD3$^+$CD8$^+$ cytotoxic T cells, or NK1.1$^+$ natural killer cell populations within the bulk culture were observed between groups (Fig. 3.4F). No differences were observed in the splenic effector cells secretion of IFN$\gamma$ in response to re-stimulation with tumor antigens (Fig. 3.4G; n=5-11/group, Kruskal-Wallis, KW=0.33, p=0.955).
**A**

Spleenocyte count (x10^5)

**B**

CD4 T cell proliferation (stimulation ratio)

**C**

IL-2 (pg/ml)

**D**

FHV (pg/ml)

**E**

NK cell cytotoxicity (percent lysis)

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**G**

Spleenotic IFN secretion (pg/ml)
**Figure 3.4.** Moderate exercise in weight stable mice altered splenic antitumor immunity. Splenocytes (n=15-20/group) were prepared into a single cell suspension and counted. (A) Splenocyte count was significantly reduced in EX+ER mice compared with both SED+AL and EX+AL mice (1-way ANOVA, F_{(3,72)}=5.60, p=0.002). (B-D) Isolated splenic CD4^+ helper T cells (0.1x10^6/well) were stimulated with increasing concentrations of anti-CD3 antibody and 1 μg/ml anti-CD28 antibody, and proliferation was quantified via [H]3 uptake. Data were converted to a stimulation index (counts per minute of stimulated wells divided by counts per minute of unstimulated [media only] wells). CD4^+ helper T cells from EX+ER mice proliferated more than CD4^+ helper T cells from EX+AL (n=15-20/group; 2-way ANOVA, F_{(3,315)}=2.99, p=0.038). (C, D) CD4^+ helper T cell supernatant was collected following 48-hour stimulation with 0.5 μg/ml anti-CD3 and 1.0 μg/ml anti-CD28 and IL-2 and IFNγ were quantified using an ELISA for the WG (i.e., SED+AL and EX+AL) vs. WM (i.e., SED+ER and EX+ER) groups. (C) IL-2 (Student’s t-test, p=0.754) and (D) IFNγ (Student’s t-test, p=0.354) secretion were not significantly different between WG and WM groups. (E) NK cell cytotoxicity was not significantly different between WG and WM groups measured via a standard 4-hour 51Cr-release assay (n=7-8/group; 2-way ANOVA, F_{(1,65)}=0.06, p=0.812). (F, G) Isolated splenocytes were depleted of Gr-1^+ cells and pulsed with irradiated 4T1.2 luc cells for five days to generate an antigen-specific T cell response to tumor antigens. (F) Post bulk culture, cells were stained with anti-CD3, -CD4, -CD8, and -NK1.1 and characterized by flow cytometry (n=5-11/group). No differences in the percentage of CD3^+ T cells, CD3^+CD4^+ helper T cells, CD3^+CD8^+ cytotoxic T cells, or NK1.1^+ natural killer cell populations within the bulk culture were observed between groups. (G) No differences were observed in the effector cells secretion of IFNγ in response to re-stimulation with tumor antigens (n=5-11/group, Kruskal-Wallis, KW=0.33, p=0.955). Significantly different from SED+AL (*) and EX+AL (†).

The percentage of splenic CD3^+ T cells (p=0.027) and CD3^+CD4^+ helper T cells (p=0.002) was significantly different between groups with the EX+ER group displaying the highest percentage (Table 3.1). The number of splenic CD19^+ B cells (p=0.007) was significantly different between groups with the EX+ER group displaying the lowest number compared with both SED+AL and EX+AL mice (Table 3.1). Splenic CD4^+CD25^+FoxP3^+ Tregs were not significantly different between groups (Fig. 3.5A; n=8-9/group; 1-way ANOVA, F_{(3,34)}=1.76, p=0.176); however group differences in splenic Gr-1^+CD11b^+ MDSCs (Fig. 3.5B; n=15-20/group, 1-way ANOVA, F_{(3,72)}=5.06, p=0.003), splenic CD11b^+Ly6C^hiLy6G^- monocytic MDSCs (Fig. 3.5C; n=15-20/group,
Kruskal-Wallis, KW = 11.50, p = 0.009), and splenic CD11b+Ly6C<sub>lo</sub>Ly6G<sup>+</sup> granulocytic MDSCs (Fig. 3.5D; n = 15-20/group, 1-way ANOVA, F(3,72) = 4.38, p = 0.007) emerged with EX+ER mice displaying the lowest number compared with either SED+AL or EX+AL groups. No group differences were observed in MDSC suppressive capacity (Fig. 3.5E).

**Table 3.1. The percentage and number of splenic immune cell populations.** The percentage of (A) splenic CD3<sup>+</sup> T cells (p = 0.027) and CD3<sup>+</sup>CD4<sup>+</sup> helper T cells (p = 0.002) was significantly different between groups with the EX+ER group displaying the highest percentage. (B) Splenocyte count (p = 0.002) and the number of splenic CD19<sup>+</sup> B cells (p = 0.007) were significantly different between groups with the EX+ER group displaying the lowest number compared with both SED+AL and EX+AL mice. Significantly different from SED+AL (*) and EX+AL (†).
Figure 3.5. Moderate exercise in weight stable mice reduced splenic protumor immunosuppressive cell populations. Splenocytes were stained with anti-CD4, -CD25, and -FoxP3 antibodies to quantify regulatory T cell (Treg) populations and anti-Gr-1, -CD11b, -Ly6G, and -Ly6C antibodies to quantify myeloid-derived suppressor cells (MDSCs) and MDSC subsets by flow cytometry. (A) Splenic CD4⁺CD25⁺FoxP3⁺ Tregs were not significantly different between groups (n=8-9/group; 1-way ANOVA, F(3,34)=1.76, p=0.176); however group differences in (B) splenic Gr-1⁺CD11b⁺ MDSCs (n=15-20/group, 1-way ANOVA, F(3,72)=5.06, p=0.003), (C) splenic CD11b⁺Ly6C⁺Ly6G⁻ monocytic MDSCs (n=15-20/group, Kruskal-Wallis, KW=11.50, p=0.009), and (D) splenic CD11b⁺Ly6C⁻Ly6G⁺ granulocytic MDSCs (n=15-20/group, 1-way ANOVA, F(3,72)=4.38, p=0.007) emerged with EX+ER mice displaying the lowest number compared with either SED+AL or EX+AL groups. Dashed line represents non-tumor bearing control. (E) The proliferative capacity of T cells with and without MDSC subsets, shown as a percentage of T cell proliferation in the presence of APC alone, i.e., in the absence of MDSC subsets, is displayed. Granulocytic and monocytic MDSCs and antigen-presenting cells were isolated from tumor-bearing BALB/c mice and T cells were isolated from control C57BL/6 mice. No group differences were observed in MDSC suppressive capacity. Significantly different from SED+AL (*) and EX+AL (†).

3.4.6. Plasma mediators

Plasma G-CSF (Fig. 3.6A; n=8-19/group; 1-way ANOVA, F(3,56)=2.37, p=0.081), IL-6 (Fig. 3.6B; n=8-20/group; Kruskal-Wallis, KW=5.60, p=0.167), and IGFBP-3 (Fig. 3.6C; n=5-7/group; Kruskal-Wallis, KW=0.73, p=0.866) were not significantly different between groups. Plasma IGF-1 was significantly different between groups (Fig. 3.6D; n=5-7/group; Kruskal-Wallis, KW=9.80, p=0.020) with EX+ER mice displaying the lowest IGF-1 concentration compared with SED+AL mice. Plasma IL-1α (Fig. 3.6E; Student’s t-test, p=0.069), MCP-1 (Fig. 3.6F; Mann-Whitney, p=0.109), leptin (Fig. 3.6G; Student’s t-test, p=0.859), and adiponectin (Fig. 3.6H; Student’s t-test, p=0.160) were not significantly different between WG (i.e., SED+AL and EX+AL) and WM (i.e., SED+ER and EX+ER) groups.
Figure 3.6. Moderate exercise in weight stable mice altered plasma mediators. Fasting blood was collected at sacrifice, centrifuged, and plasma was stored at -80°C. Milliplex Multiplex Assays quantified on Luminex-200 software were used to measure (A) G-CSF, (B) IL-6, (E) IL-1α, (F) MCP-1, (G) leptin, and (H) adiponectin. R&D Systems Quantikine ELISAs were used to measure metabolic markers for (C) IGFBP-3 and (D) IGF-1. Plasma (A) G-CSF (n=8-19/group; 1-way ANOVA, F(3,56)=2.37, p=0.081), (B) IL-6 (n=8-20/group; Kruskal-Wallis, KW=5.60, p=0.167), and (C) IGFBP-3 (n=5-7/group; Kruskal-Wallis, KW=0.73, p=0.866) were not significantly different between groups. (D) Plasma IGF-1 was significantly different between groups (n=5-7/group; Kruskal-Wallis, KW=9.80, p=0.020) with EX+ER mice displaying the lowest IGF-1 concentration compared with SED+AL mice. Analysis of the plasma profile (n=11-12/group) in WG (i.e., SED+AL and EX+AL) vs. WM (i.e., SED+ER and EX+ER) groups for (E) IL-1α (Student’s t-test, p=0.069), (F) MCP-1 (Mann-Whitney, p=0.109), (G) leptin (Student’s t-test, p=0.859), and (H) adiponectin (Student’s t-test, p=0.160) were not significantly different between groups. Significantly different from SED+AL (*).

3.4.7. The tumor microenvironment

3.4.7.1. Tumor-infiltrating immunity

The percentage (Table 3.2A) and number (Table 3.2B) of tumor-infiltrating immune cell populations were not significantly different between WG (i.e., SED+AL and EX+AL) and WM (i.e., SED+ER and EX+ER) groups.
Table 3.2. The percentage and number of tumor-infiltrating immune cell populations. The (A) percentage and (B) number of tumor-infiltrating immune cell populations were not significantly different between WG (i.e., SED+AL and EX+AL) and WM (i.e., SED+ER and EX+ER) groups.

<table>
<thead>
<tr>
<th></th>
<th>A Tumor-infiltrating immune cells Percent</th>
<th>B Tumor-infiltrating immune cells Number (x10⁶)</th>
<th>Weight gain (n=15)</th>
<th>Weight maintenance (n=10)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>B cells (CD19⁺)</td>
<td>2.2 ± 1.0</td>
<td>0.6 ± 0.6</td>
<td>24.3 ± 20.8</td>
<td>12.2 ± 6.3</td>
<td>0.258</td>
</tr>
<tr>
<td>Total T cells (CD3⁺)</td>
<td>44.6 ± 18.7</td>
<td>10.4 ± 9.4</td>
<td>6.5 ± 4.0</td>
<td>2.8 ± 1.1</td>
<td>0.056</td>
</tr>
<tr>
<td>Helper T cells (CD3⁺CD4⁺)</td>
<td>25.5 ± 9.2</td>
<td>6.5 ± 5.7</td>
<td>6.5 ± 4.0</td>
<td>2.8 ± 1.1</td>
<td>0.267</td>
</tr>
<tr>
<td>Cytotoxic T cells (CD3⁺CD8⁺)</td>
<td>13.5 ± 8.3</td>
<td>3.1 ± 2.6</td>
<td>3.2 ± 3.8</td>
<td>1.4 ± 1.4</td>
<td>0.364</td>
</tr>
<tr>
<td>NK cells (NK1.1⁺)</td>
<td>8.7 ± 5.3</td>
<td>2.8 ± 3.3</td>
<td>2.8 ± 3.2</td>
<td>1.4 ± 1.4</td>
<td>0.986</td>
</tr>
<tr>
<td>Dendritic Cells (I-Ab⁺CD11⁺c⁺)</td>
<td>10.4 ± 19.6</td>
<td>3.9 ± 10.6</td>
<td>1.7 ± 2.5</td>
<td>1.7 ± 2.5</td>
<td>0.988</td>
</tr>
<tr>
<td>Macrophages (I-Ab⁺CD11b⁺)</td>
<td>19.6 ± 20.4</td>
<td>5.8 ± 10.9</td>
<td>2.3 ± 1.7</td>
<td>2.3 ± 1.7</td>
<td>0.636</td>
</tr>
<tr>
<td>Macrophages (F4/80⁺)</td>
<td>20.0 ± 20.9</td>
<td>6.7 ± 12.8</td>
<td>4.0 ± 3.5</td>
<td>3.2 ± 3.8</td>
<td>0.228</td>
</tr>
<tr>
<td>MDSCs (Gr-1⁺CD11b⁺)</td>
<td>32.4 ± 21.4</td>
<td>32.4 ± 21.4</td>
<td>32.4 ± 21.4</td>
<td>32.4 ± 21.4</td>
<td>0.924</td>
</tr>
<tr>
<td>mMDSCs (CD11b⁺Ly6C⁺Ly6G⁻)</td>
<td>2.8 ± 5.4</td>
<td>2.8 ± 5.4</td>
<td>2.8 ± 4.4</td>
<td>2.8 ± 4.4</td>
<td>0.982</td>
</tr>
<tr>
<td>gMDSCs (CD11b⁺Ly6C⁺Ly6G⁺)</td>
<td>17.5 ± 12.9</td>
<td>17.5 ± 12.9</td>
<td>15.9 ± 13.6</td>
<td>15.9 ± 13.6</td>
<td>0.762</td>
</tr>
</tbody>
</table>
3.4.7.2. Tumor-immune crosstalk gene array

Figure 3.7. Moderate exercise in weight stable mice altered gene expression in the tumor microenvironment. Tumor homogenates were assayed using Qiagen RT² Profiler™ Mouse Cancer Inflammation & Immunity Crosstalk PCR Array. (A) Heat map displaying genes altered >2-fold. (B) Genes that were significantly altered (>2.5-fold) compared with SED+AL control.
3.4.8. High vs. low activity within the moderately exercising, weight stable mice

Mice running an average of 5.8-12.6 km/day (EX+ER HIGH) weighed significantly less than mice running an average of 2.5-5.7 km/day (EX+ER LOW) over the course of the study (Fig. 3.9A; n=17/group; 2-way ANOVA, time x treatment, $F_{(13,416)}=2.81$, $p=0.001$). Mice in the EX+ER HIGH group averaged more km/day than the EX+ER LOW group over the course of the study (Fig. 3.9B; 2-way ANOVA, $F_{(1,384)}=42.33$, $p<0.001$). Primary tumor growth was significantly reduced in EX+ER HIGH mice compared with EX+ER LOW mice (Fig. 3.9C; 2-way ANOVA, $F_{(1,224)}=3.32$, $p=0.078$); however, this failed to reach statistical significance. The Bonferroni correction for multiple comparisons was significant for the day 28 time point. Tumor weight at sacrifice (Fig. 3.9D; Student’s t-test, $p=0.261$) and lung (Fig. 3.9E; Student’s t-test, $p=0.108$) and femur (Fig. 3.9F; Mann-Whitney, $p=0.732$) spontaneous metastasis were not significantly different between groups. Total splenocyte counts were significantly different between groups (Fig. 3.9G; Student’s t-test, $p=0.012$) with EX+ER HIGH mice displaying a significant reduction compared with EX+ER LOW mice. Gr-1$^+$CD11b$^+$ MDSCs were significantly different between groups (Fig. 3.9H; Student’s t-test, $p=0.033$) with EX+ER HIGH mice displaying a reduction compared with EX+ER LOW. CD11b$^+$Ly6C$^{lo}$Ly6G$^+$ granulocytic MDSCs (Fig. 3.9I; Student’s t-test, $p=0.062$) were not significantly different between groups. Splenic effector cells from EX+ER HIGH mice harvested after a five-day bulk secreted significantly more IFN$\gamma$ in response to re-stimulation with tumor antigens as compared with effector cells harvested from EX+ER LOW mice (Fig. 3.9J; n=9/group, Student’s t-test, $p<0.001$).
Figure 3.8. High vs. low activity within moderately exercising, weight stable mice. 

(A) Mice running an average of 5.8-12.6 km/day (EX+ER HIGH) weighed significantly less than mice running an average of 2.5-5.7 km/day (EX+ER LOW) over the course of the study (n=17/group; 2-way ANOVA, time x treatment, F[13,416]=2.81, p=0.001). 

(B) Mice in the EX+ER HIGH group averaged more km/day than the EX+ER LOW group over the course of the study (2-way ANOVA, F[1,384]=42.33, p<0.001). 

(C) Primary tumor growth (measured 2-3x/week via caliper) was reduced in EX+ER HIGH mice compared with EX+ER LOW mice (2-way ANOVA, F[1,224]=3.32, p=0.078); however, this failed to reach statistical significance. 

(D) Tumor weight at sacrifice (Student's t-test, p=0.261) and (E)
lung (Student’s t-test, p=0.108) and (F) femur (Mann-Whitney, p=0.732) spontaneous metastasis were not significantly different between groups. Splenocytes (n=17/group) were prepared into a single cell suspension and counted. (G) Splenocyte counts were significantly different between groups (Student’s t-test, p=0.012) with EX+ER HIGH mice displaying a significant reduction compared with EX+ER LOW mice. (H) Splenocytes were stained with anti-Gr-1 and -CD11b to quantify myeloid-derived suppressor cells (MDSCs). Gr-1+CD11b+ MDSCs were significantly different between groups (Student’s t-test, p=0.033) with EX+ER HIGH mice displaying a reduction compared with EX+ER LOW. Dashed line represents non-tumor bearing control. (I) Splenic CD11b+Ly6CloLy6G+ granulocytic MDSCs (Student’s t-test, p=0.062) were not significantly different between groups. (J) Isolated splenocytes were depleted of Gr-1+ cells and pulsed with irradiated 4T1.2luc cells for 5 days to generate an antigen-specific T cell response to tumor antigens. Effector cells harvested from EX+ER HIGH mice secreted significantly more IFNγ in response to re-stimulation with tumor antigens as compared to effector cells harvested from EX+ER LOW mice (n=9/group, Student’s t-test, p<0.001). Dashed line represents non-tumor bearing control. Significantly different than EX+ER LOW (*).

3.5. DISCUSSION

The extent to which exercise, as opposed to changes in body weight, protect against primary tumor growth and metastatic progression remains unclear in epidemiologic, clinical, and preclinical studies. The current study was designed to determine if exercise or changes in body weight via mild dietary restriction drive protection in an orthotopic model of state IV metastatic BC. We demonstrated that moderate exercise in weight stable mice has protective effects on primary tumor and spontaneous metastasis in the lungs and bone, common sites of metastases in women, in a highly aggressive stage IV mammary tumor model. Concurrent with the protective effect of exercise on primary tumor growth and metastatic burden, tumor-bearing mice that exercised and maintained a stable body weight had reduced splenomegaly and splenic immunosuppressive cell types, enhanced CD4+ helper T cell proliferation, reduced concentration of plasma insulin-like growth factor-1 (IGF-1), and altered chemokine, proinflammatory,
immunostimulatory, and immunosuppressive gene expression in the tumor microenvironment (TME). Single interventions (i.e., dietary energy restriction-induced weight control or exercise in mice gaining weight over the course of the study) failed to show beneficial effects on primary tumor growth or metastatic burden. Moderate exercise in weight stable mice may delay primary tumor growth and metastatic progression in part, due to changes in the TME and through maintaining an antitumor immune response over a protumor immunosuppressive immune response.

Substantial clinical (308, 325, 399-406) and observational epidemiological (292, 407) evidence exists suggesting that weight control is associated with a decrease in the risk of primary BC (309, 408, 409). Studies investigating weight control often use an intervention paradigm that combines mild dietary energy restriction with physical activity (as part of the current American Cancer Society Guidelines on Nutrition and Physical Activity for Cancer Prevention (573)) making it difficult to uncouple these interventions and evaluate if the protective effects are due entirely to weight maintenance, or if the individual effects of dietary energy restriction and/or physical activity drive protection. Studies in overweight and obese postmenopausal women are beginning to separate the effects of diet alone, aerobic exercise alone, and the combination of diet and exercise on inflammatory, metabolic, and sex hormone mediators (330, 410-413). Data from these studies show that sustained weight loss is achieved via moderate dietary energy restriction alone (restriction to achieve a 10% weight loss); however, diet plus aerobic exercise results in the greatest reduction in chronic inflammation (e.g., reduced C-
reactive protein, IL-6, leptin) in overweight and obese postmenopausal women. The reduction in chronic inflammation may help explain how weight control achieved through diet and exercise reduces the risk of postmenopausal BC.

Evidence from observational, randomized controlled, and bariatric surgery studies suggest that dietary energy restriction reduces the risk of BC in women (308, 324-327, 402, 574, 575). Specifically, dietary energy restriction in premenopausal and postmenopausal years is most effective at reducing the risk of postmenopausal primary BC. Dietary energy restriction has proven benefits in terms of delaying cancer progression in mice. Preclinical studies demonstrate that moderate to severe dietary energy restriction (20%-40% reduction in energy intake) delays primary tumor growth in orthotopic (357, 358) and carcinogen-induced (341, 359, 360) mammary tumor models. The protective effect of dietary energy restriction on carcinogenesis is proportional to the degree of restriction within the range of 20-40% (341, 361, 362). However, 10% dietary energy restriction does not alter the percent of carcinogen-induced mammary lesions, or the proportion of pre-malignant to malignant lesions compared with ad libitum-fed rats (341) suggesting that restriction alone may need to exceed 10% to exert a cancer prevention effect. In contrast, 10% dietary energy restriction is beneficial in slowing weight gain in several rodent models (300, 341) since caged ad libitum-fed animals are actually overfed (576, 577). Thus, a 10% dietary energy restriction was chosen to prevent weight gain rather than induce a cancer prevention effect in the current study.
Regular physical activity is an effective intervention for the prevention of both pre and postmenopausal primary BC (301). Clinical studies (including randomized controlled trials) suggest that physical activity as part of daily life, and purposeful aerobic exercise may mediate beneficial changes in a number of physiological systems which may contribute to the primary cancer prevention effect of exercise (298-300). Numerous preclinical studies also demonstrate that exercise, achieved via access to motorized treadmill or voluntary activity wheels, reduces primary tumor growth in orthotopic (383-385), carcinogen-induced (387-392), and transgenic (394, 395) mammary tumor models. In the 4T1 model (parental cell line of the 4T1.2 cells), exercise reduces primary tumor growth when the intervention is started concomitantly with the injection of 4T1 tumor cells into the mammary gland (385). Betof, et al. (385) reports that exercise induces a reduction in primary tumor growth and is accompanied by an increase in the density of apoptotic cells, and microvessel density and maturity in the tumor. These data suggest that exercise may alter the TME resulting in a reduction in tumor mass and tumor hypoxia (385). Thus, our current finding that moderately active, weight stable mice had reduced primary tumor growth in the highly metastatic orthotopic 4T1.2 model is consistent with data collected in several preclinical models. Interestingly, exercise has no effect on primary tumor growth when MDA-MB-231 cells are subcutaneously (397) or orthotopically (398) injected into athymic mice. These data suggest that the beneficial effects of exercise on primary tumor growth may be mediated via an immune-dependent mechanism.
Few researchers have designed studies to investigate the effects of weight control in BC survivorship populations. The limited studies report generally consistent findings that weight control can reduce mortality and improve quality of life measurements in BC survivors (375, 414-417, 578). The role of dietary energy restriction on BC recurrence and BC-specific and overall survival in BC patients is understudied. The relation between weight maintenance (achieved via diet alone, exercise alone, or the combination of diet and exercise) and metastatic progression is poorly understood in preclinical studies. In two preclinical studies, mice were given an intravenous (I.V.) injection of mammary tumor cells and randomized into voluntary exercise or sedentary control groups (112, 113). In both studies, exercise following tumor cell injection did not alter the development of lung metastases. However, because tumor cells were injected I.V., many of the biological parameters that comprise the metastatic process were bypassed. Male mice orthotopically implanted with murine prostate cancer cells and randomized to sedentary or exercise groups have similar primary tumor growth rates, but exercise causes an increase in tumor vascularization concurrently with a reduction in the expression of prometastatic genes (386). Moderate to severe caloric restriction (25-40% reduction in calories) in 4T1 tumor-bearing mice reduces the total number of lung metastases that originate both spontaneously from the primary tumor and experimentally from I.V. injection of tumor cells (579). In the current study, we demonstrated that exercising, weight stable mice significantly reduced spontaneous metastasis in the lung and bone using a clinically relevant, murine metastatic model. These findings suggest a novel mechanism by which moderate
exercise in a weight stable host may contribute to the reduction in mortality in BC patients.

Evidence from epidemiological studies indicates that physical activity significantly decreases the risk of recurrence and improves BC-specific and overall survival in BC patients (298, 380). Two recent meta-analyses were completed using data from 16 cohort studies involving over 40,000 BC patients (379, 380). Physical activity was assessed using validated self-reported questionnaires and expressed as times per week, hours per week (h/week), metabolic equivalent of task (MET)-h/week, or energy expenditure in calories per week. Results of both analyses indicate that patients who participated in any amount of physical activity prior to a BC diagnosis had approximately a 20% risk reduction in BC-specific mortality when comparing the most active to the least active women. Additionally, patients who participated in physical activity after a cancer diagnosis had a 40-50% risk reduction in BC-specific mortality (379, 380). The beneficial effects of physical activity on BC-specific mortality remained significant after stratifying by body mass index or menopausal status, suggesting the effects of physical activity on cancer survival may be independent of metabolic or reproductive hormone status (379-381). A similar association between baseline cardiorespiratory fitness and reduced BC-specific mortality has also been demonstrated in the Aerobics Center Longitudinal Study, in which 14,811 women were followed for 31 years (382). It remains unclear if the protective effect of exercise on cancer outcomes (primary or secondary prevention) is due, in part, to the prevention of weight gain (i.e., obesity prevention) or to a direct effect of exercise on cancer risk and
progression. The leading cause of BC-specific mortality is due to metastatic disease (580). Thus, physical activity may be reducing BC recurrence and improving survival by delaying or preventing metastases.

Both dietary energy restriction groups (i.e., SED+ER and EX+ER) weighed significantly less than ad libitum-fed mice (i.e., SED+AL or EX+AL) over the course of the study. Importantly, body weights were not significantly different between weight gain (i.e., SED+AL and EX+AL) or weight maintenance (i.e., SED+ER and EX+ER) groups, allowing us to evaluate if the protective effects on tumor growth is due entirely to weight maintenance, or if the individual effects of dietary energy restriction and/or physical activity drive protection. SED+AL control mice gained weight over the course of the study (12% increase from baseline) and displayed growth curves comparable to 4T1.2 growth curves in previously published reports (168, 536). Exercise alone mice (EX+AL) gained weight over the course of the study comparable to the SED+AL control. No protective effect of exercise was observed on primary tumor growth or metastatic burden in mice that gained weight over the course of the study, suggesting that weight gain-induced disturbances in inflammatory, hormonal, or immunological function can override the exercise-induced benefits. Dietary energy restriction alone (SED+ER) resulted in sustained weight control (10% weight loss from baseline) over the course of the study; however, failed to drive beneficial effects on primary tumor growth. The diet and exercise group (EX+ER) maintained weight comparable to the SED+ER group suggesting that even with the additional energy expenditure of the activity wheel, the EX+ER mice remained in energy balance. EX+ER resulted in sustained weight
control and a protective effect on primary tumor growth and lung, femur, and liver spontaneous metastasis, as well as a decrease in metastatic flux measured through bioluminescent imaging. Spontaneous metastasis in the kidney, heart, and tibia was not significantly different between all groups and spontaneous metastasis in the brain and spine was not significantly different when collapsing to WG vs. WM groups. The effects of diet and exercise on the dissemination and embedding of circulating tumor cells into metastatic niches may be site specific. To our knowledge, this is the first study to show a cancer protective effect of moderate exercise in weight stable mice on spontaneous metastases to lung and bone, common sites of metastases in BC patients, in a highly aggressive stage IV BC model.

Mild dietary energy restriction and moderate physical activity could impact several host factors implicated in primary tumor growth and metastatic progression (298, 299, 418) including immune, metabolic, and inflammatory mediators. The 4T1.2 transplantable mouse model has an intact tumor-host environment, allowing for the evaluation of our interventions on the tumor-immune microenvironment (532). The 4T1 (537, 538) and 4T1.2 (figure 2.4) mammary tumor model can release endocrine signals (e.g., G-CSF) that dysregulate hematopoiesis, resulting in the expansion and accumulation of immature, myeloid-lineage cells into the blood, spleen (e.g., splenomegaly), TME, and metastatic niches. As tumors advance, there is a progressive decrease in lymphoid-lineage cells and an increase in myeloid-lineage cells within the tumor and secondary lymphoid organs. This dysregulated immune phenotype can generate an immunosuppressive
environment that dampens antitumor effector functions, which promotes tumor escape and survival. Therefore, interventions that target this shift to inhibit the expansion of immunosuppressive cells could help to maintain an antitumor immune response and aid in the elimination of transformed cells.

The combination of diet and exercise in weight stable mice had the lowest concentration of plasma G-CSF and a significant reduction in the onset of splenomegaly compared with SED+AL and EX+AL groups. EX+ER maintained splenic lymphoid-lineage populations (splenic CD3+ T cells, CD3+CD4+ helper T cells, and CD19+ B cells) while reducing the emergence of myeloid-lineage populations (splenic Gr-1+CD11b+ MDSCs, CD11b+Ly6C^hiLy6G^- monocytic MDSCs, and CD11b+Ly6C^loLy6G^ granulocytic MDSCs). MDSCs, well known suppressors of antitumor immunity which contribute to tumor progression and metastases (142), were highly correlated with tumor volume. EX+ER may not alter the immunosuppressive capacity of MDSCs on a per cell basis. However, EX+ER-induced changes in myelopoiesis and/or the recruitment and trafficking of MDSCs to secondary sites could result in a reduction in the number of these suppressive cells and could help explain the protective effect on tumor progression and metastases.

In a previous study using non-tumor-bearing mice, we demonstrate that moderate, voluntary wheel activity in weight stable mice enhances antigen-specific CD4+ T helper cell responses (562). In the current study, we report that these findings are consistent in the 4T1.2 mammary tumor model. Moderate exercise in weight stable mice enhanced splenic CD4+ helper T cell proliferation. However,
neither IL-2 and IFN\(_\gamma\) secretion from CD4\(^+\) helper T cells at 48 hours in response to 0.5 \(\mu\text{g/ml}\) anti-CD3, nor natural killer cell cytotoxicity measured via a standard 4-hour \(^{51}\text{Cr}\)-release assay, was significantly different when collapsing to WG vs. WM groups. Previous studies report exercise-induced enhancements in NK cell function (521); however, this may be model or time dependent. The terminal (day 35) evaluation of NK cell function may be too late in tumor progression to observe exercise-induced enhancements. Additionally, the percentage of splenic immune cells post bulk culture or splenic IFN\(_\gamma\) secretion in response to re-stimulation with tumor antigens was not significantly different between the four energy balance interventions. The 4T1 series is weakly immunogenic (535); therefore, without providing an immunogenic stimulus \textit{in vivo}, our assay may not be sensitive enough to detect differences in antigen-specific immune responses. However, the population shifts and functional outcomes in splenic immunity could play a role in maintaining immunosurveillance mechanisms that are critical in preventing the metastatic process via the recognition of disseminating tumor cells and promotion of immune-mediated dormancy (117). Thus, the combination of diet and exercise in weight stable mice may directly impact primary tumor growth and metastases via the augmentation of antitumor immunity and the reduction in immunosuppressive cells.

Moderate exercise in weight stable mice may protect against detrimental alterations in inflammatory and metabolic mediators which, in turn, could impact tumor growth and metastases. Numerous researchers have reported the role of inflammatory mediators (e.g., IL-1\(\alpha\), IL-6, MCP-1), adipokines (e.g., leptin,
adiponectin), and metabolic mediators (e.g., insulin, IGF-1, IGFBP-3) in tumor growth (360). Cytokines and chemokines play a critical role in cancer-related inflammation, regulating both host and malignant cells in the TME (581). Physical activity reduces inflammatory mediators associated with obesity and other chronic disease (493, 582). In the current study, we found no differences in plasma inflammatory cytokine levels (G-CSF, IL-6, IL-1α, MCP-1) between groups. These results suggest that inflammatory mediators may not be impactful at this terminal time point. Moderate exercise in weight stable mice may alter the inflammatory milieu during early tumor growth or initiation of metastasis. Once tumor burden reaches a critical mass, these differences may be less apparent. Future studies are planned to examine the effects induced by moderate exercise at both earlier time points and when tumor volumes are comparable between groups. Moderate exercise in weight stable mice could affect reproductive hormone levels, which in turn could impact BC development and progression. All mice in the current study have intact ovaries, therefore the protective effects observed in EX+ER mice could be mediated by alterations in estrogen levels. Future studies are required to evaluate if the effect of moderate exercise in weight stable mice in the 4T1.2 model is associated with estrogen status.

A reduction in primary tumor growth was observed in EX+ER mice compared with weight-matched SED+ER mice. Since body weight between groups were identical, moderate exercise-induced changes in skeletal muscle-derived cytokines, or myokines (435, 583, 584), could drive protection in EX+ER mice. Skeletal muscle is now recognized as an endocrine organ that can secrete
peptides in response to exercise-induced skeletal muscle contraction (435) to promote an anti-inflammatory milieu (e.g., IL-6, IL-10, IL-Ira) and regulate physiological processes in distant organs (493). An exercise-induced shift in circulating concentrations of pro- and anti-inflammatory cytokines may have direct effects on tumor proliferation (494), or an indirect effect through the control of systemic levels of hormones (299) or altered immune responses (e.g., acute mobilization of NK and T cells, enhancements in NK cytotoxicity) (112, 495, 496).

Regular physical activity may prevent cancer initiation and improve survival via exercise-induced changes in skeletal muscle-derived cytokines that reduce proinflammatory signaling. Interestingly, in the current study, the beneficial effects of myokines may be negated when mice participating in moderate exercise also gain weight. Although the weight gain mice (i.e., SED+AL and EX+AL) did not become classically obese over the course of the study, perhaps the positive energy balance produced an inflammatory microenvironment that overshadowed the anti-inflammatory effects induced by exercise. Future studies performing experimental blockade of exercise-induced myokines are needed to elucidate proposed metabolic effects on the control of tumor growth or enhancements in immune response.

IGF-1 is a peptide hormone involved in modulating cell growth and survival by stimulating proliferation (468). In circulation, the action of IGF-1 is regulated by a group of six IGF-binding proteins (IGFBPs) (469, 470). IGFBP-3 is the primary binding protein and can exhibit IGF-1 independent actions, such as promoting growth inhibition and apoptosis (471). Elevated circulating concentrations of IGF-
1 are firmly established as a risk factor for the development of BC, especially estrogen positive tumors (472-477). A recent meta-analysis was completed using data from 17 prospective studies quantifying pre-diagnosis circulating IGF-1 concentration and BC risk involving 4,790 BC patients (475). Results indicate that patients with the highest IGF-1 concentration had a 28% increased BC risk compared with the lowest fifth. This association was not altered when adjusting for IGFBP3 and did not vary by menopausal status; however, it does seem to be confined to estrogen-receptor-positive tumors (475). The role of IGF-1 in BC survivorship populations is emerging (478-481). Since IGF-1 is responsive to changes in energy balance, researchers are investigating the role of physical activity as a non-pharmacological intervention to reduce IGF-1 levels in BC survivors (433, 482, 483). A recent meta-analysis was completed using data from five randomized controlled trials involving 235 BC survivors (484). Aerobic exercise resulted in a significant reduction in circulating IGF-1; however, the effect on BC recurrence and survival has yet to be established. Pre-clinical studies have focused on the impact of IGF-1 in cancer cell proliferation, migration, and metastasis using in vitro and in vivo models to identify the signaling pathways involved in these processes (485). It is well established that dietary energy restriction prevents mammary tumorigenesis in rodents, and IGF-1 may play a key role in tumor reduction via activation of the Akt/mTOR pathway following dietary energy restriction (488). Emerging evidence suggests that IGF-1 may play a role in the epithelial-mesenchymal transition (EMT) process (343, 489). A number of studies show a strong correlation between EMT and high invasive and metastatic
behavior of BC (490). Results from a recent study suggest that IGF-1 levels may regulate tumor growth by modulating genes related to EMT and chemokine signaling (358). Thus, alterations in energy balance achieved via mild dietary energy restriction and moderate physical activity are likely linked to the EMT process. In the current study, the reduction in primary tumor growth and metastasis is associated with decreased levels of plasma IGF-1, which may explain the cancer prevention effect of moderate exercise in weight stable mice in the 4T1.2 model.

Moderate exercise in weight stable mice could alter the TME. The total number of isolated tumor-infiltrating immune cells in the combined WM group (12.2±6.3x10^6) was half of the WG group (24.3±20.8x10^6); however, no significant differences were observed in the percentage or number of tumor-infiltrating immune cell subsets between groups. We next investigated the effects of moderate exercise in weight stable mice on gene expression in the TME. A downward shift in the fold regulation of tumor-immune crosstalk genes was observed in the single intervention (EX+AL, SED+ER) and dual intervention (EX+ER) groups. All treatment groups experienced a downward shift in genes (\(Ccl5\) [Rantes] (585, 586), \(Cxcl1\) (124, 156, 176-178), \(Ccl20\) [Mip-3\(\alpha\)] (587), and \(Ccl22\) [Mdc] (588)) important for chemotaxis and recruitment of immunosuppressive cells, like MDSCs and Tregs, to the TME. This finding is consistent with the observed reduction in splenic MDSC subsets in the SED+ER and EX+ER groups. Also, all treatment groups experienced a downward shift in genes (\(Cxcl9\) [Mig] and \(Cxcl10\) [Inp10] (589), \(Ccr7\) (590, 591), and \(Cxcr3\) (592)) important for promoting angiogenesis, tumor cell invasion, and metastasis. A step-
wise decrease (EX+AL → SED+ER → EX+ER) was observed in the fold regulation of *ido1* (*Ido*). *Ido* is expressed by tumor cells, supporting cells within the TME, and infiltrating cells, like MDSCs, and encodes for indoleamine 2,3-dioxygenase (IDO), the first and rate-limiting enzyme of tryptophan catabolism through the kynurenine pathway (197). Depletion of tryptophan, an amino acid essential for T cell activation, results in blunted T cell proliferation, differentiation, effector functions, and viability (198). Future studies are needed to assess exercise-induced effects on the IDO pathway.

The weight maintenance groups failed to separate in the tumor gene array, therefore, since the two groups weigh the same with the only difference being sedentary vs. exercise, we performed a subset analysis of SED+ER and EX+ER groups, using SED+ER mice as the reference control to investigate the effects of exercise in weight stable mice. The EX+ER mice displayed a decrease in fold-regulation in *Csf3* and *Nfkb* and an increase in fold-regulation in *Il2*. The effects of moderate exercise in weight stable mice may be mediated through a reduction in inflammatory gene expression markers (i.e., reduced genes encoding for G-CSF and NF-κB) that drive the generation of an immunosuppressive TME. Furthermore, moderate exercise in weight stable mice may upregulate gene expression of markers that directly enhance the function of antitumor effector populations (i.e., gene encoding for IL-2) within the TME. Future studies are needed to investigate the individual effects of each gene on immune function and tumor growth; however, the current study provides essential insight into potential shifts that can occur in
immunosuppressive markers in response to moderate exercise in weight stable mice.

Lastly, characterizing the dose, duration, frequency, and type of exercise needed to drive cancer prevention is key to move the field of exercise oncology forward (551, 593, 594). Preclinical investigation of these important questions is difficult, but important to help shed light on potential mechanisms. For example, access to voluntary activity wheels for 60 days prior to tumor implantation reduces primary tumor growth in a dose-dependent manner (384), i.e., mice with higher activity before tumor cell injection have a decrease in tumor mass measured at the time of sacrifice. Subset analysis in the current study sheds light on the average quantity of activity required in the EX+ER group to drive beneficial changes on tumor growth in the 4T1.2 mammary tumor model. Mice running greater than 5.8 km/day displayed better weight control over the course of the study with a reduction in tumor growth compared with mice who ran less than 5.8 km/day. The EX+ER HIGH runners had reduced splenomegaly with less splenic MDSCs and enhanced splenic IFN\(\gamma\) secretion in response to re-stimulation with tumor antigens. This suggests that mice in the EX+ER HIGH group may have an inflammation-immune axis which is better able to reduce tumor progression through maintained immunosurveillance mechanisms. As the field of exercise oncology matures, it is essential for investigators in the fields of exercise physiology, immunology, and cancer biology to address the dose, duration, frequency, and type of exercise needed to achieve a cancer prevention effect.
Data from the current study provides critical insight into the extent to which exercise, and not just changes in body weight, underlie cancer protection. Although dietary energy restriction-induced weight control was effective at altering host splenic immunity and the expression of key genes in the TME related to immunosuppression and metastatic progression, this intervention failed to induce changes in primary tumor growth or spontaneous metastases. Interestingly the exercise-induced effects were lost when mice continued to gain weight over the course of the study, suggesting that weight gain-induced disturbances in inflammatory, hormonal, or immunological function can override the exercise-induced benefits. Moderate exercise in weight stable mice not only resulted in a reduction in primary tumor growth, but prevented spontaneous metastasis, suggesting that moderate exercise in a weight stable host may reduce BC-specific mortality by delaying metastatic occurrence or progression. This delay may be the result of lowered metabolic drivers of tumorigenesis (i.e., prevention of the toxic, immunosuppressive TME) or an enhancement in immunosurveillance mechanisms that prevent tumor cell invasion and metastatic spread. Results from the current study provide insight into potential mechanisms by which physical activity exerts primary and secondary cancer prevention effects and provides a biological rationale for future randomized controlled trials of exercise and the prevention of weight gain to prevent metastatic progression in BC survivors and ultimately improve survival outcomes.
CHAPTER 4: MODERATE EXERCISE IN WEIGHT STABLE MICE AND THE ADMINISTRATION OF A WHOLE TUMOR CELL CANCER VACCINE IN THE 4T1.2 MAMMARY TUMOR MODEL*

4.1. ABSTRACT

Regular, moderate exercise can promote weight control and reduce both the incidence and improve survival of breast cancer (BC). Numerous biological mechanism(s) are proposed to explain these beneficial clinical effects. However, little work has been done to examine the effect of moderate exercise in weight stable hosts on emerging immunomodulatory or immunotherapeutic strategies. We previously demonstrated (chapter three) a cancer protective effect (both primary tumor and metastatic burden) of moderate exercise in weight stable 4T1.2 tumor-bearing mice. Our diet and exercise intervention reduced splenomegaly and the abundance of splenic myeloid-derived suppressor cells (MDSCs) and MDSC subsets and altered chemokine, proinflammatory, immunostimulatory, and immunosuppressive gene expression in the tumor microenvironment (TME). Thus, the goal of the current study was to further characterize the prevention of weight gain through mild dietary energy restriction and moderate physical activity on tumor progression and immune responses, and determine if there were any additive effects of moderate exercise in weight stable mice and the therapeutic administration of an allogeneic, whole tumor cell cancer vaccine on the aforementioned outcomes. Female BALB/c mice were randomized into sedentary,

ad libitum-fed weight gain (WG) control or exercising, mildly calorie restricted (90% of control food intake) weight maintenance (WM) groups (n=20-24/group). Mice were provided access to standard cages (WG groups) or activity wheel cages (WM groups) for eight weeks prior to the injection of $5 \times 10^4$ luciferase-transfected 4T1.2 tumor cells into the fourth mammary fat pad. Mice were further randomized into vehicle control (n=11-12/group) or vaccination (n=9-12/group) groups and administered PBS vehicle (VEH) control or $1 \times 10^6$ irradiated 4T1.2$^{luc}$ cells (VAX) at day 7, 14, 21, and 28 post-tumor injection. Mice continued on their respective energy balance intervention until sacrifice at day 35 post-tumor implantation. WM mice, with or without VAX, weighed significantly less than WG mice throughout the study (p<0.001). There was a significant effect of both WM and VAX alone on primary tumor growth (p<0.001); and an additive effect of WM+VAX on primary tumor growth, lung (p=0.003) and heart (p=0.049) spontaneous metastases, splenocyte count at sacrifice (p=0.066), the number of total splenic MDSCs (p=0.057) and the granulocytic subset of MDSCs (p=0.029), and plasma levels of insulin-like growth factor 1 (IGF-1) (p=0.0121). Splenic interferon gamma (IFN$\gamma$) secretion in response to re-stimulation with tumor antigens was significantly elevated in response to VAX (p<0.001) and WM (p=0.017); however, there was no additive effect of WM+VAX. These results demonstrate that moderate exercise in weight stable mice in combination with a therapeutic, allogeneic whole tumor cell cancer vaccine is highly effective at delaying primary tumor growth and metastases, reducing splenomegaly and immunosuppressive cell populations, and reducing the concentration of plasma IGF-1 in the metastatic 4T1.2 mammary
tumor model. Preclinical models continue to investigate pharmacological combinatorial strategies to improve cancer vaccine efficacy. However, this is the first study to demonstrate that efficacy of a whole tumor cell cancer vaccine can be enhanced through an exercise intervention.

4.2. INTRODUCTION

Breast cancer (BC), especially the diagnosis of metastatic disease, is a global health concern (1, 2). BC is a heterogeneous disease characterized by a diverse spectrum of molecular phenotypes that are associated with different treatment modalities and clinical outcomes (21-23). Localized BC treatment typically involves breast-conserving surgery or mastectomy and is often coupled with radiotherapy, chemotherapy, endocrine therapy, and/or targeted therapy (33, 34). Metastatic BC treatment is most often radiation and/or chemotherapy (34); however, metastatic disease remains incurable and is the underlying cause of death in the majority of BC patients who die of the disease (3). Based on recent advancements in the fields of immunology and molecular biology, immunotherapy has become a promising emerging treatment for BC, especially metastatic disease (62, 63). Immunotherapy treatments include therapeutic cancer vaccines, monoclonal antibodies, adoptive cell therapies, and the administration of immunostimulatory cytokines, all of which are designed to activate or restore immune function and eliminate tumor cells in some manner (51). Due to the lack of effective treatment options, emerging immunomodulatory and immunotherapy strategies are being investigated in advanced-stage cancer patients.
Cancer vaccines can be prophylactic (i.e., preventative) (206) or therapeutic (51). Therapeutic cancer vaccines, categorized as dendritic-cell (DC) or whole tumor cell based, provide antigen-presenting cells (APCs), like DCs, with tumor-associated antigens (TAAs) to stimulate a durable antitumor effector CD8+ cytotoxic T cell response (51, 52, 207-209). Briefly, DCs phagocytose exogenous antigens and process them by proteases to peptide fragments (77). Activated DCs migrate to proximal draining lymph nodes where they can present antigens and activate CD8+ cytotoxic T cells in the context of class I major histocompatibility complex (79). Providing tumor-derived peptides (210) or full-length recombinant tumor proteins are two strategies for DC-based therapeutic cancer vaccines; however, the development of in vivo tumor-derived peptide vaccinations has proven problematic as free peptides can be rapidly cleared prior to uptake by antigen-presenting cells (51). The co-administration of immune-stimulant adjuvants (e.g., IL-2 or GM-CSF) with tumor-derived peptides to further promote the activation of either DCs or CD8+ cytotoxic T cells has increased vaccine efficacy, generating a more robust antitumor effector CD8+ cytotoxic T cell response (209, 211). An alternative strategy is to generate DCs ex vivo from peripheral blood mononuclear cells, pulse with TAAs, and inject the activated DCs back into the host for subsequent activation of a CD8+ cytotoxic T cell response (208, 212, 213). Two limitations in DC-based vaccines is that T cell epitopes against TAAs are largely undefined, making it difficult to screen and select tumor antigens (214, 215) and the ex vivo generation of DCs is laborious and costly (216).
Whole tumor cell based cancer vaccines represent an attractive alternative source of TAAs (sourced from whole tumor cells, tumor cell lysates, tumor oncolytes, apoptotic bodies, or transduced tumor cells), as this type of vaccine typically displays some intrinsic adjuvant activity and allows DCs to process and present numerous TAAs to induce a polyclonal effector CD8+ cytotoxic T cell response against numerous tumor cell populations (207, 217). Through epitope spreading, or the process by which T cells respond to peptides not present in the whole tumor cell cancer vaccine, tumor cells that are not originally targeted through TAAs within the whole tumor cell cancer vaccine can become targets through secondary priming (218-220). Whole tumor cell cancer vaccines are effective in murine models, but clinical translation has proven difficult (595). It is often difficult for autologous cancer vaccines (i.e., sourced from the same host receiving the vaccination) to obtain tumor cells in sufficient quantities in a timely fashion, expand in vitro, and administer to patients. Vaccine preparation is costly and requires standardization and regulatory oversight. Allogenic cancer vaccines (i.e., sourced from a different host or tumor cell line) can bypass cell limitations; however, may contain TAAs not present in the patient’s tumor and therefore may rely heavily on cross-priming. Limitations notwithstanding, both autologous and allogeneic whole tumor cell cancer vaccines are under preclinical (221-226) and clinical (227-229) development. Several host-related factors (e.g., tumor-induced inflammation and/or emergence of immunosuppressive cell types) can influence the efficacy of whole tumor cell based cancer vaccines (208, 230), therefore combinatorial strategies are investigating if current (e.g., chemotherapy, radiation) and emerging
(e.g., monoclonal antibodies, tyrosine kinase inhibitors) therapies can reduce tumor-induced inflammation to enhance therapeutic efficacy (224, 230-234). However, no preclinical or clinical study has examined the effects of non-pharmacological, lifestyle-based interventions on the efficacy of whole tumor cell based cancer vaccines.

Regular physical activity and the prevention of weight gain is associated with a decrease in the risk of primary BC (292, 308, 309, 325, 399-409) and a reduction in mortality and improvements in quality of life in BC survivors (375, 414-417, 578). Data from our previous findings (chapter three) show that moderately exercising, weight stable mice display a reduction in 4T1.2luc tumor growth and metastatic progression, concurrently with a reduction in the expansion of immunosuppressive cell types (e.g., MDSCs), sustained lymphoid-lineage immune phenotype, and a reduction in proinflammatory and immunosuppressive gene expression in the TME. Thus, we hypothesized that the reduction in tumor-induced inflammation and immunosuppression observed with moderate exercise in weight stable mice could enhance whole tumor cell cancer vaccine efficacy. Therefore, the goal of the current study was to determine if moderate exercise in weight stable mice could improve the efficacy of an allogeneic, whole tumor cell cancer vaccine in a clinically relevant, orthotopic model of stage IV metastatic BC.

4.3. MATERIALS AND METHODS

4.3.1. Tumor cell line and cell culture

The 4T1.2 cell line is a murine metastatic BC line derived from a spontaneously arising mammary tumor in a BALB/cfC3H mouse (566). When
implanted orthotopically, the 4T1.2 cell line mimics the metastatic progression of human BC with a tendency to metastasize to lung and bone (567). 4T1.2 cells stably expressing luciferase (4T1.2\textsubscript{luc}) were provided by Dr. Robin Anderson and maintained in Dulbecco’s modified Eagle’s medium (Life Technologies) containing 10% fetal bovine serum (Gemini Bio-Products), 2 mM glutamine (Mediatech), 1X nonessential amino acid (Mediatech), and 8 μg/ml puromycin (Mediatech).

### 4.3.2. Optimizing the whole tumor cell cancer vaccine

To prevent proliferation and to induce cell death 4T1.2\textsubscript{luc} cells were either irradiated in a cesium irradiator (10,000, 15,000, or 20,000 rad), exposed to three freeze/thaw cycles, or cultured with (50, 100, or 200 ng/ml) mitomycin (Enzo Life Science; Farmingdale, NY). After exposure to irradiation, repeated freeze/thaw cycles, or mitomycin, 4T1.2\textsubscript{luc} cells were plated in a serial dilution of cells starting at 0.1x10\textsuperscript{6} 4T1.2\textsubscript{luc} cells/well in a 96-well plate (Corning) for a 72-hour proliferation assay. Untreated 4T1.2\textsubscript{luc} cells were plated as a positive control. After 52 hours, cells were pulsed with tritiated thymidine (Perkin Elmer) and proliferation was quantified via [H\textsuperscript{3}] uptake and quantified on a Microbeta plate reader (Perkin Elmer). Each assay was performed in triplicate.

To demonstrate the immunogenicity of the 4T1.2\textsubscript{luc} cells, a cohort of mice (n=4; 14-week old) were administered 1x10\textsuperscript{6} irradiated 4T1.2\textsubscript{luc} cells (VAX) I.P. at day -28, -21, -14, and -7 pre-tumor injection followed by the orthotopic injection with 5x10\textsuperscript{4} luciferase-transfected 4T1.2 cells into the fourth mammary fat pad. Primary tumor growth was measured 2-3x/week via caliper
and all mice were removed from study by day 140 post tumor implantation. To determine the most effective route of vaccination, BALB/c mice (14-week old) were orthotopically injected with 5x10^4 luciferase-transfected 4T1.2 cells into the fourth mammary fat pad. After injection, mice were randomized into intraperitoneal (I.P.) or subcutaneous (SQ) vaccination groups (n=2/group) and administered 1x10^6 irradiated 4T1.2_luc cells (VAX) at day 7, 14, 21, and 28 post tumor injection. Mice were sacrificed at day 35 post tumor implantation. A third cohort of mice (n=8; 14-week old) were orthotopically injected with 5x10^4 luciferase-transfected 4T1.2 cells into the fourth mammary fat pad and administered 1x10^6 irradiated 4T1.2_luc cells (VAX) I.P. at day 7, 14, 21, and 28 post tumor injection or no irradiated 4T1.2_luc cells as a control. Primary tumor growth was measured 2-3x/week via caliper and mice were removed from study at 35 days post tumor implantation.

4.3.3. Optimizing splenic immune assays

Isolated splenocytes were depleted of Gr-1^+ cells and pulsed with irradiated 4T1.2_luc cells for one round (five days) or two rounds (after five days, bulk culture cells were washed and re-stimulated with irradiated 4T1.2_luc cells and fresh BALB/c antigen-presenting cells for an additional five days) of bulk culture to generate an antigen-specific T cell response to tumor antigens. Post bulk culture, 0.5x10^6 effector cells were isolated, washed, and plated in 24 well plates with a final volume of 0.5 or 1 ml of complete media. After a 24-hour period without irradiated 4T1.2_luc stimulation, cell supernatant was collected and interferon gamma (IFNγ) was quantified via Legend Max ELISA kits
(Biolegend). Splenic effector cells (0.5\times10^6) were plated alone or with irradiated 4T1.2^{luc} or Panc.02 cells (0.1\times10^6), with or without fresh BALB/c antigen-presenting cells (2.5\times10^6), in 0.5 ml complete media. After a 24-hour period without irradiated 4T1.2^{luc} stimulation, cell supernatants were collected and IFN\gamma was quantified via Legend Max ELISA kits (Biolegend). Splenic effector cells (0.5\times10^6) were plated alone and removed from antigen stimulation for 24 or 48 hours prior to cell supernatant collection. IFN\gamma was quantified via Legend Max ELISA kits (Biolegend).

### 4.3.4. Animal model

Six-week-old female BALB/c mice were obtained from Jackson Laboratory and screened for their intrinsic running potential. Intrinsic runners (14-week-old) were randomized to sedentary, weight gain (WG; n=24) or exercising, weight maintenance (WM; n=20) groups for a total of 13 weeks (Fig. 4.3A). All mice were fed AIN-76A diet (Research Diets); however, the WM mice were fed 90\% of caloric intake of WG mice to remain in energy balance (prevent weight gain) over the course of the study. After eight-weeks on study, all mice were orthotopically injected with 5\times10^4 luciferase-transfected 4T1.2 cells into the fourth mammary fat pad and continued on their intervention for 35 days. After injection, mice were further randomized into vehicle control (n=11-12/group) or vaccination (n=9-12/group) groups and administered PBS (VEH) or 1\times10^6 irradiated 4T1.2^{luc} cells (VAX) at day 7, 14, 21, and 28 post tumor injection. Mice were sacrificed at day 35 post tumor implantation. Movement was not monitored in mice that did not have access to running wheels. Food
intake, body weight, and tumor size ($v=(\text{short}^2\times\text{long})/2$) were monitored as previously reported (562, 568), and mice were observed daily for signs of ill health. All mice were housed at the Pennsylvania State University and maintained on a 12-hour light/dark cycle with free access to water. The Institutional Animal Care and Use Committee of the Pennsylvania State University approved all animal experiments.

**4.3.5. Whole tumor cell cancer vaccine**

Trypsin/EDTA (0.25%/2.21 mM) in HBSS (Corning) was added to culture flasks to harvest $4T1.2^{\text{luc}}$ cells. $4T1.2^{\text{luc}}$ cells were washed twice in $4T1.2^{\text{luc}}$ media and washed twice in PBS. $4T1.2^{\text{luc}}$ cells were counted, adjusted to $1.0\times10^6/100 \mu l$, and irradiated for 15,000 rad in a cesium irradiator. Irradiated $4T1.2^{\text{luc}}$ cells (VAX; $1.0\times10^6/100 \mu l$) or PBS control (VEH; 100 $\mu l$) were injected I.P. at day 7, 14, 21, and 28 post tumor implantation.

**4.3.6. Metastatic burden**

The lung, heart, kidney, liver, and femur were collected, flash frozen in liquid nitrogen, and stored at -80°C. Tissues were homogenized and genomic DNA was isolated using DNeasy Blood and Tissue Kit (Qiagen), per manufacturer’s instructions. DNA was quantified using a Nanodrop ND-1000 spectrophotometer (Nanodrop Products). Metastatic tumor burden of the tissues was quantified using the Taqman™ system performed on a Step-One Plus (Life Technologies) real time PCR instrument to quantify luciferase. Assay sequences for luciferase were CAGCTGCACAAAGCCATGAA (forward primer), CTGAGGTAATGTCCACCTCGATATG (reverse primer) and
TACGCCCTGGTGCCCGGC (probe with 5’ FAM reporter and 3’ BHQ quencher). The reference gene used for normalization was mouse telomerase reverse transcriptase (TERT) and was assayed using the Taqman Copy Number Reference Assay TERT (Life Technologies). The standard curve included 5-10-fold serial dilutions (200 ng to 20 pg) of DNA extracted from cultured 4T1.2lac cells. The standard curve and 200 ng of the tissue DNA samples were run in duplicate for luciferase and TERT on the Step-One Plus using the quantitative data analysis option and standard cycling parameters. Luciferase data was normalized to the quantitative values for TERT in each sample to correct for fluctuations in DNA amount, quality, and reaction efficiency.

4.3.7. Splenic and tumor-infiltrating immune cell assays

4.3.7.1. Isolation of splenic immune cells

Spleens were harvested via gross dissection and splenocytes were prepared from individual mice by mechanical dispersion, as previously described (345). Briefly, spleens were mechanically disrupted with a syringe plunger and passed through a 70 μm nylon mesh strainer (BD Biosciences), erythrocytes were lysed with ACK lysing buffer (Lonza), and remaining cells washed twice in complete medium (RPMI 1640 (Mediatech) supplemented with 10% FBS (Gemini Bio-Products), 0.1 mM non-essential amino acids (Mediatech), 1 mM sodium pyruvate (Mediatech), 2 mM glutamine (Mediatech), 10 mM HEPES (Mediatech),
100 U/mL penicillin streptomycin (Mediatech). Cell counts and viability were determined via trypan blue exclusion (Mediatech).

4.3.7.2. Splenic CD4+ helper T cell proliferation assay

Splenic CD4+ helper T cells were isolated via Dynabeads Untouched Mouse CD4 Cells Kit following manufacturer’s instructions (Life Technologies). Splenic CD4+ helper T cells (1x10^5) plus 1.0 μg/ml unlabeled anti-CD28 (BD Biosciences) were incubated in flat-bottomed, 96-well plates (Greiner Bio-One) in the presence of increasing concentrations of anti-CD3 antibody (BD Biosciences) for 72 hours (562). Proliferation data was analyzed by tritiated (H3) thymidine (Perkin Elmer) incorporation and quantified on a Microbeta plate reader (Perkin Elmer). Each assay was performed in triplicate. CD4+ helper T cell supernatant was collected following 48-hour stimulation with 0.5 μg/ml anti-CD3 and 1.0 μg/ml anti-CD28 and stored at -80°C. IL-2 was quantified using Legend Max ELISA kits (Biolegend) for the WG vs. WM groups.

4.3.7.3. Splenic IFNγ secretion post five-day bulk culture

Splenic immune cells, depleted of Gr-1+ cells via magnetic bead isolation (Miltenyi), were co-cultured for five days with 1x10^6 irradiated 4T1.2Luc cells to generate tumor antigen-specific T cells. Splenic effector cells were isolated and plated (0.5x10^6/well) in 24 well plates (Greiner Bio-One). Supernatants were harvested (48 hours post-incubation) and stored at -80°C. IFNγ was measured using Legend Max ELISA kits (Biolegend), per manufacturer instructions. Cytokines were quantified with an Epoch
Microplate Spectrophotometer (Biotek; Winooski, VT). Each assay was performed in duplicate.

**4.3.7.4. Isolation of tumor-infiltrating immune cells**

Primary tumors were harvested during dissection, weighed, minced into fine pieces (<10 mg), and incubated with 0.03 mg/ml Liberase (Roche) and 12.5 U/ml DNase I (Sigma-Aldrich) for 45 minutes at 37°C on an orbital shaker. Following the digestion, remaining pieces were mechanically disrupted with a syringe plunger, passed through a 70 μm nylon mesh strainer (BD Biosciences), layered over Lympholyte-M cell separation media (Cedarlane), and centrifuged at room temperature for 20 minutes at 1200g. Isolated cells were washed twice in cold PBS (Mediatech) and cell counts and viability of tumor immune infiltrates were determined via trypan blue exclusion.

**4.3.7.5. Flow cytometric analyses**

Single cell suspensions of splenocytes, splenic effector cells (after five-day bulk culture with irradiated tumor cells), and tumor-infiltrating immune cells were washed twice in PBS containing 0.01% bovine serum albumin (flow buffer) at 4°C. Cells were incubated with Fc block (Biolegend) and 1x10^6 cells were stained with saturating concentrations of conjugated antibodies for 30 min at 4°C, as previously described (345). Fluorescently conjugated antibodies for flow cytometry included rat α-mouse CD19 (1D3), hamster α-mouse CD3 (145-2C11), rat α-mouse CD4 (RM4-5), rat α-mouse CD8 (53-6.7), mouse α-mouse NK1.1
(PK136), mouse α-mouse I-Ab (AF6-120.1), hamster α-mouse CD11c (HL3), rat α-mouse CD11b (M1/70), rat α-mouse F4/80 (T45-2342), rat α-mouse Ly6G and Ly6C [Gr-1] (RB6-8C5), rat α-mouse Ly6C (AL-21), and rat α-mouse Ly6G (1A8). Antibodies were obtained from BD Biosciences, Biolegend, and eBioscience. Following incubation with the conjugated antibodies, cells were washed twice in flow buffer and fixed in 1% paraformaldehyde (BD Biosciences) in flow buffer for flow cytometric analysis. Lymphoid and myeloid cells were gated on forward vs. side scatter, and a total of 50,000 events were analyzed. Flow cytometric analyses were performed on a Beckman Coulter FC500 (Beckman Coulter; Indianapolis, IN) or a BD LSR-Fortessa (BD Bioscience) flow cytometer. Flow cytometric analyses were plotted and analyzed using Flow Jo software (Tree Star; Ashland, OR).

4.3.8. Plasma mediators

Fasting blood was collected at sacrifice (day 30-35) via mandibular bleed in microtainer tubes (BD Biosciences), centrifuged, and plasma was stored at -80°C. Insulin, leptin, adiponectin, Interleukin-1 alpha (IL-1α), granulocyte-colony stimulating factor (G-CSF), and Interleukin-6 (IL-6) were measured using a Milliplex MAP Multiplex or Singleplex Assay (EMD Millipore) and quantified on a Bio-plex 200 system (Bio-Rad) using Luminex-200 software (Luminex), per manufacturer’s instructions. Insulin-like growth factor-1 (IGF-1) and insulin-like growth factor binding protein-3 (IGFBP-3) were measured using
R&D Systems Quantikine ELISA kits (R&D Systems; Minneapolis, MN). Each assay was performed in duplicate.

4.3.9. Statistical analyses

Tumor weight, metastatic burden, the distribution of cells in the spleen, splenic bulk culture cells, and tumor-infiltrating immune cells, cytokine secretion, and plasma mediators were assessed for normality and equal variances; and either parametric or nonparametric analyses were used based on sample distribution to detect differences between treatment groups. Metastatic burden, cytokine secretion, and metabolic mediators were skewed; thus, the data were transformed (log or square root) prior to statistical analysis. Differences in tumor weight, metastatic burden, the distribution of cells in the spleen, splenic bulk culture cells, and tumor-infiltrating immune cells, cytokine secretion, and plasma mediators were assessed between groups via a one-way analysis of variance (ANOVA), followed by a Bonferroni correction for multiple comparisons where appropriate, or Kruskal-Wallis test, depending on normality and variance. Body weight, primary tumor volume, and CD4+ helper T cell proliferation were examined using a two-way ANOVA, followed by a Bonferroni correction for multiple comparisons where appropriate. All data are presented as the mean plus or minus the standard deviation of the mean. All analyses were conducted using GraphPad Prism 5 software (GraphPad Software; La Jolla, CA) and statistical significance was accepted at the p≤0.05 level.
4.4. RESULTS

4.4.1. Determination of method to induce tumor cell death prior to vaccination

4T1.2\textsuperscript{luc} cells were counted and plated in increasing concentrations of cells per well in a 96-well plate for a 72-hour proliferation assay. After 52 hours, cells were pulsed with tritiated thymidine and proliferation was quantified via [H]\textsuperscript{3} uptake. 4T1.2\textsuperscript{luc} cells cultured in 4T1.2\textsuperscript{luc} media served as the positive control (Fig. 4.1A). Prior to plating, cells were irradiated with 10,000, 15,000, or 20,000 rad in a cesium irradiator (Fig. 4.1B), exposed to three freeze/thaw cycles (Fig. 4.1C), or incubated with 50, 100, or 200 ng/ml mitomycin (Fig. 4.1D). All treatments significantly reduced the proliferative capacity of 4T1.2\textsuperscript{luc} cells vs. control.

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

![Graph D](image4.png)
Figure 4.1. Optimizing the induction of 4T1.2\textsuperscript{luc} cell senescence for the whole tumor cell cancer vaccine. 4T1.2\textsuperscript{luc} cells were harvested, counted, and plated in increasing concentrations of cells per well in a 96-well plate for a 72-hour proliferation assay. After 52 hours, cells were pulsed with tritiated thymidine and proliferation was quantified via \([H]^3\) uptake. (A) 4T1.2\textsuperscript{luc} cells cultured in 4T1.2\textsuperscript{luc} media as the positive control. Prior to plating, cells were (B) irradiated with 10,000, 15,000, or 20,000 rad in a cesium irradiator, (C) exposed to three freeze/thaw cycles, or (D) incubated with 50, 100, or 200 ng/ml mitomycin. All treatments significantly reduced the proliferative capacity of 4T1.2\textsuperscript{luc} cells vs. control.

4.4.2. Assessment of vaccine efficacy and splenic immune cell co-culture and bulk assays

To determine the immunogenicity of 4T1.2\textsuperscript{luc} cells, mice (n=4) were administered 1x10\textsuperscript{6} irradiated 4T1.2\textsuperscript{luc} cells (VAX) I.P. at day -28, -21, -14, and -7 pre-tumor injection. Mice were orthotopically injected with 5x10\textsuperscript{4} luciferase-transfected 4T1.2 cells into the fourth mammary fat pad. Three of four mice had a failed tumor take, whereas the fourth mouse had delayed onset of tumor growth (Fig. 4.2A). All mice were removed from study by day 140 post tumor injection.

To determine the route of administration of the whole tumor cell cancer vaccine, BALB/c mice were orthotopically injected with 5x10\textsuperscript{4} luciferase-transfected 4T1.2 cells into the fourth mammary fat pad. After injection, mice were randomized into intraperitoneal (I.P.) or subcutaneous (SQ) vaccination (n=2/group) and administered 1x10\textsuperscript{6} irradiated 4T1.2\textsuperscript{luc} cells (VAX) at day 7, 14, 21, and 28 post tumor injection. Mice were sacrificed at day 35 post tumor implantation. Tumor growth curves were not significantly different between I.P. or SQ vaccination injection methods (Fig. 4.2B; 2-way ANOVA, F\textsubscript{(1,22)}=0.05, p=0.848).
To assess the magnitude of the effect of the therapeutic whole tumor cell cancer vaccine in this model, BALB/c mice were orthotopically injected with 5x10^4 luciferase-transfected 4T1.2 cells into the fourth mammary fat pad and administered PBS vehicle control (n=8) or 1x10^6 irradiated 4T1.2^luc cells (VAX; n=8) I.P. at day 7, 14, 21, and 28 post tumor injection. Tumor growth curves were significantly reduced in all the mice vaccinated with the whole tumor cell cancer vaccine compared with unvaccinated tumor-bearing mice (Fig. 4.2C; 2-way ANOVA, time x treatment, F_{11,165}=10.35, p<0.001).

IFN\(\gamma\) secretion from effector cells plated in 1.0 ml of complete media, independent of one vs. two rounds of bulk culture, fell below detection (Fig. 4.2D). IFN\(\gamma\) secretion from effector cells plated in 0.5 ml of complete media were not significantly different between one vs. two rounds of bulk culture (Fig. 4.2D; n=4/group; Mann-Whitney, p=0.343). IFN\(\gamma\) secretion from splenic effector cells post five-day bulk was not significantly different when cultured alone or co-cultured with 4T1.2^luc or Panc.02 cells with or without fresh antigen-presenting cells (Fig. 4.2E; n=4-20/treatment; Kruskal-Wallis=4.13, p=0.389). IFN\(\gamma\) secretion was not significantly different between splenic effector cells (0.5x10^6) cultured alone for 24 or 48 hours post-bulk culture (Fig. 4.2F; n=16/group; Student’s t-test, p=0.757).
Figure 4.2. Optimizing the in vivo injection of a whole tumor cell cancer vaccine, assessing efficacy, and assay development in the 4T1.2 mammary tumor model. (A) BALB/c mice (n=4) were administered 1x10^6 irradiated 4T1.2^luc cells (VAX) I.P. at day -28, -21, -14, and -7 prior to tumor injection. Mice were orthotopically injected with 5x10^4 luciferase-transfected 4T1.2 cells into the fourth mammary fat pad at day 0. Three of four mice had a failed tumor take; whereas, the fourth mouse had delayed onset of tumor growth. All mice were removed from study by day 140 post tumor injection. (B,C) BALB/c mice were orthotopically injected with 5x10^4 luciferase-transfected 4T1.2 cells into the fourth mammary fat pad (B) After injection, mice were randomized into intraperitoneal (I.P.) or subcutaneous (SQ) vaccination (n=2/group) and administered 1x10^6 irradiated 4T1.2^luc cells (VAX) at day 7, 14, 21, and 28 post tumor injection. Mice were sacrificed at day 35 post tumor implantation. Tumor growth curves were not significantly different between I.P. or SQ vaccination injection methods (2-way ANOVA, F[1,22]=0.05, p=0.848). (C) After injection, mice were administered PBS vehicle control (n=8) or 1x10^6 irradiated 4T1.2^luc cells (VAX; n=8) I.P. at day 7, 14, 21, and 28 post tumor injection. Tumor growth curves for VAX mice were significantly reduced compared with unvaccinated mice (2-way ANOVA, F[1,16]=10.35, p<0.001). (D-F) Splenic bulk culture assay protocol development. (D) Isolated splenocytes were depleted of Gr-1+ cells and pulsed with irradiated 4T1.2^luc cells for one round (five days) or two rounds (after five days, bulk cells were washed and re-stimulated with irradiated 4T1.2^luc cells and fresh BALB/c antigen-presenting cells were added for an additional five days) to generate an antigen-specific T cell response to tumor antigens. Post bulk culture, 0.5x10^6 effector cells were collected and IFN\gamma was quantified via ELISA. IFN\gamma secretion from effector cells plated in 1.0 ml of media, independent of one vs. two rounds of bulk culture, fell below detection. IFN\gamma secretion from effector cells plated in 0.5 ml of media did not differ between one vs. two rounds of bulk culture (n=4/group; Mann-Whitney, p=0.343). (E) To test if effector cells post-bulk culture required additional co-stimulation, 0.5x10^6 effectors were plated alone or with 0.1x10^6 irradiated 4T1.2^luc or Panc.02 cells, with or without fresh BALB/c antigen-presenting cells (2.5x10^5) in 0.5 ml for 24 hours. IFN\gamma secretion from effector cells was not significantly different between various plating conditions (n=4-20/treatment; Kruskal-Wallis=4.13, p=0.389). (F) Post-bulk effector cells (0.5x10^6) were removed from antigen stimulation for 24 and 48 hours prior to cell supernatant collection. IFN\gamma secretion was not significantly different between time points (n=16/group; Student’s t-test, p=0.757).

4.4.3. Body weight and wheel activity

The experimental design is displayed in Fig. 4.3A. Briefly, female BALB/c mice (14-week-old) were randomized to sedentary, weight gain (WG; n=24) or exercising, weight maintenance (WM; n=20) for a total of 13 weeks. After eight-weeks on study, all mice were orthotopically injected with 5x10^4 luciferase-transfected 4T1.2 cells into the fourth mammary fat pad and continued on their intervention for 35 days. After injection, mice were further
randomized into vehicle control (n=11-12/group) or vaccination (n=9-12/group) groups and administered PBS (VEH) or $1 \times 10^6$ irradiated 4T1.2$^{\text{luc}}$ cells (VAX) at day 7, 14, 21, and 28 post tumor injection. Mice were sacrificed at day 35 post tumor implantation. WM mice weighed significantly less than WG mice, independent of vehicle or vaccination, throughout the course of the study (Fig. 4.3B; 2-way ANOVA, $F_{(3,325)}=41.94$, $p<0.001$). Wheel activity was maintained over the course of the study, i.e., prior to and after tumor injection. Activity was not affected by the administration of the whole tumor cell cancer vaccine (Fig. 4.3C; n=20; average distance run per day=6.7±2.7 km).
Figure 4.3. Study design, body weight, and wheel activity. (A) Experimental design. (B) WM mice weighed significantly less than WG mice, independent of vehicle or vaccination, throughout the course of the study (2-way ANOVA, $F_{(3,325)}=41.94$, $p<0.001$). (C) Wheel activity was maintained over the course of the study, i.e., prior to and after tumor injection. Activity was not affected by the administration of the whole tumor cell cancer vaccine (n=20; average distance run per day=$6.7 \pm 2.7$ km).

4.4.4. Primary tumor growth

Primary tumor growth was significantly reduced in the WM+VEH and WG+VAX groups; however the combination of WM+VAX resulted in the greatest reduction in primary tumor growth compared with WG+VEH control (Fig. 4.4A; n=9-12/group; 2-way ANOVA, time x treatment, $F_{(18,414)}=3.33$, $p<0.001$).
p<0.001). Tumor weight at sacrifice was not significantly different between groups (Fig. 4.4B; Kruskal-Wallis, KW=5.78, p=0.123).

4.4.5. Metastatic burden

Lung (Fig. 4.4C; 1-way ANOVA, F_{(3,39)}=3.44, p=0.003) and heart (Fig. 4.4D; Kruskal-Wallis, KW=7.85, p=0.049) metastatic burden were significantly different between groups with WM+VAX mice displaying the lowest metastatic burden compared with WG+VEH mice. Kidney (Fig. 4.4E; Kruskal-Wallis, KW=6.34, p=0.096), liver (Fig. 4.4F; Kruskal-Wallis, KW=0.88, p=0.829), and femur (Fig. 4.4G; Kruskal-Wallis, KW=4.84, p=0.184) metastatic burden were not significantly different between groups.
Figure 4.4. Weight maintenance, alone and in combination with a whole tumor cell cancer vaccine, reduced primary tumor growth. (A) Primary tumor growth (measured 2-3x/week via caliper) was significantly reduced in the WM+VEH and WG+VAX groups; however, the combination of WM+VAX resulted in the greatest reduction in primary tumor growth compared with WG+VEH control (n=9-12/group; 2-way ANOVA, time x treatment, $F_{(18,414)}=3.33$, $p<0.001$). (B) Tumor weight at sacrifice was not significantly different between groups (Kruskal-Wallis, $KW=5.78$, $p=0.123$). (C-G) At sacrifice, organs (n=9-12/group) were flash frozen, homogenized, and DNA was extracted. Quantitative PCR was used to detect luciferase expression as a marker of metastatic burden reported as ng of luciferase DNA normalized to mouse TERT DNA in 200 ng of sample. (C) Lung (1-way ANOVA, $F_{(3,39)}=3.44$, $p=0.003$) and (D) heart (Kruskal-Wallis, $KW=7.85$, $p=0.049$) metastatic burden were significantly different between groups with WM+VAX mice displaying the lowest metastatic burden compared with WG+VEH mice. (E) Kidney (Kruskal-Wallis, $KW=6.34$, $p=0.096$), (F) liver (Kruskal-Wallis, $KW=0.88$, $p=0.829$), and (H) femur (Kruskal-Wallis, $KW=4.84$, $p=0.184$) metastatic burden were not significantly different between groups. Significantly different from WG+VEH (*) and WM+VEH (‡).

4.4.6. Splenic immunity

Splenocyte counts were significantly different between groups (Fig. 4.5A; n=9-12/group; 1-way ANOVA, $F_{(3,43)}=2.60$, $p=0.066$) with WM+VAX mice displaying a significant reduction compared with WG+VEH mice. CD4$^+$ helper T cells from WM+VEH mice proliferated more than CD4$^+$ helper T cells from WG+VAX and WM+VAX mice (Fig. 4.5B; n=7-11/group; 2-way ANOVA, time x treatment, $F_{(15,145)}=4.71$, $p<0.001$); however, IL-2 secretion (Fig. 4.5C; 1-way ANOVA, $F_{(3,32)}=0.32$, $p=0.814$) was not significantly different between groups.

The percentages of CD3$^+$ T cells and CD3$^+$CD8$^+$ cytotoxic T cells post five-day bulk were not significantly different between groups (Fig. 4.5D; n=4-5/group). The percentage of CD3$^+$CD4$^+$ helper T cells was significantly elevated in WM+VEH mice (Fig. 4.5D; $p=0.028$) compared with WG+VAX and the percentage of NK1.1$^+$ natural killer cells was significantly reduced in the WG+VAX (Fig. 4.5D; $p=0.004$) group compared with both VEH controls. IFN$\gamma$ secretion from splenic effector cells post five-day bulk was significantly elevated (Fig. 4.5E; n=4-5/group; Kruskal-Wallis, $KW=12.07$, $p=0.007$) in response to VAX ($p<0.001$) and WM ($p=0.017$) compared with WG+VEH control; however, there was no additive effect of WM+VAX.
**D**

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Figure 4.5. The combination of weight maintenance and vaccination reduced splenomegaly and altered splenic immunity. (A) Splenocyte counts were significantly different between groups (1-way ANOVA, $F_{(3,43)}=2.60$, $p=0.066$) with WM+VAX mice displaying a significant reduction compared with WG+VEH mice. (B, C) Isolated splenic CD4$^+$ helper T cells (0.1x10$^6$/well) were stimulated with increasing concentrations of anti-CD3 antibody and 1 μg/ml anti-CD28 antibody, and proliferation was quantified via $[^{3}H]$ uptake. Data were converted to a stimulation index (counts per minute of stimulated wells divided by counts per minute of unstimulated [media only] wells). CD4$^+$ helper T cells from WM+VEH mice proliferated more than CD4$^+$ helper T cells from WG+VAX and WM+VAX mice ($n=7-11$/group; 2-way ANOVA, time x treatment, $F_{(15,145)}=4.71$, $p<0.001$). (C) CD4$^+$ helper T cell supernatant was collected following 48-hour stimulation with 0.5 μg/ml anti-CD3 and IL-2 secretion was quantified using an ELISA. IL-2 secretion was not significantly different between groups (1-way ANOVA, $F_{(3,32)}=0.32$, $p=0.814$). (D, E) Isolated splenocytes were depleted of Gr-1$^+$ cells and pulsed with irradiated 4T1.2 luc cells for five days to generate an antigen-specific T cell response to tumor antigens. (D) Post bulk culture, cells were stained with anti-CD3, -CD4, -CD8, and -NK1.1 and characterized by flow cytometry ($n=4-5$/group). The percentages of CD3$^+$ T cells and CD3$^+$CD8$^+$ cytotoxic T cells were not significantly different between groups. The percentage of CD3$^+$CD4$^+$ helper T cells was significantly elevated in WM+VEH mice ($p=0.028$) compared with WG+VAX and the percentage of NK1.1$^+$ natural killer cells was significantly reduced in the WG+VAX ($p=0.004$) group compared with both VEH controls. (E) Bulk effector cell IFN$\gamma$ secretion in response to re-stimulation with tumor antigens was significantly elevated ($n=4-5$/group; Kruskal-Wallis, KW=12.07, $p=0.007$) in response to VAX ($p<0.001$) and WM ($p=0.017$) compared with WG+VEH control; however, there was no additive effect of WM+VAX. Significantly different from WG+VEH (*), WG+VAX (†), and WM+VEH (‡).

The percentage of splenic CD3$^+$ T cells ($p=0.001$) and CD3$^+$CD4$^+$ helper T cells ($p=0.001$) was significantly different between groups with the vaccination groups (WG+VAX and WM+VAX) displaying the highest percentage compared with vehicle control (WG+VEH and WM+VEH) groups (Table 4.1). The percentage of splenic I-Ab$^+$CD11b$^+$ macrophages ($p=0.001$) was significantly different between groups with the vaccination groups (WG+VAX and WM+VAX) displaying a lower percent compared with the WG+VEH group (Table 4.1). The number of splenic CD19$^+$ B cells ($p=0.032$) was significantly different between groups with the WM+VAX group displaying the lowest number compared with the WG+VEH group (Table 4.1). The number of splenic I-Ab$^+$CD11b$^+$ macrophages ($p=0.011$) was significantly different.
between groups with the WM+VAX group displaying the lowest number compared with the WG+VEH group (Table 4.1). Splenic Gr-1+CD11b+ MDSCs (Fig. 4.6A; Kruskal-Wallis, KW=7.54, p=0.057) was reduced in WM+VAX mice compared with WG+VEH; however, this failed to reach statistical significance. The number of splenic CD11b+Ly6ChiLy6G- monocytic MDSCs (Fig. 4.6B; 1-way ANOVA, F(3,42)=1.62, p=0.200) was not significantly different between groups; however, the number of splenic CD11b+Ly6CloLy6G+ granulocytic MDSCs was significantly different between groups (Fig. 4.6C; Kruskal-Wallis, KW=9.06, p=0.029) with WM+VAX mice displaying the lowest number compared with WG+VEH mice.
Table 4.1. The percentage and number of splenic immune cell populations. (A) The percentage of splenic CD3⁺ T cells (p=0.001) and CD3⁺CD4⁺ helper T cells (p=0.001) was significantly different between groups with the vaccination groups (WG+VAX and WM+VAX) displaying the highest percentage compared with vehicle control groups (WG+VEH and WM+VEH). The percentage of splenic I-Ab⁺CD11b⁺ macrophages (p=0.001) was significantly different between groups with the vaccination groups (WG+VAX and WM+VAX) displaying a lower percentage compared with the WG+VEH group. (B) Splenocyte count (p=0.066) and the number of splenic CD19⁺ B cells (p=0.032) were significantly different between groups with the WM+VAX group displaying the lowest number compared with the WG+VEH group. The number of splenic I-Ab⁺CD11b⁺ macrophages (p=0.011) was significantly different between groups with the WM+VAX group displaying the lowest number compared with the WG+VEH group. Significantly different from WG+VEH (*), WG+VAX (†), and WM+VEH (‡).
Figure 4.6. The combination of weight maintenance and vaccination reduced splenic protumor immunosuppressive cell populations. Splenocytes (n=9-12/group) were stained with anti-Gr-1, -CD11b, -Ly6G, and -Ly6C antibodies to quantify myeloid-derived suppressor cells (MDSCs) and MDSC subsets by flow cytometry. (A) Splenic Gr-1⁺CD11b⁺ MDSCs (Kruskal-Wallis, KW=7.54, p=0.057) was reduced in WM+VAX mice compared with WG+VEH; however, this failed to reach statistical significance. (B) The number of splenic CD11b⁺Ly6CʰLy6G⁻ monocytic MDSCs (1-way ANOVA, F(3,42)=1.62, p=0.200) was not significantly different between groups; however, (C) the number of splenic CD11b⁺Ly6CʰLy6G⁺ granulocytic MDSCs was significantly different between groups (Kruskal-Wallis, KW=9.06, p=0.029) with WM+VAX mice displaying the lowest number compared with WG+VEH mice. Dashed line represents non-tumor bearing control. Significantly different from WG+VEH (*).

4.4.7. Tumor-infiltrating immunity

When examining the distribution of tumor-infiltrating immune cells as a percentage of total cells (Table 4A), on the percentage of tumor-infiltrating CD19⁺ B cells (Table 4.2; p=0.031) was significantly different between groups with the WG+VAX group displaying a higher percentage of cells compared with WG+VEH control. However, when examining the number of tumor-infiltrating immune cells, differences in several populations were detected. Total tumor-infiltrating immune cell counts were significantly different between groups (Table 4.2; p=0.042). Tumor-infiltrating Gr-1⁺CD11b⁺ MDSCs (Fig. 4.7A; 1-way ANOVA, F(3,38)=0.06, p=0.570) and CD11b⁺Ly6CʰLy6G⁻ monocytic MDSCs...
(Fig. 4.7B; Kruskal-Wallis, KW=4.37, p=0.224) were not significantly different between groups. Tumor-infiltrating CD11b<sup>+</sup>Ly6C<sup>lo</sup>Ly6G<sup>+</sup> granulocytic MDSCs were significantly different between groups (Fig. 4.7C; Kruskal-Wallis, KW=10.73, p=0.013) with WG+VAX mice displaying the lowest number compared with WG+VEH mice.

<table>
<thead>
<tr>
<th>A Tumor-infiltrating immune cells</th>
<th>Weight gain (WG)</th>
<th>Weight maintenance (WM)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B cells (CD19&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>1.4 ± 1.0</td>
<td>3.5 ± 1.5&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.031</td>
</tr>
<tr>
<td>Total T cells (CD3&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>47.4 ± 23.0</td>
<td>52.1 ± 5.0</td>
<td>0.955</td>
</tr>
<tr>
<td>Helper T cells (CD3&lt;sup&gt;+&lt;/sup&gt;CD4&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>27.8 ± 6.4</td>
<td>27.1 ± 7.4</td>
<td>0.885</td>
</tr>
<tr>
<td>Cytotoxic T cells (CD3&lt;sup&gt;+&lt;/sup&gt;CD8&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>12.7 ± 5.9</td>
<td>11.2 ± 3.8</td>
<td>0.591</td>
</tr>
<tr>
<td>NK cells (NK1.1&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>8.1 ± 6.2</td>
<td>3.3 ± 1.6</td>
<td>0.528</td>
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<tr>
<td>Dendritic Cells (I-A&lt;sup&gt;b&lt;/sup&gt;/CD11c&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>3.6 ± 2.8</td>
<td>2.1 ± 1.1</td>
<td>0.493</td>
</tr>
<tr>
<td>Macrophages (I-A&lt;sup&gt;b&lt;/sup&gt;/CD11b&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>14.4 ± 6.5</td>
<td>11.9 ± 7.3</td>
<td>0.399</td>
</tr>
<tr>
<td>Macrophages (F4/80&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>13.5 ± 6.0</td>
<td>19.3 ± 4.9</td>
<td>0.400</td>
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<table>
<thead>
<tr>
<th>B Tumor-infiltrating immune cells</th>
<th>Weight gain (WG)</th>
<th>Weight maintenance (WM)</th>
<th>p-value</th>
</tr>
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<tbody>
<tr>
<td>Number (x10&lt;sup&gt;6&lt;/sup&gt;)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Tumor-infiltrating immune cells</td>
<td>21.0 ± 15.9</td>
<td>6.1 ± 5.9</td>
<td>0.042</td>
</tr>
<tr>
<td>B cells (CD19&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>0.2 ± 0.2</td>
<td>0.3 ± 0.2</td>
<td>0.955</td>
</tr>
<tr>
<td>Total T cells (CD3&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>8.1 ± 6.3</td>
<td>4.4 ± 3.4</td>
<td>0.188</td>
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<tr>
<td>Helper T cells (CD3&lt;sup&gt;+&lt;/sup&gt;CD4&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>5.1 ± 3.4</td>
<td>2.3 ± 2.0</td>
<td>0.147</td>
</tr>
<tr>
<td>Cytotoxic T cells (CD3&lt;sup&gt;+&lt;/sup&gt;CD8&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>2.5 ± 2.3</td>
<td>0.9 ± 0.8</td>
<td>0.057</td>
</tr>
<tr>
<td>NK cells (NK1.1&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>2.2 ± 3.0</td>
<td>0.3 ± 0.2</td>
<td>0.344</td>
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<tr>
<td>Dendritic Cells (I-A&lt;sup&gt;b&lt;/sup&gt;/CD11c&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>1.0 ± 1.6</td>
<td>0.2 ± 0.1</td>
<td>0.348</td>
</tr>
<tr>
<td>Macrophages (I-A&lt;sup&gt;b&lt;/sup&gt;/CD11b&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>3.3 ± 3.5</td>
<td>1.0 ± 0.5</td>
<td>0.502</td>
</tr>
<tr>
<td>Macrophages (F4/80&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>3.3 ± 3.5</td>
<td>1.8 ± 1.2</td>
<td>0.896</td>
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</table>

Table 4.2. The percentage and number of tumor-infiltrating immune cell populations. (A) The percentage of tumor-infiltrating CD19<sup>+</sup> B cells (p=0.031) was significantly different between groups with the WG+VAX group displaying a higher percentage of cells compared with WG+VEH control. (B) Tumor-infiltrating immune cell counts were significantly different between groups (p=0.042). Significantly different from WG+VEH (*).
Figure 4.7. Vaccination reduced tumor-infiltrating protumor immunosuppressive cell populations in weight gain mice. Whole tumors were homogenized, washed, and layered over a Ficoll gradient. Tumor-infiltrating immune cells were isolated and stained for flow cytometry. (A) Tumor-infiltrating Gr-1^+CD11b^+ MDSCs (1-way ANOVA, \( F_{(3,38)}=0.06, p=0.570 \)) and (B) CD11b^+Ly6C^hiLy6G^- monocytic MDSCs (Kruskal-Wallis, \( KW=4.37, p=0.224 \)) were not significantly different between groups. (C) Tumor-infiltrating CD11b^+Ly6C^loLy6G^+ granulocytic MDSCs were significantly different between groups (Kruskal-Wallis, \( KW=10.73, p=0.013 \)) with WG+VAX mice displaying the lowest number compared with WG+VEH mice. Significantly different from WG+VEH (*).

4.4.8. Plasma mediators

Plasma insulin (Fig. 4.8A; 1-way ANOVA, \( F_{(3,36)}=0.59, p=0.629 \)), leptin (Fig. 4.8B; Kruskal-Wallis, \( KW=1.66, p=0.645 \)), adiponectin (Fig. 4.8C; Kruskal-Wallis, \( KW=7.63, p=0.054 \)), IL-1\(\alpha\) (Fig. 4.8D; 1-way ANOVA, \( F_{(3,43)}=1.72, p=0.179 \)), IL-6 (Fig. 4.8F; Kruskal-Wallis, \( KW=6.55, p=0.088 \)), or IGFBP-3 (Fig. 4.8G; 1-way ANOVA, \( F_{(3,41)}=2.15, p=0.110 \)) were not significantly different between groups. Plasma G-CSF (Fig. 4.8E; Kruskal-Wallis, \( KW=8.90, p=0.031 \)) and IGF-1 (Fig. 4.8H; 1-way ANOVA, \( F_{(3,43)}=4.13, p=0.012 \)) were significantly different between groups with WM+VAX mice displaying the lowest IGF-1 concentration compared WG+VEH mice.
**Figure 4.8. The combination of weight maintenance and vaccination reduced plasma metabolic mediators.** Fasting blood was collected at sacrifice (n=9-12/group), centrifuged, and plasma was stored at -80°C. Milliplex Multiplex Assays quantified on Luminex-200 software were used to measure (A) insulin, (B) leptin, (C) adiponectin, (D) IL-1α, (E) G-CSF, and (F) IL-6. R&D Systems Quantikine ELISAs were used to measure metabolic markers for (G) IGFBP-3 and (H) IGF-1. Plasma (A) insulin (1-way ANOVA, F(3,36)=0.59, p=0.629), (B) leptin (Kruskal-Wallis, KW=1.66, p=0.645), (C) adiponectin (Kruskal-Wallis, KW=7.63, p=0.054), (D) IL-1α (1-way ANOVA, F(3,43)=1.72, p=0.179), (F) IL-6 (Kruskal-Wallis, KW=6.55, p=0.088), or (G) IGFBP-3 (1-way ANOVA, F(3,41)=2.15, p=0.110) were not significantly different between groups. Plasma (E) G-CSF (Kruskal-Wallis, KW=8.90, p=0.031) and (H) IGF-1 (1-way ANOVA, F(3,43)=4.13, p=0.012) were significantly different between groups with WM+VAX mice displaying the lowest IGF-1 concentration compared WG+VEH mice. Significantly different from WG+VEH (*).

**4.5. DISCUSSION**

In previous work we demonstrated that moderate exercise in weight stable non-tumor bearing mice augments antigen-specific T cell response to vaccination and in response to a viral-vector based vaccine in a pancreatic cancer model (272, 273). Furthermore, we have demonstrated (chapter three) that the prevention of weight gain through exercise and mild dietary restriction can reduce primary tumor growth and metastatic burden, reduce immunosuppressive cells, augment T cell responses, and alter the tumor microenvironment in the 4T1.2 mammary tumor model. Thus, the goal of the current study was to determine if diet and exercise could enhance the efficacy of a whole tumor cell cancer vaccine via one or more of the aforementioned mechanisms.

First, the preparation of the whole tumor cell cancer vaccine must prevent proliferation and/or induce tumor cell death, yet do so in a manner that allows the processing and presentation of tumor antigens to APCs (e.g., DCs) to stimulate an antitumor effector T cell response. Tumor cells can be prepared by inducing apoptosis via ultraviolet B (UVB) ray-irradiation (non-ionizing) or gamma (γ)-irradiation (ionizing) or necrosis to generate a cell lysate via repeat cycles of
freezing and thawing (214, 596). Both apoptotic and necrotic-based strategies can generate mature DCs and elicit effective priming and antitumor efficacy in vivo; however, the best method has long been debated (597-603). DC activation via apoptosis-induced death could involve the detection of apoptotic bodies, exposure to cell surface markers (e.g., phosphatidylinositol or calreticulin), or releasing factors (e.g., GM-CSF) (600). DC activation via necrosis-induced death could involve the recognition of heat-shock proteins or degraded RNA or DNA (600). We tested both γ-irradiation and repeat cycles of freeze-thaw, as well as the DNA replication inhibitor, mitomycin, on their ability to inhibit 4T1.2 luc cell proliferation in vitro. All three methods reduced the proliferative capacity of 4T1.2 luc cells measured via a 72-hour proliferation assay. We chose γ-irradiation because it is reported to effectively penetrate cancer cells and specifically target nucleic acids, while conserving surface antigen proteins; thus, maintaining immunogenicity (602). Mitomycin is rarely used clinically and cell lysate protocols often require more extensive vaccine preparation. Additionally, γ-irradiation of 4T1.2 luc target cells for re-stimulation assays can be performed, exposing splenic or tumor-infiltrating immune cells to similar antigens experienced in vivo.

Second, we tested if the whole tumor cell cancer vaccine could be prophylactic (preventative) by treating sedentary, ad libitum-fed BALB/c mice with the γ-irradiated whole tumor cell cancer vaccine I.P. at day -28, -21, -14, and -7 prior to tumor implantation with 4T1.2 luc cells. One mouse had delayed onset of tumor and was removed from study at day 125. Three of the four mice had impalpable tumors, representing a 75% survival. All mice were removed from study
at day 140 post-tumor implantation. The fact that prophylactic vaccination of mice protected against 4T1.2\textsuperscript{luc} tumor growth was unexpected. The 4T1 parent series is reported to be poorly immunogenic and unresponsive to prophylactic vaccination with irradiated 4T1 cells alone, but protection was observed when coupling irradiated cells with adjuvant therapy or antibody blockade of immunosuppressive factors (223, 604, 605). Perhaps the 4T1 and 4T1.2 cells, while similar, have differences in cell surface tumor antigens or releasing factors post-irradiation that could generate memory cells and drive the prophylactic response observed when administering irradiated 4T1.2\textsuperscript{luc} cells alone.

Third, with the knowledge that the 4T1.2 model possesses immunogenic properties, two \textit{in vivo} studies were performed to determine injection method (intraperitoneal [I.P.] vs. subcutaneous [SQ]), as well as to assess therapeutic efficacy and optimize \textit{in vitro} recall assays. No differences were observed when the \(\gamma\)-irradiated therapeutic whole tumor cell cancer vaccine was injected I.P. vs. SQ in sedentary, \textit{ad libitum}-fed BALB/c 4T1.2\textsuperscript{luc} tumor-bearing mice at day 7, 14, 21, and 28 post tumor implantation. Therefore, we decided to test both the efficacy of the vaccine and optimize \textit{in vitro} assays in a cohort of sedentary, \textit{ad libitum}-fed BALB/c 4T1.2\textsuperscript{luc} tumor-bearing mice receiving the \(\gamma\)-irradiated therapeutic whole tumor cell cancer vaccine I.P. at day 7, 14, 21, and 28 post tumor implantation. All mice displayed a reduction in tumor growth compared with unvaccinated 4T1.2\textsuperscript{luc} tumor-bearing mice.

Recall assays were performed to test the number of rounds needed to generate effector cells via bulk culture, as well as the volume in which to plate
effector cells post-bulk culture in which to optimally detect IFN\(\gamma\) secretion. No differences were observed between one round vs. two rounds of irradiated 4T1.2\textsubscript{luc} re-stimulation; however, splenic effector cells had to be plated at 0.5x10\(^6\) cells/well in 0.5 ml for the detection of IFN\(\gamma\) secretion post 24-hour rest. To test the specificity of \textit{in vitro} re-stimulation with irradiated 4T1.2\textsubscript{luc} cells, five-day bulk cells were co-cultured with irradiated Panc.02 cells for 24-hours. IFN\(\gamma\) secretion was not statistically different between co-culture with 4T1.2\textsubscript{luc} cells or Panc.02 vs. resting the cells alone or if fresh BALB/c antigen-presenting cells were added to co-culture; therefore, we decided to plate splenic effector cells post-bulk alone for future IFN\(\gamma\) secretion assays. We further tested if splenic effector cells post-bulk secreted more IFN\(\gamma\) after a 24- or 48-hour rest and found no differences in these times. The 48-hour rest was selected for practical reasons.

Powered with this information, we designed a series of studies to investigate the effects of moderate exercise in weight stable mice on the efficacy of a therapeutic, whole tumor cell cancer vaccine (\(\gamma\)-irradiated and injected I.P.) administered at day 7, 14, 21, and 28 post tumor implantation in the 4T1.2 model. The current study was modeled to examine the effects of moderate exercise in weight stable hosts alone and in combination with a whole tumor cell cancer vaccine on primary tumor growth, metastatic progression, and immune responses. As described in chapter three, we observed a cancer prevention effect of moderate exercise in weight stable mice on primary tumor growth and metastatic burden. In addition, a vaccination effect was observed on primary tumor growth in weight gain mice. However, the whole tumor cell cancer vaccine enhanced the weight
maintenance (via diet and exercise) effects on primary tumor growth, spontaneous metastasis, splenic immunosuppressive cell types, and plasma IGF-1, and suggests that vaccination may provide an immune stimulus to further promote the protective effects of moderate exercise alone.

Cancer vaccine therapies, both tumor-derived peptide based and whole tumor cell based are under investigation in BC patients. The majority of clinical trials assessing peptide-based strategies target HER2-derived peptides alone, or in combination with additional immunostimulatory agents (235). For example, a well-studied (236) tumor-derived peptide-based vaccine targeting HER-2 (E75, also known as NeuVAX) is now being investigated clinically. NeuVAX combined with GM-CSF administration, prolongs disease-free survival in a subset of BC patients who expressed low levels of HER2 with a high risk of relapse (237). A phase II study (NCT01570036) is underway to investigate the combination of NeuVAX and targeted HER2 therapy in node positive (or node-negative if estrogen and progesterone receptor negative) BC patients who are disease-free after standard-of-care therapy. However, a study investigating the administration of NeuVAX+GM-CSF in early-stage, node-positive BC patients with low-to-intermediate HER2 expression was halted due to ineffectiveness (NCT01479244). New targets including peptides against Wilms tumor protein antigen (238) and immunization with vaccinia virus modified to express mucin-1 and IL-2 (239) can induce tumor regression in BC patients. The identification of tumor-specific antigens in which to target remains an active area of research.
Clinically, whole tumor cell cancer vaccines are rarely investigated as a monotherapy in patients with advanced stage disease. These therapies are more typically genetically modified to secrete various cytokines, such as GM-CSF, administered in combination with an immune adjuvant (214, 229), or administered in combinatorial treatment strategies coupled with chemotherapy, endocrine therapy, and/or targeted therapy (240-242). In a phase I clinical trial, the safety of a plasmid transfected/allogeneic HER2-positive cell-based GM-CSF-secreting vaccine was confirmed in metastatic BC patients. The vaccine alone or in sequence with low-dose chemotherapy induces HER2-specific T cell-mediated immunity; however, survival data was forthcoming (240). A separate Phase I trial modified MDA-MB-231 cells to express B7-1 (CD80), a costimulatory molecule required for antigen-presentation to T cells. Vaccinated stage IV metastatic BC patients had an increase in tumor-specific immune responses, but failed to show differences in tumor progression (243). The administration of a mixed vaccine composed of autologous breast tumor cells supplemented with allogeneic breast tumor cells, three additional TAAs antigens combined with IL-2 and GM-CSF to BC patients in a phase II study increases antigen-specific lymphocyte response and an improves clinical response (244). The administration of this mixed vaccine to BC patients with depressed immunity improves ten-year survival compared with historical data (228). Two active clinical trials are being carried out to investigate the safety and efficacy of modified autologous vaccinations engineered to express GM-CSF in metastatic BC patients (NCT00317603 and NCT00880464). Future studies are required to identify the optimal combinatorial or multi-antigen approach
to improve survival outcomes. However, to date, no study has investigated clinically if lifestyle-based intervention strategies can enhance peptide- or whole tumor cell-based vaccine strategies. Thus, our work may provide the rationale for collecting data in patients on body weight and physical activity patterns to determine if these factors impact vaccine efficacy in ongoing trials.

Few researchers have designed preclinical studies to investigate cancer vaccine monotherapy in murine models of mammary carcinogenesis. Cell lysate from 4T1 encapsulated in poly(lactic-co-glycolic acid) acid (PLGA) microparticles reduces spontaneous lung metastasis; however, there was no effect on primary tumor growth (222). A placental endothelial cell vaccine (ValloVAX) administered SQ for four weekly vaccinations after tumor implantation inhibits 4T1 tumor growth; however, immune effector function was not characterized (245). Combinatorial strategies are being investigated in both peptide-based and whole tumor cell based vaccines. Combinatorial immunostimulatory strategies include genetically modifying cells to express GM-CSF, CD40L, OX-40, and B7-1 or the co-administration of IL-12 in the vaccination protocol (225, 226, 234, 246). Soliman, et al. (225) vaccinated 4T1 tumor-bearing mice with irradiated B78H1 bystander cell lines engineered to secrete granulocyte macrophage-colony stimulating factor and CD40 ligand, a costimulatory ligand important for T cell activation. Vaccinated mice display a reduction in tumor growth, concurrently with an increase in the secretion of IL-2 and IFNγ from splenic T cells and an increase in tumor-infiltrating T cells. The anchoring of IL-12 and the B7-1 costimulatory molecules to 4TO7 cells results in significantly lower tumor burden compared with controls after one
treatment (246). Vaccinated mice also display a reduction in tumor angiogenesis and an increase in tumor-infiltrating CD8\(^+\) cytotoxic T cells. Data from Yan, et al. (226) demonstrates that the administration of irradiated, transfected 4T1 cells that express VEGFR2 inhibits subsequent tumor growth and lung metastasis. Lastly, the intranodal administration of autologous tumor-derived autophagosome-based therapeutic vaccine (DRibbles) with an anti-OX40 co-stimulatory antibody enhances T cell priming and antitumor efficacy in 4T1 tumor-bearing mice (234). The findings from these results indicate that anchored cytokines or the addition of co-stimulatory molecules may improve therapeutic potency. Moderate exercise in weight stable mice may create a systemic environment more conducive to a sustained antitumor immune response. Exercise training warrants further investigation as an immunostimulatory strategy to enhance vaccine efficacy.

The success of cancer vaccines is hampered by tumor-induced immunosuppression (e.g., soluble tumor-derived immunosuppressive factors and recruitment of immunosuppressive cells, including Tregs and MDSCs). Therefore, the investigation of combinatorial strategies that prevent the emergence or function of immunosuppressive factors is underway. To minimize the induction of Tregs, Monzavi-Karbassi, et al. (247) developed a vaccine composed of cell lysate lacking lectin-reactive glycoconjugates; and the vaccination of 4T1 tumor-bearing mice reduced tumor growth and spontaneous lung metastasis. In contrast, the crude lysate activated Tregs and induced their suppressive function measured via a mixed suppression assay. This suggests that minor refinements in TAAs can be
performed to weaken immune suppression and improve antitumor immune responses.

Cyclooxygenase-2 (Cox-2), a rate-limiting enzyme in the synthesis of prostaglandins from arachidonic acid, is highly upregulated in mammary cancers (248) and plays a key role in stimulating epithelial cell proliferation, inhibiting apoptosis, stimulating angiogenesis, and mediating immune suppression (248, 249). The short-term administration of celecoxib, a selective Cox-2 inhibitor, as a monotherapy can reduce 4T1 tumor growth (250-252). Celecoxib administered in combination with a tumor-lysate pulsed DC vaccine plus GM-CSF adjuvant therapy, results in the greatest reduction in 4T1 tumor growth and spontaneous lung metastasis, an increase in the secretion of IFNγ and IL-4 from re-stimulated CD4+ helper T cells, and an increase in abundance of tumor-infiltrating CD4+ helper and CD8+ cytotoxic T cells (251). These data suggest that inhibiting the expansion of Tregs or blocking Cox-2 via short-term celecoxib therapy may be safely used to increase vaccine efficacy for treating metastatic BC. Through a reduction in immunosuppressive factors within the TME and secondary lymphoid organs, moderate exercise in weight stable mice may blunt immunosuppressive cell-induced dysregulation of APCs; thus, promoting better antigen uptake and presentation by DCs, better priming of antitumor T cells, or a sustained T cell activation.

A key advantage of the administration of whole tumor cell cancer vaccines compared with tumor-derived peptides or full-length recombinant tumor proteins is that it provides numerous TAAs in which to stimulate an antitumor response; thus,
alleviating the need to identify and purify TAAs. Whole tumor antigen vaccines can use cell lysate or whole tumor cells. Strome, et al. (606) demonstrate that DCs primed with irradiated tumor cells in vitro reduces tumor outgrowth in two in vivo tumor models (E.G7 thymoma and SCCVII squamous cell carcinoma models); however, DCs primed with freeze-thaw tumor lysate is not effective at controlling tumor outgrowth and inhibits CD8+ cytotoxic T cell response in vitro. Several limitations to irradiation-induced killing for whole tumor cell cancer vaccines exist. Upon irradiation, cells can transfer phosphatidylserine, an immunosuppressive phospholipid typically found on the inner leaflet, to the outer leaflet. Phosphatidylserine can induce the secretion of immunosuppressive factors from DCs (223, 229, 607). Additionally, irradiated cells are poorly immunogenic and retain the ability to secrete immunosuppressive factors, like VEGF (229, 608, 609). The release of tumor-derived immunosuppressive factors may compromise the ability of DCs to activate T cells. Although the irradiated whole tumor cell cancer vaccine used in the current study may have had a degree of DC- and/or tumor-derived immunosuppressive factors present, we still observed a reduction in tumor growth in vaccinated mice. The observed reduction in tumor growth suggests that the stimulatory capacity to generate a robust adaptive immune response outweighed the presence of DC- and/or tumor-derived immunosuppressive signals. Additionally, the 4T1.2 cells used for the whole tumor cell cancer vaccine were tagged with luciferase. The ability of luciferase to drive an immunogenic response against transfected cells injected in vivo is a matter of debate (610-612). However, regardless of what tumor antigen(s) are driving the effects in our model,
the whole tumor cell cancer vaccine was effective at reducing primary tumor growth in both weight gain and weight maintenance groups. More importantly, the whole tumor cell cancer vaccine enhanced the efficacy of moderate exercise in weight stable mice resulting in the greatest reduction in primary tumor growth and metastatic burden.

Moderate exercise in weight stable mice may be enhancing several host factors resulting in enhanced whole tumor cell cancer vaccine efficacy. Whole tumor cell cancer vaccines can activate both CD4\(^+\) helper and CD8\(^+\) cytotoxic T cell responses (214). First, the CD4\(^+\) helper T cell proliferation of unvaccinated mice mirrored our results (chapter three) with CD4\(^+\) helper T cells from WM mice proliferating at a higher capacity than WG mice. We did observe an unexplained reduction in CD4\(^+\) helper T cell proliferative capacity in vaccinated mice; however, IL-2 cytokine secretion was not significantly different between all four intervention groups. Second, our bulk assays were depleted of Gr-1\(^+\) cells, but they contained both CD4\(^+\) helper and CD8\(^+\) cytotoxic T cells, as well as NK cells. Bulk effector cell IFN\(\gamma\) secretion in response to re-stimulation with tumor antigens was significantly elevated in response to VAX and WM compared with WG+VEH control; however, there was no additive effect of WM+VAX. This result suggests that WM and VAX-induced effects on IFN\(\gamma\) secretion mechanisms may overlap. The secretion of IFN\(\gamma\) as an antitumor cytokine is only one mechanism in which effector cells can target transformed cells (613). The killing of transformed cells can occur through direct cytotoxicity via the granzyme/perforin pathway and death receptor pathway (Fas/Fas Ligand, TNF-related Apoptosis-Inducing Ligand [TRAIL]), as well as the
release of immunomodulatory cytokines (e.g., TNFα, IL-10,) and chemokines (e.g., CCL5) (82). The contribution of CD4+ helper vs. CD8+ cytotoxic T cells, as well as the mechanisms by which effector cells induce cell death to enhance the vaccine-dependent reduction in primary tumor growth and metastatic burden, are under current investigation within our laboratory.

Similar to results reported in chapter three, we observed a reduction in plasma G-CSF and splenomegaly in the WM+VAX group. The reduction in splenomegaly was composed of less splenic MDSCs and MDSC subsets. The mechanism(s) of MDSC-mediated immunosuppression of T cells is reviewed elsewhere (125, 127, 139, 167); and likely functions through cell-surface receptors and/or through the release of short-lived soluble mediators. In general, activated MDSCs can mediate suppressive functions through multiple players, such as Arginase-1 (Arg-1) and nitric oxide synthase (iNOS), the hyper-production of nitric oxide (NO) and reactive oxygen species (ROS), induction of Tregs, as well as deregulation of Cox-2/PGE2, transforming growth factor beta (TGFβ), and IL-10 pathways. MDSCs can downregulate the ζ-chain of the TCR complex (190), and interfere with IL-2 signaling (a signal essential to T cell proliferation) (191) resulting in the desensitization of the TCR response and T cell unresponsiveness. Also, MDSCs can deplete essential nutrients (such as L-arginine, L-cysteine, and L-tryptophan) from the environment via consumption and sequestration to dysregulate APC function and inhibit T cell activation and proliferation (194, 195). Tumor-derived S100A8/A9, in addition to STAT3-induced production of S100A8/A9 from MDSCs, inhibit the maturation of DCs (200). The culmination of
these suppressive mechanisms results in less functional DCs and less activated T cells. Previous results (chapter three) indicated that the suppressive capacity of MDSCs were not significantly different between WG and WM groups. However, less MDSCs in the spleen and TME of WM mice could contribute to a less immunosuppressive phenotype resulting in better priming of CD8$^+$ cytotoxic T cells by DCs in proximal draining lymph nodes. Additionally, MDSCs play a role in driving metastases through the promotion of angiogenesis and tumor cell invasion (176). Therefore, the reduction in MDSCs in moderately exercising, weight stable mice, may in part, explain the enhanced efficacy of our whole tumor cell cancer vaccine and reduction in metastatic burden.

Moderate exercise in weight stable mice may be altering the TME. The number of tumor-infiltrating immune cells was significantly different between groups, with the number reflective of differences in end tumor weight. A vaccine effect in weight gain mice was observed with this group displaying the lowest number of tumor-infiltrating granulocytic MDSCs. Data presented in chapter three demonstrated that moderate exercise in weight stable mice downregulated several chemokines and chemokine receptor gene expression markers within the TME that may impact vaccine efficacy. Moderate exercise in weight stable mice downregulated the expression of genes ($\text{Ccl5 [Rantes]}$ (585, 586), $\text{Cxc1l}$ (124, 156, 176-178), $\text{Ccl20 [Mip-3\alpha]}$ (587), and $\text{Ccl22 [Mdc]}$ (588)) important for chemotaxis and recruitment of immunosuppressive cells, like MDSCs and Tregs, to the TME and downregulated the expression of genes ($\text{Cxc1l9 [Mig]}$ and $\text{Cxc1l10 [Inp10]}$ (589), $\text{Ccr7}$ (590, 591), and $\text{Cxc3r3}$ (592)) important for promoting angiogenesis, tumor
cell invasion, and metastasis. The downregulation of these genes correlate with the observed reduction in the expansion of immunosuppressive cell types in the spleen and TME, as well as the reduction of metastatic burden, in our dual intervention group. Moderate exercise in weight stable mice also downregulated the immunosuppressive gene expression marker, \textit{Ido1} (Ido). Ido is expressed by tumor cells, supporting cells within the TME, and infiltrating cells, like MDSCs, and encodes for indoleamine 2,3-dioxygenase (IDO), the first and rate-limiting enzyme of tryptophan catabolism through the kynurenine pathway (197). Depletion of tryptophan, an amino acid essential for T cell activation, results in blunted T cell proliferation, differentiation, effector functions, and viability (198). Data from Muller, et al. (614) indicates that IDO inhibition augments the response to chemotherapy in the MMTV-Neu murine BC model. No IDO inhibitor is approved by the FDA; however, with numerous preclinical and phase I and II clinical trials, researchers are investigating IDO inhibition in combination with cancer vaccines, chemotherapy agents, and emerging immune checkpoint blockade (615). The effects of moderate exercise in weight stable mice may be mediated through a reduction in inflammatory and immunosuppressive gene expression markers within the TME that ultimately promotes a more robust antitumor effector CD8$^+$ cytotoxic T cell response post whole tumor cell cancer vaccine. Future studies are needed to investigate the individual effects of each gene on whole tumor cell cancer vaccine efficacy. However, the current study provides essential insight into potential shifts that can occur in immunosuppressive markers in response to moderate exercise in weight stable mice.
In the current study, moderate exercise in weight stable mice combined with the whole tumor cell cancer vaccine failed to alter many plasma mediators (insulin, leptin, adiponectin, IL-1α, IL-6, and IGFBP-3); however, WM+VAX resulted in the lowest concentration of plasma IGF-1, with this group also demonstrating the lowest tumor progression and metastatic burden. IGF-1 signaling can activate PI3K/Akt and Raf-1/MEK/ERK pathways and downstream nuclear factors in cancerous cells to promote proliferation and inhibit apoptosis (486, 487). Additionally, the IGF family can activate Raf-1/MEK/ERK and decrease ERK1/2 phosphorylation and p38 dephosphorylation pathway in DCs resulting in a delay in maturation (or an induction of a tolerogenic DC phenotype) (491, 492). Reduced antigen uptake and presentation abilities in DCs will activate fewer antigen-specific CD8+ cytotoxic T cells (491, 492). IGF signaling in DCs can also induce the secretion of IL-6, TNFα, and IL-10, generating an immunosuppressive phenotype promoting tumor escape (491, 492). Clinical and epidemiologic studies show that elevated IGF-1 is a risk factor for the development of BC, especially estrogen positive tumors (472-477). However, few studies are evaluating the role of elevated IGF-1 on cancer vaccine efficacy. The enhanced efficacy in our whole tumor cell cancer vaccine may be explained, in part, through the ability of moderate exercise in weight stable mice to reduce the concentration of plasma IGF-1. A reduction in IGF-1 may result in DCs more capable of activating antigen-specific CD8+ cytotoxic T cells. Future studies are needed to explore this link, perhaps through the blockade of IGF-1 action via an IGF-1 receptor inhibitor (NVP-AEW541) (491) or
monoclonal anti-IGF1 receptor antibody in weight gain mice receiving the whole tumor cell cancer vaccine.

In conclusion, moderate exercise in weight stable mice combined with a therapeutic whole tumor cell cancer vaccine reduced splenomegaly and the abundance of MDSCs and MDSC subsets, reduced the concentration of plasma IGF-1, and ultimately reduced tumor growth and metastatic burden in 4T1.2<sup>Luc</sup> tumor-bearing mice. Preclinical and clinical combinatorial studies are underway to investigate strategies in which to enhance cancer vaccines. However, this is the first study, to our knowledge, that shows a non-pharmacological, lifestyle-based intervention strategy can blunt tumor-induced inflammation and immunosuppression, maintain lymphoid-lineage cells, and enhance the efficacy of a whole tumor cell cancer vaccine. These data suggest that moderate exercise with the goal of mild weight loss and weight stability may be an important lifestyle recommendation for patients undergoing cancer vaccine-based treatment strategies. Results from the current study provides a biological rationale for future randomized controlled trials testing if exercise strategies can enhance the efficacy of emerging cancer vaccines.
CHAPTER 5: MODERATE EXERCISE IN WEIGHT STABLE MICE AND THE DUAL ADMINISTRATION OF A WHOLE TUMOR CELL CANCER VACCINE AND PD-1 CHECKPOINT BLOCKADE IN THE 4T1.2 MAMMARY TUMOR MODEL*

5.1. ABSTRACT

Immunotherapy has become a promising treatment strategy for breast cancer (BC), especially metastatic disease. An emerging immunotherapeutic strategy is membrane programmed cell death protein-1 (PD-1) checkpoint blockade, which selectively targets PD-1 on T cells to promote a sustained, antitumor effector response. Although there is excitement in the field and successes are reported in the clinical literature, the response rate to PD-1 blockade is under 50%. Thus, there is a growing interest in improving response rates to immune checkpoint blockade through the identification of the phenotype of responders with the long-term goal of administering targeted, personalized therapy and/or combinatorial treatment strategies (e.g., chemotherapy, cancer vaccines). Moderate exercise can drive changes in metabolic, inflammatory, and immune mediators that may improve response rates of immunotherapies and improve clinical outcomes. However, few researchers have designed clinical or preclinical studies to examine the effect of exercise and weight control on the efficacy of immune checkpoint blockade. Thus, the goal of the current study was to determine if preventing weight gain through diet (10% reduction in calories) and exercise (voluntary running wheel activity) will improve the response to the dual

administration of a whole tumor cell cancer vaccine and PD-1 checkpoint blockade. Female BALB/c mice were randomized to sedentary, ad libitum weight gain (WG) control or exercising, mildly restricted (90% of control food intake) weight maintenance (WM) groups (n=32/group) and provided access to a standard cage or activity wheel cage for eight weeks prior to the injection of $5\times10^4$ luciferase-transfected 4T1.2 cells into the fourth mammary fat pad. Mice were randomized into vehicle or vaccination groups, and administered PBS vehicle (VEH) control or $1\times10^6$ irradiated 4T1.2$^{\text{luc}}$ cells (VAX) at day 7 post-tumor injection. Mice were further randomized (n=8/group) to receive isotype control (IgG) or PD-1 checkpoint blockade (10 mg/kg/mouse) at day 9 and 12 post-tumor injection. Mice continued on their respective energy balance intervention until sacrifice at day 35 post-tumor implantation. All WM groups, regardless of immunotherapy intervention, weighed significantly less than WG groups over the course of the study (p<0.001). We observed a cancer prevention effect of PD-1 checkpoint blockade in WG mice on primary tumor growth and spontaneous lung metastasis. However, moderate activity in weight stable mice, independent of PD-1 checkpoint blockade, was effective in reducing primary tumor growth and metastatic burden. The WM+PD-1 group displayed the lowest number of splenic myeloid-derived suppressor cells (MDSCs; p=0.047) and granulocytic MDSCs (p=0.017) and maintained its splenic lymphoid populations. The number of tumor-infiltrating immune cells or the effector or activation status of tumor-infiltrating CD4$^+$ helper and CD8$^+$ cytotoxic T cells was not significantly different between groups, nor were functional immune outcomes. PD-1 checkpoint blockade in WG mice, moderate exercise in weight stable mice,
and PD-1 checkpoint blockade in moderately exercising, weight stable mice had comparable, albeit subtle differences, in tumor-immune crosstalk gene expression markers that drive the expansion of immunosuppressive cell types and impact metastatic progression. The lack of responsiveness to VAX+PD-1 checkpoint blockade in WM mice suggests that moderate exercise in weight stable mice may be enhancing antitumor immunity and/or reducing protumorigenic factors (i.e., similar mechanisms mediated by VAX+PD-1 checkpoint blockade). These data demonstrate that preventing weight gain through diet and exercise may be an important recommendation to maintain prolonged antitumor effector responses and improve clinical outcomes.

5.2. INTRODUCTION

Breast cancer (BC) is the most commonly diagnosed cancer and the second leading cause of cancer-related deaths among women in the United States (1, 2). Metastatic disease remains incurable and is the underlying cause of death in the majority of BC patients who die of the disease (3). Cancer immunotherapy, deemed by Science magazine (49) as the 2013 “Breakthrough of the Year” and the 2016 and 2017 ASCO “Advance of the Year” (201, 202), has demonstrated success in treating cancer, including very advanced, metastatic diseases. Immunotherapy treatments include therapeutic cancer vaccines, monoclonal antibodies, adoptive cell therapies, and the administration of immunostimulatory cytokines, all of which are designed to stimulate a robust antitumor effector response to eliminate tumor cells through several techniques (51, 64). Therapeutic cancer vaccines provide tumor-associated antigen(s) (TAAs) to stimulate an
effector T cell response (6); whereas, immune checkpoint blockade, like anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and anti-programmed cell death protein-1 (PD-1), use specific antibodies to target inhibitory cell surface molecules to promote sustained effector cell activation (203). Studies are underway to investigate emerging immunotherapy strategies in advanced-stage cancer patients who have failed to respond to other treatments. Recent reviews have summarized clinical trials investigating checkpoint blockade in BC patients (55-58). Recent clinical BC studies target PD-1 in TNBC because it is associated with limited treatment options (55, 258), high expression of PD-L1 in the TME, and increased tumor-infiltrating immune cells (72, 259-261), making immunotherapy an attractive treatment option. Despite the recent successes of immunotherapy in advanced-stage cancer patients, less than half of patients who receive immunotherapy experience an objective, durable response (204). Thus, there is great interest in understanding the factors that contribute to the heterogeneity in response rates to immunotherapy. Areas of research include the identification of clinically meaningful biomarkers to direct treatment choices (i.e., personalized medicine) and/or through the selection of appropriate combinatorial strategies (aimed to enhance antigen presentation, reverse T cell dysfunction, and/or target immune inhibitory mechanisms) without inducing adverse effects (56-58, 205). No study, to our knowledge, has retrospectively or prospectively assessed the impact of host factors known to modulate immune function (e.g., physical activity, body mass index, or dietary and sleep patterns) on the efficacy of immunotherapeutic outcomes.
In normal physiology, inhibitory immune checkpoints are important to protect against activation events targeted toward self-antigens (i.e., autoimmunity) and to return the adaptive response to a basal level and function following the clearance of a foreign agent in a process called homeostatic control (50). However, inhibitory immune checkpoints may act as a barrier to the successful identification, activation, and eradication of malignant self-cells by effector immune cell populations. Following T cell activation by APCs, T cells migrate to specific sites and perform their effector function. Upon activation, T cells upregulate inhibitory cell surface markers, like PD-1 (53, 616). In tumor-bearing hosts, proinflammatory signals (e.g., interferon [IFN] gamma [γ]) produced in the TME, signal to tumor cells, stromal cells, and tumor infiltrating immune cells (e.g., myeloid-derived suppressor cells [MDSCs]) to increase the expression of programmed death-ligand 1 (PD-L1 or CD274) and PD-L2 (CD273), which can bind to PD-1 on T cells and shut down the antitumor response by inducing apoptosis (203). The immune checkpoint blockade approach to immunotherapy involves using antibodies to maintain T cell activation by either binding and agonizing co-stimulatory signals or binding and antagonizing co-inhibitory signals (53, 57). PD-1 is widely expressed on several cells important in antitumor immunity, including B cells, natural killer (NK) cells, and CD4+ helper and CD8+ cytotoxic T cells, making this an emerging target for cancer therapy (257).

Clinically, PD-L1 protein expression is detected in 20-30% of BC patients and single-agent PD-1 blockade can result in objective and durable clinical responses in BC patients, with responses more favorable for patients with tumors
positive for PD-L1 (257). Currently, there are close to 50 ongoing, or soon to open, clinical trials evaluating the efficacy of immunotherapy strategies alone or in combination with current and emerging therapies in the neoadjuvant and metastatic setting in BC patients (56-58). In the preclinical setting, the 4T1 series of BC cells are largely considered poor responders to single-agent PD-1 blockade due to the expansion of immunosuppressive MDSCs (262, 263). Dual interventions that target the proinflammatory TME concurrently with PD-1 or CTLA-4 checkpoint blockade result in regression of 4T1 tumors, even when disease burden is advanced and metastatic (266). However, no study has examined if moderate exercise in weight stable mice can drive a reduction in systemic inflammation and blunt the expansion of immunosuppressive cell populations to promote responsiveness to PD-1 checkpoint blockade in the 4T1 BC series.

Experimental results from previous findings (chapter three) show that moderately exercising, weight stable mice display a reduction in 4T1.2\textsuperscript{luc} tumor growth and metastatic progression, concurrently with a reduction in the expansion of immunosuppressive cell types (e.g., MDSCs), sustained lymphoid-lineage immune phenotype, and a reduction in proinflammatory and immunosuppressive gene expression in the TME. Data presented in chapter four detailed our findings that a broad-based immunogenic whole tumor cell cancer vaccine enhanced the cancer protective effects of moderate exercise in weight stable 4T1.2 tumor-bearing mice. Sanchez, et al. (57) reviewed the current BC immunotherapy literature and notes that while DC-based cancer vaccines are under clinical investigation in BC patients, no trial to date has investigated the combination of a
cancer vaccine and checkpoint blockade. Therefore, the combination of cancer vaccines and immune checkpoint blockade are two strategies to harness the immune system to treat cancer and their combination warrant further investigation. Thus, the goal of the current study was to determine if moderate exercise in weight stable mice could enhance targeted PD-1 checkpoint blockade alone, or in combination with the same whole tumor cell cancer vaccine administered in chapter four in our clinically relevant, orthotopic model of stage IV metastatic BC. It is of utmost importance to delineate new, previously unidentified combinatorial treatment strategies to attenuate tumor-induced inflammation and enhance immunotherapeutic efficacy to improve treatment responses and clinical outcomes in metastatic cancer patients.

5.3. MATERIALS AND METHODS

5.3.1. Tumor cell line and cell culture

The 4T1.2 cell line is a murine metastatic BC line derived from a spontaneously arising mammary tumor in a BALB/cfC3H mouse (566). When implanted orthotopically, the 4T1.2 cell line mimics the metastatic progression of human BC with a tendency to metastasize to lung and bone (567). 4T1.2 cells stably expressing luciferase (4T1.2lc) were provided by Dr. Robin Anderson and maintained in Dulbecco’s modified Eagle’s medium (Life Technologies) containing 10% fetal bovine serum (Gemini Bio-Products), 2 mM glutamine (Mediatech), 1X nonessential amino acid (Mediatech), and 8 μg/ml puromycin (Mediatech).
5.3.2. Animal model

Six-week old female BALB/c mice were obtained from Jackson Laboratory and screened for their intrinsic running potential. Female BALB/c mice (14-week old) were orthotopically injected with $5 \times 10^4$ luciferase-transfected 4T1.2 cells into the fourth mammary fat pad and randomized (n=4-5/group) to receive one vs. two rounds of immunotherapy. Each round of immunotherapy consisted of PBS vehicle control (VEH) or $1 \times 10^6$ irradiated 4T1.2$^{\text{luc}}$ cells vaccination (VAX), followed by two PD-1 checkpoint blockade injections (10 mg/kg/mouse). Mice were sacrificed at day 35 post-tumor implantation.

Intrinsic runners (14-week old) were randomized to sedentary, weight gain (WG; n=36) or exercising, weight maintenance (WM; n=36) groups for a total of 13 weeks. All mice were fed AIN-76A diet (Research Diets); however, the WM mice were fed 90% of caloric intake of WG mice to remain in energy balance (prevent weight gain) over the course of the study. After eight-weeks on study, all mice were orthotopically injected with $5 \times 10^4$ luciferase-transfected 4T1.2 cells into the fourth mammary fat pad and continued on their intervention for 35 days. After injection, WG and WM mice were randomized into vehicle control (VEH) or vaccination (VAX) groups and administered PBS or $1 \times 10^6$ irradiated 4T1.2$^{\text{luc}}$ cells at day 7 post-tumor injection. Mice were further randomized (n=7-11/group) to receive isotype control (IgG) or PD-1 checkpoint blockade (10 mg/kg/mouse) at day 9 and 12 post-tumor implantation. Mice were sacrificed at day 35 post-tumor implantation. A cohort of sedentary, ad
*libitum*-fed 4T1.2\textsuperscript{luc} tumor-bearing mice receiving the VEH and IgG control were sacrificed at day 24 post-tumor implantation to assess PD-1 expression on T cells. Movement was not monitored in mice that did not have access to running wheels. Food intake, body weight, and tumor size \((v=(\text{short}^2 \times \text{long})/2)\) were monitored as previously reported (562, 568), and mice were observed daily for signs of ill health. All mice were housed at the Pennsylvania State University and maintained on a 12-hour light/dark cycle with free access to water. The Institutional Animal Care and Use Committee of the Pennsylvania State University approved all animal experiments.

5.3.3. Whole tumor cell cancer vaccine

Trypsin/EDTA (0.25%\%/2.21 mM) in HBSS (Corning) was added to culture flasks to harvest 4T1.2\textsuperscript{luc} cells. 4T1.2\textsuperscript{luc} cells were washed twice in 4T1.2\textsuperscript{luc} media and washed twice in PBS. 4T1.2\textsuperscript{luc} cells were counted, adjusted to \(1.0 \times 10^6/100 \ \mu\text{l}\), and irradiated for 15,000 rad in a cesium irradiator. Irradiated 4T1.2\textsuperscript{luc} cells (VAX; \(1.0 \times 10^6/100 \ \mu\text{l}\)) or PBS control (VEH; \(100 \ \mu\text{l}\)) were injected i.P. at day 7 post-tumor implantation.

5.3.4. PD-1 checkpoint blockade

Mice were weighed and the stock concentration of *in vivo* depletion antibody was diluted to 10 mg/kg/mouse. Mice received an i.P. administration of rat IgG2a isotype control or rat α-mouse PD-1 [CD279] (RMP1-14; Bio X Cell; West Lebanon, NH) at day 9 and 12 post-tumor implantation.
5.3.5. Metastatic burden

The lung and femur were collected, flash frozen in liquid nitrogen, and stored at -80°C. Tissues were homogenized and genomic DNA was isolated using DNeasy Blood and Tissue Kit (Qiagen), per manufacturer's instructions. DNA was quantified using a Nanodrop ND-1000 spectrophotometer (Nanodrop Products). Metastatic tumor burden of the tissues was quantified using the Taqman™ system performed on a Step-One Plus (Life Technologies) real time PCR instrument to quantify luciferase. Assay sequences for luciferase were CAGCTGCACAAAGCCATGAA (forward primer), CTGAGGTAATGTCCACCTCGATATG (reverse primer) and TACGCCCTGGTGCCCGGC (probe with 5’ FAM reporter and 3’ BHQ quencher). The reference gene used for normalization was mouse telomerase reverse transcriptase (TERT) and was assayed using the Taqman Copy Number Reference Assay TERT (Life Technologies). The standard curve included 5-10-fold serial dilutions (200 ng to 20 pg) of DNA extracted from cultured 4T1.2\textsuperscript{Luc} cells. The standard curve and 200 ng of the tissue DNA samples were run in duplicate for luciferase and TERT on the Step-One Plus using the quantitative data analysis option and standard cycling parameters. Luciferase data was normalized to the quantitative values for TERT in each sample to correct for fluctuations in DNA amount, quality, and reaction efficiency.
5.3.6. Bioluminescent in vivo imaging

Mice were weighed and injected I.P. with XenoLight D-Luciferin-K\(^+\) Salt Bioluminescent Substrate (150 mg luciferin/kg body weight) (Perkin Elmer; Waltham, MA) 10 minutes prior to imaging. Anesthetized mice were imaged for a two-minute exposure in an IVIS 50 Imaging System (Perkin Elmer) and analyzed with Igor Pro - Scientific Imaging Analysis Software (Wavemetrics; Lake Oswego, OR).

5.3.7. Splenic, non-draining lymph node, and tumor-infiltrating immune cell assays

5.3.7.1. Isolation of splenic and non-draining lymph node immune cells

Spleens and non-draining lymph nodes (nDLN) were harvested via gross dissection and cells were prepared from individual mice by mechanical dispersion, as previously described (345). Briefly, spleens and nDLNs were mechanically disrupted with a syringe plunger and passed through a 70 μm nylon mesh strainer (BD Biosciences), erythrocytes were lysed with ACK lysis buffer (Lonza), and remaining cells washed twice in complete medium (RPMI 1640 (Mediatech) supplemented with 10% FBS (Gemini Bio-Products), 0.1 mM non-essential amino acids (Mediatech), 1 mM sodium pyruvate (Mediatech), 2 mM glutamine (Mediatech), 10 mM HEPES (Mediatech), 100 U/mL penicillin streptomycin (Mediatech). Cell counts and viability were determined via trypan blue exclusion (Mediatech).
5.3.7.2. Splenic CD4+ helper T cell proliferation assay

Splenic CD4+ helper T cells were isolated via Dynabeads Untouched Mouse CD4 Cells Kit following manufacturer’s instructions (Life Technologies). Splenic CD4+ helper T cells (1x10^5) plus 1.0 μg/ml unlabeled anti-CD28 (BD Biosciences) were incubated in flat-bottomed, 96-well plates (Greiner Bio-One) in the presence of increasing concentrations of anti-CD3 antibody (BD Biosciences) for 72 hours (562). Proliferation data was analyzed by tritiated (H^3) thymidine (Perkin Elmer) incorporation and quantified on a Microbeta plate reader (Perkin Elmer). Each assay was performed in triplicate.

5.3.7.3. Isolation of tumor-infiltrating immune cells

Primary tumors were harvested during dissection, weighed, minced into fine pieces (<10 mg), and incubated with 0.03 mg/ml Liberase (Roche) and 12.5 U/ml DNase I (Sigma-Aldrich) for 45 minutes at 37°C on an orbital shaker. Following the digestion, remaining pieces were mechanically disrupted with a syringe plunger, passed through a 70 μm nylon mesh strainer (BD Biosciences), layered over Lympholyte-M cell separation media (Cedarlane), and centrifuged at room temperature for 20 minutes at 1200g. Isolated cells were washed twice in cold PBS (Mediatech) and cell counts and viability of tumor immune infiltrates were determined via trypan blue exclusion.
5.3.7.4. Flow cytometric analyses

Single cell suspensions of splenocytes, splenic effector cells (after 24-hour co-culture and five-day bulk culture with irradiated tumor cells), nDLN cells, tumor-infiltrating immune cells, and tumor-infiltrating effector cell (after 24-hour co-culture with irradiated tumor cells) were washed twice in PBS containing 0.01% bovine serum albumin (flow buffer) at 4°C. Cells were incubated with Fc block (Biolegend) and 1x10^6 cells were stained with saturating concentrations of conjugated antibodies for 30 min at 4°C, as previously described (345). Fluorescently conjugated antibodies for flow cytometry included rat \( \alpha \)-mouse CD19 (1D3), hamster \( \alpha \)-mouse CD3 (145-2C11), rat \( \alpha \)-mouse CD4 (RM4-5), rat \( \alpha \)-mouse CD8 (53-6.7), mouse \( \alpha \)-mouse NK1.1 (PK136), mouse \( \alpha \)-mouse I-Ab (AF6-120.1), hamster \( \alpha \)-mouse CD11c (HL3), rat \( \alpha \)-mouse CD11b (M1/70), rat \( \alpha \)-mouse F4/80 (T45-2342), rat \( \alpha \)-mouse Ly6G and Ly6C [Gr-1] (RB6-8C5), rat \( \alpha \)-mouse Ly6C (AL-21), rat \( \alpha \)-mouse Ly6G (1A8). To differentiate between naïve, central memory (T_{CM}), effector memory (T_{EM}), and effector T cell populations, splenic, nDLN, and tumor-infiltrating immune cells were stained with rat \( \alpha \)-mouse CD4 (RM4-5), rat \( \alpha \)-mouse CD8 (53-6.7), rat \( \alpha \)-mouse CD44 (IM7), rat \( \alpha \)-mouse CD62L (MEL-14), rat \( \alpha \)-mouse CD69 (H1.2F3), rat \( \alpha \)-mouse IL-6R\( \alpha \) [CD126] (D7715A7), rat \( \alpha \)-mouse IL-7R\( \alpha \) [CD127] (SB/199), rat \( \alpha \)-mouse CCR7 [CD197] (4B12), hamster \( \alpha \)-mouse PD-1 [CD279] (J43), and hamster \( \alpha \)-mouse KLRG1 (2F1). Following incubation with the conjugated antibodies, cells were washed twice in flow
buffer and fixed in 1% paraformaldehyde (BD Biosciences) in flow buffer for flow cytometric analysis. Additionally, following the extracellular staining for CD3, CD4, CD8, and NK1.1, splenic cells, splenic effector cells, and tumor-infiltrating effector cells were permeabilized for 30 minutes, washed in a fixation/permeabilization buffer, and intracellularly stained for rat α-mouse IFNγ (XMG1.2) for 30 minutes. Following incubation with the IFNγ antibody, cells were washed twice in fixation/permeabilization buffer and read within 12 hours. Lymphoid and myeloid cells were gated on forward vs. side scatter, and a total of 50,000 events were analyzed. Flow cytometric analyses were performed on a BD LSR-Fortessa (BD Bioscience) flow cytometer. Gating for naïve, TCM, TEM, and effector T cell populations were based on expression of multiple markers using previously published studies (617-620) as a guide. Flow cytometric analyses were plotted and analyzed using Flow Jo software (Tree Star; Ashland, OR).

5.3.7.5. Splenic and tumor-infiltrating immune cell assays

Splenocytes were depleted of Gr-1+ cells and pulsed with irradiated 4T1.2luc cells for a 24-hour co-culture or a five-day bulk culture to generate an antigen-specific T cell response to tumor antigens. Tumor-infiltrating cells were pulsed with irradiated 4T1.2luc cells for a 24-hour co-culture to measure an antigen-specific T cell response to tumor antigens. Splenic effector cell supernatant was collected after the 24-hour co-culture and after a 48-hour rest following the five-day bulk culture and stored at -80°C.
Tumor-infiltrating cell supernatant was collected after the 24-hour co-culture and stored at -80°C. IFNγ was measured using Legend Max ELISA kits (Biolegend), per manufacturer instructions. Cytokines were quantified with an Epoch Microplate Spectrophotometer (Biotek; Winooski, VT). Each assay was performed in duplicate.

4T1.2\textsuperscript{luc} target cells were harvested via trypsin, washed in 4T1.2\textsuperscript{luc} media, and incubated at 37°C with Violet Proliferation Dye 450 (VPD450; BD Biosciences) for 15 minutes. Post 24-hour co-culture, splenic and tumor-infiltrating effector cells were co-cultured with VPD450-labeled 4T1.2\textsuperscript{luc} target cells. After 24 hours, cells were harvested, washed in flow buffer, permeabilized (BD Biosciences), and intracellularly stained with rabbit α-mouse active caspase-3 (CPP32) to quantify the ability of effector cells to induce caspase-3 in 4T1.2\textsuperscript{luc} target cells, as previously described (621). 4T1.2\textsuperscript{luc} target cells were cultured alone (negative controls), or with 1.0 mM camptothecin (Sigma-Aldrich) to induce caspase-3 expression (positive, spontaneous control) and the percent caspase-3 induction was calculated by: \([(\text{observed-spontaneous}) / (\text{total-spontaneous})] \times 100.

5.3.8. Gene expression in the tumor microenvironment

At sacrifice, tumor samples were incubated overnight in RNAlater (Qiagen) followed by RNA isolation from individual mice using Qiashredder columns followed by RNeasy Mini Plus Kit (Qiagen). RNA quality was assessed via Bioanalyzer analysis (Agilent Technologies; Santa Clara, CA) with samples requiring an RNA Integrity Number (RIN) above 7 to pass quality control testing
RNA sample was retrotranscribed using RT² First Strand Kit (Qiagen) according to manufacturer’s instructions and samples were loaded onto qPCR plates (Mouse Cancer Inflammation & Immunity Crosstalk PCR Array, Cat. # PAMM-181Z, Qiagen) and cycled according to the following conditions: 95°C for 10 minutes; 95°C for 15 seconds; 60°C for 1 minutes for 40 cycles on a StepOnePlus (Applied Biosystems; Foster City, CA). Qiagen’s online Data Analysis Center was used to normalize raw CT values to the housekeeping genes and fold change \(2^{\Delta \Delta CT}\) was calculated as previously described (572).

5.3.9. Statistical analyses

Tumor weight, metastatic burden, metastatic flux, the distribution of cells in the spleen, splenic bulk culture cells, and tumor-infiltrating immune cells, cytokine secretion, and caspase induction were assessed for normality and equal variances; and either parametric or nonparametric analyses were used based on sample distribution to detect differences between treatment groups. Metastatic burden, cytokine secretion, and metabolic mediators were skewed; thus, the data were transformed (log or square root) prior to statistical analysis. Differences in tumor weight, metastatic burden, metastatic flux, the distribution of cells in the spleen, splenic bulk culture cells, and tumor-infiltrating immune cells, cytokine secretion, and caspase induction were assessed between two groups via a Student’s t-test or Mann-Whitney test depending on sample distribution or between greater than two groups via a one-way analysis of variance (ANOVA), followed by a Bonferroni correction for multiple
comparisons where appropriate, or Kruskal-Wallis test, depending on normality and variance. Body weight, primary tumor volume, and CD4⁺ helper T cell proliferation were examined using a two-way ANOVA, followed by a Bonferroni correction for multiple comparisons where appropriate. All data are presented as the mean plus or minus the standard deviation of the mean. All analyses were conducted using GraphPad Prism 5 software (GraphPad Software; La Jolla, CA) and statistical significance was accepted at the p≤0.05 level.

5.4. RESULTS

5.4.1. PD-1 cell surface expression on splenic CD4⁺ helper and CD8⁺ cytotoxic T cells

Day 35 splenic CD4⁺ helper (Fig. 5.1A; p<0.001) and CD8⁺ cytotoxic (Fig. 5.1B; p<0.001) T cells expressed a lower percentage of PD-1 than day 24 cells.

Figure 5.1. PD-1 cell surface expression on splenic CD4⁺ helper and CD8⁺ cytotoxic T cells. Splenic T cells were stained for anti-CD4, -CD8, and -PD-1 at day 24 and day 35 post-tumor implantation. Day 35 splenic (A) CD4⁺ helper (p<0.001) and (B) CD8⁺ cytotoxic (p<0.001) T cells expressed a lower percentage of PD-1 than day 24 cells. Significantly different from day 24 (*).
5.4.2. Optimizing *in vivo* PD-1 checkpoint blockade

The experimental design for the PD-1 pilot study is displayed in Fig. 5.2A. Briefly, female BALB/c mice (14-week old) were orthotopically injected with \(5 \times 10^4\) luciferase-transfected 4T1.2 cells into the fourth mammary fat pad and randomized \((n=4-5/group)\) to receive one vs. two rounds of immunotherapy. Each round of immunotherapy consisted of PBS vehicle control (VEH) or \(1 \times 10^6\) irradiated 4T1.2\textsuperscript{luc} cells vaccination (VAX), followed by two PD-1 checkpoint blockade injections (10 mg/kg/mouse). Mice were sacrificed at day 35 post-tumor implantation. Body weights were not significantly different between immunotherapy groups throughout the course of the study (Fig. 5.2B; \(n=4-5/group\); 2-way ANOVA, \(F_{(3,90)}=2.84, p=0.073\)). Primary tumor growth was not significantly different between immunotherapy groups (Fig. 5.2C; 2-way ANOVA, \(F_{(3,195)}=0.16, p=0.920\)). No differences emerged between VEH or VAX groups, therefore, the vaccination intervention was collapsed to investigate one round (consisting of two PD-1 checkpoint blockade injections, \([x2]\)) vs. two rounds (consisting of four rounds of PD-1 checkpoint blockade injections, \([x4]\)) of PD-1 checkpoint blockade. Primary tumor growth was not significantly different between one vs. two rounds of PD-1 checkpoint blockade (Fig. 5.2D; \(n=9-10/group\); 2-way ANOVA, \(F_{(1,221)}=0.01, p=0.951\)). Tumor weight at sacrifice was not significantly different between groups (Fig. 5.2E; Student’s t-test, \(p=0.811\)).
Figure 5.2. Optimizing *in vivo* PD-1 checkpoint blockade in the 4T1.2 mammary tumor model. (A) Experimental design. (B) Body weights were not significantly different between immunotherapy groups throughout the course of the study (2-way ANOVA, $F_{(3,90)}=2.84$, $p=0.073$). (C) Primary tumor growth (measured 2-3x/week via caliper) was not significantly different between immunotherapy groups (2-way ANOVA, $F_{(3,195)}=0.16$, $p=0.920$). (D) No differences emerged between VEH or VAX groups, therefore, the vaccination intervention was collapsed to investigate one round (consisting of two PD-1 checkpoint blockade injections, [x2]) vs. two rounds (consisting of four PD-1 checkpoint blockade injections, [x4]) of PD-1 checkpoint blockade. Primary tumor growth was not significantly different between one vs. two rounds of PD-1 checkpoint blockade (2-way ANOVA, $F_{(1,221)}=0.01$, $p=0.951$). (E) Tumor weight at sacrifice was not significantly different between groups (Student’s t-test, $p=0.811$).
5.4.3. Splenic immunity in the PD-1 pilot study

The percentage and number of splenic immune cell populations were not significantly different between groups (Table 5.1).

![Table 5.1](image)

Table 5.1. The percentage and number of splenic immune cell populations in the PD-1 pilot study. The (A) percentage and (B) number of splenic immune cell populations were not significantly different between groups.
5.4.4. CD4+ helper and CD8+ cytotoxic T cell subpopulations and effector T cell activation and exhaustion markers in the PD-1 pilot study

CD44−CD62L−CCR7− naïve, CD44+CD62L+CCR7+ central memory (T_{CM}), CD44+CD62L−CCR7− effector memory (T_{EM}), and CD44+CD62L−CCR7− effector CD4+ helper T cell populations were not significantly different between groups in the spleen (Fig. 5.3A), non-draining lymph-node (nDLN; Fig. 5.3C), or tumor-infiltrating immune cell (Fig. 5.3E) compartments. The percent of CD44−CD62L−CCR7− effector CD4+ helper T cells expressing CD69, IL-6Rα, IL-7Rα, KLRG1, or PD-1 on their cell surface were not significantly different between groups in the spleen (Fig. 5.3B), nDLN (Fig. 5.3D), or tumor-infiltrating immune cell (Fig. 5.3F) compartment.

CD44−CD62L−CCR7− naïve, CD44+CD62L+CCR7+ T_{CM}, CD44+CD62L−CCR7− T_{EM}, and CD44+CD62L−CCR7− effector CD8+ cytotoxic T cell populations were not significantly different between groups in the spleen (Fig. 5.4A), nDLN (Fig. 5.4C), or tumor-infiltrating immune cell (Fig. 5.4E) compartments. The percent of CD44−CD62L−CCR7− effector CD8+ cytotoxic T cells expressing CD69, IL-6Rα, IL-7Rα, or PD-1 on their cell surface were not significantly different between groups in the spleen (Fig. 5.4B), nDLN (Fig. 5.4D), or tumor-infiltrating immune cell (Fig. 5.4F) compartment. The percent of CD44+CD62L−CCR7− effector CD8+ cytotoxic T cells expressing KLRG1 was not significantly different in the spleen (Fig. 5.4B) or nDLN (Fig. 5.4D); however, mice receiving (x4) PD-1 checkpoint blockade displayed a significant reduction in the cell surface expression of KLRG1 on effector CD8+ cytotoxic T cells in the tumor-infiltrating compartment (Fig. 5.4F; p=0.032).
Figure 5.3. Splenic, non-draining lymph node, and tumor-infiltrating CD4+ helper T cell populations and effector CD4+ T cell activation and exhaustion markers in the PD-1 pilot study. CD44+CD62L+CCR7+ naïve, CD44+CD62L+CCR7+ central memory (T_{CM}), CD44+CD62LCCR7+ effector memory (T_{EM}), or CD44+CD62LCCR7 effector CD4+ helper T cell populations were not significantly different between groups in the (A) spleen, (C) non-draining lymph-node (nDLN), or (E) tumor-infiltrating immune cell compartments. Effector CD4+ helper T cell populations were stained for markers of activation and exhaustion. The percent of effector CD4+ helper T cells expressing CD69, IL-6Rα, IL-7Rα, KLRG1, or PD-1 was not significantly different between groups in the (B) spleen, (D) nDLN, or (F) tumor-infiltrating immune cell compartment.
Figure 5.4. Splenic, non-draining lymph node, and tumor-infiltrating CD8+ helper T cell populations and effector CD8+ T cell activation and exhaustion markers in the PD-1 pilot study. CD44+CD62L+CCR7+ naïve, CD44+CD62L+CCR7+ central memory (T<sub>CM</sub>), CD44+CD62L+CCR7+ effector memory (T<sub>EM</sub>), or CD44-CD62L-CCR7- effector CD8+ cytotoxic T cell populations were not significantly different between groups in the (A) spleen, (C) non-draining lymph-node (nDLN), or (E) tumor-infiltrating immune cell compartments. Effector CD8+ cytotoxic T cell populations were stained for markers of activation and exhaustion. The percent of effector CD8+ cytotoxic T cells expressing CD69, IL-6Rα, IL-7Rα, or PD-1 was not significantly different between groups in the (B) spleen, (D) nDLN, or (F) tumor-infiltrating immune cell compartment. The percent of effector CD8+ cytotoxic T cells expressing KLRG1 was not significantly different in the (B) spleen or (D) nDLN; however, mice receiving (x4) PD-1 checkpoint blockade displayed a significant reduction in KLRG1 in the (F) tumor-infiltrating compartment (p=0.032). Significantly different from (x2) PD-1 checkpoint blockade (*).
5.4.5. Splenic and tumor-infiltrating immune cell co-culture and bulk assays in the PD-1 pilot study

The percentage of CD3\(^+\) T cells, CD3\(^+\)CD4\(^+\) helper T cells, CD3\(^+\)CD8\(^+\) cytotoxic T cells, and NK1.1\(^+\) natural killer cells, and the intracellular expression of IFN\(\gamma\) in helper T cells, cytotoxic T cells, and natural killer cells was not significantly different between groups receiving one vs. two rounds of vaccination + PD-1 blockade following a 24-hour co-culture (Fig. 5.5A) or a five-day bulk culture (Fig. 5.5D) with irradiated 4T1.2\(^{luc}\) cells. IFN\(\gamma\) secretion following a 24-hour co-culture was below detection (Fig. 5.5B). The percent caspase induction in 4T1.2\(^{luc}\) target cells co-cultured with 24-hour splenic effector cells was not significantly different between groups (Fig. 5.5C; 6-8/group; Mann-Whitney, p=0.284). Splenic bulk effector cell IFN\(\gamma\) secretion in response to re-stimulation with tumor antigens was not significantly different between groups (Fig. 5.5E; n=9-10/group; Student’s t-test, p=0.338). The percent caspase induction in 4T1.2\(^{luc}\) target cells co-cultured with five-day splenic bulk effector cells was not significantly different between groups (n=9-10/group; Mann-Whitney, p=0.497).
### A

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<th>Splenic 24-hour co-culture cells (percent)</th>
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<th>p-value</th>
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### B

![Graph](image)

**Below detection**

### C

![Graph](image)

**Caspase induction in 4T1.2⁺ targets (percent induction)**

### D

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<th>Splenic 5-day bulk cells (percent)</th>
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</tbody>
</table>

### E

![Graph](image)

**Splenic bulk culture IFNγ secretion (pg/ml)**

### F

![Graph](image)

**Caspase induction in 4T1.2⁺ targets (percent induction)**
Figure 5.5. Splenic immune cell 24-hour co-culture and five-day bulk assay in the PD-1 pilot study. Isolated splenocytes were depleted of Gr-1+ cells and pulsed with irradiated 4T1.2\textsuperscript{luc} cells for a 24-hour co-culture or a five-day bulk culture to generate an antigen-specific T cell response to tumor antigens. Post culture, cells were extracellularly stained with anti-CD3, -CD4, -CD8, and -NK1.1 and intracellularly stained with anti-IFN\textsubscript{γ} and characterized by flow cytometry (n=9-10/group). The percentage of CD3+ T cells, CD3+CD4+ helper T cells, CD3+CD8+ cytotoxic T cells, or NK1.1+ natural killer cells, or the intracellular expression of IFN\textsubscript{γ} in helper T cells, cytotoxic T cells, or natural killer cells was not significantly different between groups after a (A) 24-hour co-culture or (D) five-day bulk culture. (B) 24-hour co-culture cell IFN\textsubscript{γ} secretion was below detection. (C) Post 24-hour co-culture, cells were washed and co-cultured with VPD450-labeled 4T1.2\textsuperscript{luc} target cells. After 24 hours, cells were intracellularly stained for caspase expression and analyzed by flow cytometry. The percent caspase induction in 4T1.2\textsuperscript{luc} target cells was not significantly different between groups (6-8/group; Mann-Whitney, p=0.284). (E) Bulk effector cell IFN\textsubscript{γ} secretion in response to re-stimulation with tumor antigens was not significantly different between groups (n=9-10/group; Student's t-test, p=0.338). (F) The percent caspase induction in VPD450-labeled 4T1.2\textsuperscript{luc} target cells co-cultured for 24 hours with bulk effector cells was not significantly different between groups (n=9-10/group; Mann-Whitney, p=0.497).

The percentage of tumor-infiltrating CD3+ T cells, CD3+CD4+ helper T cells, CD3+CD8+ cytotoxic T cells, and NK1.1+ natural killer cells, and the intracellular expression of IFN\textsubscript{γ} in helper T cells, cytotoxic T cells, and natural killer cells were not significantly different between groups after a 24-hour co-culture (Fig. 5.6A). 24-hour co-culture cell IFN\textsubscript{γ} secretion was not significantly different between groups (Fig. 5.6B; n=6-7/group; Mann-Whitney, p=0.099). The percent caspase induction in 4T1.2\textsuperscript{luc} target cells co-cultured with 24-hour tumor-infiltrating effector cells was not significantly different between groups (Fig. 5.6C; n=4/group; Mann-Whitney, p=0.686).
Isolated tumor-infiltrating immune cells were pulsed with irradiated 4T1.2\textsuperscript{luc} cells for a 24-hour co-culture to measure an antigen-specific T cell response to tumor antigens. Post culture, cells were extracellularly stained with anti-CD3, -CD4, -CD8, and -NK1.1 and intracellularly stained with anti-IFN\textgamma and characterized by flow cytometry (n=6-7/group). (A) The percentage of tumor-infiltrating CD3\textsuperscript{+} T cells, CD3\textsuperscript{+}CD4\textsuperscript{+} helper T cells, CD3\textsuperscript{+}CD8\textsuperscript{+} cytotoxic T cells, or NK1.1\textsuperscript{+} natural killer cells, or the intracellular expression of IFN\textgamma in helper T cells, cytotoxic T cells, or natural killer cells was not significantly different between groups after a 24-hour co-culture. (B) 24-hour co-culture cell IFN\textgamma secretion was not significantly different between groups (n=6-7/group; Mann-Whitney, p=0.099). (C) Post 24-hour co-culture, cells were washed and co-cultured with VPD450-labeled 4T1.2\textsuperscript{luc} target cells. After 24 hours, cells were intracellularly stained for caspase expression and analyzed by flow cytometry. The percent caspase induction in 4T1.2\textsuperscript{luc} target cells was not significantly different between groups (n=4/group; Mann-Whitney, p=0.686).
5.4.6. Body weight and wheel activity

The experimental design is displayed in Fig. 5.7A. Briefly, female BALB/c mice (14-week old) were randomized to sedentary, weight gain (WG) or exercising, weight maintenance (WM) groups (n=36/group) for a total of 13 weeks. After eight-weeks on study, all mice were orthotopically injected with 5x10^4 luciferase-transfected 4T1.2 cells into the fourth mammary fat pad and continued on their intervention for 35 days. After injection, WG and WM mice were randomized into vaccination or vehicle control groups and administered PBS vehicle (VEH) control or 1x10^8 irradiated 4T1.2 luc cells (VAX) at day 7 post-tumor injection. Mice were further randomized (n=7-11/group) to receive isotype control (IgG) or PD-1 checkpoint blockade (10 mg/kg/mouse) at day 9 and 12 post-tumor implantation. Mice were sacrificed at day 35 post-tumor implantation WM mice weighed significantly less than WG mice, independent of immunotherapy, throughout the course of the study (Fig. 5.7B; n=7-11/group; 2-way ANOVA, F(3,741)=27.32, p<0.001). Wheel activity was maintained over the course of the study, i.e., prior to and after tumor injection. Activity was not affected by the administration of the whole tumor cell cancer vaccine or PD-1 checkpoint blockade (Fig. 5.7C; n=36; average distance run per day=5.3±2.5 km).
Figure 5.7. Study design, body weight, and wheel activity. (A) Experimental design. (B) WM mice weighed significantly less than WG mice, independent of immunotherapy, throughout the course of the study (2-way ANOVA, \( F_{(3,741)} = 27.32, p<0.001 \)). (C) Wheel activity was maintained over the course of the study, i.e., prior to and after tumor injection. Activity was not affected by the administration of the whole tumor cell cancer vaccine or PD-1 checkpoint blockade. (n=36; average distance run per day=5.3±2.5 km).
5.4.7. Primary tumor growth

Weight gain (WG) groups: Primary tumor growth was significantly reduced in the WG+VEH+PD-1 and WG+VAX+PD-1 groups compared with both WG+VEH+IgG and WG+VAX+IgG (Fig. 5.8A; n=7-11/group; 2-way ANOVA, time x treatment, $F_{(36,360)}=1.75$, $p=0.006$). Tumor weight at sacrifice was not significantly different between WG groups (Fig. 5.8B; 1-way ANOVA, $F_{(3,33)}=2.10$, $p=0.121$).

Weight maintenance (WM) groups: Primary tumor growth was not significantly different between WM groups (Fig. 5.8E; n=7-11/group; 2-way ANOVA, $F_{(3,360)}=0.37$, $p=0.777$). Tumor weight at sacrifice was not significantly different between WM groups (Fig. 5.8F; 1-way ANOVA, $F_{(3,33)}=0.36$, $p=0.782$).

Collapsing the VEH and VAX groups within weight interventions to analyze PD-1 checkpoint blockade effects (i.e., WG+IgG vs. WG+PD-1 vs. WM+IgG vs. WM+PD1): Primary tumor growth was significantly lower with PD-1 checkpoint blockade in mice in the WG group (Fig. 5.8I; n=14-20/group; 2-way ANOVA, time x treatment, $F_{(36,768)}=6.38$, $p<0.001$). WM groups had the lowest tumor volume independent of PD-1 checkpoint blockade ($p<0.001$). Tumor weight at sacrifice was reduced in both WM groups compared with WG+IgG (Fig. 5.8J; 1-way ANOVA, $F_{(3,67)}=6.00$, $p=0.001$).

5.4.8. Metastatic burden

WG groups: Lung (Fig. 5.8C; n=7-11/group; 1-way ANOVA, $F_{(3,33)}=2.10$, $p=0.121$) and femur (Fig. 5.8D; Kruskal-Wallis, KW=1.24, $p=0.745$) metastasis were not significantly different between WG groups.
WM groups: Lung (Fig. 5.8G; n=7-11/group; Kruskal-Wallis, KW=3.15, p=0.369) and femur (Fig. 5.8H; Kruskal-Wallis, KW=0.76, p=0.860) metastasis were not significantly different between WM groups.

Collapsing the vehicle and vaccination groups within weight interventions to analyze PD-1 checkpoint blockade effects (WG+IgG vs. WG+PD-1 vs. WM+IgG vs. WM+PD1): Spontaneous lung metastasis was lower with PD-1 checkpoint blockade in mice in the WG (Fig. 5.8K; n=14-30/group; 1-way ANOVA, F(3,61)=4.37, p=0.008), but not WM groups. Femur metastasis (Fig. 5.8L; 1-way ANOVA, F(3,52)=0.96, p=0.418) was not significantly different between groups. Representative IVIS images at day 34 post-tumor implantation (Fig. 5.9A). Whole-body metastatic flux (photons/sec) was not significantly different between groups at day 34 post-tumor implantation (Fig. 5.9B; 1-way ANOVA, F(3,56)=0.11, p=0.956).
Weight gain (WG) (ad-libitum + sedentary)  

Weight maintenance (WM)  
(diet + exercise)  

Collapsing VEH and VAX groups within weight intervention to analyze anti-PD-1 effects
Figure 5.8. PD-1 checkpoint blockade administration in weight gain mice reduced primary tumor growth. Weight maintenance independent of PD-1 checkpoint blockade reduced primary tumor growth. (A-D) WG groups. (A) Primary tumor growth (measured 2-3x/week via caliper) was significantly reduced in the WG+VEH+PD-1 and WG+VAX+PD-1 groups compared with both WG+VEH+IgG and WG+VAX+IgG (n=7-11/group; 2-way ANOVA, time x treatment, F(36,360)=1.75, p=0.006). (B) Tumor weight at sacrifice was not significantly different between WG groups (1-way ANOVA, F(3,33)=2.10, p=0.121). (C, D) At sacrifice, organs (n=7-11/group) were flash frozen, homogenized, and DNA was extracted. Quantitative PCR was used to detect luciferase expression as a marker of metastatic burden reported as ng of luciferase DNA normalized to mouse TERT DNA in 200 ng of sample (arbitrary units). (C) Lung (1-way ANOVA, F(3,33)=2.10, p=0.121) and (D) femur (Kruskal-Wallis, KW=1.24, p=0.745) metastasis were not significantly different between groups. (E-H) WM groups. (E) Primary tumor growth was not significantly different between WM groups (n=7-11/group; 2-way ANOVA, F(3,360)=0.37, p=0.777). (F) Tumor weight at sacrifice was not significantly different between WM groups (1-way ANOVA, F(3,33)=0.36, p=0.782). (G) Lung (Kruskal-Wallis, KW=3.15, p=0.369) and (D) femur (Kruskal-Wallis, KW=0.76, p=0.860) metastasis were not significantly different between groups. (I-L) Vehicle and vaccination groups were collapsed to investigate PD-1 checkpoint blockade effects in both WG and WM groups. (I) Primary tumor growth was significantly lower with PD-1 checkpoint blockade in mice in the WG group (n=14-20/group; 2-way ANOVA, time x treatment, F(36,768)=6.38, p<0.001). WM groups had the lowest tumor volume independent of PD-1 checkpoint blockade (p<0.001). (J) Tumor weight at sacrifice was reduced in both WM groups compared with WG+IgG (1-way ANOVA, F(3,67)=6.00, p=0.001). (K) Spontaneous lung metastasis was lower with PD-1 checkpoint blockade in mice in the WG (1-way ANOVA, F(3,61)=4.37, p=0.008), but not WM groups. (L) Femur metastasis (1-way ANOVA, F(3,52)=0.96, p=0.418) was not significantly different between groups. Significantly different from WG+VEH+IgG (*), WG+VAX+IgG (†), and WG+IgG (‡).
Figure 5.9. Bioluminescent *in vivo* imaging. (A) Representative IVIS images at day 34 post-tumor implantation. (B) Metastatic flux was calculated by applying a whole-body gate and subtracting out the primary tumor gate. Whole-body metastatic flux was not significantly different between groups at day 34 post-tumor implantation (*n*=14-20/group; 1-way ANOVA, *F*(3,56)=0.11, *p*=0.956).
5.4.9. Splenic immunity

5.4.9.1. Splenic immune cells

Splenocyte counts were not significantly different between groups (Fig. 5.10A; n=14-20/group; Kruskal-Wallis, KW=7.70, p=0.053). CD4+ helper T cell proliferation was significantly different between groups (Fig. 5.10B; n=11-16/group; 2-way ANOVA, time x treatment, $F_{(15,270)}=1.81$, p=0.033) with CD4+ helper T cells from WM mice proliferating more than CD4+ helper T cells from WG. The percentage of splenic CD3+CD4+ helper T cells was significantly different between groups (Table 5.2; p=0.048). The number of splenic immune cell populations was not significantly different between groups (Table 5.2). The number of splenic Gr-1+CD11b+ MDSCs was significantly different between groups (Fig. 5.10C; n=14-20/group; Kruskal-Wallis, KW=7.96, p=0.047) with WM+PD-1 mice displaying the lowest number compared with WG+IgG mice. The number of splenic CD11b+Ly6C^hiLy6G^- monocytic MDSCs (Fig. 5.10D; 1-way ANOVA, $F_{(3,67)}=2.28$, p=0.088) was not significantly different between groups; however, the number of splenic CD11b+Ly6C^loLy6G^+ granulocytic MDSCs was significantly different between groups (Fig. 5.10E; 1-way ANOVA, $F_{(3,67)}=3.65$, p=0.017) with WM+PD-1 mice displaying the lowest number compared with WG+VEH mice.
Figure 5.10. The combination of weight maintenance and PD-1 checkpoint blockade reduced protumor immunosuppressive cell populations. Splenocytes (n=14-20/group) were prepared into a single cell suspension and counted. (A) Splenocyte counts were not significantly different between groups (n=14-20/group; Kruskal-Wallis, KW=7.70, p=0.053). (B) Isolated splenic CD4+ helper T cells (0.1x10^6/well) were stimulated with increasing concentrations of anti-CD3 antibody and 1 μg/ml anti-CD28 antibody, and proliferation was quantified via [H]3 uptake. Data were converted to a stimulation index (counts per minute of stimulated wells divided by counts per minute of unstimulated [media only] wells). CD4+ helper T cell proliferation was significantly different between groups (n=11-16/group; 2-way ANOVA, time x treatment, F(15,270)=1.81, p=0.033). Splenocytes (n=14-20/group) were stained with anti-Gr-1, -CD11b, -Ly6G, and -Ly6C antibodies to quantify myeloid-derived suppressor cells (MDSCs) and MDSC subsets by flow cytometry. (C) The number of splenic Gr-1+CD11b+ MDSCs was significantly different between groups (n=14-20/group; Kruskal-Wallis, KW=7.96, p=0.047) with WM+PD-1 mice displaying the lowest number compared with WG+IgG mice. The number of (D) splenic CD11b+Ly6C+Ly6G+ monocytic MDSCs (n=14-20/group; 1-way ANOVA, F(3,67)=2.28, p=0.088) was not significantly different between groups; however, (E) the number of splenic CD11b+Ly6C+Ly6G+ granulocytic MDSCs was significantly different between groups (n=14-20/group; 1-way ANOVA, F(3,67)=3.65, p=0.017) with WM+PD-1 mice displaying the lowest number compared with WG+VEH mice. Dashed line represents non-tumor bearing control. Significantly different from WG+VEH+IgG (*).
Table 5.2. The percentage and number of splenic immune cell populations. (A) The percentage of splenic CD3^+CD4^+ helper T cells (p=0.048) was significantly different between groups. (B) The number of splenic immune cell populations was not significantly different between groups.

### 5.4.9.2. Splenic immune cell co-culture and bulk assays

The percentage of CD3^+ T cells, CD3^+CD4^+ helper T cells, CD3^+CD8^+ cytotoxic T cells, and NK1.1^+ natural killer cells, and the intracellular expression of IFNγ in helper T cells, cytotoxic T cells, and natural killer cells was not significantly different between groups in naïve splenocytes (Fig. 5.11A), after a 24-hour co-culture (Fig. 5.11B), or a five-
day bulk culture (Fig. 5.11E) with irradiated 4T1.2\textsuperscript{luc} cells. 24-hour co-culture cell IFN\textsubscript{\(\gamma\)} secretion was below detection (Fig. 5.11C). The percent caspase induction in 4T1.2\textsuperscript{luc} target cells co-cultured with 24-hour splenic effector cells was not significantly different between groups (Fig. 5.11D; n=14-16/group; 1-way ANOVA, \(F_{(3,60)}=1.31, p=0.281\)). Splenic bulk effector cell IFN\textsubscript{\(\gamma\)} secretion in response to re-stimulation with tumor antigens was not significantly different between groups (n=14-20/group; Kruskal-Wallis, KW=1.97, p=0.578).
### Splenic immune cells

#### A

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<th>Splenocytes (percent)</th>
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<td>WG+IgG (n=15)</td>
<td>WG+PD-1 (n=16)</td>
<td>WM+IgG (n=16)</td>
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<tr>
<td>Total T cells (CD3⁺)</td>
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<td>28.4 ± 9.3</td>
<td>31.7 ± 8.6</td>
</tr>
<tr>
<td>Helper T cells (CD3⁺CD4⁺)</td>
<td>19.8 ± 4.3</td>
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<td>13.3 ± 4.4</td>
</tr>
<tr>
<td>%IFN⁺ Helper T cells</td>
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<td>16.2 ± 8.9</td>
<td>11.2 ± 8.1</td>
</tr>
<tr>
<td>Cytotoxic T cells (CD3⁺CD8⁺)</td>
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<td>10.2 ± 3.3</td>
<td>11.5 ± 3.6</td>
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<tr>
<td>%IFN⁺ Cytotoxic T cells</td>
<td>3.5 ± 1.5</td>
<td>3.7 ± 1.8</td>
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<td>NK cells (NK1.1⁺)</td>
<td>13.5 ± 6.7</td>
<td>11.0 ± 3.3</td>
<td>13.0 ± 4.0</td>
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<tr>
<td>%IFN⁺ NK cells</td>
<td>7.0 ± 2.0</td>
<td>6.0 ± 1.5</td>
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</tbody>
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#### B

<table>
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<tr>
<th>Splenic 24-hour co-culture cells (percent)</th>
<th>Weight gain (WG)</th>
<th>Weight maintenance (WM)</th>
<th>p-value</th>
</tr>
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<td>WG+IgG (n=16)</td>
<td>WG+PD-1 (n=16)</td>
<td>WM+IgG (n=16)</td>
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<td>Total T cells (CD3⁺)</td>
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<td>Helper T cells (CD3⁺CD4⁺)</td>
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<td>16.9 ± 7.8</td>
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<tr>
<td>%IFN⁺ Helper T cells</td>
<td>15.6 ± 8.0</td>
<td>12.9 ± 6.4</td>
<td>12.5 ± 5.2</td>
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<tr>
<td>Cytotoxic T cells (CD3⁺CD8⁺)</td>
<td>16.4 ± 6.1</td>
<td>14.7 ± 4.7</td>
<td>16.0 ± 3.6</td>
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<tr>
<td>%IFN⁺ Cytotoxic T cells</td>
<td>11.4 ± 7.1</td>
<td>10.9 ± 9.7</td>
<td>9.1 ± 5.3</td>
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<tr>
<td>NK cells (NK1.1⁺)</td>
<td>19.8 ± 4.1</td>
<td>9.8 ± 3.7</td>
<td>10.3 ± 4.9</td>
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<tr>
<td>%IFN⁺ NK cells</td>
<td>59.2 ± 17.0</td>
<td>60.0 ± 11.1</td>
<td>55.7 ± 18.8</td>
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</tbody>
</table>

#### C

Splenic immune cells (depleted of Gr-1⁺ cells) co-cultured (24 hours) with irradiated 4T1.2⁺ to generate a recall response.

#### D

Splenic immune cells (depleted of Gr-1⁺ cells) co-cultured (5 days) with irradiated 4T1.2⁺ to generate a recall response.

#### E

<table>
<thead>
<tr>
<th>Splenic 5-day bulk cells (percent)</th>
<th>Weight gain (WG)</th>
<th>Weight maintenance (WM)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WG+IgG (n=15)</td>
<td>WG+PD-1 (n=16)</td>
<td>WM+IgG (n=16)</td>
</tr>
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<td>Total T cells (CD3⁺)</td>
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<td>43.4 ± 17.1</td>
<td>51.7 ± 15.5</td>
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<td>Helper T cells (CD3⁺CD4⁺)</td>
<td>12.4 ± 6.9</td>
<td>12.4 ± 6.9</td>
<td>15.6 ± 5.7</td>
</tr>
<tr>
<td>%IFN⁺ Helper T cells</td>
<td>16.4 ± 9.2</td>
<td>13.6 ± 6.9</td>
<td>15.3 ± 7.9</td>
</tr>
<tr>
<td>Cytotoxic T cells (CD3⁺CD8⁺)</td>
<td>13.9 ± 8.9</td>
<td>15.6 ± 9.6</td>
<td>20.5 ± 10.4</td>
</tr>
<tr>
<td>%IFN⁺ Cytotoxic T cells</td>
<td>12.8 ± 7.2</td>
<td>11.9 ± 6.2</td>
<td>14.7 ± 11.5</td>
</tr>
<tr>
<td>NK cells (NK1.1⁺)</td>
<td>23.7 ± 12.6</td>
<td>17.9 ± 10.1</td>
<td>15.8 ± 11.2</td>
</tr>
<tr>
<td>%IFN⁺ NK cells</td>
<td>56.3 ± 27.7</td>
<td>64.2 ± 19.4</td>
<td>63.3 ± 15.3</td>
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</table>

#### F

Splenic bulk culture (for 5 days) with irradiated 4T1.2⁺ to generate a recall response.
Figure 5.11. Splenic immune cell co-culture and bulk assays. Splenocytes (n=14-20/group) were prepared into a single cell suspension and counted. (A) Naïve splenocytes, (B) Gr-1 depleted splenocytes co-cultured for 24-hours with irradiated 4T1.2\textsuperscript{luc}, and (E) Gr-1 depleted splenocytes bulk cultured for five-days with irradiated 4T1.2\textsuperscript{luc} were extracellularly stained with anti-CD3, -CD4, -CD8, and -NK1.1 and intracellularly stained with anti-IFN\textgreek{y} and characterized by flow cytometry. No differences in cell populations or intracellular IFN\textgreek{y} staining was observed. (C) 24-hour co-culture cell IFN\textgreek{y} secretion was below detection. (D) Post 24-hour co-culture, cells were washed and co-cultured with VPD450-labeled 4T1.2\textsuperscript{luc} target cells. After 24 hours, cells were intracellularly stained for caspase expression and analyzed by flow cytometry. The percent caspase induction in 4T1.2\textsuperscript{luc} target cells was not significantly different between groups (n=14-16/group; 1-way ANOVA, F\textsubscript{3.60}=1.31, p=0.281). (F) Splenic bulk effector cell IFN\textgreek{y} secretion in response to re-stimulation with tumor antigens was not significantly different between groups (n=14-20/group; Kruskal-Wallis, KW=1.97, p=0.578).

5.4.10. The tumor microenvironment

5.4.10.1. Tumor-infiltrating immunity

The percentage and number of tumor-infiltrating immune cells were not significantly different between groups (n=6-12/group; Table 5.3).
Table 5.3. The percentage and number of tumor-infiltrating immune cell populations. The (A) percentage and (B) number of tumor-infiltrating immune cell populations were not significantly different between groups.

The percentage of CD44+CD62L+CCR7+ naïve, CD44+CD62L+CCR7+ T_{CM}, CD44+CD62L-CCR7+ T_{EM}, and CD44-CD62L-CCR7- effector CD4+ helper T cell populations was not significantly different between groups (Fig. 5.12A; n=4-6/group). The percent of tumor-infiltrating CD44-CD62L-CCR7- effector CD4+ helper T cells expressing

<table>
<thead>
<tr>
<th>A</th>
<th>Tumor-infiltrating immune cell populations</th>
<th>Weight gan (WG)</th>
<th>Weight maintenance (WM)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>W+IgG (n=11)</td>
<td>W+PD-1 (n=12)</td>
<td>WM+IgG (n=8)</td>
</tr>
<tr>
<td>B cells (CD19+)</td>
<td>27.2 ± 29.5</td>
<td>46.5 ± 12.0</td>
<td>36.3 ± 29.3</td>
<td>50.3 ± 10.9</td>
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<tr>
<td>Total T cells (CD3+)</td>
<td>61.2 ± 9.4</td>
<td>59.4 ± 10.8</td>
<td>60.7 ± 7.2</td>
<td>60.2 ± 7.1</td>
</tr>
<tr>
<td>Helper T cells (CD3+CD4+)</td>
<td>23.9 ± 12.9</td>
<td>25.8 ± 10.1</td>
<td>24.2 ± 9.0</td>
<td>25.0 ± 11.5</td>
</tr>
<tr>
<td>Cytotoxic T cells (CD3+CD8+)</td>
<td>12.9 ± 7.3</td>
<td>13.3 ± 5.3</td>
<td>11.0 ± 5.8</td>
<td>14.1 ± 2.2</td>
</tr>
<tr>
<td>NK cells (NK1.1)</td>
<td>5.7 ± 4.5</td>
<td>9.6 ± 3.5</td>
<td>9.6 ± 7.2</td>
<td>14.2 ± 7.1</td>
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<tr>
<td>Dendritic Cells (iAb’CD11c+)</td>
<td>26.2 ± 29.6</td>
<td>27.5 ± 23.9</td>
<td>22.6 ± 21.8</td>
<td>24.0 ± 11.7</td>
</tr>
<tr>
<td>Macrophages (iAb’CD11b+)</td>
<td>36.2 ± 27.5</td>
<td>34.0 ± 24.3</td>
<td>31.6 ± 20.4</td>
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<tr>
<td>Macrophages (F4/80+)</td>
<td>36.5 ± 30.3</td>
<td>38.7 ± 22.2</td>
<td>37.0 ± 22.4</td>
<td>45.7 ± 18.7</td>
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<td>mMDSCs (Gr-1+CD11b+)</td>
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<td>36.8 ± 25.1</td>
<td>40.8 ± 20.7</td>
<td>35.4 ± 15.6</td>
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<tr>
<td>gMDSCs (CD11b+Ly6C+Ly6G-)</td>
<td>20.5 ± 16.9</td>
<td>15.3 ± 15.1</td>
<td>16.4 ± 12.8</td>
<td>13.1 ± 9.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B</th>
<th>Tumor-infiltrating immune cell populations</th>
<th>Weight gan (WG)</th>
<th>Weight maintenance (WM)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>W+IgG (n=11)</td>
<td>W+PD-1 (n=12)</td>
<td>WM+IgG (n=8)</td>
</tr>
<tr>
<td>Tumor-infiltrating immune cells</td>
<td>24.8 ± 24.4</td>
<td>15.5 ± 8.6</td>
<td>13.2 ± 9.4</td>
<td>20.5 ± 15.4</td>
</tr>
<tr>
<td>B cells (CD19+)</td>
<td>14.0 ± 20.3</td>
<td>8.3 ± 4.0</td>
<td>6.3 ± 7.8</td>
<td>11.3 ± 10.1</td>
</tr>
<tr>
<td>Total T cells (CD3+)</td>
<td>16.3 ± 14.0</td>
<td>10.3 ± 4.2</td>
<td>7.7 ± 4.5</td>
<td>11.4 ± 7.3</td>
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<tr>
<td>Helper T cells (CD3+CD4+)</td>
<td>6.3 ± 7.0</td>
<td>4.6 ± 2.8</td>
<td>2.7 ± 1.0</td>
<td>3.7 ± 1.3</td>
</tr>
<tr>
<td>Cytotoxic T cells (CD3+CD8+)</td>
<td>3.1 ± 3.0</td>
<td>2.3 ± 1.2</td>
<td>1.5 ± 1.2</td>
<td>2.8 ± 2.0</td>
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<tr>
<td>NK cells (NK1.1)</td>
<td>2.4 ± 3.8</td>
<td>1.9 ± 1.6</td>
<td>1.5 ± 1.7</td>
<td>3.1 ± 2.4</td>
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<tr>
<td>Dendritic Cells (iAb’CD11c+)</td>
<td>10.1 ± 14.4</td>
<td>4.5 ± 3.6</td>
<td>3.7 ± 5.2</td>
<td>5.0 ± 3.9</td>
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<tr>
<td>Macrophages (iAb’CD11b+)</td>
<td>11.7 ± 14.6</td>
<td>5.8 ± 4.1</td>
<td>4.1 ± 3.7</td>
<td>6.4 ± 4.1</td>
</tr>
<tr>
<td>Macrophages (F4/80+)</td>
<td>13.5 ± 17.8</td>
<td>6.5 ± 3.7</td>
<td>5.8 ± 7.1</td>
<td>11.2 ± 10.9</td>
</tr>
<tr>
<td>mMDSCs (CD11b+Ly6C+Ly6G+)</td>
<td>13.6 ± 16.7</td>
<td>6.2 ± 4.0</td>
<td>6.1 ± 6.7</td>
<td>7.4 ± 5.5</td>
</tr>
<tr>
<td>gMDSCs (CD11b+Ly6C+Ly6G+)</td>
<td>3.3 ± 5.0</td>
<td>1.2 ± 1.0</td>
<td>2.3 ± 2.4</td>
<td>4.4 ± 5.8</td>
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</table>

The percentage of CD44+CD62L+CCR7+ naïve, CD44+CD62L+CCR7+ T_{CM}, CD44+CD62L-CCR7+ T_{EM}, and CD44-CD62L-CCR7- effector CD4+ helper T cell populations was not significantly different between groups (Fig. 5.12A; n=4-6/group). The percent of tumor-infiltrating CD44-CD62L-CCR7- effector CD4+ helper T cells expressing...
CD69, IL-6Rα, IL-7Rα, KLRG1, or PD-1 on their cell surface was not significantly different between groups (Fig. 5.12B).

The percentage of CD44+CD62L+CCR7+ naïve, CD44+CD62L+CCR7+ TCM, CD44+CD62L−CCR7− TEM, or CD44−CD62L−CCR7− effector CD8+ cytotoxic T cell populations was not significantly different between groups (Fig. 5.12C; n=4-6/group). The percent of tumor-infiltrating CD44+CD62L−CCR7− effector CD8+ cytotoxic T cells expressing CD69, IL-6Rα, IL-7Rα, KLRG1, or PD-1 on their cell surface was not significantly different between groups (Fig. 5.12D).
Figure 5.12. Tumor-infiltrating CD4+ and CD8+ T cell populations and effector activation and exhaustion markers. The percentage of tumor-infiltrating CD4+CD62L+CCR7+ naïve, CD4+CD62L−CCR7− central memory (T_CM), CD4+CD62L−CCR7− effector memory (T_EM), or CD44 CD62LCCR7 effector (A) CD4+ helper and (C) CD8+ cytotoxic T cell populations was not significantly different between groups. Effector CD4+ helper and CD8+ cytotoxic T cell populations were stained for markers of activation and exhaustion. The percentage of effector (B) CD4+ helper and (D) CD8+ cytotoxic T cells expressing CD69, IL-6Rα, IL-7Rα, or PD-1 on their cell surface was not significantly different between groups.
5.4.10.2. Tumor-infiltrating immune cell co-culture assay

The percentage of tumor-infiltrating CD3+ T cells, CD3+CD4+ helper T cells, CD3+CD8+ cytotoxic T cells, and NK1.1+ natural killer cells, and the intracellular expression of IFNγ in helper T cells, cytotoxic T cells, or natural killer cells was not significantly different between groups after a 24-hour co-culture (Fig. 5.13A). 24-hour co-culture cell IFNγ secretion was not significantly different between groups (Fig. 5.13B; n=5-8/group; Kruskal-Wallis, KW=0.72, p=0.868). The percent caspase induction in 4T1.2\textsuperscript{luc} target cells co-cultured with 24-hour tumor-infiltrating effector cells was not significantly different between groups (n=6-8/group; Kruskal-Wallis, KW=1.35, p=0.717).
Tumor-infiltrating immune cells co-cultured (24 hours) with irradiated 4T1.2Luc to measure a recall response

<table>
<thead>
<tr>
<th>Tumor-infiltrating immune cells 24-hour co-culture (percent)</th>
<th>Weight gain (WG)</th>
<th>Weight maintenance (WM)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total T cells (CD3+) (n=7)</td>
<td>WG+IgG</td>
<td>WG+PD-1</td>
<td>0.822</td>
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<td>Helper T cells (CD3+CD4+)</td>
<td>16.9 ± 10.4</td>
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<td>%IFNγ+ Helper T cells</td>
<td>26.7 ± 15.9</td>
<td>29.4 ± 21.9</td>
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<tr>
<td>Cytotoxic T cells (CD3+CD8+)</td>
<td>10.8 ± 7.8</td>
<td>13.3 ± 5.3</td>
<td>0.648</td>
</tr>
<tr>
<td>%IFNγ+ Cytotoxic T cells</td>
<td>21.0 ± 12.0</td>
<td>15.6 ± 13.0</td>
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</tr>
<tr>
<td>NK cells (NK1.1+)</td>
<td>7.8 ± 4.8</td>
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</tr>
<tr>
<td>%IFNγ+ NK cells</td>
<td>52.4 ± 24.2</td>
<td>69.9 ± 6.1</td>
<td>0.384</td>
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</table>

Figure 5.13. Tumor-infiltrating immune cell 24-hour co-culture assay. Isolated tumor-infiltrating immune cells were pulsed with irradiated 4T1.2Luc cells for a 24-hour co-culture to measure an antigen-specific T cell response to tumor antigens. Post culture, cells were extracellularly stained with anti-CD3, -CD4, -CD8, and -NK1.1 and intracellularly stained with anti-IFNγ and characterized by flow cytometry (n=6-8/group). (A) The percentage of tumor-infiltrating CD3+ T cells, CD3+CD4+ helper T cells, CD3+CD8+ cytotoxic T cells, or NK1.1+ natural killer cells, or the intracellular expression of IFNγ in helper T cells, cytotoxic T cells, or natural killer cells was not significantly different between groups after a 24-hour co-culture. (B) 24-hour co-culture cell IFNγ secretion was not significantly different between groups (Kruskal-Wallis, KW=0.72, p=0.868). (C) Post 24-hour co-culture, cells were washed and co-cultured with VPD450-labeled 4T1.2Luc target cells. After 24 hours, cells were intracellularly stained for caspase expression and analyzed by flow cytometry. The percent caspase induction in 4T1.2Luc target cells was not significantly different between groups (n=6-8/group; Kruskal-Wallis, KW=1.35, p=0.717).
5.4.10.3. Tumor-immune crosstalk gene array

Figure 5.14. Moderate exercise in weight stable mice, alone and in combination with PD-1 checkpoint blockade, altered gene expression in the tumor microenvironment. Tumor homogenates were assayed using Qiagen RT² Profiler™ Mouse Cancer Inflammation & Immunity Crosstalk PCR Array. (A) Heat map displaying genes altered >2-fold. (B) Genes that were significantly altered (>2.5-fold) compared with WG+IgG control.
5.5. DISCUSSION

Previous work in our lab demonstrates that moderate exercise in weight stable, non-tumor bearing mice augments antigen-specific T cell response to vaccination and in response to a viral-vector based vaccine in a pancreatic cancer model (272, 273). Furthermore, we have demonstrated (chapter three) that the prevention of weight gain through exercise and mild dietary restriction can reduce primary tumor growth and metastatic burden, reduce immunosuppressive cells, augment T cell responses, and alter the tumor microenvironment in the 4T1.2 mammary tumor model. Also, we have demonstrated (chapter four) that moderate exercise in weight stable mice in combination with the administration of an immunogenic whole tumor cell cancer vaccine significantly reduced 4T1.2 primary tumor growth and metastatic burden compared with exercise/weight maintenance alone. To our knowledge, no study has investigated if lifestyle-based intervention strategies can improve the efficacy of immune checkpoint blockade. Thus, since numerous host factors are likely to impact immunotherapeutic efficacy, we chose to examine if an exercise and weight maintenance intervention could blunt tumor-induced inflammation and immunosuppression resulting in an enhancement in the dual administration of a whole tumor cell cancer vaccine and PD-1 checkpoint blockade. Several methodological concerns were assessed in a pilot study prior to investigating the effects of moderate exercise in weight stable mice on the efficacy of the dual administration of a whole tumor cell cancer and PD-1 checkpoint blockade.
The role of PD-1 in tumor progression and the response to antibody blockade in the 4T1 series of murine BC models is emerging. Data from Hirano, et al. (264) confirm that 4T1 tumors upregulate PD-L1 in vivo and blockade of PD-L1 (100 μg) alone at day 7 and 10 post-tumor implantation partially inhibits primary tumor growth. Beavis, et al. (265) demonstrates that single agent PD-1 checkpoint blockade (200 μg, administered at day 7, 11, and 15 post-tumor implantation) is not effective at reducing 4T1.2 primary tumor growth or spontaneous lung metastasis when mice are removed from study at day 18 post-tumor implantation. This lack of effect was attributed to low levels of PD-1 cell surface expression on T cell subsets during 4T1.2 tumor progression. The exact timing of the upregulation of PD-1 on the cell surface of T cells is dependent on numerous intrinsic factors (53). Therefore, we quantified the level of PD-1 surface expression on splenic T cells from untreated (i.e., no VAX or PD-1) 4T1.2luc tumor-bearing mice at day 24 and 35 post-tumor implantation. Day 24 splenic CD4+ helper and CD8+ cytotoxic T cells expressed a higher percentage of PD-1 than day 35 T cells. Since PD-1 cell surface expression was measureable and elevated early in 4T1.2 tumor progression, this supported the inclusion of PD-1 checkpoint blockade as a viable treatment option in the 4T1.2 model.

An additional concern that is currently under clinical investigation is the optimal dosage and schedule of immunological checkpoint blockade administration (253, 622). Preclinical models use vastly different dosages and schedules of PD-1 checkpoint blockade when administered alone, or in combination with additional therapies (266-270). For example, the literature is
inconsistent if single agent PD-1 checkpoint blockade is effective at reducing established tumor growth in the 4T1 or 4T1.2 model (265, 270). Methodological differences in timing (prior to or after palpable tumor), number of administrations (one-four), or dosage of anti-PD-1 (100-250 µg) may drive the disparate findings. However, the majority of articles report that the administration of PD-1 checkpoint blockade in combination with therapies that target a reduction in systemic inflammation (e.g., celecoxib [selective Cox-2 inhibition], azacitine or entiostat [epigenetic modulating drug], and J32 [phosphoinositide 3-kinase inhibitor]) are the most effective at driving an enhancement in antitumor effector function, a reduction in established tumor growth, and a reduction in spontaneous metastasis (266-270). The number of possible combinatorial treatment strategies grows exponentially. Therefore, testing if lifestyle-based interventions, i.e., physical activity and the prevention of weight gain, which are low-cost and have known benefits across the cancer continuum (271), can augment T cell responses (272, 273) and can enhance the efficacy of immunotherapeutic strategies represents an attractive option.

Experimental results from previous findings (chapter three and four) show that moderate exercise in weight stable mice can reduce the expansion of immunosuppressive cells and enhance a broad-based immunogenic whole tumor cell cancer vaccine. We wanted to build on this work using an emerging, targeted immunotherapy, specifically PD-1 checkpoint blockade. Instead of selecting a pharmacological inhibitor of systemic inflammation and immunosuppressive cells, we decided to test if a diet and exercise intervention, coupled with the whole tumor
cell cancer vaccine as an immunogenic stimulus, could improve PD-1 checkpoint blockade in 4T1.2 tumor-bearing mice. However, prior to the dual administration of a whole tumor cell cancer vaccine and PD-1 checkpoint blockade in moderately exercising, weight stable mice, we tested the timing and rounds of treatment in an in vivo pilot study. Sedentary, ad libitum-fed BALB/c mice were orthotopically injected with luciferase-transfected 4T1.2 cells and received one vs. two rounds of immunotherapy (consisting of one or two rounds of a PBS vehicle (VEH) control or $1 \times 10^6$ irradiated 4T1.2\textsuperscript{luc} vaccine (VAX), followed by two I.P. injections of PD-1 checkpoint blockade (10 mg/kg/mouse)). Since the diet and exercise plus PD-1 checkpoint blockade study was designed to evaluate two body weight phenotypes, we administered 10 mg/kg/mouse of anti-PD-1 to adjust the dosage of anti-PD-1 antibody based on body weight, similar to Kim, et al. (266). Additionally, 10 mg/kg is the dosage currently under clinical investigation in a phase I KEYNOTE-12 trial investigating anti-PD-1 (pembrolizumab) treatment in women with PD-L1-positive TNBC (623). Primary tumor growth was not significantly different between the four treatment groups and no differences were observed between VEH or VAX within one vs. two rounds of treatment. Therefore, the VAX intervention was collapsed to investigate two (x2) vs. four (x4) PD-1 checkpoint blockade injections on primary tumor growth and immune cell populations in various immune compartments, as well as functional immune outcomes. Primary tumor growth was not significantly different between two vs. four administrations of PD-1 checkpoint blockade. Splenic, non-draining lymph node (nDLN), and tumor-infiltrating immune populations were not significantly different between groups. No differences were
observed in naïve, memory, or effector status of CD4+ helper or CD8+ cytotoxic T cell populations in the spleen, nDLN, or tumor-infiltrating immune cell compartments. The 4T1.2 model is associated with rapid in vivo tumor growth; therefore, the limited time and lack of a true dormancy period may prohibit the investigation of memory T cell populations. CD4+ helper or CD8+ cytotoxic T cell populations were stained for an activation marker (CD69), a marker of antigen-experience (KLRG1), markers with memory-forming potential (IL-6Rα, IL-7Rα), and an exhaustion marker (PD-1). The percent of tumor-infiltrating effector CD4+ helper or effector CD8+ cytotoxic T cell populations expressing CD69, IL-6Rα, IL-7Rα, or PD-1 was not significantly different between groups. Mice receiving (x2) PD-1 checkpoint blockade displayed a significant increase in the percent of tumor-infiltrating CD8+ cytotoxic T cells expressing KLRG1, a marker that is upregulated as cells undergo terminal differentiation and can promote the transition to cell senescence (624). However, this difference did not drive changes in tumor growth. Lastly, re-stimulation assays (24-hour co-culture and five-day bulk culture) assessed the tumor-killing capacity of antitumor effector cells through the characterization of IFNγ intracellular production in splenic and tumor-infiltrating lymphoid effector cells and IFNγ secretion post-re-stimulation, as well as direct characterization of the cytolytic activity of lymphoid effector cells via the quantification of cleaved-caspase-3 in 4T1.2luc target cells. Antitumor effector cells can induce apoptosis in targeted cells by releasing perforin and granzyme A and B, which cleaves caspases (e.g., caspase-3) and other downstream mediators of
apoptosis (625). Re-stimulation assay outcomes were not significantly different between groups.

Powered with this information, we selected one round of immunotherapy consisting of one PBS vehicle (VEH) control or $1 \times 10^6$ irradiated 4T1.2\textsubscript{uc} (VAX) at day 7, followed by two I.P. injections of isotype control (IgG) or PD-1 checkpoint blockade (10 mg/kg/mouse) at day 9 and 12 post-tumor implantation for use in combination with our diet and exercise intervention. The study was designed to investigate the effects of moderate exercise in weight stable mice on the dual administration of a whole tumor cell cancer vaccine and PD-1 checkpoint blockade in the 4T1.2 model. The pilot study failed to demonstrate an additive effect of whole tumor cell cancer vaccine with PD-1 checkpoint blockade on the prevention of tumor growth. The VAX intervention was included in the current study because of potential additive effects that may occur in moderately exercising, weight stable mice receiving the dual administration of VAX+PD-1. However, like the pilot study, the administration of one round of VAX or VEH were not significantly different within each energy balance (WG vs. WM) group, therefore VEH and VAX groups were collapsed to investigate PD-1 checkpoint blockade effects in both WG and WM groups (i.e., WG+IgG vs. WG+PD-1 vs. WM+IgG vs. WM+PD-1). We observed a protective effect of PD-1 checkpoint blockade in WG mice on primary tumor growth and spontaneous lung metastasis. Moderate activity in weight stable mice, independent of PD-1 checkpoint blockade, was effective at reducing primary tumor growth and metastatic burden on par with the effectiveness of PD-1 checkpoint blockade in WG mice. The lack of vaccine effect on tumor growth in the
current study may be due to the reduction from four to one round of whole tumor cell vaccine administration. Since we were combining vaccination with PD-1 checkpoint blockade, we decided to reduce the number of vaccinations to one round to allow the observation of PD-1 checkpoint blockade-specific effects. In the collapsed groups, we did observe a cancer prevention effect of PD-1 checkpoint blockade in WG mice on primary tumor growth and spontaneous lung metastasis. However, moderate exercise in weight stable mice, independent of PD-1 checkpoint blockade, was effective at reducing primary tumor growth and metastatic burden to the same extent as PD-1 checkpoint blockade in WG mice. The cancer protective effect observed in the WM groups could reflect the reduction in splenomegaly and immunosuppressive cell types (e.g., MDSCs), similar to observations in chapter three and four, and/or a shift in tumor-immune crosstalk gene expression markers (e.g., \textit{Il13} (626) and \textit{Ccl22} (588)) important in tumor invasiveness and the expansion and recruitment of immunosuppressive cell types to the TME. These mechanisms were discussed in chapter three and four and are likely still relevant as potential mechanisms underlying the cancer prevention effect of exercise in weight stable mice.

When a response to PD-1 checkpoint blockade occurs, it likely reflects some level of prior T cell-mediated antitumor response. Thus, the contrasting findings of PD-1 checkpoint blockade responsiveness between our WG and WM mice provides insight into potential mechanisms in which moderate exercise in weight stable mice may be altering host physiology to enhance antitumor immunity. These mechanisms may include: 1. An enhancement in the activation or function
of effector immune cells; 2. A reduction in the expression of PD-1 on T cells and non-T lymphocytes or a reduction in the PD-1 inhibitory signaling cascade; and/or 3. A reduction in the expression of ligands for PD-1 on tumor cells and tumor-infiltrating myeloid cells.

First, moderate exercise in weight stable mice may directly or indirectly enhance effector immune cell function resulting in improved antitumor immunity. The activation of a naïve T cell occurs through the simultaneous engagement of the T cell receptor (TCR) and co-stimulatory molecules (e.g., CD28 or inducible T cell co-stimulator [ICOS]) on the T cell surface by major histocompatibility complexes (MHC) (CD4 in the context of MHC class II and CD8 in the context of MHC class I) and co-stimulatory molecules (e.g., CD80 [B7.1] and CD86 [B7.2]) on APCs (87). TCR stimulation alone results in anergy (or T cell tolerance); whereas, co-stimulation alone has no effect on T cell activation. TCR engagement orchestrates essential changes in the gene expression profile to transition a naïve cell to an actively expanding immune effector cell. Intracellular signaling involves a myriad of molecules, with the most common being Src family kinases (e.g., Fyn, Lck) which phosphorylate tyrosines on intracellular immunoreceptor tyrosine-based activation motifs (ITAMs) on the intracellular domain of CD3 ζ-chain (88). Phosphorylation events result in downstream signaling mediated by phospholipase-C (PLC)γ, mitogen-activated protein kinase (MAPK), nuclear factor kappa B (NF-κB), and nuclear factor of activated T cell (NFAT) which results in gene transcription in the nucleus of factors that promote cell survival and proliferation (87). Co-stimulatory and cytokine signaling, especially signaling via
IL-2 (89), activate phosphoinositide-3 kinase (PI3K) and its downstream target protein kinase B (Akt), which increases expression of glucose transporters on the cellular membrane and upregulates glycolytic enzyme activity to meet the metabolic needs of the highly proliferative effector cells (90). Cytokine signaling also activates specific transcriptional factors to induce CD4+ helper T cell polarization (defined subsets include TH1, TH2, TH9, TH17, TH22, and Tregs) to refine the T cell response. Moderate exercise in weight stable mice could result in an enhancement in the ability of APCs to present antigens to T lymphocytes, improve TCR or co-stimulatory signaling mechanisms, or promote or sustain a cytokine signaling milieu to favor a robust antitumor response. For example, cytokine imbalance is one mechanism responsible for immune dysregulation within the TME and tumor-draining lymph node (627). Tumor-derived cytokines and/or cytokines produced by tumor-infiltrating immunosuppressive cells, like IL-10 and TGFβ, can condition tumor-antigen specific T cells toward a less efficacious TH2 or Treg phenotype (627). Signaling from the TME can blunt lymphocyte production of IL-2 and IFNγ (628-630); however, data from preclinical studies show an increase in lymphocyte secretion of IL-2 and IFNγ in exercising mice compared with sedentary controls (273, 522). Exercise-induced signaling via AMPK/PI3K can increase glucose uptake and fatty acid oxidation, as well as augment mitochondrial size, number, and enzymatic activity, in skeletal muscle (631). It is conceivable that these effects can also occur in immune compartments important in immunosurveillance to bypass PD-1 induced dysregulation of immunometabolism in effector cells and ultimately enhance the eradication of transformed and
malignant cells (632-634). In the current study, we observed a significant
difference in splenic CD4+ helper T cell proliferation, with WM mice having an
increased proliferative capacity. Both splenic and tumor-infiltrating immune cell
recall assays failed to show significant differences in intracellular IFNγ staining in
antitumor immune subsets or IFNγ secretion post re-stimulation. However, at day
35 post tumor implantation, we observed an increase in the fold regulation of Il2
and Il12b in the tumor-immune crosstalk gene array. These genes encode for
cytokines important in the activation of NK and T cells and promote the production
of IFNγ (635-637). Thus, the splenic and TIL bulk culture experiments may not
adequately capture what is happening in the TME. In addition, these
aforementioned functional outcomes were performed at day 35 post-tumor
implantation, but tumor growth curves began to separate at day 14 post-tumor
implantation. We may have missed the critical window to capture differences in
effector responses in the TME. Once tumor burden reaches a critical mass, the
tumor-induced cytokine imbalance could drive immune dysregulation and mask
earlier enhancements in antitumor responses. Future studies are needed to
examine the effects induced by moderate exercise at both earlier time points in
tumor growth (e.g. when the tumor growth curves start to diverge) and when tumor
volumes are comparable between groups.

Second, moderate exercise in weight stable mice may result in a reduction
in the upregulation of PD-1 transcription and/or expression of PD-1 on the cell
surface, or a reduction in the inhibitory cascade induced by PD-1 in antitumor
effector cells. Upon activation, TCR-mediated calcium influx can induce Pdcd1
transcription (the gene encoding for PD-1) via NFATc1 (254). When PD-1 is engaged by its ligand, the PD-1 intracellular domain, composed of an immunoreceptor tyrosine-based inhibition motif (ITIM), inhibits kinases involved in T cell activation through the recruitment of Src homology 2-containing tyrosine phosphatase (SHP2) (53). PD-1 induced SHP2 can directly inhibit TCR transduction by dephosphorylating and inactivating Zap70, a major integrator of TCR-mediated signaling (254), or by blocking the induction of PI3K activity and downstream Akt signaling, resulting in an inhibition in the ability of T cells to import glucose, rendering T cells more susceptible to apoptosis (255, 256). No studies to date have assessed if exercise can alter the transcription of Pdcd1 or the intracellular inhibitory signaling cascade induced by PD-1 ligation in antitumor immune cells.

Additional control of PD-1-induced inhibition can occur via cytokine signaling. For example, IL-2 signaling via the IL-2R activates Akt via signal transducer and activator of transcription (STAT) 5 to upregulate energetic mechanisms; thus, bypassing the PD-1-induced blockade of nutrient uptake (e.g., glucose uptake) and utilization within antitumor immune cells (638). Therefore, an exercise-induced increase in IL-2 can not only directly enhance T cell activation, but may override PD-1-induced inhibition, resulting in a sustained antitumor response. In the current study, we saw no difference in the cell surface expression of PD-1 on tumor-infiltrating effector CD4+ helper or CD8+ cytotoxic T cells at day 35 post-tumor implantation. The timing of PD-1 induction on immune effector cells, as well as the best time to administer PD-1 checkpoint blockade, remains an active
area of research (622, 639). Therefore, the lack of differences we observed in the
current study may reflect the time point at which cells were characterized, i.e.,
there may be differences in PD-1 expression among our WG and WM groups
earlier in tumor growth. Furthermore, PD-1 expression is not limited to T
lymphocytes. Tregs highly express PD-1 and PD-1 signaling can promote Treg
proliferation and immunosuppression (640). PD-1 is broadly expressed on non-T
lymphocyte cells, including NK cells and B cells; however, little is known about
energy balance and upregulation of PD-1 on these cells (53). PD-1 checkpoint
blockade may also enhance antitumor immunity by diminishing the number and/or
suppressive function of intratumoral Tregs or through an enhancement in NK
cytotoxicity or antibody production on PD-1+ B cells (53, 640). Future studies are
needed to investigate exercise-induced effects on the PD-1 response in T cells,
NK cells, and B cells.

Third, moderate exercise in weight stable mice could result in a reduction in
the expression of ligands for PD-1 on tumor cells and tumor-infiltrating myeloid
cells. The induction of PD-1 ligands can occur via innate mechanisms inherent to
transformed, malignant cells. Many cancer types, including TNBCs, are
characterized by constitutive oncogenic signaling and increased PD-L1 expression
(261, 641). Additionally, tumor-induced inflammation, as well as signaling from an
early phase antitumor immune response, can result in an adaptive induction of PD-
L1 on tumor cells and tumor-infiltrating myeloid cells (53, 642). The intricacies of
this system are best characterized by the duality of IFNγ signaling within the TME
(643). IFNγ is a potent antitumor cytokine that can promote tumor surveillance,
immune activation, and antitumor activity (613, 644). However, a negative feedback loop exists in which IFNγ can promote immunosuppression of PD-1+ T cells via the induction of PD-L1 expression on tumor and myeloid cells (645). Exercise could attenuate both the innate and adaptive induction of PD-L1 on tumor cells early in tumor development; thus reducing PD-1:PD-L1 interactions and sustaining a robust antitumor immune response. The current study did not observe differences in PD-1 expression on tumor-infiltrating effector CD4+ helper or CD8+ cytotoxic T cells. Future studies need to assess PD-1 expression over time, as well as PD-L1 induction in the TME. Exercise-induced enhancements in antitumor immunity could result in a more effective or timely apoptotic- or necrotic-induction of cell death in tumor cells, reducing the ability of tumor cells to upregulate PD-L1-induced suppression mechanisms. However, the assessment of splenic and tumor-infiltrating antitumor immunity via re-stimulation assays (24-hour co-culture and five-day bulk culture), including IFNγ intracellular production and IFNγ secretion post-re-stimulation, as well as direct characterization of the cytolytic activity of lymphoid effector cells via the quantification of cleaved-caspase-3 in 4T1.2\textsuperscript{Luc} target cells, were not significantly different between groups. The lack of effect could be due to in vitro assay limitations, the terminal timing of this endpoint, and/or exercise induces enhanced antitumor immune killing of target cells via an alternative mechanism. The mechanisms in which antitumor effector cells can kill transformed and malignant cells is extensively reviewed (95, 646-649). Future studies are needed to assess possible exercise-induced mechanisms on the
induction of PD-1 ligands in the TME and the timing and alternative mechanisms of immune-mediated antitumor responses.

In conclusion, the contrasting findings of PD-1 checkpoint blockade responsiveness between our WG and WM mice provides extensive clues by which exercise in weight stable mice may be altering host physiology to enhance antitumor immunity. The observed response to PD-1 checkpoint blockade in WG mice suggests that some level of prior T cell-mediated antitumor response occurred in the WG mice. The lack of PD-1 checkpoint blockade effect in WM mice, in combination with the comparable tumor outcomes to WG+PD-1 mice, suggests that the T cell-mediated antitumor response was maintained in the WM mice via an exercise-induced reduction in tumor-mediated immunosuppressive mechanisms and/or a direct enhancement in antitumor effector mechanisms. These data demonstrate that moderate exercise and weight control may be an important intervention to maintain prolonged antitumor effector responses and improve clinical outcomes.
CHAPTER 6: RESEARCH SUMMARY AND FUTURE DIRECTIONS

6.1. SUMMARY OF RESEARCH

My dissertation studies were designed to model women who remain weight stable throughout adulthood via two lifestyle-based strategies, a modest reduction in calories (10% of caloric intake) and moderate physical activity, to investigate these energy balance interventions using a clinically relevant murine metastatic BC model. The goal of these studies was to determine 1) the extent to which exercise, as opposed to changes in body weight, protect against primary tumor growth and metastatic progression and 2) if moderate exercise in weight stable mice improves responses to two immunotherapeutic interventions, whole tumor cell cancer vaccine and PD-1 checkpoint blockade.

The study presented in chapter three was designed to examine the effects of exercise, mild dietary restriction, or the combination of diet and exercise on tumor progression and the inflammation-immune axis in a preclinical metastatic BC model to determine the extent to which exercise or body weight contribute to a cancer prevention effect. Although the prevention of weight gain via dietary energy restriction (i.e., SED+ER mice) was effective at altering host splenic immunity and the expression of key genes in the tumor microenvironment (TME) related to immunosuppression and metastatic progression, this intervention failed to induce changes in primary tumor growth or spontaneous metastases.

Body weight in the moderately exercising, weight stable mice (i.e., EX+ER) was matched to SED+ER mice, allowing the comparison of exercise-induced vs. body weight-induced effects on tumor growth and immune endpoints. Moderate
exercise in weight stable mice resulted in a reduction in immunosuppressive and metastatic genes in the TME similar to the SED+ER mice; however, EX+ER mice had the greatest reduction in splenic immunosuppressive cells, including a reduction in MDSCs and MDSC subsets, as well as a reduction in plasma insulin-like growth factor 1 (IGF-1). The effects of moderate exercise in weight stable mice culminated in a significant delay in primary tumor growth and spontaneous metastases, suggesting that exercise-induced alterations, not simply a change in body weight, underlie the protective effects of the combined intervention on tumor growth. The exercise-induced effects could include the reduction in hormonal, inflammatory, metabolic and/or proliferative signals and/or the prevention of the acquisition of a toxic, immunosuppressive TME. These events may promote sustained immunosurveillance mechanisms that prevent tumor cell invasion and metastatic spread.

Metastatic progression is a multi-step process that involves the interaction between tumor cells, stromal cells, and tumor-infiltrating immune cells to alter tumor angiogenesis, cell invasion, and cell migration pathways to promote the dissemination of tumor cells to distant sites. The reduction in spontaneous metastases suggests that moderate exercise in a weight stable host may impact this multi-step process, perhaps through a reduction in MDSCs or plasma IGF-1. Emerging data suggests MDSCs and IGF-1 play a role in metastatic progression by modulating genes related to epithelial-mesenchymal transition (EMT) and chemokine signaling, as well as altering the vasculature of the TME. MDSCs can further promote metastases by infiltrating distant sites and generating a niche more
favorable to the seeding of disseminated cancer cells. The specific mechanism by which exercise in weight stable mice reduces tumor growth and metastatic progression remains unclear and warrants further investigation. However, experimental results (chapter three) suggest a shift in tumor-induced inflammation and immunosuppression, as well as a reduction in plasma IGF-1, resulting in a sustained antitumor response.

Interestingly, the exercise-induced protective effect on tumor growth and reduction in immunosuppressive factors (both immunosuppressive cell types [e.g., MDSCs] and reduced expression levels of immunosuppressive genes in the TME) was lost when exercising mice continued to gain weight over the course of the study via *ad libitum* feeding. Over the course of the 13-week study, *ad libitum*-fed mice progressed to an overweight phenotype. The current findings suggest that weight gain-induced disturbances in hormonal, inflammatory, and/or immunological function can override the exercise-induced benefits on tumor growth and metastatic progression observed in weight stable mice. Weight gain-induced disturbances likely occur in a continuum. This further emphasizes the importance of the current American Cancer Society guidelines to maintain a healthy weight throughout life, or lose weight and/or maintain weight if overweight or obese by balancing caloric intake and engaging in physical activity to reduce the risk of developing breast cancer. Additionally, the “metabolically healthy obese” phenotype may still result in disturbances in the inflammation-immune axis that reduce immunosurveillance mechanisms. Therefore, weight maintenance may be
the safest public health recommendation to prevent breast cancer until more is
known about weight maintenance vs. weight gain in this population.

Study one provides a deeper understanding of the extent to which exercise,
and not solely changes in body weight, underlie cancer protection. It provides
further evidence that exercise can act via the inflammation-immune axis to
attenuate the generation of a protumorigenic and immunosuppressive TME.
Results from the current study provide insight into potential mechanisms by which
exercise in weight stable hosts exerts primary and secondary cancer prevention
effects. Furthermore, these observations in a preclinical model provide a biological
rationale for future randomized controlled trials of exercise and the prevention of
weight gain to prevent metastatic progression in BC survivors and ultimately
improve survival outcomes.

To investigate potential mechanisms and determine if moderate exercise in
weight stable mice can improve therapeutic responses, we combined two different
emerging cancer therapies with our lifestyle-based, diet and exercise intervention.
An allogeneic, whole tumor cell cancer vaccine is a broad-based immunogenic
stimulus that provides a wide array of TAAs and drives a polyclonal T cell
response. This response involves antigen uptake, processing, and presentation
from APCs to T cells to induce antitumor effector cell function. Conversely,
targeted PD-1 checkpoint blockade reactivates the T cell-mediated antitumor
response by antagonizing the co-inhibitory PD-1 immune checkpoint via antibody
blockade. Upon T cell activation, the PD-1 inhibitory molecule is upregulated on
the T cell surface and plays a role in downregulating the antitumor response by
inducing apoptosis. PD-1 is widely expressed on several cell types important in antitumor immunity, including B cells, NK cells, and CD4+ helper and CD8+ cytotoxic T cells, making this an emerging target for cancer therapy. The efficacy of both therapies can be compromised by tumor-secreted proinflammatory factors that promote the expansion and accumulation of immunosuppressive immune cell types within the TME and peripheral organs. Study one (chapter three) demonstrated that the prevention of weight gain through mild dietary energy restriction and moderate exercise can reduce primary tumor growth and metastatic burden, reduce immunosuppressive cells, augment T cell responses, and alter the TME in the 4T1.2 mammary tumor model. Therefore, we wanted to determine if moderate exercise in weight stable mice could enhance the efficacy of the whole tumor cell cancer vaccine or PD-1 checkpoint blockade. Numerous researchers are investigating pharmacological blockade of immunosuppressive mechanisms; however, the studies presented in chapter four and five are among the first to show that exercise can impact the inflammation-immune axis resulting in a better response to immunotherapy. In chapter four, we observed an additive effect of moderate exercise in weight stable mice and the administration of the whole tumor cell cancer vaccine. However, in chapter five, we saw no additive effect of our lifestyle intervention and PD-1 checkpoint blockade. The disparate responses of therapies to moderate exercise provide insight into potential mechanisms by which exercise confers protection.

Whole tumor cell cancer vaccines utilize numerous immune mechanisms to induce a robust antitumor response and exercise-induced changes in hormonal,
metabolic, and inflammatory mediators could impact one or multiple mechanisms. The whole tumor cell cancer vaccine provided APCs, like DCs, with a broad array of TAAs. The DCs phagocytose exogenous antigens, process them by proteases to peptide fragments, migrate to proximal draining lymph nodes, present antigens, and activate CD8$^+$ cytotoxic T cells in the context of class I major histocompatibility complex. Activated T cells migrate to the TME and induce targeted killing of cancerous cells. The exercise-induced reduction in immunosuppressive cells and inflammatory TME, as well as the reduction in plasma IGF-1, could enhance antigen presentation, T cell activation, and/or effector function. An exercise-induced enhancement in antitumor cytotoxicity could improve vaccine efficacy via epitope spreading, or the process in which T cells respond to tumor-associated peptides not present in the original vaccine. These results indicated that the whole tumor cell cancer vaccine can augment the weight maintenance (via diet and exercise) effects on primary tumor growth and spontaneous metastasis and suggest that vaccination may provide an immune stimulus to further promote the protective effects of moderate exercise alone.

PD-1 checkpoint blockade is a more targeted approach that antagonizes co-inhibitory signals on immune cells that induce inhibition of immune responses, which in turn promotes prolonged tumor-fighting capabilities. We observed a protective effect of PD-1 checkpoint blockade in WG mice on primary tumor growth and spontaneous lung metastasis, suggesting that PD-1 checkpoint blockade was effective at reactivating the immune system and/or preventing effector cell inhibition in WG mice. It was hypothesized that PD-1 checkpoint blockade would
allow the immune system to completely eradicate the tumor and prevent spontaneous metastases in moderately exercising, weight stable mice. However, moderate exercise in weight stable mice, independent of PD-1 checkpoint blockade, was effective at reducing primary tumor growth and metastatic burden with the same effectiveness as PD-1 checkpoint blockade in WG mice. The lack of PD-1 checkpoint blockade effect in WM mice suggests that moderate exercise in weight stable mice is generating a systemic environment that favors prolonged activation of antitumor effector cells (i.e., PD-1 checkpoint blockade can only be effective if it can bind to PD-1 on antitumor cells).

The protective effect on tumor growth observed in the WM groups could be a consequence of the reduction in splenomegaly and immunosuppressive cell types (e.g., MDSCs), similar to observations reported in chapter three and four, and/or a shift in tumor-immune crosstalk gene expression markers important in tumor invasiveness and the expansion and recruitment of immunosuppressive cell types to the TME. This exercise-induced shift in inflammatory status could delay the upregulation of the PD-1 checkpoint on antitumor cells, reduce the inhibitory signaling by PD-1 ligation, and/or reduce PD-L1 expression on tumor and supporting cells within the TME, resulting in sustained antitumor immune cells. These data demonstrate that moderate exercise and weight control may be an important recommendation to maintain prolonged antitumor effector responses and improve clinical outcomes.

Exercise effects are pleiotropic and likely involve complex and multifaceted interactions between numerous physiological systems. Although the current study
does not definitively prove what specific host systems are involved, it does suggest that exercise, and not merely the prevention of weight gain, is necessary for cancer prevention. These studies demonstrate that moderate exercise in a weight stable host can prevent spontaneous metastases, a substantial finding considering the leading cause of BC-specific mortality is due to metastatic disease. Results from the current study provide insight into potential mechanisms (Fig. 6.1) by which exercise acts over the cancer continuum and provides a biological rationale for clinical studies of exercise strategies to prevent metastatic progression in BC survivors and ultimately improve survival outcomes. Lastly, the results provide evidence that emerging immunotherapy interventions could be coupled with lifestyle-based intervention strategies to improve clinical outcomes.
A. Decrease mutational rate in normal breast

B. Enhance immunosurveillance mechanisms

C. Reduce the generation of the proinflammatory tumor microenvironment

D. Reduce immunosuppressive mechanisms

E. Reduce metastatic progression
Figure 6.1. Exercise-induced effects along the cancer continuum. (A) In a normal breast cell, exercise-induced effects could regulate the cell cycle, increase inflammatory resolving capacity, decrease the rate of intrinsic oncogenic mutations, and/or alter epigenetic modulation favoring tumor suppressor gene expression, culminating to a reduction in cancer cell initiation events. (B) Exercise-induced effects could directly or indirectly improve immunosurveillance mechanisms resulting in the elimination of transformed cells. (C) In early tumor progression, exercise-induced effects could reduce the secretion of tumor-derived suppressive factors and delay the generation of the proinflammatory tumor microenvironment. Exercise could reduce the expression of immune inhibitory ligands on tumor and tumor-infiltrating immune cells or reduce the expression of inhibitory immune checkpoints on antitumor effector cells. (D) Exercise-induced effects could reduce the emergence of immunosuppressive cell populations (e.g., myeloid-derived suppressor cells [MDSCs] and/or tumor-associated macrophages [TAMs]) through a reduction in the expansion of immature myeloid-lineage cells (iMC) from hematopoietic stem cell (HSC) progenitors, decrease Treg polarization, and decrease the recruitment of immunosuppressive cells through a reduction in chemokine signaling (e.g., Ccl5, Cxcl1). Furthermore, exercise-induced effects could blunt immunosuppressive mechanisms (e.g., release of suppressive factors, suppressive enzymes). (E) Lastly, exercise-induced effects could alter tumor angiogenesis, epithelial-mesenchymal transition, cell invasiveness, the ability of circulating tumor cells to extravasate into secondary sites, and/or the metastatic niche.

6.2. FUTURE DIRECTIONS

As exercise oncology matures, it is essential for investigators in the fields of exercise physiology, immunology, and cancer biology to understand the mechanisms by which exercise induces beneficial changes in the inflammation-immune response and harness these mechanisms to enhance therapeutic efficacy. Numerous questions remain unanswered.

Study one (chapter three) provides a deeper understanding of the extent to which exercise, and not only changes in body weight, contributes to cancer prevention. Exercise induces pleiotropic effects on numerous physiological systems. Goh, et al. (509) hypothesizes that exercise can affect the immune system by 1) altering inflammatory tone and tumor-immune crosstalk, or 2) through the release of muscle-derived cytokines, called myokines. Both mechanisms can
result in a host environment more suitable to prolonged immunosurveillance and tumor clearance. Identifying the specific molecules and mechanisms driving the exercise-induced benefit in cancer prevention is an active area of research (435, 509-511). In the current study, body weight in moderately exercising, weight stable mice (i.e., EX+ER) was matched to SED+ER mice, allowing the comparison of exercise-induced vs. body weight-induced effects. Additionally, the exercise-induced protective effect on the emergence of immunosuppressive factors and reduced tumor burden was lost when mice continued to gain weight over the course of the study. This result suggests that weight gain-induced disturbances on hormonal, inflammatory, and/or immunological function can override the exercise-induced benefits. Therefore, contrasting the EX+AL and EX+ER groups could shed light onto how moderate weight gain negates exercise-induced benefits.

A more thorough exploration of muscle-derived myokines (e.g., leukemia-inhibitory factor, IL-6, IL-7, brain-derived neurotrophic factor, IGF-1, fibroblast growth factor-2, follistatin-related protein 1) could shed light on potential pathways by which exercise (i.e., contraction of skeletal muscle) induces cancer prevention benefits (510). Taking mice off study at different times after tumor implantation could provide insight into myokine signaling on the immune response over time, as well as detail the deleterious effects of progressive tumor growth and/or ad libitum-feeding on myokine-immune crosstalk. Additionally, follow-up studies could selectively knock out or inhibit select myokines and observe if exercise-induced benefits are lost.
In the current study, mice were sacrificed at day 35 post-tumor implantation. Several plasma (e.g., G-CSF, IL-6, IL-1α, MCP-1) and immune outcomes (e.g., NKCC) were not significantly different between groups, which may suggest that these mediators are not be impactful at this terminal time point. However, primary tumor growth curves diverged at day 14 post-tumor implantation. Moderate exercise in weight stable mice may alter the inflammatory milieu during early tumor growth or initiation of metastasis. Once tumor burden reaches a critical mass, these differences may be less apparent. Ongoing studies in the Rogers lab are planned to examine the effects induced by moderate exercise at both earlier time points and when tumor volumes are comparable between groups.

The weight maintenance-induced reduction in immunosuppressive cells (MDSCs, Tregs) and tumor-immune gene expression important in the expansion of immunosuppressive cells warrants further investigation. Clinical studies reveal that BC stage and metastatic tumor burden is positively correlated with the percentage of circulating MDSCs (162) and intratumoral Tregs (650); and elevated levels of immunosuppressive cell types are indicative of a poor prognosis (118, 119, 588). Additionally, both MDSCs and Tregs play a role in promoting metastatic progression and establishing prometastatic niches within peripheral organs (91, 120, 176). For example, an exercise-induced reduction in lung-infiltrating MDSCs may result in a microenvironment less favorable for the embedding of disseminating tumor cells. The expansion of immunosuppressive cells, driven by the release of tumor-derived factors, is independent of BC biomarker classification (i.e. estrogen receptors, progesterone receptors, and/or the human epidermal
growth factor receptor 2 (HER2)). Therefore, regardless of biomarker-based treatment, novel therapies targeting MDSCs or Tregs are an attractive strategy to overcome the immunosuppression associated with the TME and enhance conventional therapy and novel immunotherapy to prevent or slow metastatic disease and improve clinical outcomes.

Immunosuppressive cells utilize numerous, complex, and often redundant mechanisms to promote tumor progression. For example, various pharmacological strategies are being investigated to reduce the expansion and accumulation of MDSCs, inhibit their suppressive functions, or promote their differentiation into non-suppressive cell types. Markowitz, et al. (651) has recently reviewed clinical studies targeting MDSCs in BC patients; however, no researchers are investigating if lifestyle-based interventions could reduce MDSC expansion or inhibitory function. A new appreciation for the role of diet-induced obesity on the emergence of MDSC populations and Tregs is reported in preclinical models (652, 653). However, few researchers have designed studies to investigate if exercise-induced changes in the inflammatory milieu can reduce the expansion and accumulation of MDSCs and Tregs in the TME or peripheral organs. Subsets of MDSCs, with varying suppressive function, are identified based on Gr-1 (Ly6G or Ly6C) and CD11b expression (125). Granulocytic MDSCs, defined as Gr-1\textsuperscript{hi}CD11b\textsuperscript{+} (or CD11b\textsuperscript{+}Ly6C\textsuperscript{lo}Ly6G\textsuperscript{+}) are terminally differentiated; whereas, monocytic MDSCs, defined as Gr-1\textsuperscript{lo}CD11b\textsuperscript{+} (or CD11b\textsuperscript{+}Ly6C\textsuperscript{hi}Ly6G\textsuperscript{-}), can further differentiate into macrophages or dendritic cells. Tregs can develop in the thymus (i.e., natural Tregs) or be generated in the periphery from conventional CD4\textsuperscript{+}
helper T cells (inducible Tregs) (144). It remains unexplored if exercise has subset-specific effects on MDSC subpopulation expansion or function or alters the generation or function of natural Treg or inducible Treg populations. Improving cancer immunotherapy through the development of MDSC- or Treg-targeted strategies is a high priority and may be the next breakthrough for clinical cancer treatment.

The weight maintenance-induced reduction in tumor-immune gene expression related to immunosuppressive pathways (e.g., Ido1) is interesting and deserves additional investigation. Moderate exercise in weight stable mice may not only reduce the expansion and accumulation of immunosuppressive cell types, but also blunt their ability to suppress by directly altering the gene expression of inhibitory enzymes. For example, Ido1 is expressed by tumor cells, supporting cells within the TME, and infiltrating cells, like MDSCs, and encodes for indoleamine 2,3-dioxygenase (IDO). IDO is the first and rate-limiting enzyme of tryptophan catabolism through the kynurenine pathway (197). Depletion of tryptophan, an amino acid essential for T cell activation, results in blunted T cell proliferation, differentiation, effector functions, and viability in several preclinical models (198). No IDO inhibitor is approved by the FDA; however, IDO inhibition in combination with cancer vaccines, chemotherapy agents, and emerging immune checkpoint blockade is under investigation in preclinical and phase I and II clinical trials (199, 615). To date, no group is investigating if lifestyle interventions, such as moderate exercise, can reduce IDO-mediated inhibition to promote a more robust antitumor effector CD8$^+$ cytotoxic T cell response. Furthermore, weight maintenance altered
tumor-immune expression of several genes related to immunosuppressive cell expansion and metastatic progression. Future studies are needed to investigate the individual effects of each chemokine gene (through knockout or small molecule inhibitors) on subsequent expansion of immunosuppressive cells, antitumor immune responses, and tumor progression.

It is well established that tumor-induced or immunosuppressive cell-induced dysregulation of metabolic pathways in antitumor effector cells can promote tumor escape. Exercise-induced enhancement of immunometabolism could bypass the induction of T cell anergy or apoptosis, resulting in a sustained antitumor response. Exercise-induced enhancements in skeletal muscle metabolism via IL-6 and other acute phase myokines are well studied (631) and involve signaling via AMPK/PI3K to increase glucose uptake and fatty acid oxidation, as well as augment mitochondrial size, number, and enzymatic activity in myocytes. It is conceivable that these effects can also occur in immune compartments important in immunosurveillance and maintain antitumor activity. Future studies are needed to assess exercise-induced effects on energy uptake (e.g., glucose transporters) and metabolic pathways in antitumor immune cells and to determine if metabolic alterations associated with weight change impact these mechanisms.

Metastatic progression is a dynamic process and remains poorly understood (40). In the current experiments, it remains unknown if the exercise-induced reduction in spontaneous metastasis is a consequence of reduced primary tumor growth or a direct inhibition in metastatic progression. Exercise-induced effects could alter tumor angiogenesis, epithelial-mesenchymal transition (EMT),
cell invasiveness, the ability of circulating tumor cells to extravasate into secondary sites, and/or the metastatic niche. Additionally, an exercise-induced enhancement in immunosurveillance (e.g., greater clearance of primary tumor cells, circulating tumor cells, or cells within a metastatic lesion) could result in a reduction in metastasis. Lastly, exercise could improve mortality outcomes associated with BC metastases by altering the inflammation-immune axis or directly enhancing immunosurveillance mechanisms to maintain pressure on disseminated tumor cells to favor a dormant, non-proliferative state. Future studies, perhaps resecting the primary 4T1.2 tumor in the current model to allow for greater metastatic outgrowth or utilizing a dormancy model, are needed to elucidate the exercise-induced protective effect on metastasis. To identify what immune compartment is mediating the exercise-induced effects on immunosurveillance, immune cell depletion experiments (e.g., CD8+ cytotoxic T cells or NK cells) are required.

The current model applied exercise for eight weeks prior to the administration of luciferase-transfected 4T1.2 cells into the fourth mammary fat pad. Chronic exercise exposure could generate a host with better inflammation resolving capacity and a better functional reserve to combat the tumor insult. However, studying exercise in the therapeutic window remains clinically relevant. The aggressiveness of the 4T1.2 model may prohibit the study of exercise in the therapeutic window. Future studies could assess if exercise could result in a reduction in primary tumor growth and spontaneous metastasis if administered once the 4T1.2 tumor was palpable or utilize transgenic models with a longer tumor latency period.
Characterizing the dose, duration, frequency, and type of exercise needed to drive cancer prevention is key to move the field of exercise oncology forward. Subset analysis in the current study sheds light on the average quantity of activity required in the EX+ER group (greater than 5.8 km/day) to drive beneficial changes on tumor growth in the 4T1.2 mammary tumor model. Using speed to estimate the percent of maximal oxygen consumption (%VO_{2\text{max}}) or relative intensity of exercise, we determined, based on a regression analysis reported by Fernando, et al. (654), that mice were exercising at 60-70% of VO_{2\text{max}}, which is indicative of moderate exercise training. Studies implementing an exercise routine via treadmill or automated running wheel cages are needed to further assess duration and intensity of aerobic exercise on both immune and tumor outcomes. As the field of exercise oncology matures, it is essential for investigators in the fields of exercise physiology, immunology, and cancer biology to address the dose, duration, frequency, and type of exercise needed to achieve a cancer prevention effect.

It is well accepted that participating in moderate physical activity during and after active treatment can improve quality of life measurements, including alleviating fatigue and maintaining physical functioning, emotional, and/or social wellbeing (655, 656). Exercise-induced benefits on the response to therapy are studied to a lesser extent. The observational data, combined with preclinical studies, suggest that exercise-induced effects can also enhance the inflammation-immune axis. Despite the recent successes of immunotherapy in advanced-stage cancer patients, less than half of patients who receive immunotherapy experience an objective, durable response. An active area of research attempts to identify
ways to increase immunotherapy efficacy through the identification of clinically meaningful biomarkers to direct treatment choices (i.e., personalized medicine) and/or through the selection of appropriate combinatorial strategies (aimed to enhance antigen presentation, reverse T cell dysfunction, and/or target immune inhibitory mechanisms) without inducing adverse effects. The number of possible combinatorial treatment strategies grows exponentially, therefore, testing if lifestyle-based interventions, like physical activity, which is low-cost and has known benefits across the cancer continuum, can enhance immunotherapy represents an attractive option. For example, immune checkpoint blockade relies on the reactivation of an immune response that targets the cancer and therapeutic efficacy is correlated with the presence of tumor-infiltrating immune cell populations. Immunotherapy typically fails in patients with tumors lacking a pre-existing immune response. No study, to our knowledge, has retrospectively or prospectively assessed the impact of lifestyle (e.g., physical activity, weight control) on the efficacy of immunotherapeutic outcomes. Future studies are needed to determine if exercise-induced changes in the inflammation-immune axis could promote immune-infiltration of the TME and convert non-responders into responders. It remains unknown if this requires exercise over the course of one’s lifetime, or if exercise could be started in an adjuvant setting. For example, if exercise could be applied in the adjuvant setting, could immunotherapy be delayed (e.g., by two or three weeks) allowing time for patients to be administered a supervised exercise routine to increase functional reserve and create a host environment more responsive to the therapy?
An emerging area of research is investigating the role of the gastrointestinal microbiome on cancer development and response to therapy (657), with estrogen metabolism, diet, immune modulation, and inflammatory milieu acting as potential mediators. Monda et al. (658) reports that exercise can enhance the number of beneficial microbial species, enrich the microflora diversity, and improve the development of commensal bacteria. Although the exact mechanisms by which the microbiome interacts with BC remain to be discovered, exercise-induced changes in the inflammation-immune axis could be mediated through changes within the gut microbiome.

6.3. CONCLUDING REMARKS

Collectively, study one provides a deeper understanding of the extent to which exercise, and not changes in body weight, underlie cancer protection. Study one demonstrated that moderate exercise in weight stable mice resulted in a reduction in splenomegaly and the accumulation of splenic immunosuppressive cell types, as well as a reduction in immunosuppressive and metastatic genes in the TME. The efficacy of emerging cancer therapies can be compromised by tumor-secreted proinflammatory factors that promote the expansion and accumulation of immunosuppressive immune cell types within the TME and peripheral organs. Therefore, we investigated if moderate exercise in weight stable mice can blunt the acquisition of a toxic, immunosuppressive TME and improve the therapeutic responses of two emerging immunotherapeutic strategies. We observed (chapter four) an additive effect of moderate exercise in weight stable mice and the administration of the whole tumor cell cancer vaccine; however, we saw no additive effect of our lifestyle intervention and PD-1 checkpoint blockade
(chapter five). The disparate response of therapies to moderate exercise provide insight into potential mechanisms by which exercise confers protection and suggests an exercise-induced maintenance in immunosurveillance mechanisms. Lastly, we demonstrated that exercising, weight stable mice significantly reduced spontaneous metastasis, which suggest a novel mechanism by which moderate exercise in a weight stable host may contribute to the reduction in mortality in BC patients.
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2. Activity-induced weight maintenance in combination with a whole tumor cell vaccine delays mammary
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POSTER PRESENTATIONS:
1. Diet and exercise-induced weight maintenance may be preventing mammary tumor growth and metastatic
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2. Weight maintenance alters the tumor microenvironment and delays murine mammary tumor growth and
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3. The dual administration of immunotherapy is enhanced by activity-induced weight maintenance in the
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