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Immunological Effects of Berberine and Physical Activity in a Murine Breast Cancer Model

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IMMUNOLOGICAL EFFECTS OF BERBERINE AND PHYSICAL ACTIVITY IN A MURINE BREAST CANCER MODEL

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ABSTRACT

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Berberine (BBR) is a plant-derived alkaloid popularly used in a complementary context to treat splenomegaly and chronic disease states such as rheumatoid arthritis, diabetes, and cancer. Recent research has examined BBR as a regulator of lipid and glucose metabolism in association with the AMPK pathway; but little is known concerning its immunological impacts. It is also known that exercise is a potent AMPK activator exhibiting immunological benefits, but nothing is known about the combination of these complementary approaches for treating breast cancer. The purpose of this study is to determine if oral consumption of BBR when paired with physical activity will increase T lymphocyte activation while decreasing the presence of myeloid-derived suppressor cells (MDSC) within the tumor microenvironment, spleen, bone marrow, and blood of the immunocompetent BALB/c 4T1 mammary adenocarcinoma model**.** We hypothesize that subjects treated with this combination will have reduced MDSC counts, and increased T cell infiltration and activation in tissues of interest, effector, and regulatory subsets. Our data indicates no intervention specific change in NUR77 presence—a transcription factor expressed in antigen specific activation of T cells—in the spleen, lungs and tumor microenvironment of tumor bearing mice. However, T regulatory lymphocyte FOXP3 was also assessed and found to increase significantly in the lungs of tumor-bearing mice. These findings suggest BBR paired with physical activity may have significant immunological implications on

T cell activation broadly, and infiltration into the tumor microenvironment. These findings directly inform those who practice complementary and alternative supplementation, an area lacking immunological analysis, including raising concerns for the impact on aberrant, potentially undesirable Treg function.

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TABLE OF CONTENTS

CHAPTER

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

Historical Progression of Cancer Treatment

Recent statistical measures suggest that progress has been made in the fight against cancer. However, much of the observed reduction in metastatic cancer-related death is associated with preventative efforts. For example, improved testing sensitivity leading to increased rates of early detection, and community health measures such as reduced smoking rates (Liu et al., 2015; Simon, 2020). While these efforts have led to some reduction in cancer related deaths, neoplastic disease evading these efforts are recalcitrant to decades-old standard of care (chemo- and radiotherapy with surgery). In response to this realization, clinical treatments are shifting from these older practices alone to integrative and immunotherapy-based interventions. This new focus on complimentary and nutraceutical interventions is leading to a subsequent shift in research efforts. Integrative (sometimes referred to as complimentary) interventions include administration of isolated nutrients, potential cancer vaccines, whole diet interventions, and prescription exercise. However, the highly mutagenic and metastatic nature of many cancers makes studying the mechanistic impact of integrative interventions difficult (Hanahan & Weinberg, 2011).

Herbal Interventions

Herbal interventions are the foundation of historical medical practice and continue to exert a marked influence on modern treatments. Berberine (BBR) is a plant-derived alkaloid that is popularly used in a complementary context to treat splenomegaly and chronic disease states such

as rheumatoid arthritis, hyperinsulinemia, diabetes, and cancer (Malhotra et al., 2021; Vita et al., 2021). Recent research has examined BBR as a regulator of lipid and glucose metabolism in association with the AMPK pathway, but little is known concerning its immunological impacts. However, recent evidence shows immunosuppressive functions on specific effector T lymphocyte subsets such as $CD4^+$ T helper cells (T_h) (Vita et al., 2021). T regulatory (T_{reg}) subsets of T_h cells are of great interest in the cancer immunology field as T_{regs} pose a problem due to their negative impact in the tumor microenvironment and subsequent vital function in peripheral tissues for maintaining immunological homeostasis (Weinhold et al., 2016). Consistent long-term exercise is thought to increase T_{reg} frequency in a healthy population and is supposed to possibly suppress T_{reg} tumor recruitment (Hagar et al., 2019).

Immune Modulating Influence of Exercise and Physical Activity

It is also known that exercise is a potent AMPK activator exhibiting immunological benefits, but nothing is known about combination of these two complementary approaches for treating breast cancer. Exercise is also a rising topic of research within the field of cancer immunology. Epidemiological data suggest that physical inactivity is increasing on a global level which contributes to concerning levels of neoplastic evading disease, obesity epidemics, and increased risk of autoimmunity. As a solution to widespread disease risk, consistent moderate intensity physical activity has been found to modulate immune activity effectively decreasing a patient's risk for multiple disease states including breast cancer (McTiernan et al., 2003).

Study Purpose and Hypothesis

The purpose of this study is to determine if oral consumption of BBR when paired with physical activity will increase T lymphocyte presence and activation status while decreasing the presence of myeloid-derived suppressor cells (MDSC) within the tumor microenvironment,

spleen, bone marrow, and blood of both a healthy population of immunocompetent BALB/c mice and a BALB/c 4T1 mammary carcinoma model. The impact of BBR on voluntary exercise volume will be examined and lastly, the impact of voluntary oral BBR supplementation with or without physical activity on metastasis to the lungs of mice inflicted with 4T1 mammary carcinoma will be assessed. The findings of this study will directly inform those who practice complementary and functional medicine, an area lacking immunological analysis, including raising concerns for the impact on aberrant, potentially undesirable T_{reg} function.

Research Hypotheses

H1 Mice treated with BBR while concurrently engaging in an extended period of physical activity will exhibit reduced MDSC counts in addition to increased T cell counts and activation response in the lungs, tumor microenvironment, spleen, and blood.

H2 Voluntary wheel running will decrease in the presence of oral BBR supplementation.

H3 A combination of physical activity and BBR treatments will additively decrease 4T1 metastasis to the lungs.

CHAPTER II

REVIEW OF THE LITERATURE

Immunologically and Metabolically Relevant Modulatory Impact of Berberine and Physical Activity

Immune Mediated Neoplastic Modulation

T Lymphocytes

Tumor infiltrating lymphocytes have been extensively studied in the context of cancer, however minimal research has paired BBR with physical activity in a neoplastic context. Within the tumor microenvironment the presence of cells among the lymphocyte lineage of the adaptive immune system performs various immune-tumor mediated interactions including immune suppression and cytotoxicity. A large proportion of these immune-neoplasm interactions revolve around a component of the immune system known as T lymphocytes. T lymphocytes, also known as T cells, are distinguished from other lymphocytes by the $CD3^+$ T cell receptor complex (TCRC) present on mature T cells. T cells can be further identified by co-receptor markers on their surface. CD4 is a marker present on T helper (T_h) lymphocytes that plays a role in the activation of antibody producing B Lymphocytes as well as gives rise to a subset of *fork-head box protein P3* (Fox-P3) transcription factor expressing T_{regs}. $CD4^+CD24^+$ $T_{regs,}$ and a myeloid lineage of cells known as MDSC's, along with tumor-associated macrophages, are generally accepted as the prominent immunosuppressive agents within the immune system (Chuckran et al., 2021). As such, T_{regs} play an important role in a healthy immune response by maintaining homeostatic control through suppressing overactivity of the immune system. This function can

be exploited in the tumor microenvironment where T_{regs} are actively promoted and their immunoprotective function is hijacked to inhibit anti-tumor immunity (Chuckran et al., 2021). Cytotoxic $CD8^+$ T-lymphocytes have the opposite function and are well characterized as immune cells with anti-tumor properties. $CD8^+$ T-lymphocytes play an important role in protecting the host from neoplastic disease and viral infection. These cells are thought to increase significantly in circulation after moderate exercise (Garritson et al., 2020). However, this increase is short lived following physical activity which supports the idea that consistent exercise is key to enhanced immunity and therefore greater protection against cancerous disease.

Myeloid-Derived Suppressor Cells

Cancer is infamous for its ability to circumvent natural immune defenses by emulating "self" and thereby effectively hiding from the immune system. This guise is accomplished, in part, through the recruitment of a lymphoid population known as $CD11B^+$ myeloid-derived suppressor cells (MDSC's). MDSC's are a host of immature immune cells stemming from myeloid origin. MDSC's works in concert with each other to suppress the activity of the immune system in specific contexts such as cancer or pregnancy (Ostrand-Rosenberg & Fenselau, 2018). Phenotyping paired with identification of suppression in immune cell populations, such as $CD4^+$ T-helper cells, is one of the most effective ways to identify this mixture of cells (Ostrand-Rosenberg & Fenselau, 2018). Indeed, MDSC's immunosuppressive nature plays a significant role in metastatic dissemination of cancer cells while also creating an environment that is favorable for tumor growth (Ugel et al., 2015).

Berberine: The 'Poor Man's' Metformin?

In the past 5 years nutraceuticals have risen in popularity and are quickly becoming a pharmacological phenomenon. Nutraceutical is a term referring to naturally occurring

compounds that claim to alter physiology in a similar manner to a specific pharmaceutical and therefore can be used alongside or as a replacement for a traditional medication. Berberine (BBR), a common herbal supplement, has been prescribed through the ages in Eastern cultures as a non-pharmaceutical prescription. Much of this age-old use of BBR relates to its pleiotropic properties that have led to widespread use within the lay public, complementary medicine community and even some traditional medicine practices specifically for treatment of conditions with significant metabolic impact, such as diabetes and PCOS (Malhotra et al., 2021). As a supplement, the lay public utilizes BBR to increase athletic performance, enhance immunity, manage glucose metabolism, increase insulin sensitivity in PCOS and type 2 diabetes, and as treatment for bacterial diarrhea. Moreover, the general American population may be consuming BBR unknowingly as the compound also termed as *Natural Yellow-18* and utilized as a color additive in the food industry (Pereira et al., 2008). Within the scientific community BBR may also have utility in staining for secretory activity of mast cells (Berlin & Enerbäck, 1983) It is well characterized that the antimicrobial properties of BBR impacts the gut microbiome which in turn may account for some of the metabolic and immunological influence BBR exhibits within the body (L. Zhang et al., 2021).

Structurally, BBR is a hydrophobic isoqualine alkaloid and possesses molecular characteristics resembling cholesterol (el Khalki et al., 2020). It has been widely studied in the context of diabetes as well as lipid metabolism, and more recently has appeared in cancer and autoimmunity research. BBR exhibits similar physiological mechanisms to the common pharmacological insulin sensitizer commonly known as Metformin, which functions as a mild inhibitor of cytochrome complex 1 in the mitochondrial electron transport chain (Andrzejewski et al., 2014; Bridges et al., 2014; Griss et al., 2015). While the molecular mechanisms will be

described below, BBR is well characterized as an insulin sensitizer through its ability to upregulate insulin receptor expression in muscle and hepatic tissue which allows for increased response to circulating insulin and greater glucose absorption (H. Zhang et al., 2010). Due to its metformin-like glucose regulating properties along with its inexpensive cost and easy non-prescription access, berberine has been coined the "poor man's metformin". Furthermore, clinical European guidelines have already approved BBR as a long-term non-prescription nutraceutical for dyslipidemia patients with low tolerance for statins (Rondanelli et al., 2020). BBR has also been found to desensitize doxorubicin (DOX) resistant cancer cells. When administered alongside this common chemotherapeutic there was a dose-dependent incidence of apoptosis among breast cancer cells that were thought to be DOX resistant (Pan et al., 2017).

Metabolic Influence of Berberine

Both BBR and Metformin alter the function of a metabolic pathway known as AMP-activated protein kinase (AMPK) and exert a similar physiological influence through upregulation of this pathway which primarily alters energy metabolism (Xu et al., 2020; Zhao et al., 2021). Increases in AMPK activity artificially mimic metabolic processes that naturally occur during fasting and result in a marked influence on fatty acid oxidation, as well as hepatic and muscular sensitization to glucose and insulin (Gillespie et al., 2016). These pathways are hijacked in the tumor microenvironment and utilized for the benefit of a growing tumor, making pathways such as AMPK popular targets for anti-cancer interventions. AMPK also influences metabolism within T-cells in response to nutrient availability (E. H. Ma et al., 2017). Metformin has been found to increase the efficacy of $CD8^+$ T-cells and the development of memory T cells when administered alongside an experimental anti-cancer vaccine (Pearce et al., 2009).

Metformin's insulin sensitizing action can also decrease risk of tumorigenesis through decreased insulin levels, and down regulation of nuclear factor-κ B (NF-κ B) (Podhorecka et al., 2017).

Although Metformin is currently considered a first line treatment for type 2 Diabetes Mellitus, recent studies are elucidating the drug's potential as an anti-aging intervention as well as possible use in specific cancers, such as mammary carcinoma (Campbell et al., 2017). Research has suggested that Metformin's may possess life extending properties that stem from its ability to mimic calorie restriction without fasting or introduction of malnutrition (Campbell et al., 2017; Gillespie et al., 2016). The mechanisms activated by calorie restriction—including inhibition of MTOR, decreased insulin signaling, and activation of AMPK—are extensively studied and have been found to promote longevity through cellular disease evading strategies such as autophagy leading to apoptosis of cancer cells (Knapowski et al., 2002). Furthermore, calory restrictive mechanisms have an inherently negative impact on diseases, such as cancer, that depend on accessibility of biomolecules and energy generation to fuel rapid proliferation. In line with this theory, patients taking metformin as a diabetes treatment have been found to exhibit reduced development of breast cancer which further validates its theorized oncoprotective properties (Gillespie et al., 2016). Increased cancer rates are paired with poor nutritional quality and excessive calorie intake within developed countries makes the study of calorie restricting mimetics and their possible life extending benefits within a healthy population of great scientific interest. This supports the need for research efforts exploring if BBR supplementation may possess similar promise for both diseased and healthy patients as a non-prescription intervention with metformin like effects (Podhorecka et al., 2017).

Breast cancer is currently the most prevalent cancer type in both developed and developing countries alike (el Khalki et al., 2020). As of 2018 approximately 18% of breast

cancer cases were an aggressive triple negative form of cancer with very limited treatment options (el Khalki et al., 2020). The primary non-hormonal treatment for this type of breast cancer is known as cytotoxic chemotherapy (el Khalki et al., 2020). One classes of cytotoxic chemotherapy is anthracycline drugs such as doxorubicin (Lipodox) and valrubicin (Valstar). This therapy type is systemically devastating and varies in efficacy. In an effort to uncover treatment options with fewer negative side effects and also to reduce the need for polypharmacy, researchers are delving into the possibilities of BBR as a complementary treatment option. Although metformin and exercise both exert influence on AMPK, there is thought that when these two interventions are combined metformin may decrease exercise volume (Ramos et al., 2020). It is currently unknown if BBR exhibits the same reduction in exercise volume.

Adenosine Monophosphate-Activated Protein Kinase and Energy Metabolism in Cancer

As stated above, BBR is thought to be a stimulator of AMP-Activated Protein Kinase (AMPK). AMPK functions as a metabolic cellular ATP/ADP sensor recognizing and playing a role in maintaining energy levels within the cell (Pan et al., 2017). The findings of Faubert et al., suggest that AMPK activity inhibits tumor development through its role in the metabolic cycle of the cell which effectively reverses Warburg metabolism (Faubert et al., 2013). The Warburg effect is a metabolic shift unique to cancer cells and required for tumor expansion (Liberti & Locasale, 2016). Healthy cells within an oxygen rich environment will conduct aerobic glycolysis and undergo oxidative phosphorylation to produce high levels of ATP. However, in this metabolic phenomenon cancer cells take in exorbitant amounts of glucose and process it via the same mechanism required for anaerobic glycolysis regardless of oxygen sufficiency. Although in the context of cancer this aberrant metabolic process is referred to as aerobic, similar

to anaerobic metabolism, it results in the production of lactate and minimal amounts of ATP. The Warburg Effect is a pro-tumor mechanism, but its impacts are believed to be combated by increased AMPK which exerts anti-tumor properties in cancer models through reversal of the Warberg effect (Faubert et al., 2013). The Ketogenic diet has also been studied in breast cancer patients and found to successfully discourage the pro-tumor effects of this metabolic shift by balancing insulin spikes and reducing glucose levels (Khodabakhshi et al., 2021). However, the ketogenic diet is not always sustainable for cancer patients or those wishing to utilize this eating pattern for its insulin stabilizing benefits.

Furthermore, physical activity requires muscle contraction which increases the energetic demands on the mitochondria within myofibrils. Similar to the function of BBR, Metformin, and the ketogenic diet, it has been previously demonstrated that the energy consumed by this contraction process skews ATP/AMP ratios effectively mimicking cellular fasting mechanisms that upregulate the AMPK pathway, leading to reduced cell proliferation, and decreased insulin levels(Drake et al., 2021). Indeed, reduction in cell proliferation is beneficial for slowing neoplastic evading disease states such as cancer, which places exercise at the forefront of promising cancer treatment and rehabilitation interventions.

Physical Activity and Cancer in the Immune System

A phenomenon known as the “inverted J” is a term used to describe immune function under the influence of consistent moderate exercise (Gustafson et al., 2017). The inverted J hypothesis suggests that too little, or too much physical activity may lead to lowered immune function and even immune suppression, such as in the case of overtraining. Furthermore, studies suggest exercise immune stimulating properties may relate to its ability to increase insulin sensitivity (McTiernan, 2008) However, one exception is found in aging populations. Older

populations experience a decrease in the benefits of exercise by an observed reduction in the number of circulating post exercise lymphocytes as well as a shortening in the duration of their presence within peripheral blood (Freidenreich & Volek, 2012). As the effects of exercise begin to fade with age, additional supplementary interventions with potential anti-aging or life extending properties, such as BBR, become of the utmost importance.

Consistent physical activity has also been proven to decrease the activity of MDSC's. Exercise has been found to combat the rate of cancer growth by reducing circulating total MDSC count (Garritson et al., 2020). A recent study explored the impact of physical activity on the immune function of a 4T1 murine breast cancer model and found that consistent wheel running, over a 10-week period significantly decreased MDSC infiltration in the spleen, blood and tumor. However, no reduction in tumor size was observed. Furthermore, a non- significant reduction in metastasis of breast cancer to the lungs was observed in wheel run animals (Garritson et al., 2020).

MDSC's also promote suppression of anti-tumor immune function and cancer development through reduction of $CD4^+$ T_h cells and by increasing production of T_{regs} through secretion TGF-β1 (Veglia et al., 2018). Garritson et al., suggests that treatments which decrease the activity and/or presence of MDSC's will increase therapeutic outcomes for patients with any form of breast cancer (Garritson et al., 2020).

Breast Cancer in the Context of Berberine and Physical Activity

Due to its vast metabolic effects, BBR has been studied in the context of many cancers such as esophageal cancer, nasopharyngeal carcinoma, GI cancers, and within cancer cell lines such as HELA cells, and 4T1 mammary carcinoma (Iizuka et al., 2000; Kim et al., 2010; W. Ma et al., 2020; Tang et al., 2009). It is estimated that 2/3 of all breast cancer cases could be

attributed to insufficient physical activity and obesity—suggesting insulin resistance may be a factor in development of neoplastic disease. McTiernan states that women who engage in moderate-intensity physical activity for 3-4 hours/week experience a 30-40% reduction in breast cancer as compared to sedentary females (McTiernan, 2008). Moderate physical activity, such as brisk walking was found to significantly decrease breast cancer risk in women who engaged in this type of physical activity for approximately 2.5-7 hours per week as compared to sedentary women (McTiernan et al., 2003).

For many disease states and health conditions, exercise is a common standard of care with many known physiological benefits. IL-6 is known to be secreted from breast cancer tissues and is associated with poor prognosis. IL-6 is a pleiotropic cytokine that can function as both a pro and anti-inflammatory agent. In the cancer environment IL-6 promotes inflammation and is thought to play a role in tumor development by increasing angiogenesis as well as cellular adhesion and tumor metastasis through activation of Rho, a protein known to stimulate cell growth and migration (Ravishankaran & Karunanithi, 2011). IL-6 is also an indicator of breast cancer progression and appears to increase in expression as the stage of cancer severity increases (Ravishankaran & Karunanithi, 2011). Interestingly, IL-6 is also released from contracting muscles as a myokine. As a myokine IL-6 exemplifies favorable glucose regulation outcomes in healthy animals (Chow et al., 2022). One aspect of its glucose regulatory function stem from its ability to increase insulin secretion from the pancreas which could have a negative effect on cancer (Chow et al., 2022). However, as a myokine, IL-6 is also found to mobilize natural killer cells which have an anti-tumor impact within the immune system (Chow et al., 2022).

Berberine and Immune Activity

Increasing casual and voluntary consumption of BBR makes the impact of orally administered BBR on immune activity of great interest. One way to determine BBR's influence on immune function is to assess T-cell activity. $CD4^+$ helper T cells and their cytotoxic counterparts $CD8^+$ T-cells are vital within the immune system for protection against a host of pathogenic factors such as viruses and cancer. A recent study conducted by Vita et al. found cause for concern regarding the prophylactic use of BBR and possible immunosuppressive properties in T cell populations (Vita et al., 2021). Specifically, BBR's anti-inflammatory function can be in part linked to suppression of effector $CD4^+$ T_h cells which directly reduces B cell autoantibody production. Overall, this suggests that consumption of BBR may reduce the T_h cell's capacity to provide the help needed for B cell autoantibody production lowering overall immune response which is a function akin to some Rheumatoid Arthritis (RA) pharmaceuticals (Vita et al., 2021). The same study found that T_{regs} also appear to be impacted by BBR consumption with a higher overall proportion of $FOXP3^+$ $CD4^+$ T_{reg} than $FOXP3^-CD4^+$ in BBR treated groups as compared to PBS controls (Vita et al., 2021). Increased levels of Tregs and lower Th cells contribute to an anti-inflammatory environment, which may be helpful in the case of autoimmunity but potentially harmful for cancer patients.

Immune Cell Activation

When the effector T cells listed previously are activated via antigen specific binding, they produce the transcription factor NUR77, also known as NR4A1 of the NR4A human nuclear hormone receptor family. Coded by the gene *NR4A,* the presence of this transcription factor allows discrimination between T cells by ruling out those that have been activated by the inflammatory milieu and thus do not produce NUR77 and T cells that are actively engaged in the

immune response as a result of antigen presentation (Ashouri & Weiss, 2017; Wyss et al., 2016). CD69 is also commonly used as an extracellular marker of T cell activation, however it does not distinguish between T cell activation based on antigen specific involvement and thus is not as precise of a measure of immune activation (Ashouri & Weiss, 2017). Higher levels of NUR77 expression in the cytosol correspond to a greater level of TCR stimulation. However, Nur77 production in the presence of BBR is not well studied. Specific T cell activity can be assessed by staining for the transcription factor NUR77 (NR4A1 of the NR4A human nuclear hormone receptor family).

Study Rationale

The ever-growing popularity of BBR, a potent and biologically active plant-derived compound, is a cause for concern. The lack of human clinical trials, or studies that define its biological effects means that consumers are unaware of potential consequences, and thus assume lopsided, risk free, benefit of this compound. This study aims to begin to clarify the biological effects of BBR in preclinical models.

It is important to note that very little research has been conducted connecting the dots between the possible cooperative effects of berberine and exercise in the context of cancer. The rising prevalence of both cancer and autoimmunity begs urgent need for additional research. Medical practitioners, the supplement industry, as well as the food production field would benefit from understanding the impact this compound may have on the immune system. Moreover, understanding BBR's immunological impact within the body, specifically in the context of cancer paired with physical activity, may inform practitioners of evidence-based uses for this compound. As stated before, the purpose of this study is to determine if oral consumption of BBR when paired with physical activity will increase T lymphocyte presence and activation

status while decreasing the presence of myeloid-derived suppressor cells (MDSC) within the tumor microenvironment, spleen, bone marrow, and blood of both a healthy population of immunocompetent BALB/c mice and a BALB/c 4T1 mammary carcinoma model.

CHAPTER III

METHODOLOGY

Materials and Protocol Models

Animal Care

Female BALB/c mice (n=53) were housed in a temperature-controlled facility running on a 12:12 (6am-6pm) light dark cycle. Mice were housed in separate cages, fed standard chow and had access to water *ad libitum*. All procedures were performed in accordance with the University of Northern Colorado's Institutional Animal Care and Use Committee protocol 2005C-NP-M-23 and protocol 1511CE-RM-RH-18 as well as the animal welfare act guidelines.

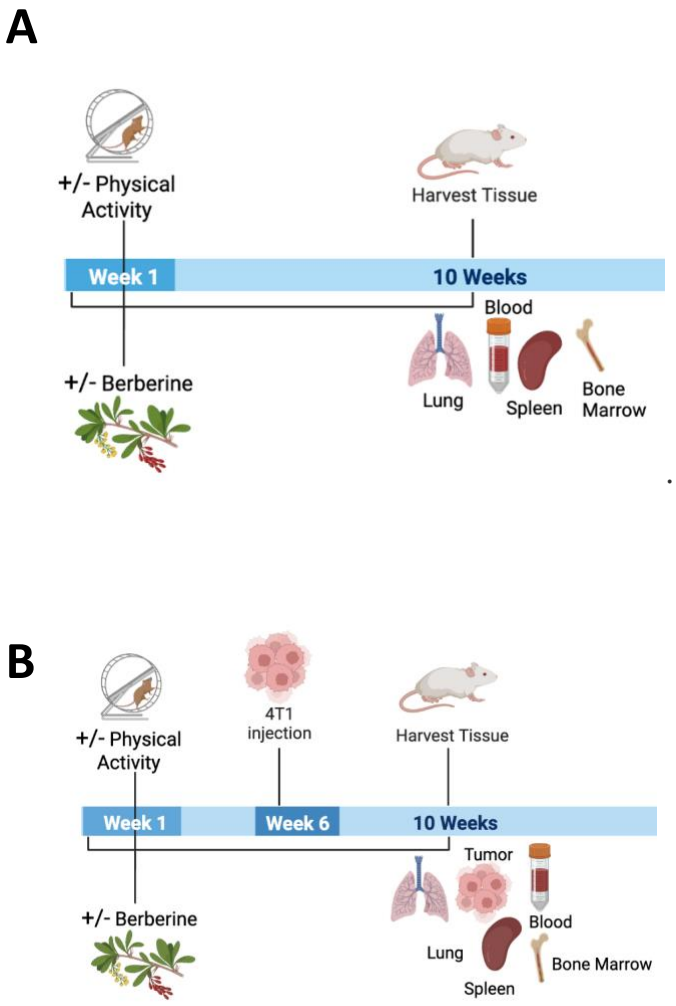
Berberine Supplementation

Voluntary, oral BBR supplementation was achieved by suspending BBR hydrochloride (purity > 98%; Sigma-Aldrich, St. Louis, MO, USA) into a berberine gummy made of an 8% gelatin solution containing 2% sucralose solution (L. Zhang, 2021). Sucralose is a non-nutritive sweetener chosen for its sweet flavor without increasing blood glucose levels upon consumption, therefore preventing promotion of neoplasm due to increased insulin levels. Mice are inherently neophobic, so a training period was necessary to desensitize the mice to the BBR gummy as well as a small serving platter. Mice were fasted overnight before a vehicle gummy/platter was placed in their cage. Mice were returned to their normal feeding schedule and a second vehicle jelly was administered during the same time of day. After the training period, mice consumed 1 BBR gummy (4.06 mg BBR) within 30 seconds of placing the gummy in their cage. This ensured the peak concentration of BBR entering their system and emulated oral human consumption. Past

studies found that a dose of 145 mg/kg of BBR administered via gavage to BALB/c was the most effective in decreasing tumor size of tumor bearing mice (W. Ma et al., 2020). Mice typically eat during their active dark cycle, thus, BBR was administered five days per week ten minutes before the beginning of the dark cycle on a serving platter to prevent contamination with bedding or excrement (figure 1).

Figure 1

Study Design and Interventions



Note. This figure demonstrates an overview of study methods. All mice were fed either a BBR gummy (sucralose vehicle) or a sucralose control gummy (A,B). Mice were randomly assigned to a wheel running group or a sedentary group. One cohort of Balb/c mice were inoculated with 4T1 cells expressing tdT RFP mammary carcinoma cells on week 6 of the study (A). Spleen, blood, bone marrow, lungs and tumor were harvested on day 70 which fell on week 4 after 4T1 inoculation. Tissue was processed, stained, and analyzed via flow cytometry.

Exercise Wheel Run Model

Exercise volume was assessed by subjecting mice to a 10-week endurance voluntary exercise wheel run protocol. This model is considered the most common animal research physical activity protocol and mimics endurance training in humans (Manzanares et al., 2019). Mice 6-8 weeks of age were randomly assigned to one of 8 control and intervention groups (Table 1). Exercise mice were provided with a cage mounted running wheel (11.5cm diameter) for the duration of the study and non-exercise mice were restricted to normal cage activity. Running volume was monitored utilizing magnetic probes that tracked wheel revolutions every 15 seconds through Vital View data acquisition system (MiniMitter, Bend, OR).

Table 1

Outline of Intervention and Control Groups, Descriptive Abbreviations, and Sample Size

Group	Control	Berberine	Exercise	Berberine & Exercise	Cancer	Berberine & Cancer	Exercise & Cancer	Berberine, Exercise & Cancer
Abbrev-iation	Cont.	BBR	EX	BBR+EX	4T1	BBR+4T1	EX+4T1	BBR+EX+4T1
Sample Size	N=3	N=3	N=4	N=3	N=8	N=8	N=8	N=8

Fluorescent 4T1 Tumor Inoculation and Measurement

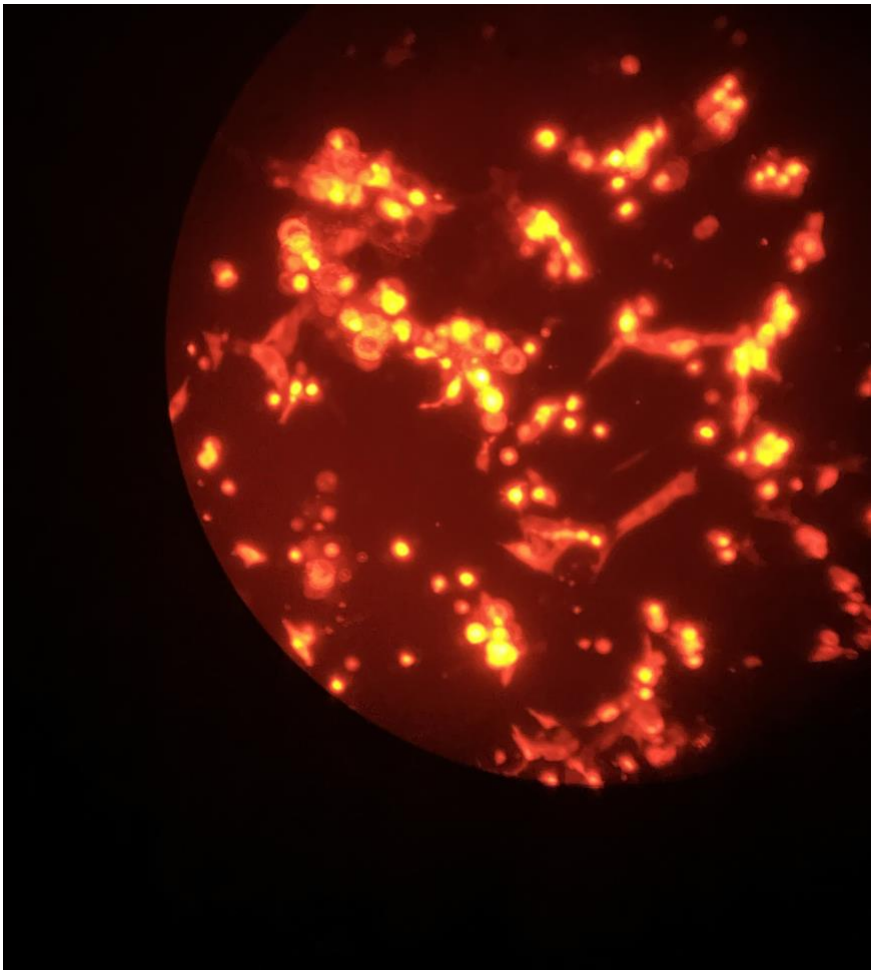
Cell Growth and Stable Plasmid Insertion

Fluorescent Model

4T1 mammary carcinoma cells were originally purchased from ATCC (Manassas, VA) and cultured in RPMI 1640 (ThermoFisher) supplemented with 1% pen/strep, 2mM L-Glutamine, 1mM Sodium Pyruvate, 10mM HEPES, 0.05mM β-mercaptoethanol, and 10% (by volume) fetal bovine serum (FBS). To achieve syngeneic tumor growth BALB/c female mice (The Jackson Laboratory, Bar Harbor, ME) were selected for injection with 4T1 tdTomato expressing cells. Cells containing the pcDNA 3.1 tdT plasmid (figure 3) successfully produce a fluorescent protein that, unlike other luminescent models, do not require a complementary substrate for activation (Patel et al., 2010). In this model synthetic firefly luciferase is paired with a protein known as Tandem Tomato (tdTomato) which emits photons that appear red when stimulated with a blue-yellow laser during flow cytometry or fluorescence microscopy (figure 2).

Figure 2

tdT Expressing 4T1 Cells



Note. This figure demonstrates the fluorescence intensity of cells tranfected with the tdT plasmid. Cells were analyzed utilizing fluorescent microscopy with a blue-yellow laser.

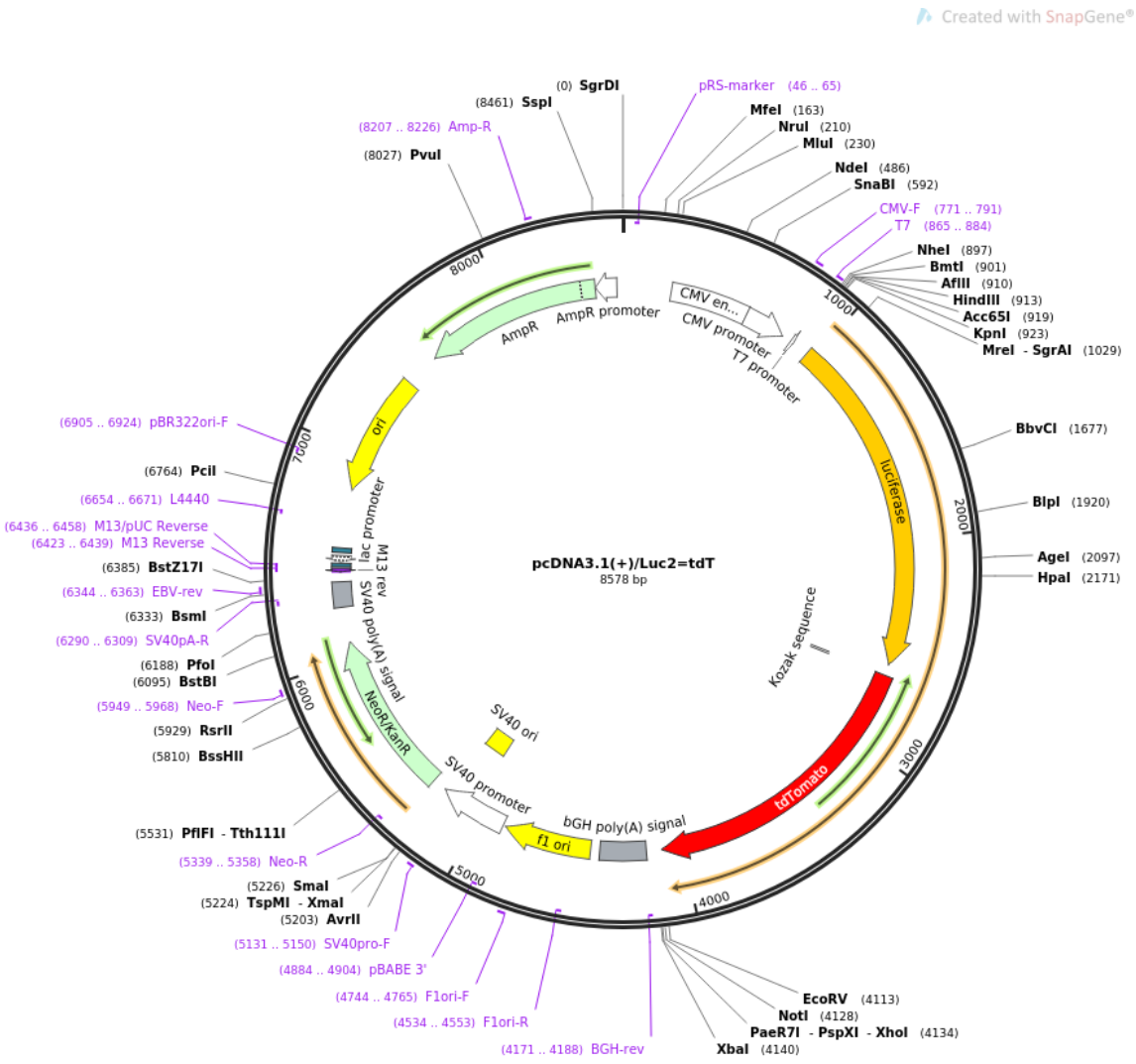
Inoculation and Tumor Progression

The plasmid insert also expresses neomycin antibiotic resistance, allowing tdT containing cells to be cultured under antibiotic selection (Lith et al., 2020). RPMI cell growth media was used to culture 4T1/tdT plasmid containing cells and was changed every three to four days. Antibiotic selection was achieved by the addition of 30μL (50mg/mL) of neomycin G418 (Sigma), added on day two after each medium replacement. On the 6th week of the study, animals were inoculated with 10^5 4T1 mammary carcinoma cells containing the pcDNA 3.1

tdT plasmid. G418-Neomycin was removed a day before inoculation and cells were rinsed to remove any lingering antibiotic and provided fresh C-RPMI. On the day of inoculation, media was removed, and cells were rinsed again and suspended in 100 µL HBSS (Ca^{2+}/Mg^{2+} free, ThermoFisher) before injection with a 25-gauge syringe needle into the nipple of the right, fourth mammary fat pad. Mice were monitored for any signs of infection around the site of injection. Upon euthanasia, at day 28, tumor mass was obtained, and tumor volume was calculated using the equation $\pi/6 \times width \times length\text{^}2$ where length refers to the area of largest diameter and width was found by measuring 90 degrees from length. Length and width were assessed via digital calipers.

Figure 3

Red Fluorescent Protein Addition to the Genome of 4T1 Mammary Carcinoma Cells



Note. This figure represents the pcDNA 3.1 Tdt. Plasmid Map and was adapted from "TdTomato and EGFP identification in histological sections: insight and alternatives," by L. Morris, C. Klanke, S. Lang, F-Y. Lim, & T. Crombleholme,. 2010. Biotechnic & Histochemistry, 85(6), 379–387. https://doi.org/10.3109/10520290903504753.

Euthanasia

On week 10 mice were euthanized via CO_2 asphyxiation, and tissues were harvested. Running wheels were removed from the cage 24 hours before euthanasia, and a feces sample was collected directly before euthanasia for PCR analysis.

Tissue Preparation and Analysis

Aortic puncture was utilized for collection of blood from the chest cavity and 100 µL of heparin was infused locally to prevent clotting. Blood was then incubated with 2 mL ACK lysis buffer for 3-5 minutes, centrifuged at 0.2 RCF for 5-6 minutes before a second incubation with ACK lysis buffer to remove any remaining erythrocytes. Finally, blood was centrifuged at 0.2 xg for 5-6 before resuspension in 1mL of 1X HBSS and placed on ice for staining. Whole spleens were weighed and dissociated in 500 µL 1X HBSS through pulverization by a rubber policeman. The spleens of tumor bearing mice were weighed whole and then divided in half for dissociation. Lungs were harvested and mechanically homogenized using a cell dissociation sieve fitted with a 100 µm mesh screen. Tumors were extracted and weighed following the removal of extraneous tissue before undergoing mechanical homogenization via the same method used for lung tissue. To achieve a single cell suspension lung and tumor pulp were re-suspended in HBSS and enzymatically digested in Type IV collagenase (2 mg/mL) and DNase (0.1 mg/mL; Worthington) on a platform rocker for 45minutes at 37 degrees Celsius at 225rmp. Remaining erythrocytes from various tissues were cleared utilizing ACK lysis buffer (Quality Biological). Bone marrow was isolated from the femur and tibia of both hind legs. All tissues were strained through a 100 µm cell strainer (VWR) in preparation for Fc blocking and antibody staining.

Cellular Permeabilization, Flow Cytometry and Antibody Staining

Spleen, blood, lung, and tumor cell suspensions were aliquoted to appropriate cell concentrations (~10^6 cells/mL) for staining. Cells were Fc blocked (101302) on ice for 10 minutes prior to staining for intracellular and extracellular markers. All cells were stained for extracellular surface markers in flow cytometry buffer containing 0.5% BSA in Ca^{2+}/Mg^{2+} free 1X Dulbecco's PBS (ThermoFisher). Following extracellular staining, approximately half of all cells were set aside for intracellular permeabilization by the True-Nuclear Transcription Factor Buffer set (Biolegend) before staining for intracellular markers. Unless otherwise indicated, flow reagents and antibodies were sourced from Biolegend (San Diego, Ca). Samples were fluorescently analyzed on the Attune NxT Cytometer (ThermoFisher); and raw data were analyzed using Flowjo v10. Antibodies used to define cell types and biomarker expressions in these studies are outlined in Table 2.

Table 2

Biomarker Profiles, Antibody Catalog Numbers and Supplies

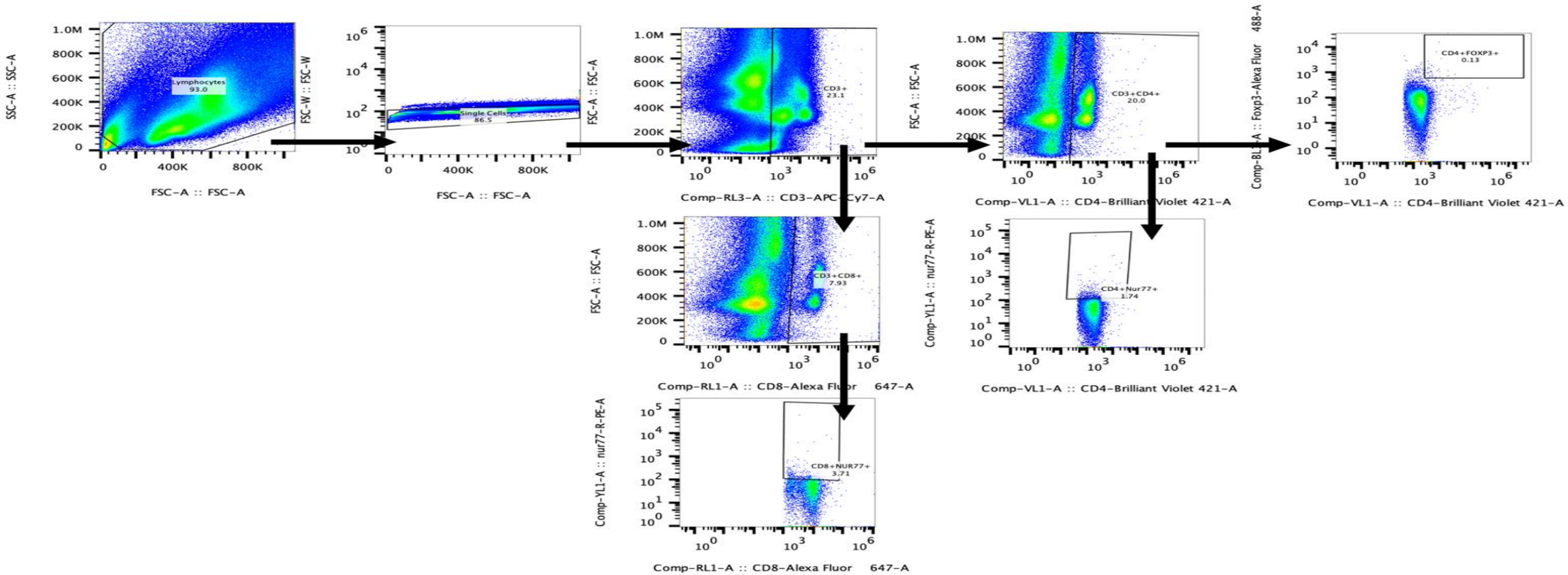
Cell Type	Lot/Cat Number	Supplier
MDSC ($CD11b^+Ly6G^+Ly\text{-}6C^{+)}$	147001	BioLegend
$CD3^+$	b346120/100222	BioLegend
$CD4^+$	b266324/100438	BioLegend
$CD8^+$	2162149/12-5965-82	BioLegend
$Nur77^+$	2162149/12-5965-82	ThermoFisher
$FoxP3^+$	b331607/126406	BioLegend

Gating for Flow Cytometry

Initial forward and side scatter gating was used to exclude any possible cellular debris. Following acquisition, each sample is plotted with forward-scatter (FSC) (which measures size of each event) vs side-scatter (SSC) (which measures the internal complexity of each event). Next, doublet discrimination was performed to exclude any clumped cells. Remaining cells were plotted based on respective extracellular or intracellular markers (see table 2). Unstained controls were used to exclude background fluoresce.

Figure 4

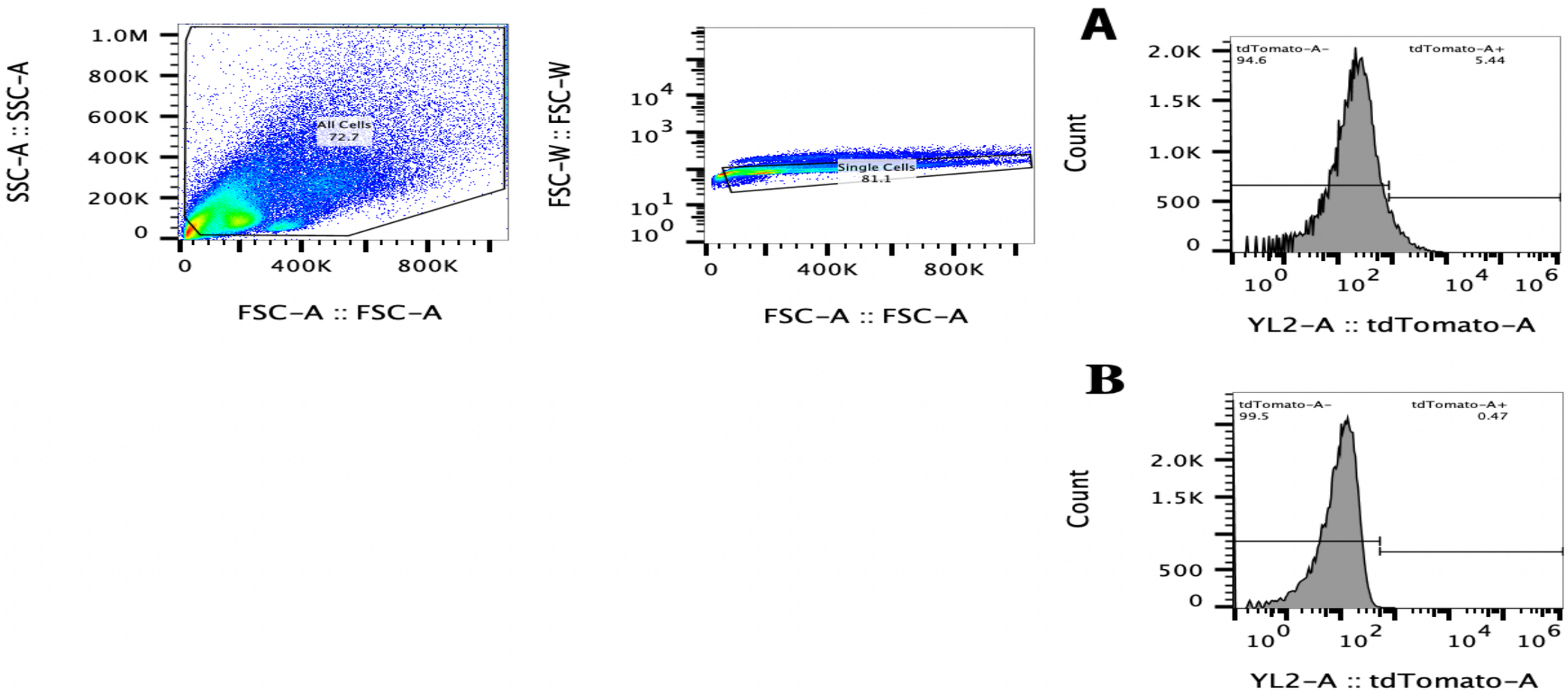
Extracellular and Intracellular Gating Strategy Represented by Spleen Tissue



Note. Cellular debris were gated out and single cells excluded. $CD3^+$, $CD4^+$, $FOXP3^+$, $CD8^+$, and NUR77 expression are all represented within this gating strategy. $CD4^+$ and $CD8^+$ are expressed as frequency of the $CD3^+$ gate.

Figure 5

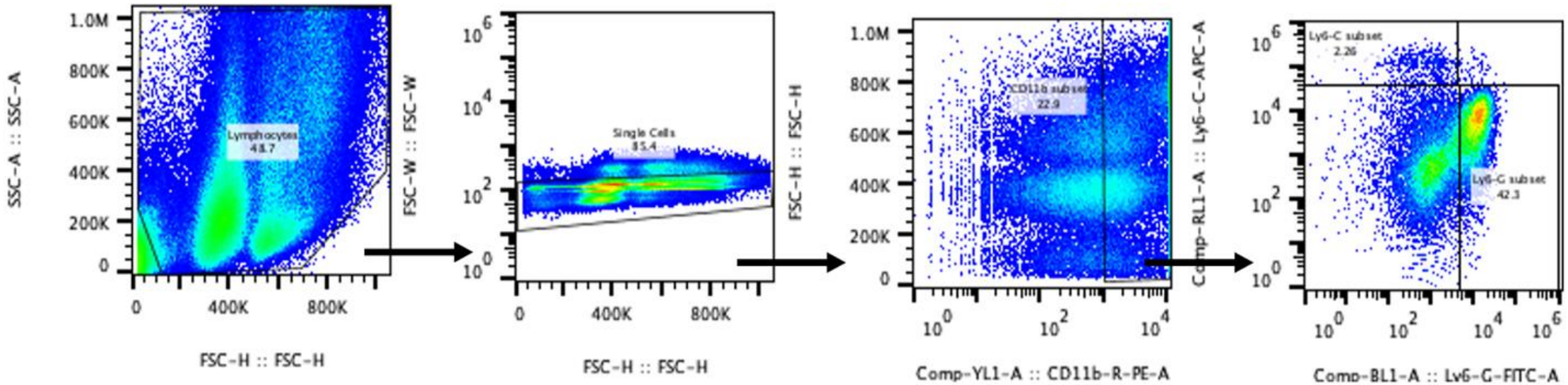
Lung Fluorescence Gating Strategy



Note. For all samples cellular debris were gated out and doublet discrimination was performed. Tumor bearing lungs (A) showed increased red fluorescence as compared to non-tumor bearing lungs (B).

Figure 6

MDSC Gating Strategy



Note. Spleen samples from tumor bearing animal showing total CD11b frequency as well as gating for $Ly6C^{-}/LY6C^{+}$ and $Ly6G^{-}/Ly6G^{+}$.

Statistical Analysis

One-way ANOVAs utilizing Tukey's post hoc comparison method of locating significant pairwise variations were run for each tissue type to compare the means between each group. P-values that fell within $p < 0.05$ were considered representative of significant variation within immune marker frequency. All statistical analyses were conducted in Prism version 9.4.1 (GraphPad). Unless otherwise indicated all samples are reported as means ± SEM.

CHAPTER IV

RESULTS

Impacts of Berberine Supplementation on Cancer Progression and Physical Activity

The primary purpose of this study was to investigate immune cell accumulation within tumor-bearing and healthy host subjects after voluntary oral BBR supplementation and/or voluntary physical activity. A secondary purpose of this study was to measure wheel running volume in animal subjects supplemented with voluntary oral BBR. The final purpose of this study was to assess the impact of voluntary oral BBR supplementation with or without physical activity on 4T1 metastasis to the lungs. We hypothesized that oral consumption of BBR, when paired with physical activity, would increase T lymphocyte presence and activation status while decreasing the presence of MDSC's within the tumor microenvironment, spleen, bone marrow, and blood of both a healthy population of immunocompetent BALB/c mice and a BALB/c 4T1 mammary carcinoma model. We also suspected that BBR would have a negative impact on voluntary exercise volume, and that the impact of voluntary oral BBR supplementation with or without physical activity would decrease metastasis to the lungs of mice inflicted with 4T1 mammary carcinoma.

Physical Activity and Wheel Running Volume

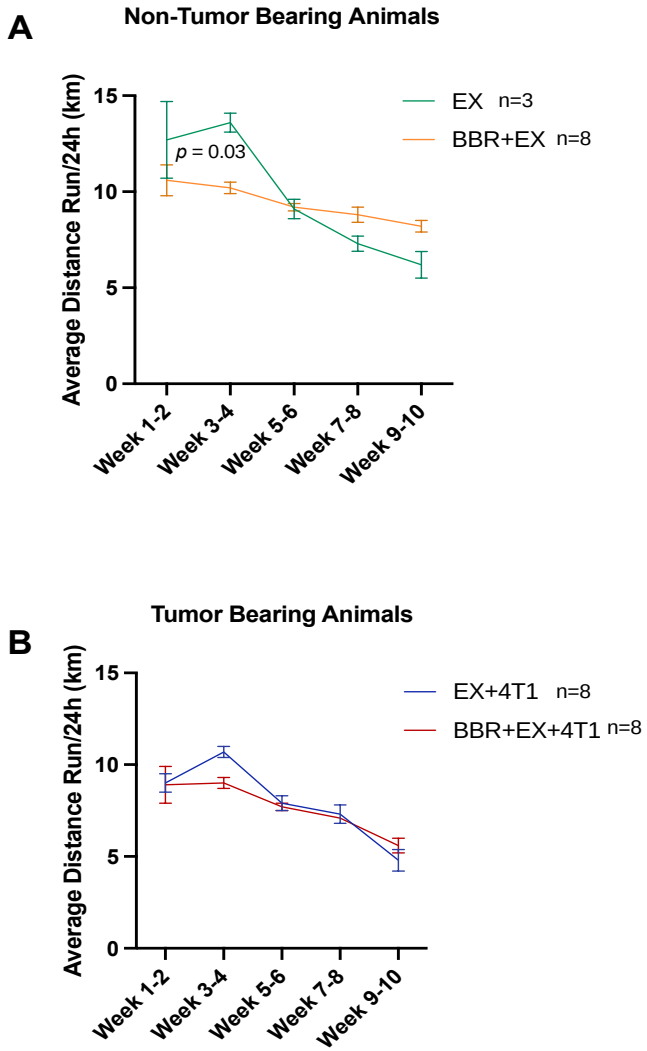
Mice were randomly assigned to a control non-tumor-bearing-sedentary group (Control n=3), a wheel run group (EX, n=3), a BBR wheel run group (BBR+EX, n=3) a control-4T1-tumor-bearing-sedentary group (4T1, n=8), or a tumor bearing, BBR, wheel run group (BBR+EX+4T1, n=8). Mice were given approximately 7 days to adjust to their new environment before running data were collected. Wheel running data were recorded at 15 second intervals and compiled into 2-week averages based on treatment groups (figures 7A and 7B). Non-tumor bearing animals not treated with BBR exhibit a relatively normal wheel running pattern with a steady decrease after the first few weeks of wheel access. BBR+EX animals exhibited a slightly lower initial exercise volume but did not experience the same weekly decrease as animals not receiving BBR treatment. The distance run by each of the two treatment groups intersected at weeks 5-6 with BBR averaging at 9.1 km/day ± 0.5 SEM and BBR+EX with a mean distance of 9.2 km/day ± 0.2 SEM. The following weeks resulted in a steady decline on average in the EX group whereas the BBR+EX group exhibited a minimal decrease in km run. Comparatively, between weeks 5-6 and weeks 9-10 the exercise group distance had reduced by 3km and the BBR+EX group had only reduced by approximately 1km.

Within the tumor bearing groups very little change was noted in both the BBR and non-BBR supplemented groups exhibiting similar patterns of early increase in distance ran before subsequent decrease in progression over the following weeks. The only notable divergence was found in weeks 3-4 where the mean running distance of the BBR+EX+4T1 group (10.2km/day ± 0.3) did not exhibit a marked increase in distance run like the 4T1+EX group, which exhibited a temporary increase (figure 7B). When the BBR+EX group was compared to the BBR+EX+4T1, and the EX-group compared to the EX+4T1 group, similar trends were observed. In the animals

supplemented with BBR an overall change from week one to week ten showed an approximate total decrease of 2.1 km in the BBR+EX group and 3.3 km in the BBR+EX+4T1 group. In the EX and EX+4T1 groups a greater overall decrease was observed at 6.5 km and 4.2 km respectively.

Figure 7

Wheel Running Volume in BALB/c 4T1 Tumor-Bearing Female Mice



Note. Average wheel run volume in km/day over a 70-day period (divided into 2-week time increments). Non-tumor bearing animals **(A)** represented separately from tumor-bearing groups **(B).** Time points represented as mean ± SEM.

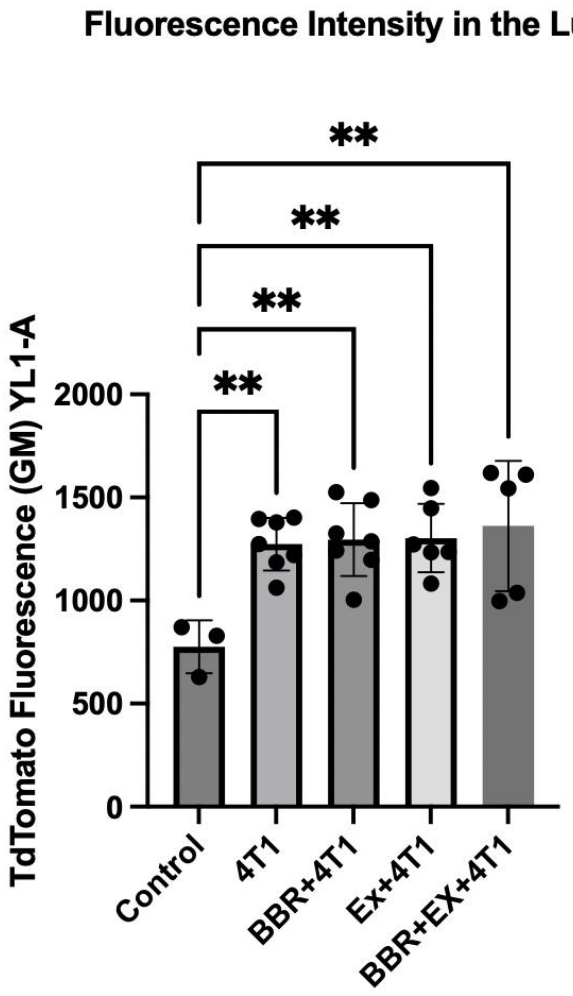
Metastasis to the Lungs

To assess metastasis of mammary carcinoma with or without EX and BBR we utilized a 4T1 Mammary Carcinoma cell line that we enhanced with the monomeric tandem dimer (Td) Tomato fluorescent protein. This method has been previously used to tag specific cells for in-vivo observations in mice (Takahashi et al., 2015). However, to our knowledge it has not been utilized to assess cancer metastasis. Tdtomato 4T1 cells produce a red protein that is fluorescent in the YL1-A channel and possesses an extremely bright emission wavelength of 581nm.

Between treatment groups no significant change was observed from baseline control, non-tumor bearing animal (figure 8). However, a significant increase (**$p<0.01$) in MFI between the non-tumor bearing control lungs and tumor bearing lungs was observed (figure 8).

Figure 8

Fluorescence Intensity Within the Lungs of Tumor Bearing BALB/c Mice at 4 Weeks Post Injection with 4T1 Mammary Carcinoma



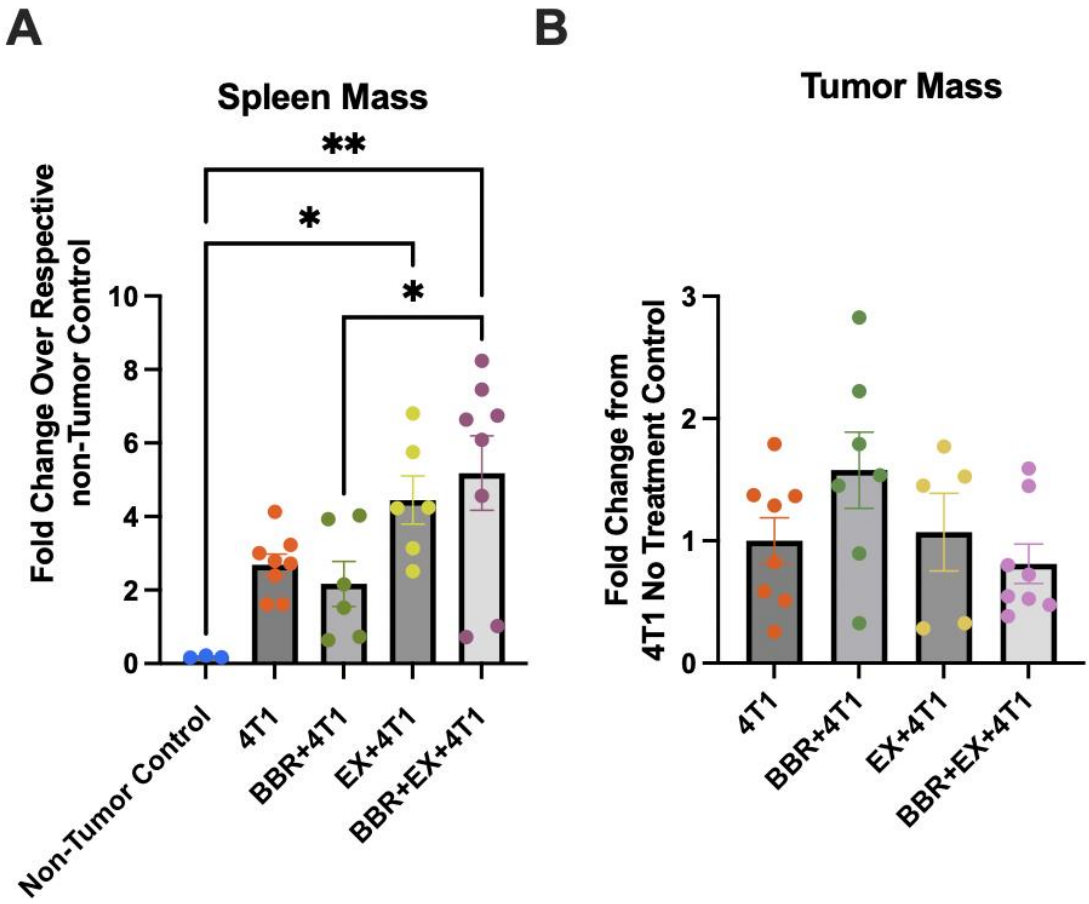
Note. Lung Fluorescence in tumor bearing animals as compared to the lung fluorescence of a non-tumor bearing animal (ctrl). Fluoresce measured as mean fluorescence intensity (MIF) *p<0.05, **p<0.01, ***p<0.001.

Tumor and Spleen Mass

Whole tumors and spleens were extracted from each subject and mass was collected (mg). Fold change was calculated for the spleens of each group by dividing the mass of individual spleens in each of the respective groups by the average mass of the spleens from non-tumor bearing animals. The same method was utilized for calculating fold change of tumor mass, with the exception of a tumor-bearing animal that had not been assigned to either the BBR or physical activity groups functioning as the control devisor (figure 9). The most notable change is seen in the BBR+4T1 groups spleen when compared to the same group's tumor mass. The BBR+4T1 group (n=8) exhibited the smallest fold change in spleen mass, but the largest fold change in tumor mass. The BBR+EX+4T1 spleen (n=8) experienced the largest fold change in splenic mass but a negative fold change compared to the sedentary 4T1 control group tumor mass.

Figure 9

Comparative Spleen and Tumor Mass Between Treatment Groups



Note. Spleen mass, represented as fold change over respective non tumor control ± SEM. Tumor mass, represented in fold change from 4T1 no treatment control ± SEM *p<0.05, **p<0.01.

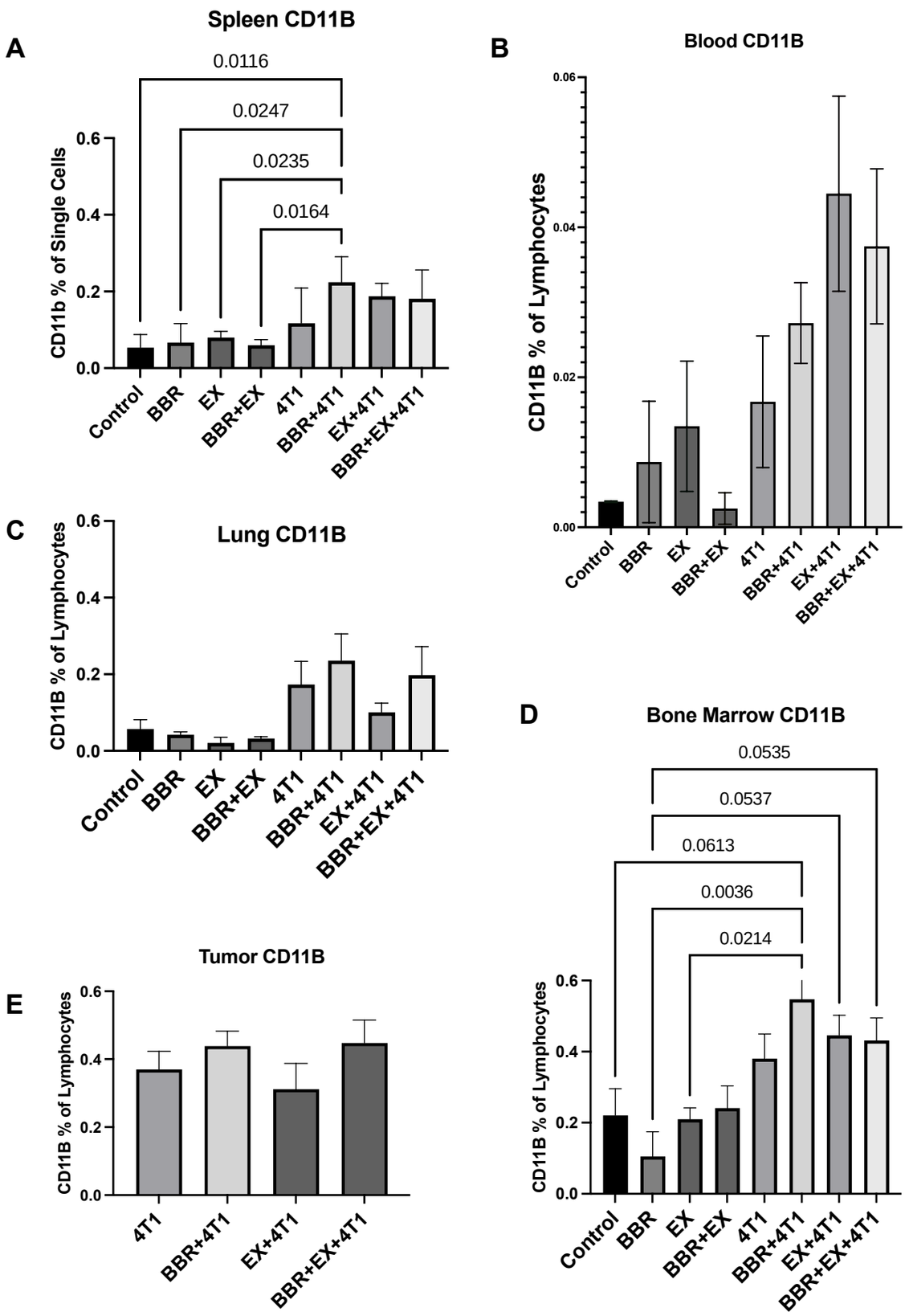
Immune Markers

CD11B Leukocytic Immune Populations

CD11B is a marker expressed on Leukocytic cells such as monocytes, natural killer cells, and granulocytes. Significant expansion of CD11B was seen in the spleen and bone marrow between a control non-tumor bearing animal and a tumor bearing animals. However, no treatment effect was seen within the control and tumor bearing groups. Across most tissues (figures 10 A and D), on average there was a trend suggesting slight non-significant elevation in CD11B counts within the BBR+4T1 group.

Figure 10

Accumulation of Total CD11B Count Across All Tissue Samples



Note. All non-tumor bearing samples were compared to non-tumor bearing samples along with comparisons within the tumor bearing samples and control groups. No significant intervention specific change was seen within the tumor bearing group. *P* values numerically represented to show samples that are nearing significance. **p*<0.05, ***p*<0.01.

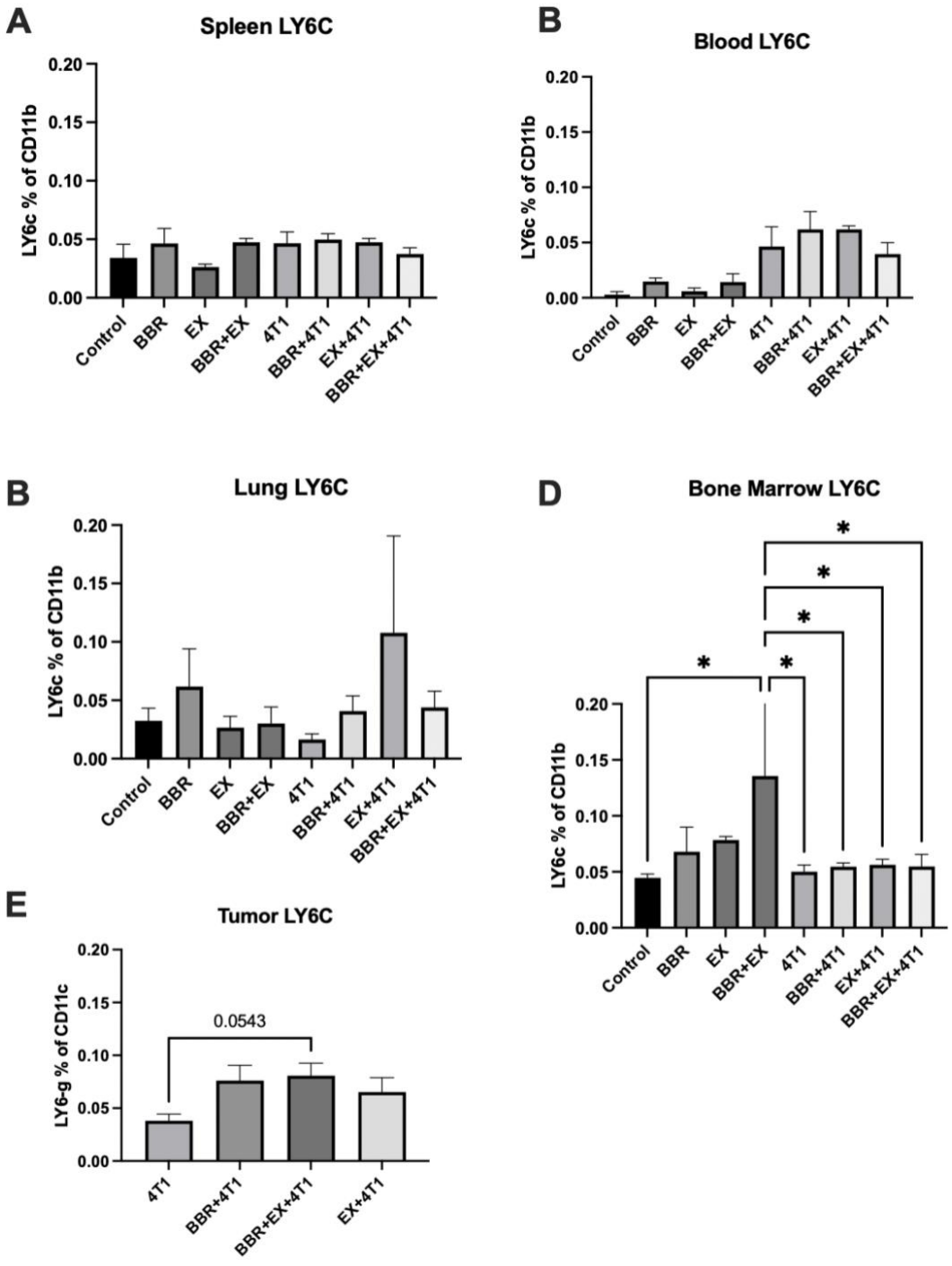
Myeloid-Derived Suppressor Cell Immune Subsets

$CD11B^{+}LY6C^{+}$ and $LY6G^{+}$ Myeloid-derived subsets were analyzed within each tissue of each mouse. Within the $LY6C^{+}$ subsets minimal variations were observed not only across treatment groups but also between tumor bearing and non-tumor bearing animals. On average the blood showed an observable increase in circulating $LY6C^{+}$ populations within tumor bearing animals (figure 11B). The inverse was true within the bone marrow with an observable increase in the $LY6C^{+}$ non-tumor bearing population (figure 11D).

$LY6G^{+}$ MDSC's exhibited significant increases across the tumor bearing group as compared to non-tumor bearing groups in all tissues (figure 12). However, within the tumor bearing and non-tumor bearing groups no statistically significant treatment specific effects were observed. Exercised animals in the non-tumor bearing bone marrow group were an exception to this observation exhibiting a significant increase when compared to the bone marrow of all other non-tumor bearing animals.

Figure 11

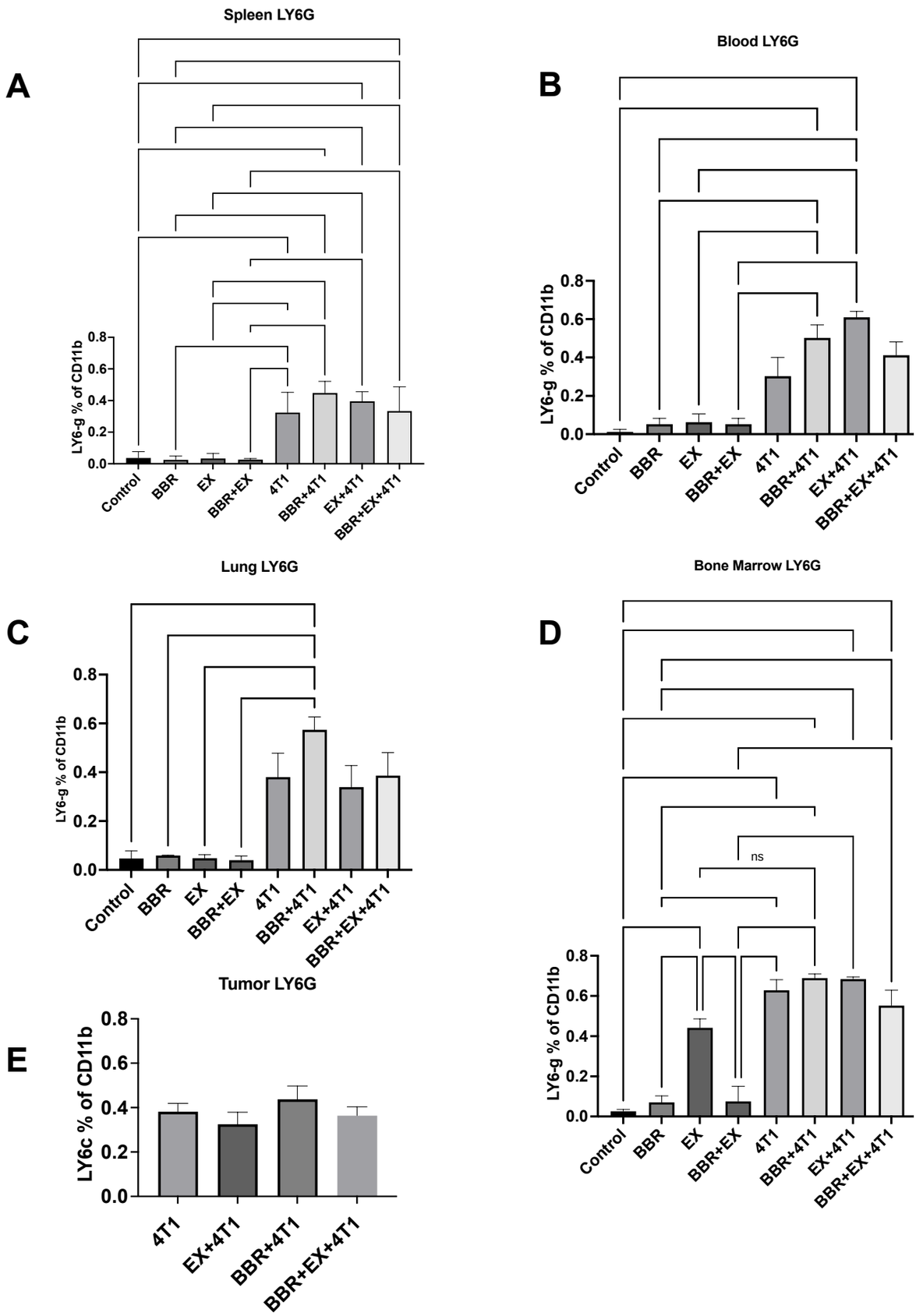
LY6C MDSC Cell Counts Represented Across All Tissues



Note. Very minimal significant change in $LY6C^{+}$ subsets were observed across intervention groups.

Figure 12

LY6G MDSC Cell Counts Represented Across All Tissues



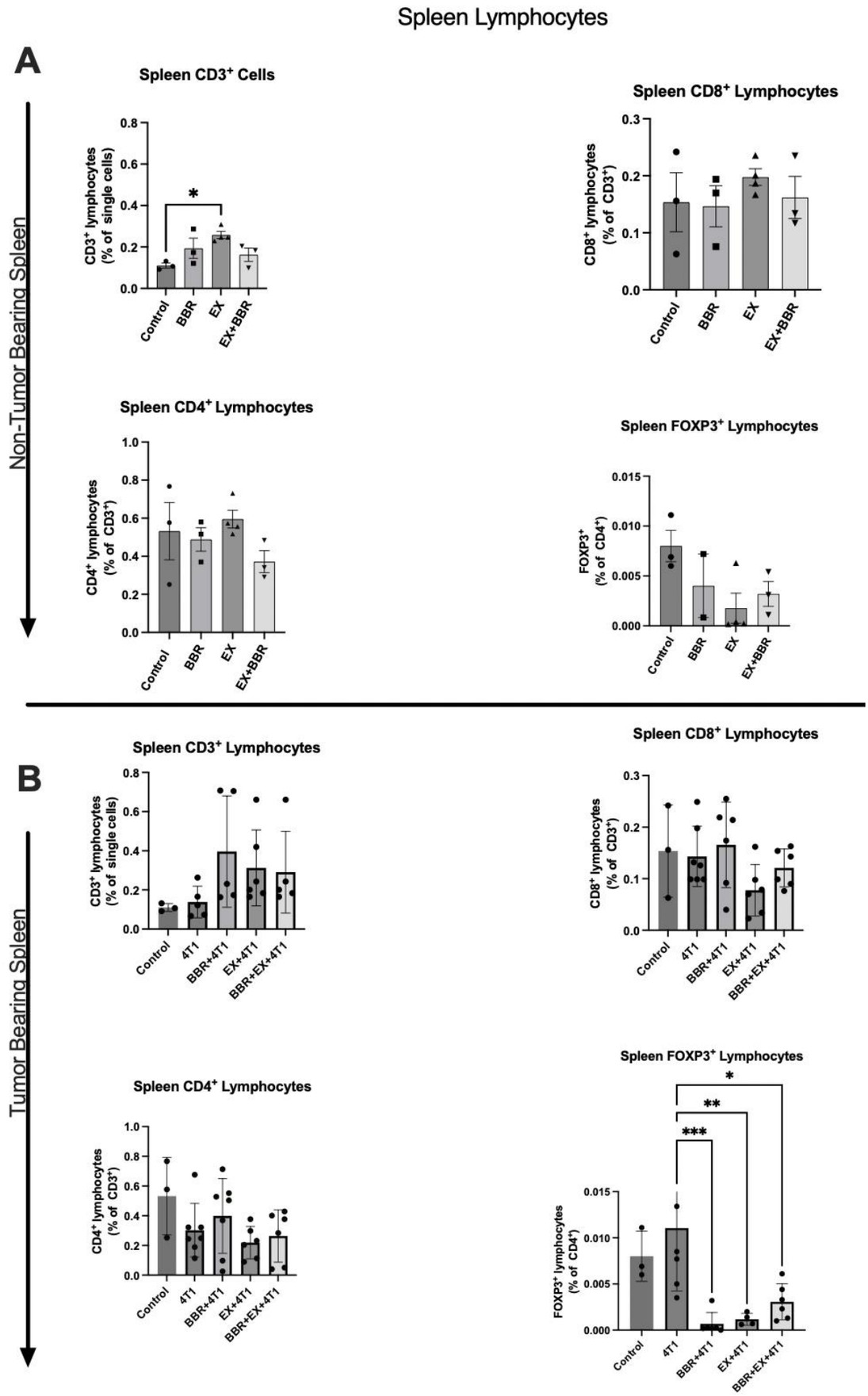
Note. Very minimal significant change in LY6G^{+} subsets were observed across intervention groups. However, there was a significant increase in LY6G subsets across most tissues between the non-tumor bearing and the tumor bearing animals. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

$CD3^+$ and $CD4^+$ T Effector Subsets

Various $CD3^+$ T cell phenotypes were assessed in the spleen, blood, bone marrow, and tumor of each intervention and control group. On week 10, 28 days post tumor inoculation the change in frequency of all $CD4^+$ cells remained relatively moderate across the tissue types with no statistically relevant findings suggesting a lack of intervention specific suppression and/or stimulation of T_h cells (figures 13 through 18). However, some $CD3^+$ intervention specific variance was noted between control and intervention groups. We compared the spleens of 4T1 sedentary controls to the spleen of tumor and non-tumor bearing EX mice resulting in a significant increase ($p < 0.05$) in $CD3^+$ cells residing in the spleens of non-tumor bearing EX mice (figure 13A). The same non-significant general trend was noted in the spleens of exercised tumor bearing animals. Within the same tissue, non-significant $CD3^+$ increase was also noted in both the BBR+4T1 and EX+4T1 groups when compared to both tumor bearing and non-tumor bearing (healthy) controls (figure 13 A and B).

Figure 13

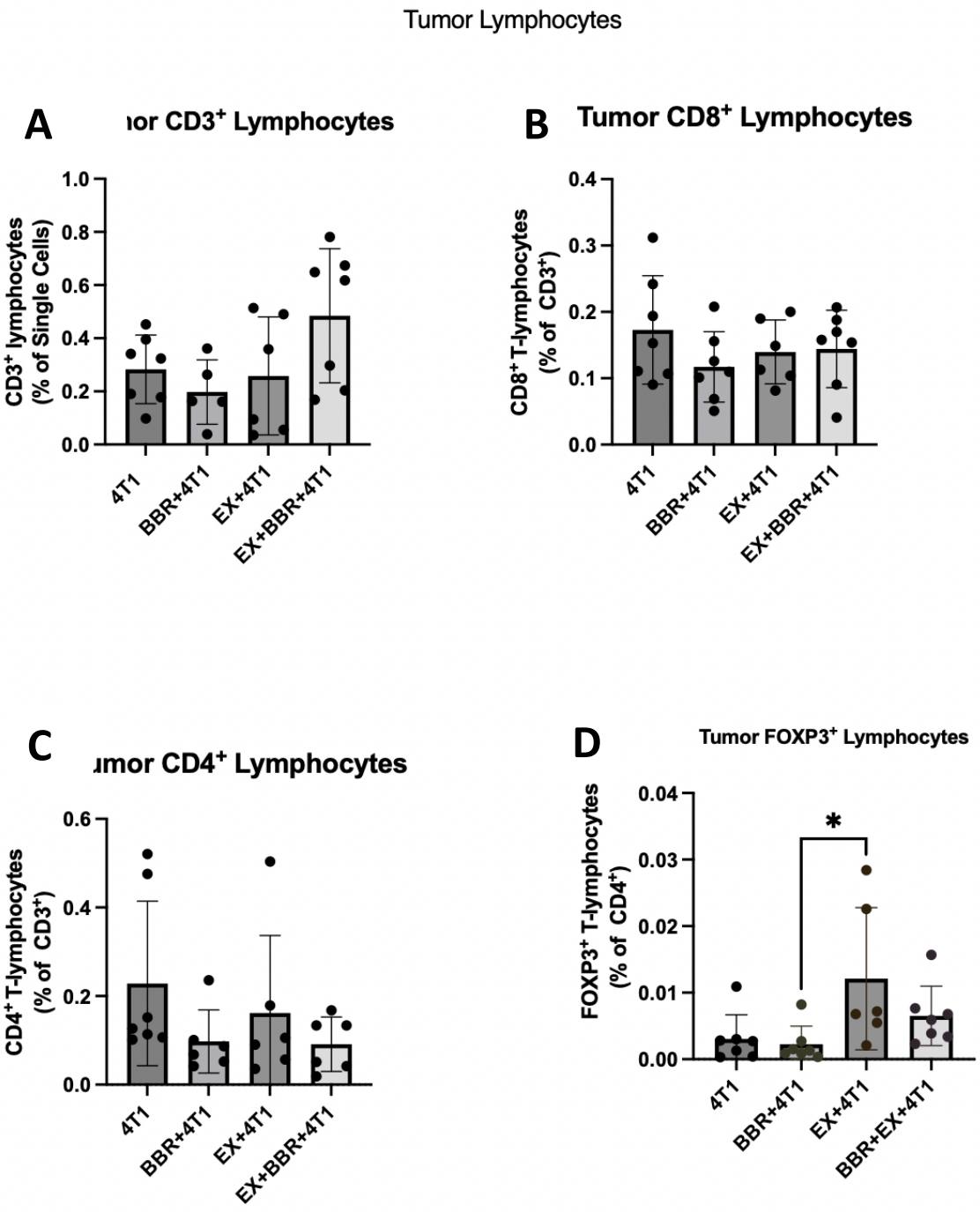
Frequency of Lymphocytes Within the Spleen of Tumor and Non-Tumor Bearing BALB/c Mice



Note. Lymphocytes isolated from splenic tissue in non-tumor bearing **(A)** and tumor bearing **(B)** animals. The presence of resident CD3+, CD8+, CD4+ and FOXP3+ lymphocytes ls is represented as frequency (%) ± SEM; n= 3-8 mice per group. *p<0.05.

Figure 14

Frequency of Lymphocytes Within Tumors of BALB/c Mice



Note. 4T1 mammary carcinoma cells (10^5) were injected into the right mammary gland of BALB/c mice and harvested after 28 days. The presence of $CD3^+$, $CD8^+$, CD4+ and $FOXP3^+$ lymphocytes isolated from tumor bearing animals is represented as frequency (%) ± SEM; n= 3-8 mice per group. *p<0.05, **p<0.01.

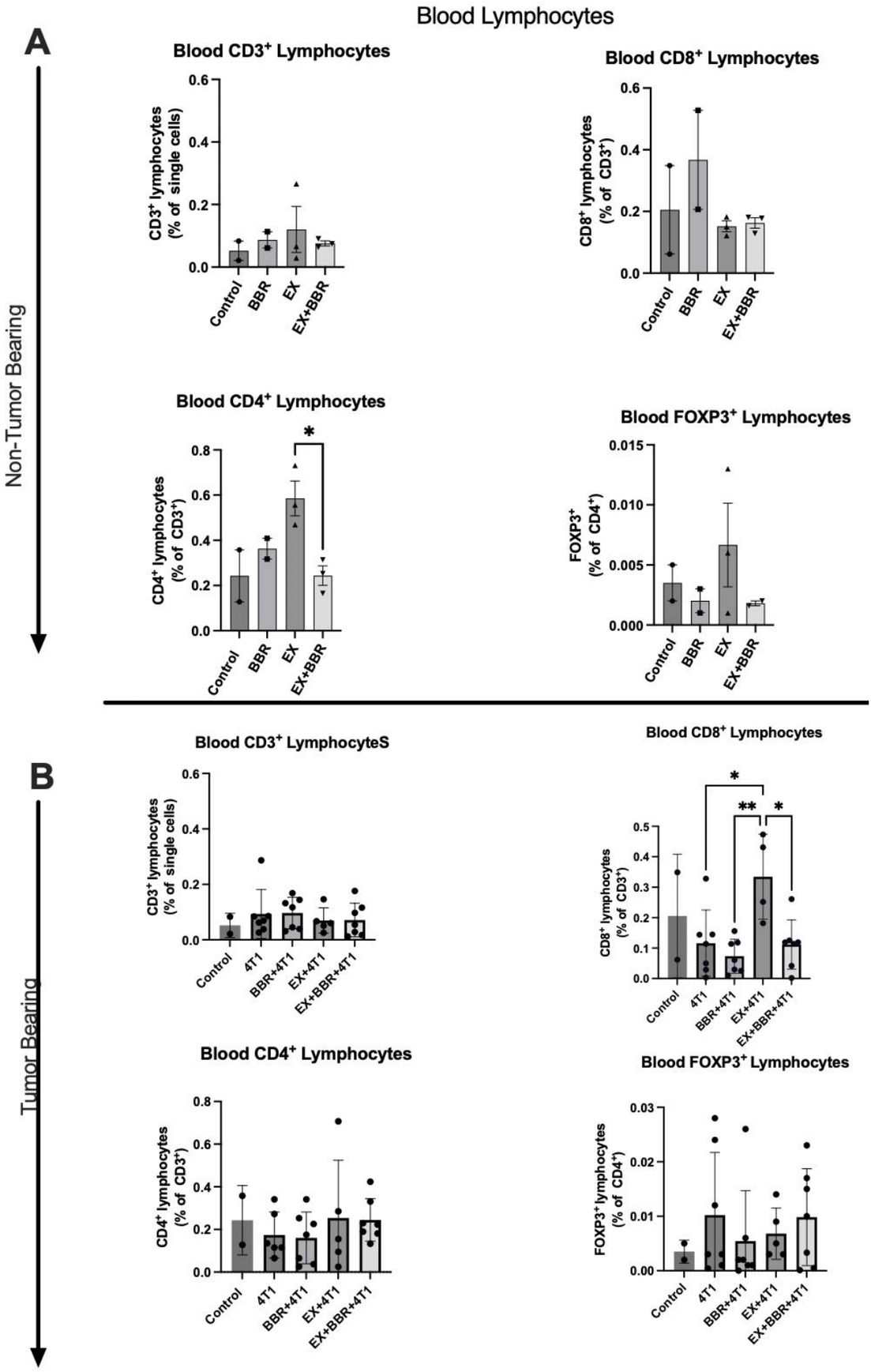
Resident and Circulating Cytotoxic T Lymphocytes

$CD8^+$ T lymphocytes were found to significantly increase within the blood of tumor bearing mice (figure 15B). This increase was observed specifically in tumor bearing wheel run mice when compared to blood taken from sedentary tumor bearing controls ($p<0.01$). Interestingly, when BBR was paired with wheel running in tumor bearing mice (BBR+EX+4T1), frequency of $CD8^+$ T cells circulating in the blood were found to decrease significantly ($p<0.05$).

The most significant variation across tissues was observed in $CD4^+$ $FOXP3^+$ subsets (figure 16 and 17) with the lungs exemplifying the greatest amount of variation.

Figure 15

Frequency of Lymphocytes Within the Blood of Tumor and Non-Tumor Bearing BALB/c Mice



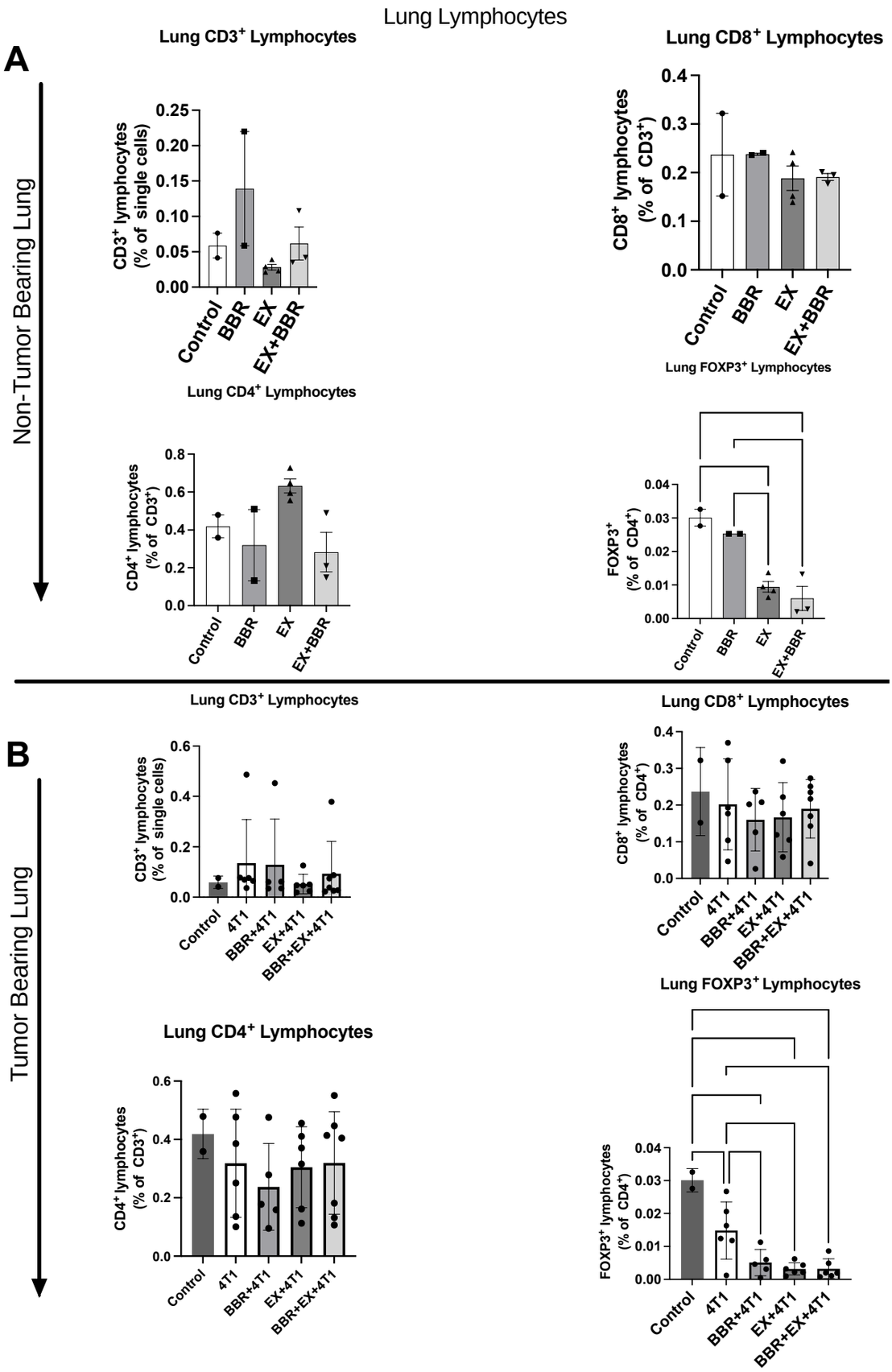
Note. Lymphocytes isolated from blood obtained via cardiac puncture in non-tumor bearing **(A)** and tumor bearing **(B)** animals. The presence of circulating $CD3^{+,}$ $CD8^{+}$, $CD4^{+}$ and $FOXP3^{+}$ lymphocytes is represented as frequency (%) ± SEM; n= 3-8 mice per group. *p<0.05.

Tissue Specific Infiltration of $CD4^+CD24^+$ $FoxP3^+$ T-regulatory Cells

EX+4T1 mice were observed to possess the highest frequency of tumor resident T_{regs}. Intriguingly, sedentary BBR+4T1 mice had significantly less T_{regs} within tumors than EX+4T1 mice ($p<0.05$) and when BBR and physical activity were administered together to tumor bearing mice (BBR+EX+4T1) the frequency of tumor resident T_{regs} decreased noticeably (figure 14). The tumors of EX mice were also approaching a significantly ($p=0.054$) greater number of T_{regs} than those found in sedentary 4T1 mice. Our studies found circulating Tregs at the highest concentration in the blood of sedentary 4T1 and non-tumor bearing EX animals (mean of 1.0% ± SD of all circulating $CD4^+$ cells). But this variation between groups was not found to be a significant increase when compared to a healthy or tumor bearing control respectively (fig 14). The highest frequency of T_{regs} was found within the lungs of control non-tumor bearing animals making up 3.0% ± SD of all $CD4^+$ expressing cells (figure 16).

Figure 16

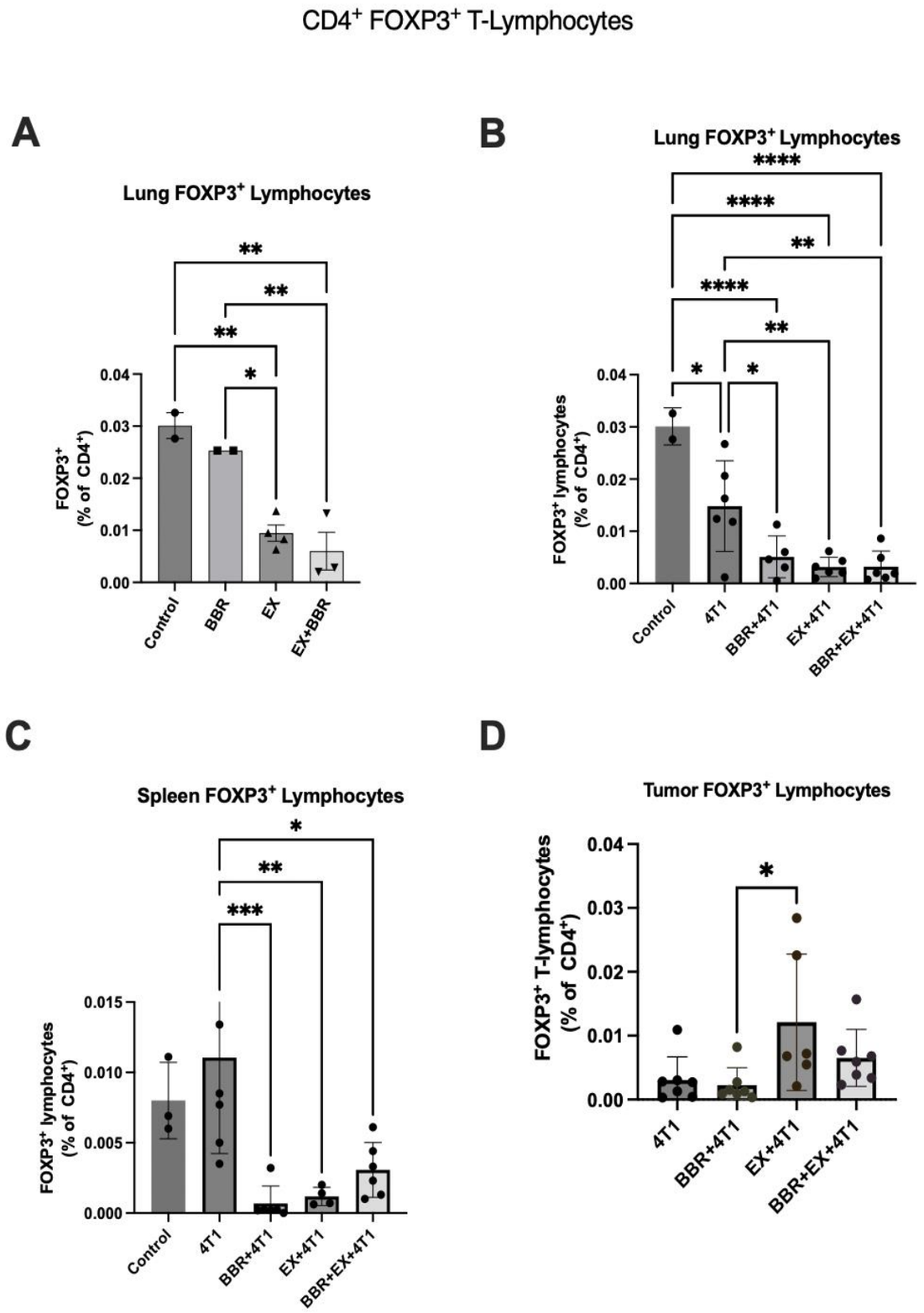
Frequency of Lymphocytes Within the Lung of Tumor and Non-Tumor Bearing BALB/c Mice



Note. Lymphocytes isolated from the lung via mechanical and enzymatic tissue dissociation in non-tumor bearing **(A)** and tumor bearing **(B)** animals. The presence of tissue resident $CD3^+$, $CD8^+$, $CD4^+$ and $FOXP3^+$ lymphocytes is represented as frequency (%) ± SEM; n= 3-8 mice per group. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

Figure 17

FOXP3 Specific Variation Within Various Tissues



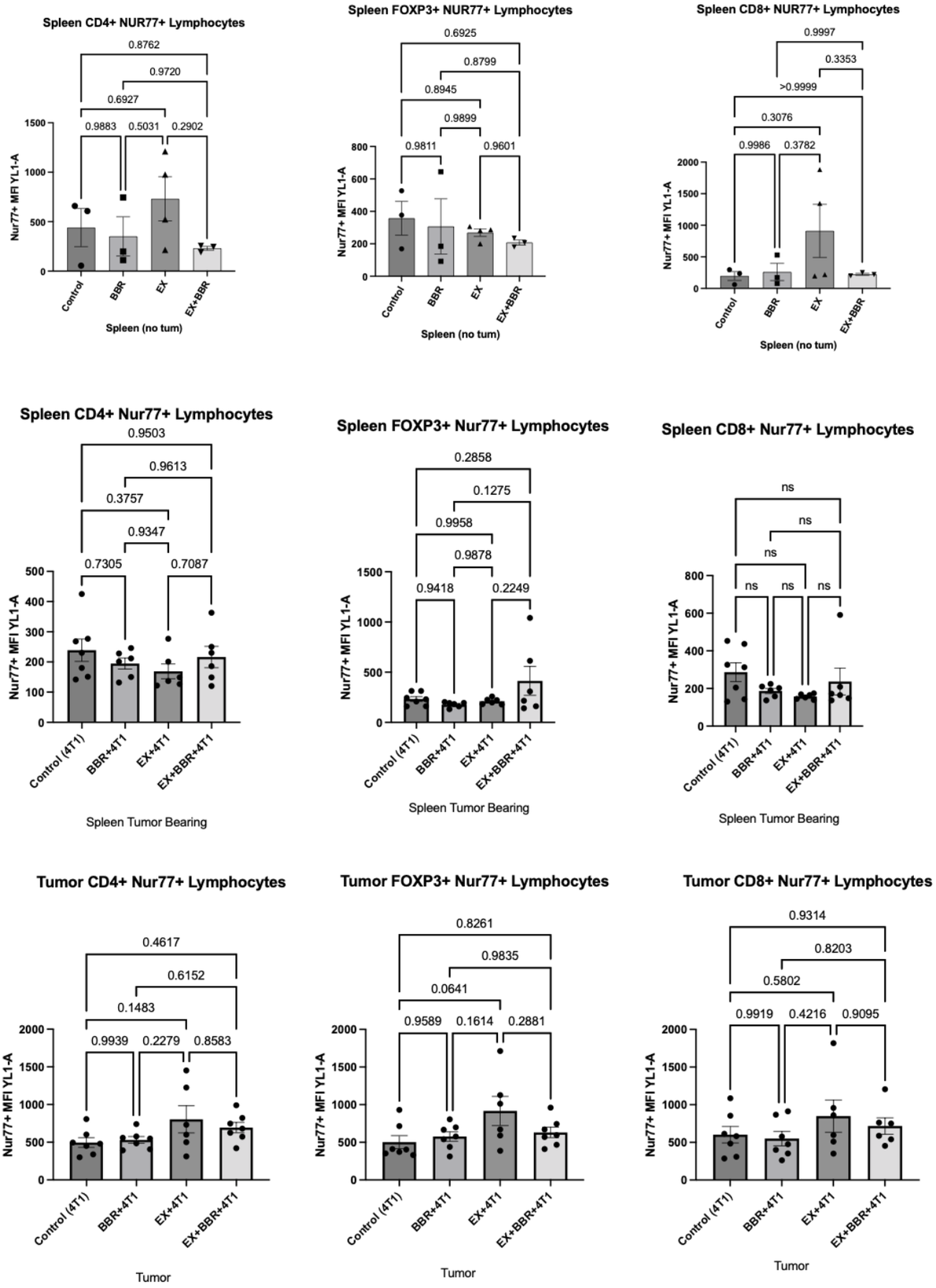
Note. Significant variation between $CD4^+CD24^+T_{reg}^+$ lymphocyte frequencies isolated from non-tumor bearing lung **(A),** tumor bearing lung **(B),** Tumor bearing spleen **(C)** and Tumor **(D).** The presence of tissue resident $FOXP3^+$ cells is represented as % of $CD4^+$ frequency ± SEM; n= 3-8 mice per group. **p*<0.05, ***p*<0.01, ****p*<0.001 and *****p*<0.0001.

Activation Status (Nur77)

Activation status was assessed for $CD4^+$, $CD8^+$, and $FOXP3^+$ lymphocytes in the spleen, tumor, lungs and blood, of all treatment and control groups (Figures 18 and 19). Modest variation was seen between groups with a notably higher expression of NUR77 in the tumor bearing groups than in the non-tumor bearing group comparatively (Figure 18). Statistical analysis utilizing one way ANOVA's and Tukey's multiple comparisons test suggested no significant variation between groups (figure 18).

Figure 18

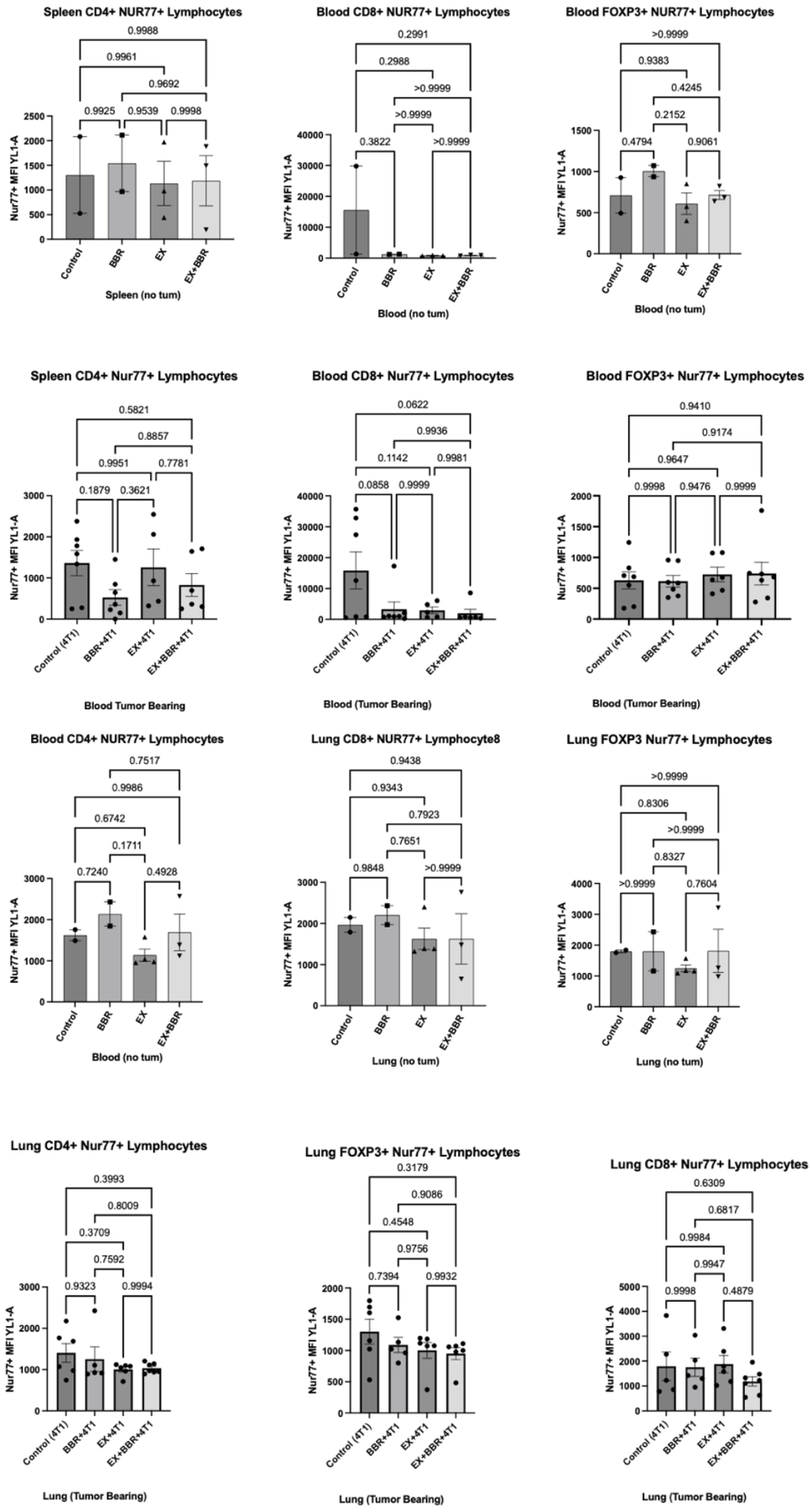
Nurr77 Expression Within the Spleen and Tumor of Each Control and Intervention Group



Note. Activation status measured in Mean fluorescence intensity (MFI) ± SEM. Non-significant treatment effects can be observed throughout each immune marker subset. *P* values represented above bracket.

Figure 19

Nurr77 expression within the blood and lung of each control and intervention group



Note. Activation status measured in Mean fluorescence intensity (MFI) ± SEM. Non-significant treatment effects can be observed throughout each immune marker subset. *P* values represented above brackets.

CHAPTER V

DISCUSSION AND CONCLUSIONS

Discussion

This study was one of the first to assess the combined impact of BBR and physical activity on immunological impact within a breast cancer model. It is also one of the first studies to explore exercise volume under BBR supplementation in tumor bearing and/or healthy subjects. The purpose of this study revolved around elucidation of the impact oral consumption of BBR when paired with physical activity may have on increasing T lymphocyte presence and activation status while decreasing the presence of MDSC's within the tumor microenvironment, spleen, bone marrow, and blood of both a healthy population of immunocompetent BALB/c mice and a BALB/c 4T1 mammary carcinoma model.

With increasing accessibility and utilization of BBR as an over-the-counter nutraceutical, as well as an herbal intervention with growing popularity in the medical and health realms it is important to understand the impact BBR may have on the immune system as well as how it might impact aspects of daily living such as physical activity. Physical activity has been extensively studied in the context of breast cancer, however BBR and its immunomodulatory effects are not as widely understood. The impact BBR may have on physical activity volume is of special interest to both healthy and diseased populations, as well as any utility it may have in prevention or co-treatment of breast cancer.

To evaluate overall immune function, both presence of specific T lymphocytes as well as activation status were assessed. Information on total $CD3^+$ T lymphocyte frequency along with

$CD8^+$ Cytotoxic, $CD4^+$ T-helper, and $CD4^+CD24^+FOXP3^+$ T-regulatory frequency and activation status in the blood, spleen, and lung of healthy and tumor bearing animals was gathered. We aimed to monitor metastasis to the lungs of tumor bearing animals in BBR and/or physical activity treatment groups while also considering the impact these treatments may have on spleen and tumor growth in both healthy and tumor bearing subjects.

Berberine May Impact Physical Activity and Wheel Running Volume

A marked decrease in wheel run volume could be seen in the weeks following 4T1 injection which may suggest discomfort when running from the developing tumor mass, or systemic inflammation leading to reduced energy levels. Normal healthy mice will run approximately 4-20 km/day with the greatest distance run in the first few weeks of being housed with a wheel and decreasing distances each subsequent week (Manzanares et al., 2019; Turner et al., 2005). Our data generally replicated this trend with some exceptions to this rule within the non-tumor bearing BBR supplemented groups. These subjects, on average, maintained wheel run volume than non-BBR treated non-tumor bearing mice. This phenomenon is noteworthy due to the fact that BBR is thought to function in a similar manner to metformin—a drug that is well known to decrease exercise volume in patients (Wong et al., 2012). It thus may be assumed that BBR would follow a similar pattern. However, our data suggested BBR may have a stabilizing effect on wheel running volume in mice. This possible conclusion will require further investigation as to whether BBR may have a metabolically favorable effect on exercise volume, over time, within a healthy population.

Metastasis to the Lungs

Fluorescent and luminescent cancer models have been used in the past to track tumor metastasis. However, to our knowledge, they have never been used in conjunction with BBR treatments. Luciferase expressing cancer cell lines are one example of a luminescent cell model. The genome of 4T1 mammary carcinoma cells may be manipulated to express fluorescent luciferase proteins, or the same cell line may be edited to transcribe other fluorescent proteins within their genome, such as TdTomato. 4T1 Mammary Carcinoma cell lines have been commonly used to assess metastasis to the lungs of mice (Takahashi et al., 2015). Dyes have also been used to differentiate healthy lung cells from infiltrating tumor cells, but the accuracy of this method is less than desirable (Garritson et al., 2020).

For the purpose of tracking metastasis to the lungs, red fluorescent protein (RFP) expressing cells were injected into the tumor groups. 4T1 cells have a special affinity for the lungs, which is why this specific tissue was chosen to represent metastatic variance among intervention groups (Pulaski & Ostrand-Rosenberg, 1998). Between treatment groups no significant change was observed that would suggest treatment specific effects.

However, a significant increase in MFI between the non-tumor bearing control lungs and tumor bearing lungs regardless of intervention was observed (figure 8). This suggests that the fluorescent 4T1 cells did indeed migrate to the lungs with the tdT protein intact. However, it appears that metastasis of the red 4T1's is not impacted by BBR, physical activity or a combination of the two. Alternatively, a greater number of 4T1 cells may have spread to the lungs but fluorescence was not observed because they had discarded the RFP from their genome through mutation.

Spleen and Tumor Mass

In ancient times BBR was utilized as a treatment for splenomegaly. In the modern era BBR is still claimed to treat this condition but there is very little evidence supporting these claims.

The data collected from the spleen and tumors of tumor bearing vs healthy mice revealed the BBR+4T1 group to exhibit the smallest fold change in spleen mass, but the largest fold change in tumor mass (figure 9). This could indicate that BBR was suppressing extramedullary hematopoiesis within the spleen. Extramedullary hematopoiesis is common in diseased states such as advanced tumor progression or viral infections like Epstein Barr Virus (Yang et al., 2020). This significant decrease in spleen mass may validate claims of BBR as an intervention for splenomegaly. However, the associated increase in tumor mass suggests that BBR may suppress the immune system to a point where it is not able to adequately respond to the challenge of a growing tumor. BBR may indeed remediate some of the effects of splenomegaly, but further research will be required to assess if the anti-inflammatory cost is too high for BBR to be utilized as an effective anti-inflammatory treatment in cancer patients.

Overall, EX did not appear to influence spleen or tumor mass. On average spleens from the BBR+EX+4T1 group experienced the largest fold change which resulted in less massive tumors (figure 9). This observation begs the question if exercise and BBR together increased immune function and extramedullary hematopoiesis through immune enhancing properties that neither BBR nor EX possess alone. Our immune marker data suggests that a slight increase in $CD3^+$ lymphocytes could be in part responsible for the increased spleen mass (figure 9). However, the lack of significant increase of other immune cell groups within the spleen may

suggest that other non-$CD3^+$ cells are being produced and leading to significant increase in spleen mass variation from the control.

Myeloid-Derived Immune Populations

$LY6C^+LY6G^{low}$ and $LY6G^+LY6C^{low}$ cells are considered monocytic and granulocytic, respectively (Höchst et al., 2015). These cells have been found to exert immunosuppressive effects on T cell populations. MDSC'S are immature monocytic cells that arise within the body in states of extreme inflammation, cancer, or pregnancy (Höchst et al., 2015; Youn et al., 2008). Some studies suggest that MDSC expansion is influenced by soluble factors produced by the tumor microenvironment (Youn et al., 2008) This would explain the well elucidated increase seen within MDSC's in tumor bearing animals compared to non-tumor bearing animals (Garritson et al., 2020; Youn et al., 2008). This overall expansion of MDSC's may also account for splenic expansion observed within tumor bearing mice (figure 9). One observation that has not been well elucidated is the expansion of $LY6G^+$ MDSC's in the spleens of tumor bearing mice (figure 12). Our study found $LY6G^+$ MDSC's to be larger in size than LY6C. Thus, it leads to the hypothesis that an expansion of $LY6G^+$ cells would significantly increase spleen mass. Interestingly, the spleens of the sedentary, tumor bearing animal that received BBR supplementation were the smallest of the tumor bearing spleens (figure 12). If MDSC expansion accounted for the additional splenic mass observed in tumor bearing spleens, we would expect that $LY6G^+$ MDSC expansion would increase. But this was not the case. Rather, there was a non-significant increase in $LY6G^+$ count within the spleen of this treatment group but the spleens of this group were on average smaller than relative tumor bearing controls that did not receive BBR. Another reason for the spleen size reduction within the 4T1+BBR group could be mobilization of MDSC's from the spleen into the blood. Within the blood of tumor bearing

exercised animals there was a significant increase in $LY6G^+$ cells compared to a non-tumor bearing control, however, there was not a significant decrease in this subset from 4T1 controls. Within the lungs of tumor bearing animals there is an increase in LY6G as well as T_{regs}. Interestingly, there is an established connection between MDSC and Treg cross talk in contributing to suppression of anti-tumor immunity (Siret et al., 2020).

This does not follow the trend of decreased total circulating and tumor resident MDSC's seen in other studies where exercise was found to decrease MDSC counts in the blood and spleen of tumor bearing animals (Garritson et al., 2020). However, these studies assessed total MDSC count at various time points and did not evaluate the specific subsets. Reduction in total MDSC count was observed in the blood at day 16, however this trend was not maintained up to day 28 (Garritson et al., 2020). Our described study only measured MDSC counts on day 28 which may have impacted intervention specific effects.

Lymphoid Derived Immune Populations

Very little statistical significance was observed within Lymphoid populations across the tissue types. This may indicate that by day 28, when mice were euthanized, BBR and EX had reduced impact on the frequency of immune cells within a cancer population. However, the same trend was seen within a healthy population with only minimal increase or decrease among specific immune markers. This may suggest that BBR and exercise had minimal statistically relevant impact. However, a generalized non-significant decrease was observed within BBR treated $CD4^+$ T cells across each of the tumor bearing tissue groups and the same trend was observed within $CD8^+$ T cells excluding a significant $CD8^+$ increase in tumor bearing tissues. These findings align with the discoveries of Turbitt et al., who observed a significant decrease in $CD4^+$ and $CD8^+$ T cells within physically active tumor bearing mice (Turbitt et al., 2019).

Alternatively, the lack of significance could be owing to the fact that at time of sacrifice, the tumors had aggressively progressed, and T cell exhaustion could have played a role in decreasing the frequency and activity of these T effector subsets. Studies suggest that $CD8^+$ T-cells may become exhausted within a cancer patient due to persistent antigen recognition leading to over stimulation of the TCR and long-term activation (Kwon et al., 2022). This exhausted state leads to progressive loss of function and proliferation lowering numbers of T effector subsets (Kwon et al., 2022).

Wheel running appeared to have the most significant effect on $CD3^+$ cells. A significant increase from baseline in the spleen of wheel run non-tumor bearing animals was observed on day 28 of the study. However, this $CD3^+$ increase did not translate into increased $CD4^+$ or $CD8^+$ counts within healthy wheel run mice. This begs the question of what may be causing splenic growth if not a T effector subset. MDSC expansion could be one answer to this question, increased production of blood cells to meet the increased demands of tumor growth is also theorized.

It is well understood that healthy animals will not produce high numbers of $CD8^+$ T cells; this means that increased $CD8^+$ frequency within overall residential or circulating T cell numbers may indicate an active immune response against viral infection, or cancer. Modest statistically relevant fluctuations in frequency of circulating $CD8^+$ T cells were found in the blood of tumor bearing animals (figure 6). The greatest increase compared to a baseline tumor bearing control group was seen in the EX+4T1 group. These findings align with the findings of Garritson et al., who observed suppression of $CD3^+CD8^+$ T cells by MDSC's to be ameliorated within a physically active tumor bearing population (Garritson et al., 2020).

T Regulatory Cells in the Context of Berberine, Physical Activity and Cancer

The airways and lungs are considered an external surface with robust innate and adaptive defenses against a constant stream of external allergens and pathogens. In response to these external challenges, the lungs are immunologically very active and T_{regs} are of vital importance for maintaining homeostatic control and preventing excessive inflammatory responses. Furthermore, as stated before, 4T1 cells have a specific affinity for the lungs and are a common site for metastasis making the pulmonary space an environment rich in T_{regs} (Pulaski & Ostrand-Rosenberg, 1998).

The tumor microenvironment, regardless of tissue, can evade immunosurveillance by hijacking the function of immunosuppressive cells such as T_{regs}. Increased T_{reg} function within a tumor may foster a favorable environment for tumor growth and metastasis by effectively hiding the neoplasm from adaptive immune response. The concept of decreasing T_{reg} presence and function within a tumor without dampening the overall impact of Tregs in other bodily tissues is of the utmost value.

Intentional alteration of T_{reg} function would revolutionize immunotherapy. T_{regs} are central in the theoretical ability to manipulate immunological homeostasis within the body as well as the tumor microenvironment (Huang et al., 2004). Our study isolated $CD4^+CD24^+T_{reg}^+$ cells by gating for $FOXP3^+$ events within a $CD4^+$ positive population.

In line with this concept of immunity in the lungs, the overall greatest variation in T_{reg} frequency could be observed in the lungs of both healthy and tumor bearing animals (figure 16). Across the tissue types BBR and EX, when administered separately, seem to have the greatest influence on T_{reg} frequency (figure 17). But these interventions showed opposite impacts across the tissue types with BBR generally decreasing T_{reg} frequency and EX often increasing T_{reg}

frequency. This non-significant increase could be attributed to the fact that physical activity increases respiration and therefore the number of foreign particles inhaled may cause an inflammatory response.

EX+4T1 mice were also observed to possess the highest frequency of tumor resident T_{regs} (figure 8D). Intriguingly, sedentary BBR+4T1 mice (figure 8D) had significantly less T_{regs} within tumors than EX+4T1 mice ($p < 0.05$) and when BBR and physical activity were administered together within tumor bearing mice (BBR+EX+4T1) the frequency of T_{regs} decreased noticeably suggesting BBR may ameliorate some of the impact of exercise on T_{reg} accumulation within the tumor (figure 14). BBR alone within a tumor did not appear to vary in effect from the 4T1 control group. This suggests that BBR alone may exert minimal influence on T_{reg} infiltration into the tumor microenvironment. However, physically active animals did see a T_{reg} increase approaching significance ($p=0.054$) of T reg infiltration into the tumor compared to T_{regs} in the tumor of a sedentary 4T1 mouse (figure 14). The *P* value for the comparison of EX+BBR to 4T1 tumors was well over 0.05 which may confirm the idea that BBR reduces the impact of EX on T_{reg} infiltration into the tumor. This is an important finding to further explore because it would allow cancer patients to experience greater immunological benefits from consistent physical activity.

Furthermore, when physically active tumor bearing spleen T_{reg} frequency is compared to T_{reg} frequency within a physically active tumor group an inverse relationship of increased frequency within the tumor and decreased frequency within the spleen can be observed. However, T_{reg} function within the tumor of BBR treated mice vs non-BBR treated mice showed a slight non-significant difference within the BBR treated mice (figure 14D). Within the spleen, when BBR and EX were administered separately, there was a significant decrease in T_{reg}

frequency within the control spleen T_{reg} numbers (figure 13). Treg function in the presence of BBR and physical activity is conflicted, warranting more investigation to elucidate both the frequency and function of Tregs under the influence of BBR in various tissues. These findings again point to the suggestion that BBR may not influence immune response when taken orally by healthy or cancer patients.

Bioavailability of Orally Administered Berberine

It is important to note when considering the impact of BBR within the immune system that most studies utilize findings that were derived from research methods that have exclusively administered BBR via routes dissimilar to the oral consumption of BBR seen within the lay public. Route of administration is an important consideration in BBR supplementation because many sources have recognized BBR as significantly hydrophobic with an oral bioavailability of approximately 1% (Feng et al., 2015). As is similar for many orally administered drugs (L. Zhang et al., 2021) to be absorbed across the epithelial brush, border BBR must be first converted via microbial metabolism into dihydroberberine. After entering the bloodstream, dihydroberberine is oxidized back into its original form (Feng et al., 2015). The low bioavailability and required microbial metabolism of BBR requires that more research be conducted with orally administered BBR. The low bioavailability may also influence its seemingly low impact on immune marker frequencies as well as activation status and tumor metastasis.

The anti-microbial properties of BBR lead to another supposition for why very little BBR specific significance was seen across treatment groups. As stated above, BBR requires microbial metabolism in the gut for absorption. Within the functional medicine community, health care practitioners often prescribe BBR cyclically, with patients consuming the compound for a few

weeks before engaging in a short wash-out period to allow the gut microbiome to replenish. However, if BBR was reducing microbial activity within the gut, its absorption rate over time may have been slowed. By day 28 the anti-microbial effects of BBR could have significantly reduced the impact that BBR had on immune markers in the later stages of tumor development by reducing absorption rate through the intestinal epithelium. Later studies may find it advantageous to investigate pairing a probiotic with BBR supplementation.

This study was limited by small sample sizes within control groups that may have reduced the significance of possible variation between treatment groups. Further investigation is warranted to confirm that BBR does indeed impact T_{reg} infiltration into tissue specific environments as well as any impact EX+BR may have on $CD8^{+}$ frequency within the blood of tumor bearing animals. Furthermore, it is of special interest to elucidate the possibility of BBR's impact on sustained long term physical activity outside of a cancer context.

Conclusions

In the presented study we hypothesized that subjects treated with this combination of voluntary oral BBR and voluntary physical activity will have reduced MDSC counts, and increased T cell infiltration and activation in tissues of interest for both effector, and regulatory subsets. Overall, very little intervention specific effect was observed within immune populations across tissue types. However, T regulatory lymphocyte FOXP3 was also assessed and found be significantly altered in the lungs of tumor-bearing mice, suggesting BBR paired with physical activity may have significant immunological implications on T_{reg} subset presence in various tissues. Observations of MDSC infiltration into the spleen, lung, and tumor, as well as circulating MDSC's and production within the bone marrow showed minimal intervention specific effects. There were non-significant increases in MDSC's within the lungs of tumor bearing mice treated

with BBR. It is interesting to note that both Tumor bearing animals treated with BBR had minimal increase of Tregs and MDSC's in the lungs. Tregs and MDSC's are known to participate in crosstalk, which contributes to suppression of anti-tumor immunity. Whether this suggests a BBR specific effect within the lungs of tumor bearing animals will require more research.

Our findings suggest that BBR may help decrease infiltration of T_{regs} into the tumor microenvironment. Furthermore, the findings of this study suggest a possible relationship between BBR and sustaining consistent long term exercise volume. The general lack of effect seen within BBR and BBR+EX intervention groups suggests that BBR may have a very minimal immunological influence when taken by a healthy subject.

These findings will directly inform those who practice complementary medicine and nutraceutical supplementation, an area lacking in immunological analysis, including raising concerns for the impact on aberrant, potentially undesirable T_{reg} function.

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APPENDIX A

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL

UNIVERSITY OF
NORTHERN COLORADO

Institutional Animal Care and Use Committee

Date: January 15, 2021

Principal Investigator: Dr. Nicholas Pullen

Committee Action: **IACUC Protocol- Amendment Approval**
Action Date: January 14, 2021

Protocol Number: 2005C-NP-M-23
Protocol Title: *A Primary Source of Immune System Tissues*

Expiration Date: May 12, 2023

The University of Northern Colorado Institutional Animal Care and Use Committee (IACUC) APPROVED your amendment (addition of personnel) to animal use protocol "*A Primary Source of Immune System Tissues*"– 2005C-NP-M-23. All requested changes are incorporated into this protocol effective January 14, 2021

The committee's review was based on the requirements of the Government Principles, the Public Health Policy, the USDA Animal Welfare Act and Regulations, and the Guide for the Care and Use of Laboratory Animals, as well as university policies and procedures related to the care and use of animals at the UNC. Based on the review, the IACUC has determined that all review criteria have been adequately addressed. The PI/PD is approved to perform the experiments or procedures as described in the amendment request as approved by the committee.

If you have any questions, please contact the Animal Care and Use Program (ACUP) Director, Laura Martin, at 734-730-6631 or via e-mail at laura.martin@unco.edu. Additional information concerning the requirements for the protection and use of animal subjects at UNC may be found at the ACUP website, https://www.unco.edu/research/research-integrity-and-compliance/iacuc/, or at the Office of Laboratory Animal Welfare website, https://olaw.nih.gov/.

Sincerely,

Laura W. Martin
Director of Compliance and Operations
Animal Care and Use Program

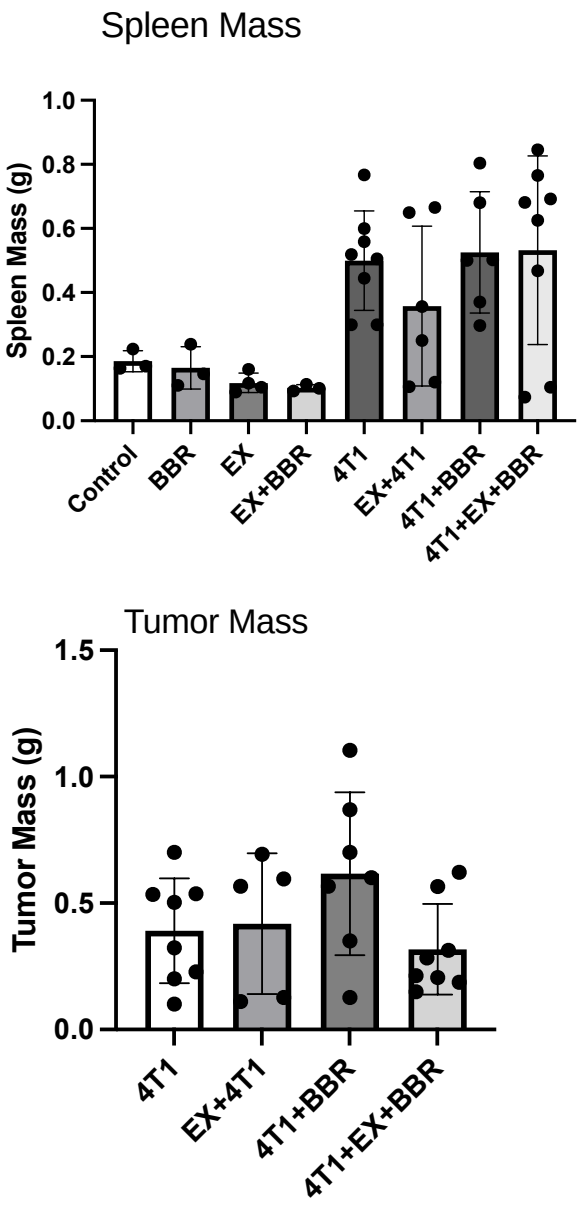
OLAW Assurance: D16-00579
USDA Registration: 84-R-0008

APPENDIX B

SUPPLEMENTAL FIGURES

Figure 20

Mean spleen and tumor mass represented in grams ± SD across treatment group.



Note. Spleen mass, represented as fold change over respective non tumor control ± SEM. Tumor mass, represented in fold change from 4T1 no treatment control in both tumor and non-tumor-bearing animals. ± SEM *p<0.05, **p<0.01.