THE ROLE OF IMMUNE EFFECTOR CELLS IN THE 4T1.2 MURINE MAMMARY TUMOR MODEL

A Thesis in
Nutritional Sciences
by
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ABSTRACT

Breast cancer is the most commonly diagnosed cancer among women and the second leading cause of cancer related death in the US (1). Despite recent advances made in breast cancer research, triple negative breast cancer (TNBC) remains the subtype with the poorest prognosis due to limited treatment options. The 4T1 murine mammary metastatic model has been widely used as a model for TNBC, because of the similarities in hormone receptor profile to human TNBC (2). One caveat of the 4T1 model is that there are few known tumor associated antigens which has limited the use of this model in preclinical immunotherapeutic studies (3, 4). The firefly luciferase gene, *luc2*, has been transfected into various tumor cell lines as a reporter gene for imaging studies. However, previous studies demonstrate that the luciferase epitope can induce CD8+ cytotoxic T cell specific responses, thus may be serving as a tumor antigen for immune recognition (5-7). The goal of the current study was to explore the role of luciferase in 4T1.2 cells, a clone that was selected for greater metastatic potential, and a model used to evaluate nutrition and exercise interventions in our laboratory. Specifically, we compared *in vitro* and *in vivo* growth rates, the role of immune components in tumor growth, metastatic progression and survival rate in the parental 4T1.2 and luciferase transfected 4T1.2 clone (4T1.2\textsuperscript{lux}).

The goal of the first study was to determine if luciferase expression by the 4T1.2\textsuperscript{lux} cell line altered the *in vitro* proliferative capacity compared to the parental 4T1.2 cell line. We cultured both 4T1.2 and 4T1.2\textsuperscript{lux} mammary tumor cell lines
(2,500 cells/well in serial dilution) in triplicate for 72 hours, and assessed in vitro proliferation using the MTS assay. The proliferation rate of 4T1.2\textsubscript{luc} cell line did not differ significantly from 4T1.2 cell line, which indicated luciferase expressed by the 4T1.2\textsubscript{luc} cell line is not actively involved in signaling cascades mediating cell proliferation.

In the second study, we examined in vivo growth rates of 4T1.2 and 4T1.2\textsubscript{luc} cells in immunocompetent BALB/c mice. We demonstrated that 4T1.2\textsubscript{luc} tumor-bearing mice had a significantly reduced primary tumor growth compared to 4T1.2 tumor-bearing mice. Next, we wanted to determine which immune subtype was responsible for the delayed tumor growth observed in 4T1.2\textsubscript{luc} tumor-bearing mice, and if any of these immune population were important in controlling metastatic progression or survival. To this end, we depleted the major effector immune cell populations, CD4\textsuperscript{+} T cells, CD8\textsuperscript{+} T cells and NK cells, in both 4T1.2 and 4T1.2\textsubscript{luc} tumor-bearing animals. We observed that CD4\textsuperscript{+} T cell, CD8\textsuperscript{+} T cell and NK cell depletion did not affect primary tumor growth in 4T1.2 tumor-bearing mice. However, CD8\textsuperscript{+} T cell depletion increased primary tumor growth and NK cell depletion decreased primary tumor growth in 4T1.2\textsubscript{luc} tumor-bearing mice. Metastatic burden in the lung and femur was not significantly different among isotype control, CD4\textsuperscript{+} T cell-depleted and CD8\textsuperscript{+} T cell-depleted, and NK cell-depleted 4T1.2\textsubscript{luc} tumor-bearing mice.

The final aim of this study was to determine the role of CD4\textsuperscript{+} T cells, CD8\textsuperscript{+} T cells and NK cells in the survival of 4T1.2 and 4T1.2\textsubscript{luc} tumor-bearing mice. We
observed that $4T1.2^{\text{luc}}$ tumor-bearing mice treated with PBS or the isotype control antibody (LTF-2) had a significantly longer median survival than PBS or isotype control antibody-treated $4T1.2$ tumor-bearing mice. In $4T1.2$ tumor-bearing mice, CD4$^+$ T cell, CD8$^+$ T cell and NK cell depletion did not alter median survival. However, CD4$^+$ T cell, CD8$^+$ T cell depletion reduced median survival in $4T1.2^{\text{luc}}$ tumor-bearing mice in comparison to control groups.

In conclusion, luciferase expression in the $4T1.2^{\text{luc}}$ cell line did not alter the in vitro proliferation rate but potentially served as an antigen for immune cell recognition. In $4T1.2^{\text{luc}}$ tumor-bearing mice, CD8$^+$ T cells are important in controlling primary tumor growth. Depletion of CD4$^+$ T cells, CD8$^+$ T cells and NK cells did not significantly affect lung and femur metastatic burden. However, both CD4$^+$ T cell and CD8$^+$ T cell populations are important for survival. In contrast, the absence of CD4$^+$ T cells, CD8$^+$ T cells or NK cells did not significantly influence primary tumor growth or survival in $4T1.2$ model. Results from the current study provide important information about the interaction between host immune components and $4T1.2$ and $4T1.2^{\text{luc}}$ tumor cells.
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<thead>
<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>2.43</td>
<td>Anti-mouse CD8α monoclonal antibody</td>
</tr>
<tr>
<td>4T1</td>
<td>Murine metastatic breast cancer model</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>Asialo GM1</td>
<td>Anti Asialo ganglio-N-tetraosylceramide polyclonal antibody</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BRCA</td>
<td>Breast cancer susceptibility gene</td>
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<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CLP</td>
<td>Common lymphoid progenitor</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T-lymphocyte-associated protein 4</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FoxP3</td>
<td>Forkhead box O3</td>
</tr>
<tr>
<td>GK1.5</td>
<td>Anti-mouse CD4 monoclonal antibody</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epidermal growth factor 2</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
</tr>
<tr>
<td>HR</td>
<td>Hormone receptor</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IDO</td>
<td>Indoleamine 2,3-dioxygenase</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G, isotype control</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LTF-2</td>
<td>Rat IgG2b Isotype Control</td>
</tr>
<tr>
<td>MDSC</td>
<td>Myeloid-derived suppressor cell</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloprotease</td>
</tr>
<tr>
<td>MMTV</td>
<td>Murine mammary tumor virus</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>NKG2D</td>
<td>Natural-killer group 2, member D</td>
</tr>
<tr>
<td>NLR</td>
<td>Nod-like receptor</td>
</tr>
<tr>
<td>ORR</td>
<td>Objective response rate</td>
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<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PD-1</td>
<td>Programmed cell death protein-1</td>
</tr>
<tr>
<td>PD-L1</td>
<td>Programmed death-ligand 1</td>
</tr>
<tr>
<td>PFS</td>
<td>Progression-free survival</td>
</tr>
<tr>
<td>PGE</td>
<td>Prostaglandin E</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>PRR</td>
<td>Pathogen recognition receptor</td>
</tr>
<tr>
<td>PTHrp</td>
<td>Parathyroid hormone-related protein</td>
</tr>
<tr>
<td>RLR</td>
<td>RIG-like receptor</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SHBG</td>
<td>Sex hormone binding globulin</td>
</tr>
<tr>
<td>TAA</td>
<td>Tumor-associated antigens</td>
</tr>
<tr>
<td>TAM</td>
<td>Tumor-associated macrophage</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TERT</td>
<td>Telomerase reverse transcriptase</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TH</td>
<td>Helper T cell</td>
</tr>
<tr>
<td>TIL</td>
<td>Tumor infiltrating lymphocyte</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TME</td>
<td>Tumor microenvironment</td>
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</table>
TNBC  Triple-negative breast cancer
TNF\(\alpha\)  Tumor necrosis factor alpha
Treg  Regulatory T cell (CD4\(^{+}\)CD25\(^{+}\)FoxP3\(^{+}\) TH cells)
VEGF  Vascular endothelial growth factor
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CHAPTER 1
LITERATURE REVIEW

1.1. Breast Cancer

1.1.1. Overview of Mammary Glands

Mammary glands are composed of various types of cells including epithelial cells, adipocytes, fibroblasts, vascular endothelial cells and immune cells (8, 9). Histologically, mammary tissue contains epithelium and stroma. Mammary epithelium is primarily composed of luminal, basal epithelium and a small population of stem cells. The inner luminal layer of epithelium faces a central apical cavity and is surrounded by outer basal myoepithelial cells (10, 11). Morphologically, the mammary gland consists of 15 to 20 lobes, each lobe is made up of milk production units called lobules that are built up by ductal and alveolar luminal epithelial cells (12). Growth and development of the mammary gland is a complex process that is tightly controlled by hormones and growth factors, which induce cell differentiation and proliferation (13). For example, postnatal development is regulated by growth hormone and insulin-like growth factor-1 (IGF-1) and estrogen plays an important role in breast development during puberty. After reaching adulthood, progesterone is responsible for alveologensis that prepares the breast tissue for pregnancy and lactation (9). Mammary gland epithelium undergoes dramatic morphogenetic changes over
the lifespan. Exposure to carcinogens or/and genetic predisposition can disrupt intracellular signaling cascades that could trigger tumorigenesis (11).

1.1.2. Breast Cancer

1.1.2.1. Overview

Breast cancer is the most common diagnosed cancer among women in the US and the second leading cause of cancer related death (1). It is estimated 266,120 new cases of breast cancer (15.3% of all new cancer cases) and 40,920 breast cancer deaths (6.7% of all cancer deaths) are to occur among US women in 2018 (1, 14). Breast cancer, originates from the epithelial cells of the milk ducts, is a heterogeneous disease in terms of histology, response to treatments, metastatic patterns, prognosis and clinical outcome (15). Molecular subtypes of breast cancer are primarily based on the expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor 2 (HER2 also known as ERBB2), which can provide important information in treatment selection and prognosis (16-18). These subtypes include luminal A (ER+, PR+), luminal B (ER+, PR+, HER+), HER2+ (ER+, PR-, HER2+) and Basal-like (Triple-negative). Luminal A has the best prognosis mainly because this subtype is less aggressive and more responsive to endocrine therapy, followed by luminal B subtype (19-21). HER2+ subtype is negative in hormone receptors expression but overexpresses human epidermal growth factor 2 (HER2+), which has been
exploited for targeted therapy development (21-23). Basal-like subtype is usually considered a synonym of the triple-negative subtype (TNBC), and lack expression of sex hormone receptors and HER2. Treatment options are limited to conventional chemotherapy and radiation therapy and has the poorest prognosis relative to other subtypes (21, 23, 24). TNBC subtype itself is a group of heterogeneous tumors that can be, based on molecular expression profile, further classified into basal-like, mesenchymal, luminal androgen receptor, and immune-enriched subtypes. Subclassification of TNBC provides crucial information that may be important in determining the most effective treatment and in exploring new therapeutic targets (25).

### 1.1.2. Grade and Stage of Breast Cancer

Tumor grade is a pathological method of tumor classification which indicates the tendency of tumor cells to grow and spread based on the resemblance of tumor cells appearance compared to the tissue of origin (26). In general, tissues obtained from biopsy or surgical resection are graded as G1 through G4, with G1 tumor cells being low grade and well differentiated meaning, while G4 tumor cells being poorly differentiated or undifferentiated that are morphologically abnormal (tubule formation, nuclear morphology, mitotic figures etc.) and may lack normal tissue structure (26). The most widely used grading system for breast cancer is Nottingham grading system (27, 28), which classifies tumors into three grades namely, low (Grade I), intermediate (Grade II), high
(Grade III) based on differentiation or tubule formation, pleomorphism (shape and size of tumor cells), hyperchromatic and mitotic nuclei (28).

1.1.2.3. Risk Factors

The etiology of breast cancer usually involves combinations of multiple contributing factors (29, 30). Population based epidemiological studies have identified a number of breast cancer risk factors including age, age at menarche and menopause, age at first pregnancy, family history, lifestyle factors (diet, weight, alcohol intake), smoking and hormone replacement therapy etc. (31, 32).

Breast cancer risk factors mentioned above can be classified into nonmodifiable and modifiable (or lifestyle related) risk factors (29). The nonmodifiable risk factors include age, sex, family and personal BC history, race and ethnicity, genetic alterations, higher breast density, early menstruation or late menopause and environmental exposures such as ionizing radiation (29, 33). Among these factors, age is the most significant non-modifiable risk factor. Female breast cancer is most frequently diagnosed in women aged from 55 to 64 years old with the median age of 61-years-old (14). Being female significantly increases the risk of breast cancer as less than 1% of all breast cancer cases are diagnosed in males (34). Family history, especially having a first-degree relative (mother, sister or daughter) with breast cancer is associated with a two to three fold increase in breast cancer risk (35).
Disparities of breast cancer occurrence among ethnic groups are significant: i.e. African American women tend to develop more aggressive breast cancer and poorer prognosis than Caucasian women. In contrast, Asian, Hispanic, and Native American women have a lower risk of developing and dying from breast cancer than African and Caucasian American women (33, 36).

The most common genetic alterations associated with breast cancer is an inheritable mutation in the BRCA1 or BRCA2 genes which are involved in DNA repair (33). Mutation of either is associated with increased breast cancer and ovarian cancer risks (37, 38). Higher breast density, characterized by higher percentage of fibroglandular tissue and less adipose tissue, is also associated with higher breast cancer risk (39). Starting menstruation younger than age 12 or going through menopause older than 55 increase risk of breast cancer later in life possibly due to increased exposure to ovarian hormones (40, 41).

Modifiable risk factors include diets, alcohol consumption, overweight or obesity, physical inactivity, nulliparity, breast feeding, menopausal hormone replace therapy (29, 33). Alcohol consumption, even at moderate level, is a risk factor for multiple cancer types including breast cancer, and the association can be extended to multiple ethnic groups (42, 43). An increase in BMI is associated with postmenopausal breast cancer but does not increase breast cancer risk in premenopausal women (33, 44), which can be partially explained by obesity induced insulin resistance. Hyperinsulinemia followed by obesity decreases circulating insulin-like growth factor binding proteins (IGFBPs) and sex hormone
binding globulin (SHBG), which in turn, increase the availability of insulin-like growth factor (IGF) and estrogen in blood circulation. These hormones together with increased circulating insulin, significantly promote cell proliferation which might lead to mutation of breast epithelial cells into malignancy (33, 45, 46). Obesity induced low-grade chronic inflammation is associated with increased proinflammatory cytokines, enhanced aromatase expression and hormone receptors expression which also contribute to the development of breast cancer (47). Physical inactivity together with increased BMI elevate breast cancer risk by negatively affecting energy balance in terms of energy intake and expenditure, insulin sensitivity, circulating IGF and IGFBPs and steroid sex hormones (48, 49). Reproductive factors such as early full-term pregnancy and breast feeding have been associated with decreased breast cancer (33, 50).

1.1.2.4. Current Treatment Strategies

1.1.2.4.1 Overview

Multiple therapeutic options are available for breast cancer patients and can be classified into local and systemic treatments. Surgery and radiation therapy are considered local treatments while chemotherapy, hormone therapy, targeted therapy and immunotherapy are classified as systemic treatments due to their treatment agents being transported throughout the body via the bloodstream.
1.1.2.4.2 Surgical Resection

Surgical approaches to remove breast cancer include breast-conserving surgery (also known as lumpectomy) and mastectomy (51). Lumpectomy or partial mastectomy is a surgical procedure that only the cancer and some surrounding normal tissue are removed while mastectomy is a radical surgical procedure in which the entire breast and sometimes other nearby tissues are resected (51, 52). Clinical trials indicate that the combination of lumpectomy with radiation therapy (using ionizing radiation to damage DNA of fast dividing cancer cells) is associated with reduced recurrence and improved survival over lumpectomy or mastectomy alone; therefore, lumpectomy with radiation is an appropriate therapeutic strategy for early stage breast cancer patients (53, 54).

1.1.2.4.3 Chemotherapy

Chemotherapy is one of the systemic therapy strategies for breast cancer, aiming to control the primary tumor growth and eliminate distant metastasized microscopic tumors or single cancer cells circulating within the body. Based on the mechanism of action and chemical structure, chemotherapy can be classified into alkylating agents, antimetabolites, anti-microtubule, and topoisomerase inhibitors (55-58). Despite the side effects and toxicity, chemotherapy remains a standard systemic therapeutic strategy for triple-negative breast cancer (TNBC) in neoadjuvant, adjuvant and metastatic settings (24, 59).
1.1.2.4.4 Targeted Therapy

With better understanding of the underlying breast cancer biology and molecular subtypes of breast cancer, a great number of targeted therapeutic agents have been developed in recent years (21, 60). Based on hormone receptor expression (i.e. ER+, PR+) of breast cancer patients, hormone receptor antagonists such as ER inhibitor tamoxifen and degrading agents such as fulvestrant (by binding to the hormone receptor and causing the cell’s normal protein degradation processes) can be used to block sex hormones from binding to their receptors (60). HER2+ subtypes are generally resistant to hormone-based therapies despite the expression of hormone receptors (HR). HER2 blockers such as monoclonal antibodies pertuzumab and trastuzumab (Herceptin) are effective in HER2+ subtype patients, and combination strategies such as HER2 blocker with HR inhibitors significantly reduces the risk of progression (18, 61).

1.1.2.4.5 Immunotherapy

Breast cancer was originally considered non-immunogenic or less inflamed than those cancer types with high somatic mutation rates such as lung squamous carcinoma (62). However, after decades of research focused on the role of the immune system in breast cancer development and progression, the field of breast cancer immunotherapy has been expanding dramatically (63).

ER+ or luminal breast cancer subtypes are less immunogenic compared to TNBC subtype. Some successful immunotherapeutic approaches on other
cancer types including checkpoint inhibitors, and adoptive T cell transfer are being investigated pre-clinically (64).

HER2+ subtype is considered more immunogenic and has more tumor infiltrating lymphocytes (TILs) compared to luminal subtypes (65) which led to the investigation efficacy of several immunotherapeutic strategies on HER2+ breast cancer, including immune checkpoint inhibitors. Immune checkpoints are molecules expressed by immune cells that are crucial for self-tolerance which prevent autoimmunity but can also limit immune cells from killing cancer cells. Preclinical studies have shown that antibodies against immune checkpoint (checkpoint inhibitors) such as anti-PD-1 can increase the effectiveness of anti-HER2 therapy, which led to the initiation of several phase I and II trials (66, 67), to evaluate the potential superiorities of checkpoint inhibitors and anti-HER2 monoclonal antibodies (mAbs) combinations versus single anti-HER2 antibody treatment (65, 68).

Among subtypes of breast cancer, TNBC is considered most immunogenic among all the breast cancer subtypes due to the significant amount of genetic mutations and subsequent immunogenic protein products such as MAGE-A3 and NY-ESO-1 (69, 70). These neoantigens can be recognized by the immune system as foreign invaders therefore more likely to attract greater number of antigen specific lymphocytes (tumor-infiltrating lymphocytes or TILs) into tumor microenvironment (TME) (65, 71). Efficacy of Immune checkpoint inhibitors in breast cancer have been clinically investigated including monoclonal antibodies
(mAb) targeting Programmed Cell Death 1 (PD-1) or Programmed Cell Death Ligand 1 (PD-L1) (72, 73), showing preliminary evidence of clinical activity and acceptable safety profile; there is an ongoing phase III trial aimed to compare overall survival (OS) and progression-free survival (PFS) between participants receiving pembrolizumab and chemotherapy (74). In addition to single agent immunotherapy, combination approaches of chemotherapy and checkpoint inhibitors have also been extensively studied. Some chemotherapeutic drugs can selectively inhibit the proliferation of regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) so that relieving the suppression of antigen specific response against cancer cells. Moreover, by causing cancer cell death, chemotherapy promotes the release of neoantigens that can be taken up and presented by antigen presenting cells (APCs) and therefore increases antigen specific CD8+ T cell infiltration and activation (75-77). Based on these findings, several phase I and II clinical trials were conducted to evaluate the combination of different chemotherapy drugs and PD-1 or PD-L1 inhibitors in metastatic TNBC patients, preliminary results showed promising objective response rate (ORR) and good tolerance, which paved the way for the ongoing phase III double-blinded randomized controlled trials (78, 79).
1.2 Immune System

1.2.1. Overview

The immune system is a host defense system comprising various specialized organs, tissues, cells, and proteins that protect against foreign pathogenic agents and transformed self-cells (80). Specialized immune cells are differentiated from bone-marrow-dwelling hematopoietic precursor cells, and the differentiation processes are a tightly controlled intricate series of highly regulated signaling events. The immune system is divided into two parts determined by the rapidity and specificity of the reaction, namely innate and adaptive immune systems (81).

1.2.2. Innate and Adaptive Immunity

1.2.2.1. Innate Immunity

1.2.2.1.1 Overview

Innate immunity is a primitive host defense mechanism and the first-line of defense against foreign invaders. After breaching host’s anatomic barriers, pathogens will encounter a series of antimicrobial enzymes and peptides that can digest bacterial structural components such as cell wall and membrane and the complement system will target pathogens that facilitate phagocytosis by cellular components of innate immunity. Most of the cellular components of the innate
immune system are derived from the common myeloid progenitor cells, including macrophages (mature form of monocytes), granulocytes (including neutrophils, eosinophils, basophils and mast cells) and dendritic cells. Natural Killer cells (NK cells) of lymphoid lineage are also part of innate immunity (82). These immune cells recognize pathogens by various mechanisms including Fc receptors, scavenger receptors and pathogen recognition receptors (PRRs). PRRs include Toll-like receptors (TLRs), Nod-like receptors (NLRs), C type lectin receptors and RIG-like receptors (RLRs). Microbes contain classes of molecules including lipopolysaccharides, endotoxins, viral DNA and double-stranded RNA; these molecules, called pathogen-associated molecular patterns (PAMPs), can be recognized by innate immunity as foreign via their cell surface or cytoplasm PRRs to trigger phagocytosis and subsequent inflammatory responses that are characterized by proinflammatory cytokine and chemokine production (81, 83). Besides eliminating pathogens at the sites of infection, professional antigen presentation cells will migrate to lymphoid organs and present antigens to activate adaptive immune cell populations including T cells and B cells will be activated to carry out prolonged antigen-specific immune responses (81).

1.2.2.1.2 Innate Immunity Components

Macrophages, together with granulocytes and dendritic cells, comprises phagocyte types of immune system and also play an important role in activating adaptive immunity by presenting antigens (82). These cell populations can recognize pathogens (bacteria, virus and transformed cells) and pathogen
components (LPS, viral DNA, and neoantigen etc.) via cell surface receptors including PRRs, Fc receptors, C-type lectin receptors and scavenger receptors and ingest pathogens via phagocytosis. Despite their similarities, macrophages are usually the first defensive cell population that pathogens encounter after breaching physical barrier. Upon recognition and phagocytosis, macrophages are activated, pathogens are killed and degraded by lysozymes and reactive oxygen species (ROS) contained in lysosomes. Pathogen components are processed into small immunogenic molecules and conjugated with MHCII (major histocompatibility complex II) to present on the cell surface. Simultaneously, activated macrophages secrete pro-inflammatory cytokines and chemokines such as IL-1β, IL-6, TNF-α and GM-CSF to initiate inflammatory response by recruiting other immune cells to the site of infection for pathogen clearance (81, 82).

Granulocytes (include neutrophils, eosinophils, basophils and mast cells) contain specialized granules that are either low in pH or holding reactive oxygen species (ROS), lysozymes and antimicrobial peptides to kill microbes or initiate allergic reactions (81). Neutrophils are the most abundant granulocytes in circulation and the first to be recruited to the site of infection, primarily in response to GM-CSF secreted by macrophages (81, 82). Besides the importance in killing pathogens, neutrophils are also professional antigen presenting cells (APC) that present antigens via MHCII in response to cytokines such as GM-CSF, IFN-γ, TNF-α (84). Eosinophils and basophils are less abundant than neutrophils and
eosinophils mainly focus on battling parasitic infection while basophils and mast cells are involved in allergic reactions (82).

Dendritic cells (DCs), of either lymphoid or myeloid lineage, are also phagocytes, their functions are more focused on antigen presentation rather than pathogen clearance (85, 86). A large number of dendritic cells migrate to infection sites in response to the cytokines and chemokines secreted by macrophages and other tissue residence immune cell types (81, 85). After encountering infectious agents, dendritic cells recognize pathogens via various surface receptors including opsonin receptors, Fc receptors, scavenger receptors and pattern recognition receptors (PRRs) (86). After encountering pathogens, dendritic cells mature into potent antigen presenting cells that are characterized by increased expression of cell adhesion molecules such as E-selectin, ICAM-1 and VCAM-1 that facilitate dendritic cells’ migration to secondary lymphoid organs, expression of antigen-conjugated MHC molecules and costimulatory ligands such as B7 that are crucial to prime and activate naïve T cells (83, 85, 87).

Common lymphoid progenitor (CLP) derived Natural Killer cells (NKs) are important first line innate effector cells against foreign or mutated cells (81). NK cells are nonspecific effector cells that recognize pathogens and transformed cells via multiple mechanisms including Fc receptor binding to antibody coated cells and NK activating receptors such as natural-killer group 2, member D (NKG2D) binding to NKG2D ligands (81, 88). Healthy body cells express major histocompatibility complex I (MHC I) on their surface that can bind to inhibitory
receptors of NK cells thus prevent NK cells from attacking healthy tissue, while transformed cells or pathogens that lack self MHCI can activate NK cell (88). The pathogen-killing effects of NK cell are carried out by their cytoplasmic granules that contain cytotoxic proteins such as granzyme and perforin (89). After binding of activating receptors to target cells which lack MHCI expression, and in response to macrophage derived cytokines such as IFNs and IL-12, NK cells release cytotoxic proteins to induce apoptosis of the targets and secrete large amount of IFN-γ to contain infection before adaptive immunity is activated (89-91).

1.2.2.2. Adaptive Immunity

1.2.2.2.1 Overview

The adaptive immune system is composed of T and B lymphocytes and is characterized by their ability to specifically respond to immunogenic proteins expressed on pathogenic foreign microorganisms and transformed cells (63). The pathogen recognition receptors of innate immunity undergo somatic hypermutation that reshuffles the genetic coding of variable region of T cell receptors (TCR) and B cell receptors (BCR), which enable them to recognize specific antigenic molecules (92).
1.2.2.2 B Lymphocytes and Humoral Immunity

B cells are derived from common lymphoid progenitor cells (CLPs) that can be traced back to hematopoietic stem cells (HSCs). In the bone marrow, in response to various cytokine signals, CLPs give rise to pre-B cells which undergo several developmental stages that involve specific transcription factors into immature B cells and leave the bone marrow; Immature B cells will arrive in spleen and proceed through transitional stages namely T1 and T2, then into mature B cells in germinal center and migrate to lymphatic system. In this process, heavy chain and light chain genes of pre-BCRs proceed through somatic hypermutation and transcribe into antigen specific BCRs. Mature/ naïve B cells express IgM and IgD on their cell surface (81, 92, 93).

B cells are activated after encounter with antigen and become either memory B cells or plasma cells, the activation processes are either T cell dependent or independent (92). B cells can recognize antigen by the surface BCRs and then internalize, process and present the antigen on MHCII that can be recognized by T cells (T follicular helper cells) in secondary lymphoid organs, T cells in turn produce cytokines that are essential for B cells maturation and activation into memory or plasma cells (94). On the other hand, some molecules can interact with BCRs without the assistance of T cells and elicit an activating signal, with additional cytokines or other cells (such as DCs) in contact, B cells can be activated into memory or plasma cells (95).
Antibodies produced by B cells are the key mediators of humoral immunity. Antibodies can prevent pathogens such as viruses and intracellular bacteria from entering host cells by binding to their surface molecules (96). Antibodies also facilitate phagocytosis by coating pathogen surfaces (97). Last but not the least, antibodies binding to pathogens can activate the complement system to enhance phagocytes recognition via complement receptors (97).

1.2.2.2.3 T Lymphocytes and Cellular Immunity

T cells are also derived from CLPs from bone marrow but the maturation process takes place in the thymus where individual T cells with specific binding capacity are formed.

In order to be activated, mature yet naïve T cells need to interact with antigenic molecules in the context of MHC molecules on APCs. There are two types of MHC that T cell receptors bind: MHC I expressed on all nucleated cell surfaces that presents endogenous such as viral or tumor antigens, and MHC II on APCs that present exogenous antigen by endocytosis (92). Besides interaction between TCRs and MHC/peptide complex, costimulatory molecules such as CD28 (binds to CD80/CD86 (B7)) from APCs are also required to activate T cells (81). Lastly, various cytokines can drive the differentiation of CD4+ T cells into various subsets with distinct functions, including T helper 1 (T\textsubscript{H}1), T helper 2 (T\textsubscript{H}2), T helper 9 (T\textsubscript{H}9), T helper 17 (T\textsubscript{H}17), T helper 22 (T\textsubscript{H}22), follicular helper T cells (T\textsubscript{FH}), and regulatory T cells (Tregs) (92).
The major functions of CD4+ helper T cells are regulating immune responses via cytokine secretion, including B cell antibody class switching, cytotoxic T cell activation, and phagocytes pathogen clearing activities (81, 92). Activated CD8+ cytotoxic T cell population can eliminate bacteria or virus infected, stressed, and tumor cells by recognizing antigens presented on MHC class I molecules and inducing apoptosis via the release of cytotoxins, perforins, and granzymes or through the expression of surface proteins such as Fas ligand (92, 98).

1.2.3. Immune Response to Malignancies

The interaction between the immune system and cancer is a complex process that involves the balance between pro- and anti-tumor mediators. Based on the initial concept of ‘immune surveillance’ introduced by Burnett and Thomas, the interaction of the immune system and cancer now includes three phases: elimination, equilibrium and escape phase (99-102).

Elimination phase of cancer immunoeediting is the process that innate and adaptive immune components recognize and eliminate transformed cells from developing into visible tumors. Both innate and adaptive immune cells are involved in killing of cancer cells, major effectors include NK cell of innate immunity, and adaptive immunity mainly CD8+ cytotoxic T cell with the assistance of CD4+ Helper T, especially T_{H1} cells (99). NK cells are activated when tumor cells (usually with reduced MHCI expression) are recognized via surface binding.
of activating receptor NKG2D to NKG2D ligands such as MICA/B of human and H60, Rae-1 of mouse (99, 103). Initial recognition of cancer cells by innate immune cells such as NK, NKT, macrophages and dendritic cells (DCs) triggers the production of proinflammatory cytokines such as IFN-γ, which increases anti-tumor activity in multiple ways such as upregulating tumor cells’ MHCI expression that facilitates CD8+ T cell’s recognition and killing process (99). Activated DCs migrate to secondary lymphoid organs to initiate the production of tumor-specific T cells (99). Classic tumor antigens include differentiation (i.e. melanocyte differentiation antigen), mutation (i.e. mutated p53), overexpressed (i.e. HER2/neu) testis (i.e. MAGE) and viral antigens (104-106). Activated NK, CD8+ T cell and other immune cell populations target and kill tumor cells by releasing perforin, granzyme from secretory lysosome, increasing expression of TNF-related apoptosis-inducing ligand (TRAIL) and First apoptosis signal Ligand (FasL) inducing apoptosis of tumor cells (99, 107).

The equilibrium phase is the immunity-tumor interaction where cancer is stably controlled by immune system or becomes dormant but not eliminated. Tumor cells in the equilibrium phase will eventually be eliminated or escape from immunosurveillance and develop into visible tumors (99).

The escape phase describes the failure of the immune system to eradicate transformed cells and the outgrowth of these cells into clinically detectable tumors. Cancer cells achieve evasion of destruction by the immune system via a combination of various mechanisms. Cancer cells are rapidly mutating due to the
unstable genome that will eventually contribute to heterogeneity of tumor cells, therefore it is likely that the immune system fails to recognize and eliminate all the variants, such as the ones that lack TAAs, NKG2D ligands expression, or the variants with increased expression of PD-L1 including breast cancer cells (108, 109). Tumor cells together with tumor associated macrophages (TAMs) can also generate an immunosuppressive microenvironment by producing anti-inflammatory cytokines, such as TGF-β, IL-10, and recruit regulatory T cells (Tregs) that can prevent the activation of DCs, NK cells, T cells and B cells (99, 110). Cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-1β (IL-1β), vascular endothelial growth factor (VEGF), and prostaglandin E2 (PGE2) are responsible to recruit myeloid-derived suppressor cells (MDSCs) to TME, which are another source of immunosuppressive cytokines such as TGF-β. MDSCs can also deplete arginine (essential for T cell function) by secreting arginase, and debilitate T cell receptor (TCR) function by nitrosylation (80, 111). Lastly, tumor cells can directly inhibit T cell proliferation by producing indoleamine 2,3-dioxygenase (IDO) to deplete tryptophan leading to cell cycle arrest (112).
1.3 Animal Models of Breast Cancer

1.3.1 Overview

For decades, animal models have been used to study primary tumor growth and metastatic spread with and without therapeutic interventions (113). Current available in vivo murine models for metastatic breast cancer can be classified into two categories: experimental metastasis models and spontaneous (metastasis arises spontaneously from primary tumor) models including transgenic and transplantable models (114). Experimental metastatic models are designated for the investigation of tumor colonization mechanisms at a secondary site, by injecting tumor cells into circulation or specific organs. Transgenic models have several advantages that allow researchers to study the entire process of cancer progression including tumor initiation, pre-metastatic niche as well as distant colonization, additional oncogene or tumor suppressor genes can also be inserted to study oncogenic pathways (113-116). Compared to transgenic models, transplantable models allow researchers to quickly assess metastatic process, because of their short metastatic onset latency and multiple metastatic sites such as brain, liver and bone that most transgenic models lack. Tumor cell lines of transplantable models usually can be modified in vitro before inoculation, such as transfection of a foreign gene (114).

Murine transplantable models can be divided into two groups, xenograft and syngeneic transplantable models (113). Xenograft models involve
transplanting human cell lines or cells isolated directly from patients into immunocompromised hosts. However, several limitations weaken the predictive power of xenograft models as pre-clinical cancer models. First, species specificity sets a boundary for tumor-stromal interaction which is critical for clinically relevant cancer progression and metastasis (113, 117). Secondly, in order to prevent host rejection of human cell implantation, the murine host must be immunocompromised which makes it impossible to investigate the role of immune system in tumor progression (118). Lastly, immunocompromised mice such as nude mice have been reported containing impaired or altered angiogenesis within transplanted tumors (119, 120). Syngeneic transplantable model tumor cell lines are isolated from spontaneous tumors triggered by carcinogens and can be repeatedly and orthotopically injected into the host animal to initiate primary tumor growth and metastasis (121, 122). Currently, the 4T1 series is the most widely used transplantable breast cancer model. Tumor cells can be injected into mammary gland as allograft with genetic and immunological compatibility while spontaneous metastasis to distant organs can be achieved quickly (2, 114, 121).

1.3.2 4T1 Breast Cancer Model Series

The 4T1 transplantable model mimics human stage IV triple negative breast cancer; the 4T1 cell line was originally isolated by Miller et al. and was one of four sublines (67NR, 168FARN, 4T07, and 4T1) derived from 410.4 tumor
The 410.4 cell line was isolated from a spontaneously initiated mammary tumor of a BALB/c female mouse triggered by murine mammary tumor virus (MMTV) infection (123). These four cell lines display different metastatic capacities: with 67NR being nonmetastatic, 168FARN being local micrometastatic, 4TO7 being weakly metastatic to distant organs but unable to form metastatic nodules and 4T1 cell line being highly tumorigenic and can spontaneously metastasize from primary mammary tumor also form visible nodules in distant organs including lung, liver, bone and brain (121, 124, 125).

The 4T1.2 transplantable tumor cell line is a single cell clone derived from the 4T1 cell line as a site-specific metastatic breast cancer model to the bone (122). 4T1.2 is currently one of a few transplantable models with molecular features of a human triple-negative and basal like phenotype (ERα-, PR-, HER2- and EGFR+) (2), which makes it a valuable model for investigating bone metastasis and developing new therapeutic strategies to the triple-negative and basal like human breast cancer patients (20). Interaction with surrounding stroma is important to initiate metastasis. 4T1.2 cell adhere to collagen I, IV, fibronectin and especially stronger to vitronectin and lamin-511 than its non-metastatic cousin 67NR (126). Matrix metalloproteinases (MMPs) are an enzyme family that are crucial for cancer metastasis by degrading basement membrane in human breast cancer, it was reported 4T1.2 cells produce more active MMP-9 than 67NR with the stimulation of its substrate laminin-511 (127, 128). It has also been reported that compared to 67NR, metabolic plasticity and adaptivity enables 4T1
cells to better adapt in the tumor microenvironment which is important to establish metastasis (125). As a bone metastatic model and similar to human breast cancer cells, 4T1.2 cell line is also characterized by elevated parathyroid hormone-related protein (PTHrP) secretion compare to other 4T1 family cell lines, and Ca\(^2+\) circulation of 4T1.2 tumor bearing mice is also significantly increased relative to other 4T1 family tumor bearing mice (122, 129).

4T1 transplantable model family are valuable tools to study breast cancer metastasis due to their similarity with human TNBC in molecular profile; yet unlike human TNBC, mouse 4T1.2 breast cancer model lacks identifiable tumor associated antigen (3, 4) which limits the model’s applicability in investigating the interaction of immune system especially antigen-specific immunity with tumor cells. To preclinically examine immunotherapeutic approaches against TNBC, modifications to 4T1 series were done aimed to increase the immunogenicity, such as introducing human TAAs to these cell lines (130).

4T1.2\textsuperscript{luc} cell line was originally developed by Lou and Dedhar (131) by transfecting a plasmid shuttle vector containing luciferase reporter gene \textit{luc}2 into 4T1.2 cells and thus established a luciferase-expressing cell line (132). Firefly luciferase gene \textit{luc}2, as a reporter gene, was first reported more than three decades ago and later adopted by scientists as an approach to design informative, noninvasive yet cost-effective \textit{in vivo} studies (133). In the field of cancer research, transfection of \textit{luc}2 into transplantable tumor cell line allows scientists to track metastasis and micro-metastasis noninvasively with the aids of
bioluminescent imaging and PCR technologies (134-136). Despite the benefits brought by luciferase reporter gene in the application of orthotopic transplantable models, it has been suggested that reporter transgenes including β-galactosidase, Enhanced green fluorescent protein (EGFP) and luciferase epitopes can induce CD8+ T cell specific response that is characterized by increased interferon-γ (IFN-γ) secretion (5). Moreover, it has been reported that 4T1 cell lines labeled with luciferase reporter gene displayed a reduced tumor growth rate and a suppressed metastatic activity in immunocompetent BALB/c mice, which could potentially compromise the utility of these models, but it may also provide important information for immunotherapy development (6, 7).

Until now, no study has been published characterizing the in vivo tumorigenesis, metastasis and immunogenicity of 4T1.2 luc cell line compared to the parental 4T1.2 cell line. With the preliminary evidence that firefly luciferase may be immunogenic in the 4T1 model, it is reasonable to hypothesize similar antigenic effect of luciferase expressed by 4T1.2 cells will be observed.

1.4 Rationale of Current Study

The 4T1.2 transplantable model is the most widely used for modeling human triple negative breast cancer (TNBC) due to the similarities in molecular profile (Lacking in ER, PR and HER2 expression) and sites of metastases. Unlike human breast cancer, however, the 4T1.2 tumor cell line lacks defined tumor associated antigens (TAAs) which limits its utility in immunotherapeutic research.
Previous studies demonstrate that luciferase (as a foreign protein) can elicit CD8+ T cell specific response \textit{in vitro} and reduce primary tumor growth in both the 4T1 and other tumor models (5, 7), but no studies have been done to directly characterize the role of luciferase in the luciferase-expressing 4T1.2\textsuperscript{Luc} model. Therefore, the primary goal of this study was to explore the \textit{in vitro} and \textit{in vivo} growth rates of 4T1.2 vs. the 4T1.2\textsuperscript{Luc} mammary tumor cells. In addition, we also investigated the role of major cancer immunosurveillance components (CD4+ T cells, CD8+ T cells and NK cells) in primary tumor growth, metastatic progression and survival rate. Findings of this study can provide important information for future pre-clinical research in immunotherapeutic strategies targeting TNBC.
CHAPTER 2
AIMS AND HYPOTHESES

2.1. Aim 1

Determine if the expression of luciferase in the 4T1.2 clone (4T1.2\textsuperscript{luc}), alters the \textit{in vitro} or \textit{in vivo} proliferative capacities compared to the parental cell line 4T1.2.

\textbf{Hypothesis 1:} Luciferase serves only as an antigen and has no known functions in mediating signaling cascades that govern tumor cell proliferation. Therefore, we hypothesize 4T1.2 and 4T1.2\textsuperscript{luc} cell lines will have similar \textit{in vitro} proliferation rates.

\textbf{Hypothesis 2:} In an immunocompetent host, luciferase will be recognized as a foreign antigen. Thus, primary tumor growth of 4T1.2\textsuperscript{luc} cells will be reduced compared to the growth of 4T1.2 cells.

2.2. Aim 2

Determine the role of CD4\textsuperscript{+} T cells, CD8\textsuperscript{+} T cells and NK cells in controlling primary tumor growth and metastatic progression in 4T1.2\textsuperscript{luc} and 4T1.2 tumor-bearing mice.

\textbf{Hypothesis 1:} Depletion of CD4\textsuperscript{+} T cells, CD8\textsuperscript{+} T cells and NK cells in 4T1.2\textsuperscript{luc} tumor-bearing mice will enhance 4T1.2\textsuperscript{luc} tumor growth and metastatic burden.
**Hypothesis 2:** Depletion of CD4+ T cells, CD8+ T cells and NK cells in 4T1.2 tumor-bearing mice will have no effect on 4T1.2 tumor growth and metastatic burden.

**2.3. Aim 3**

Determine if the absence of CD4+ T cells, CD8+ T cells and NK cells alters the survival rate of 4T1.2 and 4T1.2luc tumor-bearing mice respectively.

**Hypothesis 1:** Immunocompetent 4T1.2luc tumor-bearing mice (i.e. mice treated with PBS or isotype control antibody, LTF-2) will have a significantly higher survival rate compared to 4T1.2 tumor-bearing mice.

**Hypothesis 2:** Depletion of CD4+ T cells, CD8+ T cells or NK cells will not significantly affect the survival rate of 4T1.2 tumor-bearing mice.

**Hypothesis 3:** Depletion of CD4+ T cells, CD8+ T cells or NK cells will significantly decrease the survival rate of 4T1.2luc tumor-bearing mice.
CHAPTER 3
MATERIAL AND METHODS

3.1. Experimental Design

The study was designed to investigate the role of CD4$^+$ T cells, CD8$^+$ T cells and NK cells in controlling primary tumor growth, metastatic burden, and survival in 4T1.2 and 4T1.2$^{\text{luc}}$ tumor-bearing mice. Two cohorts of female BALB/c mice were used for either 4T1.2 and 4T1.2$^{\text{luc}}$ tumor cell inoculation. Each cohort was randomized into five treatment groups, including PBS control (intraperitoneal injection [i.p.] with PBS), isotype control (i.p. injected with 100 µg LTF-2 antibody), CD4$^+$ T cell depletion (i.p. injected with 100 µg GK1.5 antibody), CD8$^+$ T cell depletion (i.p. injected with 100 µg 2.43 antibody) and NK cell depletion (i.p. injected with 20 µl anti-asialo GM1 antibody). Injection of PBS and the aforementioned antibodies started three days prior to tumor cell inoculation and was maintained every three days until day 23 post tumor implantation. 4T1.2 or 4T1.2$^{\text{luc}}$ cells ($5 \times 10^4$ tumor cells in 50 µl of PBS) were implanted into the fourth mammary gland of each mouse at day zero. Primary tumor growth was measured two-three times per week using digital caliper until the day 35 post tumor implantation. The experimental design and sample size of each group are shown in Figure 3.1.
Figure 3.1. Experimental design. Two groups of BALB/c mice were orthotopically injected with either $5 \times 10^4$ 4T1.2 or 4T1.2$^{luc}$ tumor cells into the fourth mammary gland. Each treatment group was injected with depleting antibodies for specific immune cell population starting three days before tumor cell inoculation and maintained every three days until day 23 post tumor implantation. Primary tumor volume was measured every two-three days. Mice were sacrificed on day 35, spleens were collected to confirm immune cell depletion, lungs and femurs of 4T1.2$^{luc}$ tumor bearing mice were collected to quantify metastatic burden.
3.2. Materials and Methods

3.2.1. Cell Lines and Cell Culture

The 4T1.2 murine breast cancer cell line was maintained in α minimum essential medium (Life Technologies; Grand Island, NY) supplemented with 10% fetal bovine serum (Gemini Bio-Products, Sacramento, CA), 2 mM L-glutamine (Mediatech; Manassas, VA) 100U/ml penicillin (Mediatech) and 100 μg/ml streptomycin (Mediatech) and incubated at 37°C with 5% CO₂. The 4T1.2\textsuperscript{luc} cell line was 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 L-glutamine (Mediatech; Manassas, VA), nonessential amino acid (Mediatech), and 8 μg/ml puromycin (Mediatech) and incubated at 37°C with 5% CO₂.

3.2.2. In Vitro Cell Proliferation Assay

Trypsin/EDTA (0.25%/2.21 mM) in HBSS was added to culture flasks to harvest 4T1.2 and 4T1.2\textsuperscript{luc} cells. Tumor cells were washed twice in their respective media, adjusted to 2.5×10\textsuperscript{3}/ml, and plated using serial dilutions in a 96 well plate. Cells were allowed to proliferate for 48 prior to the removal of 70 μl of supernatant for cytokine analysis. 50 μl media and 20μl/well of CellTiter 96\textsuperscript{®} AQueous One Solution Reagent (Promega) were added back to well to quantify proliferative capacity of the cells. After incubation for 90 minutes the plate was
read using an Epoch™ microplate spectrophotometer (BioTek, Winooski, VT) to quantify absorbance at 490nm. Each assay was performed in triplicate.

### 3.2.3. Animal Model

Six-week-old female BALB/c mice were purchased from Jackson Laboratory (Bar Harbor, MA). Mice were orthotopically injected either with $5 \times 10^4$ 4T1.2 cells (parental cell line) or 4T1.2 cells transfected with luciferase (4T1.2\textsuperscript{luc}) into the fourth mammary gland. Primary tumor growth was measured two-three times/week using digital caliper until the endpoint day 35 post tumor implantation and tumor volume was calculated following the equation of $v=(\text{short}^2 \times \text{long})/2$. For the survival study, mice were sacrificed when either the tumor size reached the ethical limit ($1.5\text{cm}^3$) or if the mice were moribund. All mice were housed at The Pennsylvania State University in the Chandlee Laboratory animal facility and maintained on a 12-hour light/dark cycle with free access to AIN-76A diet (Research Diets, New Brunswick, NJ) and water. The Institutional Animal Care and Use Committee of The Pennsylvania State University approved all animal experiments.
3.2.4. Depletion of immune Cells

Two cohorts of female BALB/c mice were orthotopically injected into the fourth mammary gland with either $5 \times 10^4$ 4T1.2 or 4T1.2$^{\text{luc}}$ tumor cells (Figure 4.2). Within each cohort mice were randomized into five treatment groups: control (i.p. injected with PBS), isotype control (i.p. injected with 100 µg LTF-2 antibody), CD4$^+$ T cell depletion (i.p. injected with 100 µg GK1.5 antibody), CD8 T cell depletion (i.p. injected with 100 µg 2.43 antibody) and NK cell depletion (i.p. injected with 20 µl anti asialo GM1 antibody). Injection of PBS and antibodies started three days prior to tumor cell inoculation and was maintained every three days until day 23 post tumor implantation. Primary tumor growth was measured two-three times per week using digital caliper until the day 35 post tumor injection.

3.2.5. Flow Cytometric Analyses

Single cell suspensions of splenocytes were washed twice in PBS containing 0.01% bovine serum albumin (flow buffer) at 4°C. Cells were incubated with Fc block (Biolegend) and $1 \times 10^6$ cells were stained with saturating concentrations of conjugated antibodies for 30 min at 4°C. Fluorescent dye conjugated antibodies used in flow cytometric analyses were 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); hamster IgG1 (A19-3), Rat IgG2a (RTK2758) and mouse IgG2a (MG2a-53) were used as isotype controls. Following incubation with the conjugated antibodies, cell samples were washed twice in flow buffer.
and fixed in 1% paraformaldehyde (BD Biosciences) in flow buffer and analyzed on a BD LSR-Fortessa (BD Bioscience) flow cytometer by collecting 50,000 events for each sample analyzed. Flow cytometric data were analyzed using FlowJo software (Tree Star; Ashland, OR).

3.2.6. Metastatic Burden Quantification

At sacrifice, lungs and femurs of mice 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) according to manufacturer’s instructions. DNA concentration of each sample was measured using Nanodrop ND-2000c spectrophotometer (Thermo Scientific; Wilmington, DE). Luciferase copies for each individual tissue were quantified using TaqMan™ assay on StepOnePlus™ (Life Technologies) real-time PCR system to determine metastatic burden. Forward and reverse primers for luciferase sequence were CAGCTGCACAAAGCCATGAA, and CTGAGGTAATGTCCACCTCGATATG and probe was TACGCCCTGGTGCCCGGC with 5’ FAM reporter and 3’ BHQ quencher. Mouse telomerase reverse transcriptase (TERT) was used as reference gene for normalizing luciferase data and was analyzed using the TaqMan™ Copy Number Reference Assay TERT (Life Technologies). The standard curve was performed with five 10-fold serial dilutions (200ng-20pg) of DNA extracted from cultured 4T1.2^Luc cells. TERT and luciferase reactions for
Standard curve and 200ng of each tissue sample were performed in duplicate on StepOnePlus™ real-time PCR system.

3.3. Statistical Analyses

All data are presented as the mean plus or minus the standard error mean. Tumor volume and metastatic burden 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. In vitro proliferation of 4T1.2 and 4T1.2\textsuperscript{Luc}, and primary tumor growth was assessed using 2-way ANOVA, followed by Bonferroni correction for multiple comparisons where appropriate. Metastatic burden among treatment groups was assessed using Kruskal-Wallis test, followed by Dunn's multiple comparisons test. Survival of tumor-bearing mice in all treatment groups were analyzed using Kaplan-Meier curves with subsequent log rank (Mantel-Cox) test. Significance was accepted at p<0.05.
CHAPTER 4
RESULTS

4.1. In Vitro and in vivo Proliferation of 4T1.2 and 4T1.2\textsuperscript{luc} tumor cells

The expression of luciferase in the 4T1.2 cell line did not alter the \textit{in vitro} proliferation rate as the proliferation rates of 4T1.2 and 4T1.2\textsuperscript{luc} cells were similar (Fig. 4.1; 2-way ANOVA, time × cell number, F\textsubscript{(7, 28)} = 1.768, p = 0.137).

Mean tumor volume of PBS-treated 4T1.2\textsuperscript{luc} tumor-bearing mice was significantly reduced compared to PBS treated 4T1.2 tumor-bearing mice (Fig 4.2; n=5/group; 2-way ANOVA, time × treatment, F\textsubscript{(12, 96)} = 1.876, p = 0.047).
Figure 4.1. *In vitro* proliferation of 4T1.2 and 4T1.2\textsubscript{luc} cell lines. 4T1.2 or 4T1.2\textsubscript{luc} mammary tumor cells (2,500 cells/well in serial dilution) were cultured for 72 hours and quantified proliferation. The *in vitro* proliferation rates were similar between 4T1.2 and 4T1.2\textsubscript{luc} cell lines (2-way ANOVA, time × cell number, $F_{(7, 28)} = 1.768$, $p = 0.137$).

Figure 4.2. *In vivo* tumor growth of 4T1.2 and 4T1.2\textsubscript{luc} mammary tumor cell lines. Primary tumor growth of PBS-treated 4T1.2\textsubscript{luc} tumor-bearing mice was significantly reduced compared to PBS-treated 4T1.2 tumor-bearing mice (n=5/group; 2-way ANOVA, time × treatment, $F_{(12, 96)} = 1.876$, $p = 0.047$).
4.2. Immune Cell Depletion

Splenocytes from each treatment group were stained with anti-CD3, anti-CD4, anti-CD8 or NK1.1 to quantify the number of CD4\(^+\) T cells, CD8\(^+\) T cells and NK cells, respectively, in the spleens of either PBS or antibody-depleted mice (Figure 4.3). In PBS-treated mice, 27.8% of splenocytes were CD3\(^+\)/CD4\(^+\) T cells, whereas in GK1.5-treated mice, only 0.28% of splenocytes were CD3\(^+\)/CD4\(^+\) T cells (Figure 4.3A). In PBS-treated mice, 13.3% of splenocytes were CD3\(^+\)/CD8\(^+\) T cells, whereas in 2.43-treated mice 1.42% of splenocytes were CD3\(^+\)/CD8\(^+\) T cells (Figure 4.3B). Lastly, in PBS-treated mice 12.28% of splenocytes were NK1.1\(^+\), whereas in anti-asialo GM-treated mice 1.91% of splenocytes were NK1.1\(^+\) cells (Figure 4.3C).
Figure 4.3. Splenic immune cell depletion in control and antibody depleted mice. At sacrifice on day 35 post tumor implantation, spleens were harvested and single cell suspension of splenocytes were prepared for flow cytometric analysis. CD3⁺/CD4⁺ helper T cells (A), CD3⁺/CD8⁺ cytotoxic T cells (B) and NK1.1⁺ NK cells (C) populations were quantified in PBS treated (left column) and antibody-depleted mice (right column).
4.3. Primary Tumor Growth

Individual tumor growth curves demonstrate the variability that was observed in 4T1.2 and 4T1.2\textsuperscript{Luc} tumor growth among different animals (Fig.4.4).

In 4T1.2 tumor bearing mice, administration of an isotype control antibody (Figure 4.5A; ANOVA, time × treatment, \(F_{(9, 99)} = 1.184, p=0.313\)); depletion of CD4\textsuperscript{+} T cells (Figure 4.5B; 2-way ANOVA, time × treatment, \(F_{(7, 77)} = 1.763, p=0.107\)); depletion of CD8\textsuperscript{+} T cells (Figure 4.5C; 2-way ANOVA, time × treatment, \(F_{(9, 90)} = 0.9577, p=0.480\)); and depletion of NK cells (Figure 4.5D; 2-way ANOVA, time × treatment, \(F_{(9, 99)} = 1.874, p=0.065\)) did not significantly alter primary tumor growth compared to PBS treated 4T1.2 tumor-bearing mice.

In 4T1.2\textsuperscript{Luc} tumor bearing mice, administration of an isotype control antibody (Figure 4.6A; 2-way ANOVA, time × treatment, \(F_{(12, 384)} = 0.2934, p=0.990\)) and depletion of CD4\textsuperscript{+} T cells (Figure 4.6B; 2-way ANOVA, time × treatment, \(F_{(12, 420)} = 0.5773, p=0.861\)) did not significantly alter tumor growth compared to PBS treated mice. However, depletion of CD8\textsuperscript{+} T cells (Figure 4.6C; 2-way ANOVA, time × treatment, \(F_{(12, 408)} = 1.847, p=0.039\)) significantly enhanced 4T1.2\textsuperscript{Luc} tumor growth while NK cell depletion significantly reduced 4T1.2\textsuperscript{Luc} tumor growth (Figure 4.6D; 2-way ANOVA, time × treatment, \(F_{(12, 408)} = 1.878, p=0.035\)).

When 4T1.2 and 4T1.2\textsuperscript{Luc} tumor growth was compared within each antibody treatment group, 4T1.2\textsuperscript{Luc} tumor growth was significantly lower than
4T1.2 tumor growth in PBS-treated (Figure 4.7A; PBS; n=5/group; 2-way ANOVA, time × treatment, F(12, 96)=1.876, p= 0.047); isotype control-treated (Figure 4.7B; LTF-2; n=8/group; 2-way ANOVA, time × treatment, F (12, 168)=4.845, p<0.001); CD4⁺ T cell depleted (Figure 4.7C; GK1.5; n=8/group; 2-way ANOVA, time × treatment, F(12, 168)=3.118, p<0.001) and NK cell depleted (Figure 4.7E; anti-asialo GM1; n=7-8/group; 2-way ANOVA, time × treatment, F(12, 156)=3.560, p<0.001) mice. However, 4T1.2<sup>luc</sup> tumor growth was similar to 4T1.2 tumor growth in CD8⁺ T cell depleted mice.
Figure 4.4. Tumor growth of individual mice in each treatment group by tumor cell types. Individual tumor growth curves of BALB/c mice inoculated with (A-E) 5X10⁴ 4T1.2 or (F-J) 5X10⁴ 4T1.2 luc cells and treated with either PBS (n=5-6/group), isotype control antibody (LTF-2; n=8/group), anti-CD4 antibody (GK1.5; n=8/group), anti-CD8α antibody (2.43; n=8/group) or anti-asialo GM1 antibody (n=8/group) antibodies.
Figure 4.5. Primary tumor growth of 4T1.2 cells in control and immune cell depleted mice. (A) Isotype control (LTF-2; 2-way ANOVA, time × treatment, F (9, 99) = 1.184, p=0.313), (B) CD4$^+$ T cell depletion (GK1.5; 2-way ANOVA, time × treatment, F (7, 77) = 1.763, p=0.107), (C) CD8$^+$ T cell depletion (2.43; 2-way ANOVA, time × treatment, F (9, 90) = 0.9577, p=0.480) and (D) NK cell depletion (anti-asialo GM1; 2-way ANOVA, time × treatment, F (9, 99) = 1.874, p=0.065) did not significantly change 4T1.2 tumor growth compared to PBS-treated mice.
Figure 4.6. Primary tumor growth of 4T1.2<sup>luc</sup> cells in control and immune cell depleted mice. In 4T1.2<sup>luc</sup> tumor bearing mice, (A) Isotype control (LTF-2; 2-way ANOVA, time × treatment, $F_{(12, 420)} = 0.3230$, $p=0.985$), and (B) CD4<sup>+</sup> T cell depletion (GK1.5; 2-way ANOVA, time × treatment, $F_{(12, 420)} = 0.5773$, $p=0.861$) did not alter tumor growth. However, (C) CD8<sup>+</sup> T cell depletion significantly increased primary tumor growth ($2.43$; 2-way ANOVA, time × treatment, $F_{(12, 408)} = 1.765$, $p=0.039$) and (D) NK cell depletion significantly reduced tumor growth compared to the PBS-treated control group (anti-asialo GM1; 2-way ANOVA, time × treatment, $F_{(12, 408)} = 1.878$, $p=0.035$).
Figure 4.7. Primary tumor growth in 4T1.2 and 4T1.2\textsuperscript{2uc} tumor bearing mice receiving control or immune cell depleting antibodies. (A) control (PBS; n=5/group; 2-way ANOVA, time × treatment, F (12, 96) = 1.876, p=0.047), (B) isotype control (LTF-2; n=8/group; 2-way ANOVA, time × treatment, F (12, 168) = 4.85, p<0.001), (C) CD4\textsuperscript{+} depletion (GK1.5; n=8/group; 2-way ANOVA, time × treatment, F (12, 168) = 3.118, p<0.001), (D) CD8\textsuperscript{+} T cell depletion (2.43; n=6-8/group; 2-way ANOVA, time × treatment, F (12, 144) = 0.5127, p=0.904) and (E) NK cell depletion (anti-asialo GM1; n=7-8/group; 2-way ANOVA, time × treatment, F (12, 156) = 3.560, p<0.001) in 4T1.2 and 4T1.2\textsuperscript{2uc} tumor bearing mice. Tumor growth was significantly reduced in 4T1.2\textsuperscript{2uc} tumor-bearing mice compared to 4T1.2 tumor-bearing mice in all treatment groups except in CD8\textsuperscript{+} T cell depleted mice.
4.4. Metastatic Burden

Metastatic burden in the lung (Figure. 4.8A; Kruskal-Wallis, KW=8.452, p=0.076) and the femur (Figure. 4.8B; Kruskal-Wallis, KW=3.073, p=0.546) was not significantly different among treatment groups.
Figure 4.8. Metastatic burden in the lung and femur in 4T1.2\textsuperscript{luc} tumor-bearing mice. Lung (A) metastatic burden was not significantly different among treatment group (Kruskal-Wallis, KW=8.452, p=0.076). Femur (B) metastatic burden was not significantly different among treatment groups (Kruskal-Wallis, KW=3.073, p=0.546).
4.5. Survival Analyses

The median survival of PBS-treated 4T1.2\textsubscript{luc} tumor-bearing mice was significantly longer than 4T1.2 tumor-bearing mice (Figure 4.9; n=6-18, log-rank test, p=0.045).

In 4T1.2 tumor bearing mice, the median survival of PBS and LTF-2 treated were not significantly different (Figure 4.10A; n=6-8/group, log-rank test, p=0.431). Depletion of CD4\textsuperscript{+} T cells (Figure 4.10B; GK1.5; n=6-8/group, log-rank test, p=0.385), CD8\textsuperscript{+} T cells (Figure 4.10C; 2.43; n=6-8/group, log-rank test, p=0.135) or NK cells (Figure 4.10D; anti-asialo GM1, n=6-8, log-rank test, p=0.709) did not significantly change the median survival in 4T1.2 tumor-bearing mice compared to PBS treated mice.

In 4T1.2\textsubscript{luc} tumor-bearing mice, the median survival of isotype control (Figure 4.11A; LTF-2; n=5-8/group, log-rank test, p=0.899) and NK cell depleted (Figure 4.11D; anti-asialo GM1, n=5-8, log-rank test, p=0.825) mice were not significantly different than PBS–treated mice. CD4\textsuperscript{+} T cell depletion (Figure 4.11B; GK1.5; n=8-16/group, log-rank test, p=0.004) and CD8\textsuperscript{+} T cell depletion (Figure 4.11C; 2.43; n=8-16/group, log-rank test, p=0.025) significantly reduced median survival in 4T1.2\textsubscript{luc} tumor bearing mice compared to PBS-treated mice.
Figure 4.9. Median survival of 4T1.2 and 4T1.2\textsuperscript{luc} tumor-bearing mice. Median survival of PBS-treated 4T1.2\textsuperscript{luc} tumor-bearing mice was significantly higher than 4T1.2 tumor-bearing mice (PBS, n=6-18, log-rank test, p=0.045).
Figure 4.10. Median survival in antibody depleted 4T1.2 tumor-bearing mice compared to PBS treated mice. Median survival of (A) isotype control (LTF-2; n=6-8/group, log-rank test, p=0.431), (B) CD4+ T cell depleted (GK1.5; n=6-8/group, log-rank test, p=0.385), (C) CD8+ T cell depleted (2.43; n=6-8/group, log-rank test, p=0.135) and (D) NK cell depleted (anti-asialo GM1, n=6-8, log-rank test, p=0.709) mice were not significantly different than PBS-treated mice.
Figure 4.11. Median survival of antibody-treated 4T1.2\textsuperscript{luc} tumor-bearing mice compared to PBS-treated control mice. Median survival of (A) isotype control (LTF-2; n=8-16/group, log-rank test, p=0.899), (B) CD4\textsuperscript{+} T cell depleted (GK1.5; n=8-16/group, log-rank test, p=0.004), (C) CD8\textsuperscript{+} T cell depleted (2.43; n=8-16/group, log-rank test, p=0.025) and (D) NK cell depleted (anti-asialo GM1, n=8-16, log-rank test, p=0.825) mice. CD4\textsuperscript{+} and CD8\textsuperscript{+} T cell depletion significantly reduced median survival compared to PBS-treatment.
CHAPTER 5
DISCUSSION

Breast cancer is the most commonly diagnosed cancer and the second leading cause of cancer related deaths among American women (1). Despite recent advances in breast cancer therapeutics, triple negative breast cancer (TNBC) remains the subtype with poorest prognosis due to limited treatment options. The 4T1 murine mammary metastatic model has been widely used as a model for TNBC because of the similarities in hormone receptor profile to human TNBC (2). One caveat of the 4T1 model is that there are few known tumor associated antigens which has limited the use of this model in preclinical immunotherapeutic studies (3, 4). The firefly luciferase gene, luc2, has been transfected into various tumor cell lines as a reporter gene for imaging studies. However, previous studies demonstrate that the luciferase epitope can induce CD8+ cytotoxic T cell specific responses, thus may be serving as a tumor antigen for immune recognition (5-7). Therefore, the first goal of this study was to explore the in vitro and in vivo growth rates of the parental 4T1.2 cell line and the luciferase transfected clone (4T1.2\textsuperscript{luc}). In addition, we also investigated the role of CD4+ T cells, CD8+ T cells, and NK cells in primary tumor growth, metastatic progression and survival.

Consistent with our hypothesis, we found that 4T1.2 and 4T1.2\textsuperscript{luc} cell lines had similar in vitro proliferation rates suggesting that luciferase expression by 4T1.2\textsuperscript{luc} cells does not interfere with pathways important in cell proliferation.
However, *in vivo* tumor growth differed in 4T1.2 and 4T1.2\textsuperscript{luc} tumor-bearing mice. 4T1.2\textsuperscript{luc} tumor-bearing mice had significantly reduced primary tumor growth compared to 4T1.2 tumor-bearing mice. Thus, in an immunocompetent host, the 4T1.2\textsuperscript{luc} cell line may be more immunogenic than the 4T1.2 cell line, i.e. 4T1.2\textsuperscript{luc} cell line was more effectively recognized by the host immune system than the parental 4T1.2 cell line. Baklaushev *et al.* reported similar *in vitro* growth rates between the parental 4T1 cell line and two luciferase-expressing 4T1 cell lines. However, significantly lower growth rates were observed in luciferase-expressing 4T1 tumor cells compared to the parental 4T1 *in vivo* in BALB/c mice (7). Moreover, luciferase-specific IFN-γ production was detected in the two luciferase-expressing clones of 4T1 in tumor-bearing mice, but not in the mice implanted with the parental 4T1 cell line (7). In summary, our data are similar to the results reported by Baklaushev *et al.*, and demonstrate that expression of luciferase did not alter the *in vitro* proliferative capacity of 4T1.2\textsuperscript{luc} cell line, but luciferase could be recognized by the immune system as a foreign protein *in vivo*. This recognition likely influences tumor growth as 4T1.2\textsuperscript{luc} tumor growth was slower than 4T1.2 tumor growth in BALB/c mice.

Both innate and adaptive immune components are actively involved in the elimination phase of cancer immunosurveillance and the major immune cell populations involved are CD4\textsuperscript{+} helper T cells, CD8\textsuperscript{+} cytotoxic T cells and NK cells (99). By depleting each immune cell type in 4T1.2 tumor-bearing mice, we found that the absence of any aforementioned immune cell types did not significantly
alter the trajectory of primary tumor growth compared to immunocompetent controls. However, in 4T1.2\textsuperscript{luc} tumor-bearing mice, depletion of CD8\textsuperscript{+} cytotoxic T cells significantly increased primary tumor growth in comparison to the PBS-treated control group. Furthermore, we demonstrated that NK cell depletion significantly reduced primary tumor growth compared to PBS-treated group.

Our results confirm that CD8\textsuperscript{+} T cells are key effector cells of anti-tumor immunity in the 4T1.2\textsuperscript{luc} model. In breast cancer patients, tumor-infiltrating CD8\textsuperscript{+} T cells are significantly inversely correlated with advanced tumor stages, positively correlated with relapse free survival (RFS) and overall survival (OS), while the CD4\textsuperscript{+}:CD8\textsuperscript{+} ratio is negatively correlated with RFS and OS (137-139). Baklaushev \textit{et al.} demonstrated that immunization of mice using a epitope from luciferase that is recognized by CD8\textsuperscript{+} T cells resulted in growth inhibition of luciferase-expressing 4T1 primary tumors (7). In the current study, the absence of CD8\textsuperscript{+} T cells augmented 4T1.2\textsuperscript{luc} primary tumor growth suggesting that CD8\textsuperscript{+} cytotoxic T cells are important in mediating host anti-tumor effector immune responses in the 4T1.2\textsuperscript{luc} tumor model.

Contrary to our hypothesis, depletion of CD4\textsuperscript{+} T cells did not result in increased primary tumor growth in both 4T1.2 and 4T1.2\textsuperscript{luc} tumor-bearing mice. CD4\textsuperscript{+} T cells are a heterogeneous group of immune cells including T helper 1 (T\textsubscript{H1}), T helper 2 (T\textsubscript{H2}), T helper 9 (T\textsubscript{H9}), T helper 17 (T\textsubscript{H17}), T helper 22 (T\textsubscript{H22}), follicular helper T cell (T\textsubscript{FH}), and regulatory T cells (Tregs). Among these CD4\textsuperscript{+} T cell subsets, T\textsubscript{H} 1 provide help to activate CD8\textsuperscript{+} cytotoxic T cells, while Tregs are
potent inhibitors of antigen-specific immunity (140). Huang et al. demonstrated that in the 4T1 and EO771 mammary tumor models, both tumor-infiltrating CD4+ and CD8+ T cells accumulated with the progression of tumor, but CD4+ TILs became increasingly high in the CD4+CD25+Foxp3+ Treg subset late in tumor progression, and the CD4+:CD8+ ratio also increased with tumor progression (139). It is possible that TH1 plays an important role in the 4T1.2luc model, but depleting all subsets may have masked the effect of the loss of TH1 cells. Therefore, depletion of TH1 cells and Tregs separately in subsequent experiments may reveal more about the role CD4+ cell subsets in the 4T1.2 and 4T1.2luc tumor models.

In the current study, depletion of NK cells did not affect 4T1.2 primary tumor growth which supported our hypothesis. However, depletion of NK cells resulted in decreased tumor growth in 4T1.2luc tumor bearing mice, suggesting a pro-tumorigenic role for NK cells in this model. These findings contradict the evidence in some tumor models that NK cells exert an anti-tumor effect. Based on previous data collected in the Rogers laboratory, the expression of major histocompatibility complex I (MHC1) by 4T1.2 and 4T1.2luc cell lines is negligible. All healthy nucleated cells express MHC1 on their surface which can bind to inhibitory receptors of NK cells, thus preventing NK cells from attacking healthy tissue. In contrast, transformed, tumor cells often lack MHC1 which can activate NK cell recognition of tumors (88). Therefore, since 4T1.2 and 4T1.2luc lack MHC1 expression, we do not believe these tumors are recognized by NK cells. One
possible explanation for our findings is that NK cells can inhibit CD8^T cell activity (141) and depletion of NK cells may allow a greater CD8^T cell cytotoxicity against 4T1.2\textsuperscript{Luc} tumors. It is reported that NK cells can regulate T cell immunity via secretion of cytokines or direct lysing of T cells (142, 143). NK cells have been reported to increase IL-10 secretion after various infections (144, 145). NK cells can also interact with T cells by engaging NKG2D to NKG2D ligands on T cells and can kill activated T cells via cytolytic granules or death ligand pathways such as TRAIL or FasL (141, 142). Therefore, examining the interaction between NK cells and CD8^T cells in the 4T1.2\textsuperscript{a} model is necessary to explain the protumorigenic role of NK cells we observed in this study.

In 4T1.2\textsuperscript{Luc} tumor bearing mice, no statistically significant increase in lung and femur metastatic burden was observed in CD4^T cell, CD8^T cell and NK cell depleted groups. The current study was designed to examine the role of key immune components in 4T1.2 and 4T1.2\textsuperscript{Luc} models. Thus, we collected lungs and femurs at the end of study (day 35) based on our previous metastatic burden data. However, there were a large number of mice that were taken off prior to day 35 because their tumors reached the ethical limit or they were moribund. This may have introduced a bias in that animals with the lowest metastatic burden may have survived until day 35 post tumor implantation. Future studies are aimed at examining the metastatic burden of immune-depleted mice at an earlier time point to avoid a survival bias in the data. Also, we did not examine the metastatic burden of 4T1.2 tumor bearing mice, therefore we cannot compare to what extent
does immune system influence metastases between 4T1.2 and 4T1.2\textsuperscript{luc} tumor-bearing mice.

PBS-treated 4T1.2\textsuperscript{luc} tumor-bearing mice had a significantly longer median survival than PBS-treated 4T1.2 tumor-bearing mice. One reason that PBS-treated 4T1.2\textsuperscript{luc} tumor-bearing mice exhibit a significantly greater median survival compared to 4T1.2 group may be greater recognition of the tumor by the immune system due to the expression of luciferase. This immune control of the tumor may prevent accelerated tumor growth and/or the initiation of cachexia which contribute to survival. Consistent with our hypothesis, depletion of CD4\textsuperscript{+} T cells, CD8\textsuperscript{-} T cells and NK cells did not alter the median survival in 4T1.2 tumor-bearing mice compared to PBS-treated group. These results can also be explained by the lack of tumor antigens of 4T1.2 tumors, thus the importance of CD4\textsuperscript{+} T cells, and CD8\textsuperscript{+} T cells is less prominent in this tumor model.

In 4T1.2\textsuperscript{luc} tumor-bearing mice, CD4\textsuperscript{+} T cell and CD8\textsuperscript{+} T cell depletion groups had significantly reduced median survival compared to the PBS-treated group. NK cell depleted mice had identical survival rate with PBS-treated 4T1.2\textsuperscript{luc} tumor-bearing mice. It is reported CD8\textsuperscript{+} T cells and NK cells are crucial in 4T1 tumor-bearing mice survival, while CD4\textsuperscript{+} T cells are negatively associated with patient overall survival and the inhibitory CD4\textsuperscript{+}/FOXP3\textsuperscript{+} Treg subset is reported to be dominant in the late tumor stage (139, 146, 147). However, our data demonstrated that CD4\textsuperscript{+} T cells and CD8\textsuperscript{+} T cells are important immune cell populations to maintain survival in the 4T1.2\textsuperscript{luc} tumor model. Even though our
results contradict published data that CD4$^+$ T cells are negatively associated with survival, it is possible that total number and percentage of Treg cells are low in the CD4$^+$ T cell population in the 4T1.2$^{\text{luc}}$ model, so that most of the CD4$^+$ T cells may be performing a protective role. Future experiments need to be conducted to confirm the proportion of pro- or anti-tumor CD4$^+$ T cell subsets at sacrifice and the effect of depleting these CD4$^+$ T cell subsets on tumor growth and survival in 4T1.2$^{\text{luc}}$ tumor-bearing mice.
CHAPTER 6

CONCLUSION

In summary, 4T1.2 and 4T1.2\textsubscript{luc} tumor cell lines are widely used as TNBC models due to the similarity of cell surface expression of hormone receptors to human TNBC. Here we found both cell lines have comparable \textit{in vitro} proliferation rates. More importantly 4T1.2\textsubscript{luc} had significantly reduced \textit{in vivo} proliferation rate compared to 4T1.2 tumor cells and we demonstrated that this is due to recognition of luciferase on 4T1.2\textsubscript{luc} cells by CD8\textsuperscript{+} T cells. As a TNBC model, 4T1.2 cell line lacks defined tumor associated antigens, but 4T1.2\textsubscript{luc} cell line is more immunogenic which potentially expands its use to immunotherapeutic studies. Our data also suggest a pro-tumorigenic role of NK cells in this model. Future studies need to be conducted to clarify the interaction between NK cells, CD8\textsuperscript{+} T cells and tumor cells. We did not observe a significant increase in metastatic burden in the lung or femur in immune cell depleted 4T1.2\textsubscript{luc} tumor-bearing mice. Additional studies with a larger sample size are needed to clarify the roles that CD4\textsuperscript{+}, CD8\textsuperscript{+} T cells and NK cells in controlling metastases in 4T1.2\textsubscript{luc} tumor bearing mice. Lastly, we demonstrated that CD4\textsuperscript{+} T cells and CD8\textsuperscript{+} T cells are crucial for the survival of 4T1.2\textsubscript{luc} tumor-bearing mice but not for 4T1.2 tumor-bearing which suggests an important role for antigen-specific immunity in tumor growth and survival.
Results from the current study have solidified our understanding of the role of CD4$^+$ and CD8$^+$ T cells and NK cells in tumor growth, metastases, and survival in the 4T1.2$^{\text{Luc}}$ model. This information will enable our laboratory to investigate the immune mechanisms underlying the diet and exercise effects on tumor progression and survival previously reported. Specifically, future studies are aimed at determining if our diet and exercise interventions are enhancing the anti-luciferase response to 4T1.2$^{\text{Luc}}$ tumors, or may be preventing the emergence of immune suppressive cells, such as regulatory T cells.
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