Turning Up Antitumor Immunity Against Breast Cancer

Johnie Hodge

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TUNING UP ANTITUMOR IMMUNITY AGAINST BREAST CANCER

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ABSTRACT

Breast cancer is the most common cancer in women worldwide, and is the second leading cause of cancer-related death in spite of significant advances in treatment and emphasis on early diagnosis. While treatment of localized disease is often successful, metastatic breast cancer, especially of the triple negative molecular subtype, carries a much poorer prognosis. The significant role of the immune system in the progression from localized to metastatic disease is becoming more and more appreciated. Tumor escape from immune surveillance and immune suppression in the tumor microenvironment have become therapeutic targets in addition to the traditional goals of directly killing tumor cells. A number of immune therapeutic strategies have been proposed and tested, but few have seen clinical application in the treatment of breast cancer. The development of successful breast cancer immunotherapeutic strategies depends on the rapid identification of immunogenic antigens, timely delivery of these antigens by the optimal vehicle to induce a robust immune response, and appropriate combination with other therapies such as adjuvants, innate immune therapies, and the current standard-of-care treatments. This will allow for more comprehensive targeting of the breast cancer immune regulatory network and likely lead to improved therapeutic success. In the following experiments, two immune therapies which address elements of this multipronged approach will be investigated. The models in which these therapies are tested for gross phenotypic effect are intended to bring basic murine models of experimentation closer to the patterns of future clinical application. The first of these
therapies is emodin, a natural, small molecule compound derived from several Chinese herbs. Its immune modulatory properties have been demonstrated in a number of disease models, but our lab was the first to show that it is capable of attenuating breast cancer progression by blocking the interactions between tumor-associated macrophages and cancer cells. Here emodin is tested as an adjuvant therapy following primary tumor surgical removal for the purpose of preventing metastatic recurrence in a model of murine triple negative breast cancer. We show that trough interfering with TGF-β1 signaling, emodin is capable of decreasing the ability of tumor cells to invade and establish themselves at metastatic sites, thereby reducing metastatic recurrence and increasing overall survival. In addition to its ability to disrupt the reciprocal signaling between tumor cells and macrophages, emodin’s low cost and proven low toxicity make it a promising innate immune therapy. The second therapeutic strategy focuses on improving the effectiveness of dendritic cell-based therapeutic cancer vaccines. Our lab was the first to reveal the role of microRNA-155 (miR155) as a key regulator of dendritic cell function. Our previous work has shown that total-body knockout of miR155 increased both tumor growth and metastasis in an orthotopic model of murine breast cancer. Our therapeutic goal is to increase the presence of miR155 in the immune cells of the tumor microenvironment, most importantly in dendritic cells. Here, total immune system overexpression of miR155 and a miR155-overexpressed DC vaccination therapy are shown to reduce breast tumor growth and metastasis by boosting cytotoxic T cell activity through it multiple positive effects on dendritic cell maturation and migration to tumor-draining lymph nodes. From the results detailed here, both of these strategies hold promise as valuable elements of future combination immune therapies for breast cancer.
TABLE OF CONTENTS

Acknowledgements ........................................................................................................... ii

Abstract .............................................................................................................................. iii

List of tables .......................................................................................................................... vii

List of figures ........................................................................................................................ viii

Chapter 1: Introduction ...................................................................................................... 1
  1.1 Breast cancer .................................................................................................................. 1
  1.2 Breast cancer classification and characterization ......................................................... 2
  1.3 Breast cancer treatment ............................................................................................... 4
  1.4 Cancer immunotherapy ............................................................................................... 8

Chapter 2: Generation of a mouse model for triple-negative breast cancer post-surgery metastatic recurrence ........................................................................... 19
  2.1 Background .................................................................................................................. 19
  2.2 Preliminary test of metastatic recurrence model ......................................................... 21
  2.3 4T1-luc2-RFP metastasis time-point and tropism ....................................................... 29
  2.4 9th mammary fat pad 4T1-luc2 metastasis model ....................................................... 35
  2.5 Final 4T1-luc2 metastatic recurrence model test ....................................................... 38

Chapter 3: Emodin as an agent to prevent breast cancer post-surgery metastatic recurrence ........................................................................................................... 57
  3.1 Background .................................................................................................................. 57
  3.2 Detailed methods ......................................................................................................... 59
  3.3 Results .......................................................................................................................... 67
  3.4 Discussion .................................................................................................................... 76

Chapter 4: Generation of a microRNA-155 transgenic mouse model .............................. 94
  4.1 Background .................................................................................................................. 94
4.2 Detailed methods .................................................................98
4.3 Results .............................................................................105
4.4 Discussion ..........................................................................111

Chapter 5: miR155 overexpression boosts dendritic cell vaccine efficacy

for breast cancer ........................................................................122

  5.1 Background .........................................................................122
  5.2 Detailed methods ..................................................................123
  5.3 Results ................................................................................133
  5.4 Discussion ...........................................................................138

Chapter 6: Conclusions and future work .....................................153

  6.1 Emodin adjuvant therapy conclusions ..................................153
  6.2 Emodin adjuvant therapy future work ...................................155
  6.3 miR155tg dendritic cell vaccine conclusions .........................156
  6.4 miR155tg dendritic cell vaccine future work .........................158

References ..................................................................................160
LIST OF TABLES

Table 2.1 Antibodies for flow cytometry .............................................................. 43
Table 3.1 Antibodies for flow cytometry .............................................................. 80
Table 3.2 Antibodies for western blot/Immunohistochemistry .............................. 80
Table 3.3 Primers for RT-qPCR ................................................................. 80
Table 4.1 Antibodies for flow cytometry ............................................................ 114
Table 4.2 Primers for RT-qPCR ................................................................. 114
Table 5.1 Antibodies for flow cytometry ............................................................ 143
Table 5.2 Primers for RT-qPCR ................................................................. 143
LIST OF FIGURES

Figure 2.1 10 days of tumor growth was insufficient to achieve an appropriately high metastatic efficiency with 4T1-luc2-RFP cells.........................................................44

Figure 2.2 Metastases did not occur preferentially in the lungs ........................................46

Figure 2.3 Mice were able to recover from surgery and metastases appeared quickly ....46

Figure 2.4 Emodin treatment showed a decreasing trend in metastatic burden and an increasing trend in overall survival.................................................................47

Figure 2.5 The metastatic tropism of 4T1-luc2-RFP cells following primary tumor resection is not lung-specific .................................................................48

Figure 2.6 Gross lung metastases only developed after 26 days of tumor growth before resection ........................................................................................................50

Figure 2.7 4T1-luc2 cells metastasize almost exclusively to the lungs ......................51

Figure 2.8 Emodin increased overall survival in lung metastasis bearing mice ..........52

Figure 2.9 4T1-luc2 cells implanted in the 9th mammary fat pad metastasize efficiently to the lungs ........................................................................................................53

Figure 2.10 Two weeks of emodin treatment decreased both metastasis number and metastatic burden ....................................................................................................55

Figure 3.1 Adjuvant emodin treatment decreases metastatic recurrence of TNBC after surgical resection ..........................................................................................82

Figure 3.2 Emodin modulates the immune microenvironment of the metastatic site ....85

Figure 3.3 Emodin inhibits breast tumor cell EMT through modulating macrophage phenotype and TGFβ secretion .................................................................87

Figure 3.4 Emodin inhibits TGF-β and tumor-associated macrophage-induced migration and invasion of tumor cells ...............................................................88

Figure 3.5 Emodin interferes with both TGF-β canonical and non-canonical signaling in tumor cells .....................................................................................................91

Figure 4.1 The miR155-containing transgene is present, expressed, and functional in one founder line ......................................................................................................115
Figure 4.2 Total immune system miR155 overexpression reduces breast tumor growth and lung metastatic burden .................................................................116

Figure 4.3 Total immune system miR155 overexpression increases dendritic cell frequency, maturation, and T cell activation in tumor tissue and tumor draining lymph nodes ..................................................................................................................118

Figure 5.1 Dendritic cell maturation is promoted by miR155 overexpression ..........144

Figure 5.2 miR155tg dendritic cells exhibit increased migration in vitro and in vivo ....145

Figure 5.3 miR155tg dendritic cells elicit increased T cell activation in vitro and in vivo. Wild-type and miR155tg DCs were pulsed with tumor antigen, then co-cultured with naïve wild-type T cells ........................................................................................................146

Figure 5.4 miR155tg dendritic cells reduce systemic immune suppression ............148

Figure 5.5 miR155 overexpression increased the efficacy of a dendritic cell-based vaccine for breast cancer .................................................................................149

Figure 5.6 Effects of miR155 transgenic overexpression on molecules relevant to dendritic cell function ........................................................................................................152
CHAPTER 1

INTRODUCTION

1.1 BREAST CANCER

The most prevalent non-skin malignancy worldwide is breast cancer. Though second to lung cancer in terms of mortality, its much higher prevalence makes it the most expensive malignancy to treat [1]. The annual cost is expected to rise to $20.5 billion by 2020 [2]. 12.3% of women will be diagnosed with breast cancer at some point in their lives, and metastatic breast cancer remains largely incurable even with recent therapeutic advances [2]. Five-year survival of patients with metastatic disease is only 21% while that of patients with non-metastatic disease is 89-100%, and 1/3 of patients deemed metastasis-free at diagnosis develop distant metastasis after surgical removal of the primary tumor [3]. These sobering facts remain even as the overall breast cancer death rate has decreased by 36% from 1989 to 2012 in the United States [2]. This decrease has largely been due to increased emphasis on early detection and some improvements in the effectiveness of available therapies [4]. However, there is still a great need for further development of more effective therapies, especially for triple negative tumors, and to reduce the harmful side effects associated with systemic chemotherapy and radiotherapy, such as cardiomyopathy, peripheral neuropathy, neurocognitive dysfunction, decreased fertility, and the induction of secondary cancers among others [5]. Immune therapies present promising alternatives with a theoretically more targeted activity and more durable clinical response with fewer long-term negative effects.
1.2 BREAST CANCER CLASSIFICATION AND CHARACTERIZATION

Human breast cancer is divided into two overarching categories based on whether or not the disease has spread; namely, in situ carcinoma, which consists of cellular abnormalities and resulting distortions of ductal and lobular tissue, and invasive carcinoma, which has breached the basement membrane of the glands from which it originated. The majority of clinically identified cancers are invasive, and prognosis is largely determined by staging and subtype [2].

Staging is the extent to which the invasive carcinoma has spread at the time of diagnosis, and there are two widely used systems for quantifying this: the Surveillance, Epidemiology, and End Results (SEER) Summary Stage and TNM Staging. The TNM classification is most often used in clinical settings and is determined by the following three factors: how far the primary tumor has spread within the breast and adjacent tissues (T), the extent to which tumor cells have colonized draining lymph nodes (N), and the absence or presence of metastasis in distant organs (M) [6]. A stage of 0 to IV is then assigned based on the TNM assessment with 0 being in situ and IV being advanced metastatic disease.

There are two major subtype classifications: histological, of which there are 21, and molecular, of which there are at least four [7, 8]. These molecular subtypes are most often determined by the presence or absence of hormone (estrogen and progesterone) receptors (HR+/HR−) and the overproduction of human epidermal growth factor (HER2). Conveniently these biological markers can also be used as therapeutic targets, and often to great effect, making certain molecular subtypes demonstrably more treatable than
others. These four subtypes are generally as follows: luminal A (HR+/HER2-), luminal B (HR+/HER2+), HER2-enriched (HR-/HER2+), and triple negative (HR-/HER2-) [9-12].

Luminal A is the most common (74% of tumors), least aggressive, and the most quickly responsive to therapy, especially hormonal therapy, and is associated with the best prognosis of the four subtypes [13]. Luminal B tumors make up about 10% of breast cancers and are more aggressive than luminal A, often containing a high proportion of actively dividing cells [14]. With the recent advent of HER2-targeted therapies in addition to hormone therapies, the prognosis for luminal B tumors is improving. HER2-enriched tumors make up only 4% of all diagnosed breast cancers, and tend to be very aggressive with a poor prognosis in the absence of HER2-targeted therapies, which have proven highly effective [13]. Triple negative cancers are the most difficult to treat. Triple negative breast cancer is more common in younger women, African Americans, and those with BRCA1 gene mutations, though recent studies have shown that the increased prevalence among African Americans is not related to BRCA1 [15]. The vast majority (~86%) of triple negative breast cancer is basal-like based on gene expression profile in contrast to the other three molecular subtypes, none of which are more than 11% basal-like [16]. Basal-like breast cancers are so named because they express a pattern of markers consistent with the basal layer of the breast ductal epithelium [17]. With no ER, PR, or HER2 to target, clinicians are limited to surgical resection, radiotherapy, and cytotoxic chemotherapy. There are currently few targeted therapies for triple negative tumors, and thus this breast cancer subtype has the least favorable short-term prognosis, but is the focus of a great deal of study in order to understand its molecular background and translate this to targeted therapy [13, 18-20].
1.3 BREAST CANCER TREATMENT

Treatment regimens for breast cancer are largely determined by the stage and molecular subtype as well as patient characteristics, such as age and menopausal status [2]. In general, tumors discovered at earlier stages merit some type of surgical intervention combined with adjuvant therapies to reduce the likelihood of recurrence. Successful treatment options for late-stage, metastatic disease are more limited, and systemic chemotherapies or targeted therapies are key due to the fact that removal of the primary tumor has little beneficial impact on overall survival once the cancer has spread [2].

Surgical intervention often serves a dual purpose, to both remove the dysplastic tissue and aid in accurately staging the disease. The most common two surgical procedures currently performed are mastectomy (complete removal of the breast) and breast-conserving surgery (BCS), which involves removal of the tumor itself and a margin of normal tissue. Both BCS and mastectomy are often accompanied by removal of a few axillary lymph nodes, sentinel lymph node biopsy (SLNB), in order to determine staging, whether or not to perform further surgery, and to guide chemotherapy decision-making [21]. Patients are increasingly opting for mastectomy over BCS even though long-term outcomes are very similar for these two interventions, while BCS does yield a higher rate of local recurrence [22-24]. Patient studies have found that this is because of reluctance to undergo radiation therapy, which is more often recommended with BCS, and patient fear of recurrence after less aggressive BCS surgery [25]. Modified radical mastectomy, removal of the entire breast and axillary lymph nodes, is also performed in some cases if patients elect to pursue this route, but the risk of lymphedema, severe
swelling of the arm due to impaired lymph drainage, discourages some, though there are successful physical therapies for this condition [26, 27]. Traditional radical mastectomies, in which the muscles of the chest wall are also removed, are very rarely performed and are not necessary to adequately remove most cancers. However, the rate of bilateral mastectomy, or contralateral prophylactic mastectomy (CPM) is increasing. While this does eliminate the risk of developing future breast cancer, it has not been shown to increase long-term survival and is more closely associated with a number of harmful outcomes including mortality [28-33].

Radiation therapy often accompanies surgery, especially BCS or when a small number of tumor cells are found upon SNLB, and full axillary lymph node dissection is not performed [34, 35]. It employs appropriately shaped beams of ionizing radiation or an implanted radioactive source (brachytherapy) to damage the DNA of and kill tumor cells, often using intersecting low-energy beams to limit damage to adjacent tissue [2]. In the case of BCS, adjuvant radiation therapy has been demonstrated to reduce breast cancer recurrence by about 50% and the relative risk of death due to recurrence by about 20% [36]. The most common implementation is whole breast radiation for three to seven weeks [37]. Brachytherapy has been shown to be more likely to have complications, such as infection, and still requires a subsequent full mastectomy more often than whole breast external radiation, though any radiotherapy is not without side effects.

The other common component of breast cancer treatment is systemic therapy, referring to any treatment to which the entire body is subjected due to its distribution via the circulation. This includes chemotherapy, targeted therapy, immunotherapy, and hormone therapy. These treatments are usually administered either before primary
surgical intervention (preoperative or neoadjuvant) or after (adjuvant). It is often advisable to attempt to shrink larger tumors via neoadjuvant therapy in order to reduce the complexity of resection. Additionally, systemic neoadjuvant therapy has been shown to be as effective as adjuvant therapy at increasing survival, and decreasing metastatic recurrence [38]. After all, the main purpose of adjuvant chemotherapy is to destroy disseminated cancer cells and micrometastases, and neoadjuvant therapy likely has a similar effect. Due to this ability to kill tumor tissue throughout the body, high stage, metastatic disease is primarily treated with systemic therapy, as primary tumor surgical resection has little impact on the total disease burden. The decisions about which systemic therapies are appropriate are governed by tumor stage, molecular subtype, and occasionally genetic panels such as Oncotype DX and PAM 50, which can help to predict the risk of metastatic recurrence [2].

Traditional chemotherapies are more deadly to rapidly proliferating cells than more quiescent ones. This makes them generally more effective against more aggressive tumors such as those overexpressing HER2 and triple negative cancers [39]. Chemotherapy is the most commonly prescribed therapy for triple negative tumors regardless of stage due to the complete lack of targeted therapies, with platinum compounds, such as cisplatin, demonstrating clinical efficacy. However, recent massively parallel sequencing has revealed that triple negative breast cancer itself is highly heterogeneous and some forms may express molecules that could be potential therapeutic targets such as androgen receptors [40]. No systemic chemotherapy is free of potentially life-altering toxicities, driving the development of targeted/immune therapies to reduce this collateral morbidity [41].
The goal of hormone therapy is to either lower systemic levels of estrogen or block its pro-tumor effects. Hormone therapy is of course most effective on luminal tumors, especially luminal A tumors expressing both estrogen (ER+) and progesterone (PR+) receptors. Until recently it was not known why such “double positive” tumors were more susceptible to hormone therapy, because the progesterone receptor was not directly targeted by most therapies, and ER+/PR+ patients received the same treatments as ER+/PR- ones. Evidence now points to increased progesterone receptor activity naturally counteracting the estrogen-mediated proliferation, and progesterone receptor agonism may boost the effectiveness of estrogen receptor antagonism [42]. Compared to chemotherapies, hormone therapy is usually implemented for much longer after the primary surgical intervention takes place. Tamoxifen, a selective estrogen receptor modulator (SERM) and one of the most common hormonal therapies, when given for at least five years post-surgery, reduced the rate of recurrence by 40-50% over the 10-year period following surgery and reduced mortality by about 30% 15 years after diagnosis [43]. More recent studies have demonstrated that even longer use of tamoxifen yields even greater benefit [44, 45]. In addition to SERMs, aromatase inhibitors (AIs) are commonly used to treat HR+ breast cancer. The aromatase enzyme converts testosterone to estradiol and androstenedione to estrone, thus inhibiting this enzyme, reduces total available estrogens produced in the adrenal glands, but has no effect on ovarian estrogen. For this reason AIs are more effective in post-menopausal women and often considered an adjunct therapy to SERMs. These hormonal therapies, while effective for HR+ tumors, are not without side effects related to starving the body or specific tissues of estrogen stimulation such as menopausal symptoms and osteoporosis.
A number of targeted therapies are now available for tumors overexpressing molecules related to increased proliferative activity. Several of these target HER2; trastuzumab and pertuzumab are monoclonal antibodies against different regions of the HER2 protein. Trastuzumab has been demonstrated to reduce the risk of recurrence in early stage, HER2-enriched tumors by about 50% relative to chemotherapy alone [46]. Ado-trastuzumab emtansine is a combination molecule consisting of the same antibody found in trastuzumab and the chemotherapy drug emtansine (DM-1). The HER2 antibody preferentially carries what would be a systemic chemotherapy to the tumor, thus decreasing side effects and increasing the tumoricidal effect [47]. Other approved targeted therapies block cyclin-dependent kinases (CDKs) involved in cell proliferation and mTOR, a protein that promotes angiogenesis and proliferation, and there are many more currently in development [48-51]. A noteworthy issue with these therapies is that all of these targets are the body’s native molecules which are just aberrantly expressed in breast cancers, thus even these therapies are not targeted to only the tumor cells.

1.4 CANCER IMMUNOTHERAPY

The targeted therapies discussed in the previous section are all antibodies, and are thus referred to as passive immunotherapies [52]. Any directly tumor-targeting antibody or adoptive cell therapy is considered a passive immunotherapy, while cancer vaccines and directly immune modulatory antibodies (such as checkpoint blockade) are considered active immunotherapy [52]. The only immune modulatory antibody currently approved for the treatment of breast cancers with some genetic characteristics is pembrolizumab, a humanized IgG4 isotype antibody against lymphocyte PD-1. In 2017 the FDA approved the use of pembrolizumab for metastatic solid tumors with genetic anomalies such as
microsatellite instability and mismatch repair deficiency [53]. Two of the most significant reasons why the subset of tumors for which this drug can be used is so small are: (a) not all tumors express elevated levels of PD-L1 and (b) blocking the interaction between PD-1 on leukocytes and PD-L1 fosters the immune-mediated destruction of the PD-L1 expressing tumor, but also can induce severe autoimmunity resulting in conditions such as Crohn’s disease, lupus erythematosus, and rheumatoid arthritis [54]. Other PD-1 and PD-L1 antibodies have shown promising results in breast cancer studies and several are currently undergoing breast cancer-specific clinical trials, but response rates remain at about 15-30% when used as a monotherapy. Modifications to PD-L1 antibodies, such glycosylation via B3GNT3 glycosyltransferase, have increased this response rate significantly, but so far only outside the clinical setting [55]. CTLA-4 blocking antibodies, such as ipilimumab, have been approved for some cancers, but not breast cancer, and present a higher risk for life-threatening autoimmunity than PD-1/PD-L1 interference [56]. Several other checkpoint inhibitors have been investigated, and some, including TIM-3 blocking antibodies, have demonstrated potential to increase proliferation and cytokine release from tumor antigen-specific T cells, but none have been approved for clinical use [57, 58].

Another emerging immune therapy for solid tumors involves adoptive transfer of tumor specific T cells. There are several such strategies including expansion of tumor-infiltrating lymphocytes (TILs) and retrovirus-encoded T cell receptors, but most commonly this is achieved by genetically modifying the patient’s own T cells to express a chimeric antigen receptor (CAR-T cells) [59]. The CAR itself often consists of the variable region of a monoclonal antibody to a tumor-expressed antigen which has been
grafted onto the internal signaling domains of the T cell receptor and, in newer iterations, other costimulatory molecules such as CD28 [60]. This strategy has proven quite effective against leukemia, but many solid tumors, including breast cancer, do not respond significantly to such therapies [61]. Some recent studies, however, have uncovered effective CAR-T targets, even on TNBC, and show promising results, including regression of primary tumors and lung metastases of human cancers implanted in immune deficient mice [62]. The promise of CAR-T cell therapy is not without its drawbacks. The most significant of these stems from the fact that the engineered T cells are highly effective at killing the tumor. In malignancies for which CAR-T cell therapy has been approved, especially if there is a high disease burden, such targeted cells can sometimes elicit the release of enormous amounts of pro-inflammatory cytokines, termed cytokine release syndrome (CRS). Release of large quantities of cytokines such as IFN-γ, IL-6, and GM-CSF can cause fever, shock, and liver failure. It is for this and other reasons that CAR-T cell therapy is often seen as a “double-edged sword”, and co-administered inhibitors of some of these off-target effects may prove useful [63].

Cancer vaccines move a step beyond checkpoint blockade in complexity of implementation, and hold promise as both adjuvant and long-term preventive therapies for patients with higher likelihoods of recurrence. The goal of these therapeutic vaccines is to sensitize the patient’s immune system to specific epitopes either unique to or enriched on the tumor cells, thus allowing the relatively specific targeting and destruction of the cancer by the patient’s own immune cells. Tumor cell destruction then releases additional cytokines and antigens that further boost and focus the immune response, which can be sustained long after treatment has ended [64]. This immunologic memory
allows the body to seek out and destroy the cancer systemically and stave off recurrence from disseminated micrometastases. The main challenges with this approach are to identify ideal tumor associated antigens (TAAs) which both are unique and produce an effective immune response. The identification of TAAs has been taking place since the late 1970s. The majority of these early endeavors were focused on mutations in what were then newly discovered cancer-causing genes and the aberrant proteins transcribed from them. Vaccines based on the proteins derived from oncogenes, and fusion proteins (such as BCR-ABL-1) were shown to be effective in mouse models and were tested clinically with mixed results. Proteins from oncogenic viruses such as human papilloma virus (HPV) and Epstein-Barr virus (EBV) have also been investigated. Patients with relevant virus-mediated cancers did possess limited numbers of T cells specific for viral proteins, but while results in mouse models were promising, none of these vaccines demonstrated clinical efficacy [65-67]. Since that time, more traditional preventive vaccines have decreased the incidence of several important oncogenic viruses and the malignancies often arising from chronic infection. Hepatitis B virus (HBV) vaccination efforts promise to prevent 1.5 million hepatocellular carcinoma (HCC) related deaths over the next 12 years, and human papilloma virus (HPV) vaccines have not only demonstrated drastic decreases in the incidence of both cervical and oral cancers, but have also resulted in the regression of extant neoplasia in some clinical trials (100% regression among some patient cohorts) [68-73]. Where these and other vaccines have previously only been tested as treatments, improvements in technology and a change in perspective have led to overwhelming success as preventative measures. There have also been efforts to use TAAs in the development of preventative vaccines for non-viral
mediated cancers in high risk patient populations. More recent efforts along these lines have been met with some success, especially with the TAAs MUC1 (here in ovarian cancer, though also implicated in some breast cancers) and HER2 [74]. This is likely because both of these antigens are required for the growth and development of their respective malignancies, and losing them to escape immune destruction would likely bring little selective advantage for the tumor.

One problem with the early therapeutic vaccine efforts is the fact that the vast majority of tumor antigens to which there is an adaptive immune response are non-mutated proteins which are overexpressed in tumors. The early focus was on peptide and peptide plus adjuvant designs, similar to preventative vaccines, but since 1990 overexpressed TAAs have become the focus of vaccine design. Some of the first attempts at breast cancer vaccination were peptide vaccines using the familiar marker HER2, and at least one such HER2-peptide vaccine therapy, nelipepimut-S, is currently undergoing phase III clinical trials. Nelipepimut-S is a nine amino-acid peptide (E75) from the extracellular domain of HER2 [75]. Many patients with HER2-expressing breast cancer have been found to already have some level of immunity to E75 antigen, and in this subset of cancers, it has demonstrated clinical benefit [76].

As the importance of dendritic cells (DCs) as professional antigen presenting cells became clear over this time period, and the techniques required to generate large quantities of autologous DCs were developed, these cells came to be viewed as the best vehicles for antigen delivery and initiation of a tumor-specific adaptive immune response. Hundreds of DC-based vaccine trials were carried out for many different cancers [77, 78]. Viruses, virus-like particles, and bacterial vectors were also engineered to carry
tumor antigens as vaccines [75, 79, 80]. Emerging knowledge of the stimulatory effects of toll-like receptor (TLR) agonism on antigen presenting cells led to the inclusion of TLR ligands as adjuvants in vaccines to more robustly activate the immune system. In spite of all of these advances and refinements, most of these vaccines had little impact on disease-free survival, overall survival, or recurrence [81]. In order to shed light on the reasons for these shortcomings and guide future vaccine development, several meta-analyses have been performed on hundreds of these trials. A key conclusion from these meta-analyses is that the vast majority of vaccines phase II trials did not demonstrate statistically significant benefit and never moved on to phase III (only 4% did). This likely may have been due to the limited duration of outcome monitoring, and none of the studies analyzed contained long term (>10 years) follow-up statistics. However, 12-year survival rates have recently been reported for an ex vivo matured autologous DC vaccine against non-resectable metastatic melanoma. 19% of these patients were alive after this lengthy period of time, an impressive result for an aggressive disease with a normally very poor prognosis. This result is comparable to treating similarly severe melanoma with FDA-approved CTLA4 checkpoint blockade therapy [82].

Though no breast cancer vaccines are currently available, many either have been or are currently under development. The first, and only true, FDA-approved cancer vaccine, sipuleucel-T, was approved for the treatment of hormone-resistant metastatic prostate cancer in 2010, but no such vaccines have been approved thus far for any other malignancies [83]. Sipuleucel-T is a DC-based vaccine. Its implementation involves the extraction of the patient’s own circulating monocytes, which are then incubated with a fusion protein called PA2024 made up of granulocyte monocyte stimulating factor (GM-
CSF) and prostatic acid phosphatase (PAP), an antigen present in almost all prostate
cancer cells. This stimulates the differentiation of the immature monocytic cells into DCs
as well as their maturation into effective presenters of the provided tumor antigen, PAP.
These cells are then re-infused into the patient three times over a 6-week period [84]. To
date, two phase III clinical trials have demonstrated a modest, but statistically significant
increase in patient survival after sipuleucel-T treatment.

Another possible reason for the failure of these vaccines could have been the fact
that the participants in most of these trials were patients with advanced disease and,
likely, significant immune suppression. Even mature DCs bearing potently immunogenic
antigen were insufficient to overcome this environment and carry out the desired
tumoricidal activity. To combat this, there are three obvious avenues by which better
vaccine success rates may be achieved, all of which may in the end be combined for
maximal effect. First, more complex vaccines containing many potentially effective
tumor antigens and adjuvants may provide more robust immune stimulation than the
single antigens tested in the majority of these trials. Second, enrolling patients with early
stage disease might allow the introduction of vaccine material into a less immune-
suppressive micro-environment, where it stands a better chance of beneficially increasing
the patient’s own antitumor immune response. Third, ex vivo genetic manipulations of
autologous DCs may increase their capacity for maturation and decrease their
vulnerability to intratumoral immunosuppression, again allowing the vaccine to better
carry out its function as intended [85].

Several more recent clinical trials of DC-based vaccines in patients with pre-
malignant breast lesions, ductal carcinoma in situ (DCIS), have demonstrated favorable
results. These have taken advantage of the effectively immunogenic TAA, HER2. One such trial used the following strategy. Four weekly autologous DC vaccinations were administered to 13 patients prior to surgery. These DCs, loaded with HLA class I-binding and HLA class II-binding HER2-derived peptides, were matured and activated in vitro. In order to maximize the immune response and ideally place the DCs for successful tumor-primed T cell interaction, the vaccine was injection into regional lymph nodes likely to be draining the tumor bed. All patients developed HER2 peptide-specific CD4+ and CD8+ cells as well as a robust tumor-lytic antibody response. ~64% of patients were found to have a decreased DCIS burden at surgery and remaining DCIS contained a significant T and B cell infiltrate. Accompanying this decrease in tumor size, HER2 expression was also decreased, which also likely had a direct impact on tumor cell survival [86]. A second trial, in which 27 patients with DCIS participated, replicated these favorable results. After following the same vaccination schedule, five of the 27 patients were found to have no evidence of DCIS at surgery. Half of the remaining 22 patients had lost their HER2 expression [87]. Thus far, there has not been follow-up or disease outcome data collected for these studies, but as the prognosis for DCIS is usually quite good, a high long-term survival rate and low incidence of recurrence would be expected even without this increased tumoricidal immune response.

During the early period of cancer therapeutic vaccine development, the induction of systemic immune suppression by the tumor was largely unknown, but since that time, many mechanisms of immune suppression have been discovered and provide potential immune therapeutic targets. It is very likely that groups of these strategies will be shown to function well together, and just as tumor cells take advantage of many mechanisms to
compromise host immunity, treatments will need to counteract these efforts on multiple fronts simultaneously. Some of the primary immune suppressive cell populations that have been targeted are monocyte-derived suppressor cells (MDSCs) and tumor associated macrophages (TAMs). Multiple aspects of these cells have become the focus of proposed therapeutic perturbations whether through blocking growth factor receptors such as CSF-1 [88], altering metabolic pathways such as fatty acid oxidation or glycolysis [89], or blocking the activity of certain key enzymes such as indoleamine 2,3-dioxygenase 1 (IDO1) [2, 90-92]. Combinations of these innate immune therapies, checkpoint inhibition, T cell transfer, and therapeutic vaccines may have the most significant and universal antitumor effects [93]. The relative successes of checkpoint inhibition and CAR-T cells, coupled with new data supporting the inclusion of therapeutic vaccines as part of combination immunotherapy, has led to a significant recent increase in enthusiasm for therapeutic vaccine development as a part of a multi-pronged approach.

As the breast cancer molecular subtype with the most limited number of treatment options, TNBC is often the focus of new combination immunotherapeutic approaches. The only completed clinical trial to date testing the efficacy of immune checkpoint inhibitors on TNBC, specifically, demonstrated a benefit to only a limited number of patients [94]. Oncolytic virotherapy has been found to enhance general antitumor immunity, reduce forced recurrence, and render TNBC more vulnerable to checkpoint blockade in murine studies [95, 96]. Studies into TNBC genomic data has yielded a surprising, and fortuitous finding. Almost all of the immune activation pathways were enriched to a significantly higher degree in TNBC versus non-TNBC [97]. This means that it may be the case that while TNBC expresses no traditionally targeted antigens, it is
the most immunogenic molecular subtype and may be the most vulnerable to immune therapy. The majority of immune checkpoint genes, including CTLA4 and PD-1, are overexpressed in TNBC compared to non-TNBC and normal tissue. There is also a much higher density of tumor-infiltrating lymphocytes in TNBC than non-TNBC, though it is important to remember that many of these cells are immune suppressive and would be negative prognostic indicators. However, it was found that CD57 expression was more abundant in TNBC than in non-TNBC; CD57 is a marker of activated T and NK cells. Other proinflammatory genes such as IFNγ and IRF1 are also enriched in triple negative tumors [97]. Overall the genomic profiling of these tumor samples paints a picture of TNBC as both a highly immunogenic and highly metastatic tumor which may prove a fruitful target for checkpoint inhibition, therapeutic vaccination, and various combination immunotherapies.

The future success of cancer immunotherapy will depend on the rapid identification and/or use of novel immunogenic antigens. Delivery of these antigens by the optimal vehicle, such as DCs, viruses, or some other yet to be discovered mechanisms will also be key to future therapeutic success. Coupling these therapies with adjuvants and immune modulatory treatments such as checkpoint blockade, IDO1 inhibition, or other innate immune therapies will allow for the more comprehensive targeting of the breast cancer immune regulatory network and improved therapeutic success. In the experiments described here, the development of two immune therapeutic modalities and strategies are discussed. Each is intended to both bring basic murine models of experimentation closer to the patterns of clinical application and to further the development of two immune therapies toward use in human patients. The first of these
therapies is emodin, a natural compound derived from several herbs used in traditional Chinese medicine (TCM) [98]. It has been shown to have numerous immune modulatory properties [99-102], but our lab was the first to demonstrate its ability to attenuate breast tumor progression through its effect on macrophage-cancer cell interaction [103]. Here emodin is tested in a murine model of tumor resection as an adjuvant therapy to prevent metastatic recurrence of triple negative breast cancer. Its proven low toxicity and ability to disrupt the reciprocal signaling between tumor cells and macrophages make it a promising innate immunotherapy [104]. The second therapeutic strategy involves genetic modification of DCs to increase their immune stimulatory capabilities and the implementation of these modified DCs as therapeutic breast cancer vaccine. Our lab was again the first to reveal the role of microRNA-155 (miR155) as a key regulator of DC function and mobility [105]. We have previously demonstrated that total-body knockout of miR155 increased both tumor growth and metastasis in an orthotopic model of murine breast cancer; here total immune system overexpression of miR155 and a miR155-overexpressed DC vaccination therapy are shown to suppress murine breast tumor progression. Some of the relevant parameters of DC maturation, migration to draining lymph nodes, and activation of T cells are also investigated using these miR155-overexpressed DCs.
CHAPTER 2

GENERATION OF A MOUSE MODEL FOR TRIPLE-NEGATIVE BREAST CANCER POST-SURGERY METASTATIC RECURRENCE

2.1 BACKGROUND

Historically, the drug development process for cancer therapeutics has relied heavily on preclinical murine models to determine the effectiveness of treatments in vivo. Especially in the case of using immune deficient mice bearing human cancer cell lines or explants, these models have had difficulty in predicting treatment efficacy for patients [106, 107]. The tumor microenvironment in a largely immune competent human is often found to respond very differently to treatments leading to many failed clinical trials [108, 109]. In order for mouse models to be more predictive of clinical success, whenever possible it is advantageous to use immune competent mice. The interplays between tumor cells, immune cells, and other stromal cell populations determine the efficacy of therapies in patients where these interactions also shape disease progression. This is particularly true when testing targeted therapies and immunotherapies [108, 109]. It is also very common for studies to not focus on the most appropriate measures of therapeutic success or endpoints. Many murine studies of cancer progression focus on inhibiting tumor growth and monitor it as the key phenotypic finding, with a reduction in final tumor weight indicating positive therapeutic benefit. While reduction in tumor size is a welcome clinical outcome, it is usually only a minor component of overall therapeutic goals related to easing surgical excision or a step toward eradication of a highly
invasive/disseminated malignancy (which has a relatively low success rate).

Overemphasizing primary tumor growth retardation can sometimes lead to the use of therapies that simultaneously encourage metastatic spread and local recurrence. This is the case with some systemic cytotoxic therapies, and has been largely attributed to the induction of a pro-tumor immune response to the dying tumor cells [110-113]. Clinical outcomes are usually measured in terms of progression-free survival and overall survival, criteria that often largely depend on the degree of tumor metastatic progression after treatment [114]. Preclinical, immune-competent murine models mirroring the treatment strategies and schedules actually used to treat human breast cancer should serve as a better predictor of patient outcomes when/if therapies progress to clinical testing [115].

In an attempt to do just this, we have established a murine model of highly metastatic TNBC, which as closely as possible, seeks to imitate the progression from of diagnosis to surgical excision and subsequent adjuvant therapy to prevent metastatic recurrence experienced by breast cancer patients [116]. Non-invasive molecular imaging was used to estimate the extent of metastatic dissemination leading up to and after surgical resection. As the eventual purpose of the model is to test the effect of emodin treatment on metastatic recurrence, the optimal traceable cell line, injection site, and time-point for tumor resection have to be determined where metastases would eventually arise in untreated mice but are not detectable at the time of surgery. This recurrence could be the result of existing micrometastases or circulating tumor cells present at the time of tumor removal, but exhaustive studies to parse this out have not been performed. At this time for our phenotypic analysis, the broader measures of clinical outcome are most relevant.
2.2 PRELIMINARY TEST OF METASTATIC RECURRENCE MODEL

2.2.1 DETAILED METHODS

Tumor models

7 week-old, female BALB/c mice were purchased from Jackson Labs (Bar Harbor, ME), and were housed at the University of South Carolina Animal Research Facility for one week prior to experiments. All procedures were approved by the University of South Carolina Institutional Animal Care and Use Committee. 4T1-luc2-RFP cells were generated by transducing 4T1-luc2 cells (PerkinElmer, Waltham, MA) with pWPI-RFP lentivirus. The resulting 4T1-luc2-RFP cells were sorted using a FACS Aria II (BD Biosciences, San Jose California) and stored in liquid nitrogen. 4T1-luc2-RFP cells were recovered from liquid nitrogen storage and passaged not more than three times to expand before inoculating BALB/c mice. 4T1-luc2-RFP cells were washed twice in PBS after harvesting with 0.05% Trypsin/EDTA and resuspended in PBS at a density of 10x10^6 cells/mL. On Day 0, 30 8-week-old female Balb/c mice were bilaterally injected with 2x10^5 4T1-luc2-RFP tumor cells in the 4th pair of mammary fat pads by creating a 1 mm-long incision about 1 mm inferior to the nipple and using a short, beveled needle for cell implantation. This blunt needle method was used to help ensure delivery of the tumor cells into the fat pad tissue at a shallow depth without entering the peritoneum. On Day 6, mice were separated into 3 groups based on equal average tumor volume. On Day 8, hair was removed from the surgical field using a depilatory cream in preparation for surgery. Tumors were allowed to grow for 10 days to a volume of ~200 mm^3 before removal. Tumor volume was calculated using the following formula: (short axis)^2 x (long axis)/2 = volume. The tumors were removed on Day 10. On Day 11, the
three groups of mice received i.p. injections of PBS with 2% DMSO by volume containing either 0 mg/kg, 20 mg/kg or 40 mg/kg emodin. The volume of these injections was ~800 µL per mouse. These i.p. injections were administered daily for the next two weeks. After two weeks of daily injections, injections were given every other day for two more weeks. When mice died during the course of this study or exhibited significant moribundity and were sacrificed, their lungs and other metastatic sites identified on IVIS Spectrum (PerkinElmer) imaging were taken for counting of metastatic nodules/determination of metastatic burden. On Day 42 the lungs of surviving mice were removed for metastatic and immune cell population analyses.

*Tumor removal procedure*

Each mouse was anesthetized using an isoflurane vaporizer and maintained under anesthesia for the duration of the surgery with the vaporizer set to deliver 2% isoflurane. The surgical field was cleaned liberally with 70% ethanol and betadine swabs. A 1-2 cm incision was made medial to the tumor, and the tumor, with as much surrounding fat pad as possible, was resected. An electrocautery unit was on hand to stop excess bleeding if necessary. Incisions were closed with stainless steel clips and suture. Mice were allowed to recover in a cage heated to 30˚C.

*IVIS Imaging*

BALB/c mice bearing 4T1-luc2-RFP tumors were imaged using an IVIS Spectrum (PerkinElmer). Prior to data collection, a kinetic curve was collected. To do this, D-luciferin (Sigma-Aldrich Corp., St. Louis, MO) was dissolved in PBS at a concentration of 15 mg/mL. Mice received 10 µL of this solution per gram body weight intraperitoneally (i.p.), were anesthetized using an isoflurane vaporizer, and imaged at
600 nm wavelength beginning 10 min after having received the D-luciferin injection. Images were obtained once per minute for the next 30 min while the mice were maintained under anesthesia at 1.5% isoflurane. Metastatic recurrence was monitored using the IVIS Spectrum to image the luciferase activity in the 4T1-luc2-RFP cells using the same imaging procedure described for kinetic curve determination, but only one image was collected at 10 min. IVIS imaging began on Day 13 and continued weekly until the termination of the experiment.

Flow Cytometry

Lung tissue was collected from the surviving mice on Day 42, cut into small fragments and digested using the following mixture of enzymes in serum-free RPMI at 37°C for 45 min: 10 mg collagenase IV (Worthington Biochemical Corp., Lakewood, NJ), 10 µg hyaluronidase (Sigma-Aldrich Corp.), and 100 µg DNAse I (Sigma-Aldrich Corp.). Red blood cells were then lysed in 3 mL RBC lysing Buffer (Sigma-Aldrich Corp.) for 5 min at RT. 25 mL PBS was added and each sample pelleted by centrifugation at 325 x g for 5 min. Cell pellets were resuspended in 10 mL PBS, passed through a 70-µm cell strainer, and pelleted by centrifugation. Cells were counted and 1x10⁶ cells from each sample was resuspended in 100 µL staining buffer (PBS 2% FBS). Cells were blocked with Fc blocking antibody (Biolegend, San Diego, CA) for 10 min at 4°C, then cells were stained with anti-CD3 FITC, anti-CD8 APC, anti-F4/80 FITC, and anti-CD206 APC (Biolegend) for 30 min at 4°C. Cells were then washed twice with PBS and resuspended in 500 µL PBS for flow cytometry using a BD FACS Aria II and FACS Diva software (BD Biosciences). 20,000 events were collected for each sample.
Statistical Analysis

Data were shown as mean ± standard error of mean (SEM) whenever the mean was the primary value representative of a sample group’s behavior. Two group comparison of means was accomplished using a 2-tailed Student’s t test. All such comparisons were performed using GraphPad Prism 5 software (Graphpad Software Inc., San Diego, CA). Differences between proportions were determined using a two tailed N-1 Chi-Square test. Three group comparison of means was accomplished using a two tailed ANOVA followed by Tukey multiple comparisons test. p≤0.05 was considered statistically significant for all tests.

2.2.2 RESULTS

The goals of this preliminary study were to (1) determine whether 10 days post-orthotopic 4T1-luc2-RFP cell inoculation is a good time-point for tumor removal that ensures appropriate metastatic recurrence rate; (2) test the general metastatic potential of the 4T1-luc2-RFP cells and their tropism for lung tissue; (3) assess the survivability of our surgical technique; (4) determine the timescale for metastatic recurrence; and (5) determine whether emodin has an effect on metastatic recurrence. How well this model met or shed light on each of these objectives will be discussed as well as the future studies needed to fine-tune the method.

10 days of tumor growth was insufficient to achieve an appropriately high metastatic rate with 4T1-luc2-RFP cells

There were no statistically significant differences between average tumor volume or radiance between the groups prior to tumor resection as intended (Figure 2.1B&C). Three days after tumor resection, and after only three treatments, two mice in the 0 mg/kg
emodin group had obvious lung metastasis, and one of the mice in the 40 mg/kg emodin group exhibited signs of local recurrence or residual tumor (Figure 2.1D). We intended the surgery to completely eliminate the primary tumor, so this early local recurrence, likely due to incomplete removal, was not ideal, but others have experienced significant local recurrence when using a similar model [115]. The metastatic efficiency, or total percentage of mice in each group that developed metastasis, was 40% for the control-treated group and 20% for both the 20 mg/kg and 40 mg/kg emodin-treated groups, meaning in total only 26.6% of the mice developed metastases in any part of their bodies (Figure 2.1E&F). Due to this very low metastatic efficiency across all groups, no statistically significant differences could be determined.

Metastases did not occur preferentially in the lungs

From our IVIS imaging, in addition to very few of the mice ever developing metastases, the majority of these metastases did not occur in the lung tissue. The majority of highly bioluminescent regions that were obviously not local recurrence seemed to be located in the jaw and shoulder areas of the mice (Figure 2.2A&2.1E). It was not possible to determine whether these growths originated in lymph node or bone upon necropsy or autopsy. Also what appeared on IVIS to be local recurrence on IVIS were found at sacrifice to largely be metastases to the inguinal and hind limb region (Figure 2.2A&2.1E). Metastases occurred unpredictably with 4T1-luc2-RFP cells; other cell lines with more reliable lung tropism may be better for our purpose. Allowing tumors to grow longer in the mice before resection will also be considered.
Mice were able to recover from surgery and metastases appeared quickly

None of the mice died as a direct result of the surgery in spite of the fact that two of the mice did remove their wound clips during the recovery period immediately following surgery and required re-suturing of their incisions. Also, all of the mice in which metastases did arise developed these by two weeks post-surgery (Figure 2.3A). This two week timeframe (~3 weeks since tumor implantation) is consistent with other studies tracking the progression of 4T1 tumors [117].

Emodin treatment showed a decreasing trend in metastatic burden and an increasing trend in overall survival

Our previous studies have shown that emodin not only reduces metastases when administered shortly after tumor cell implantation but also when tumors are already established [103, 118]. Here, though our metastatic efficiency is low and not specific to a single location after the orthotopic tumors were removed, we show that emodin treatment does appear to exhibit some trends toward decreasing metastatic recurrence after surgical resection of the primary tumor and increasing overall survival. The observation of this trend in this particular study is obscured by the fact that the only mouse that developed what appeared to be local recurrence was in the 40 mg/kg (high dose) emodin group (Figure 2.1E). This is most likely due to lack of experience performing the surgery and not due to the treatment itself. In this high dose emodin group, this mouse was the only one that developed lung metastases and may never have developed these had the primary tumor been completely removed. When only chest radiance is considered as a measure of metastatic burden, the high dose emodin group had the lowest overall metastatic burden by a large margin, and the low dose emodin group fell between the control and high dose
emodin groups in chest radiance until mice began to die due to the progression of their disease (Figure 2.4A). Metastasis-free survival also exhibited a trend toward emodin reducing metastatic recurrence, though it did not reach statistical significance (Figure 2.4B). Overall survival paralleled metastasis-free survival, but the low mortality rates and small sample sizes yielded insufficient statistical power for differences to manifest (Figure 2.4C). The surviving mice at the conclusion of the study were not bearing any lung metastases (Figure 2.4D); nonetheless flow cytometric analysis was performed of lung macrophages and CD8+ T cells. There was unsurprisingly no difference in the total T cell burden in the lungs and only a slight, statistically insignificant increase in CD8 positivity among the mice receiving emodin treatment (Figure 2.4E). The F4/80 staining yielded no positive cells, which further study revealed was due to excessively harsh enzymatic digestion destroying the F4/80 expressed on the surface of the lung macrophages.

2.2.3 DISCUSSION

The data from this experiment show that many of our objectives for this model were not met, but do point toward ways of improving these deficiencies. First and most importantly, 10 days of tumor growth prior to resection was not adequate to induce enough metastases to be able to study the potential impact of therapies on metastatic recurrence with reasonable sample size. Future studies will monitor lung fields with IVIS imaging more frequently in order to attempt to gage when metastases begin to be visible. Then a study similar to this one will be repeated with tumors being removed a few days prior to this determined time of macro-metastasis establishment.
This particular strain of 4T1-luc2-RFP cell also does not appear to have a highly specific lung tropism in this fat pad injection followed by surgical resection model. The original purpose of using this cell line was to (1) have a highly aggressive model of TNBC, (2) be able to take advantage of the non-invasive molecular imaging afforded by the luciferin to monitor metastatic recurrence, and (3) to be able to analyze the level of red fluorescent protein (RFP) expression in a single cell suspension of lung tissue to easily determine metastatic burden. For the purposes of this study, the least important of these objectives is the presence of RFP to measure metastatic burden. This can be determined by counting and quantifying metastatic area on serial sections taken at measured intervals and stained with hematoxylin and eosin (H&E). It may be possible to perform a metastatic kinetics study using 4T1-luc2 cells without the RFP in order to see if these cells metastasize more predictably while still be monitored in vivo using the IVIS Spectrum.

The surgical method seems adequate for yielding high survivability and relatively low local recurrence. In future experiments a little more normal tissue around the tumor will be taken in order to ensure clean margins, and it remains to be seen how feasible this will be if tumors are allowed to grow larger to ensure a higher percentage of metastasis-bearing mice. It may be more difficult to effectively close the larger incisions.

With macrometastases appearing within one to two weeks after surgical resection, this timetable is quicker than expected, but adequate to administer several doses of treatment before the development of overt metastases. It remains to be seen if allowing the tumors to grow longer will affect the rate of metastatic spread. It is possible that the trauma of surgical resection itself may aid in metastasis, so once again improvement of
surgical technique may improve the overall outcome of studies using this tumor resection model.

Due to the deficits in the model, it is not possible to definitively determine whether or not emodin treatment affected metastatic recurrence. Once further studies are performed to improve this model, it may be possible to observe more definitive results in metastatic burden, progression-free survival, overall survival, and immune cell infiltrate at the metastatic site due to adjuvant therapy with emodin.

2.3 4T1-LUC2-RFP METASTASIS TIME-POINT AND TROPISM
2.3.1 DETAILED METHODS

Tumor Model

BALB/c mice were housed at the University of South Carolina Animal Research facility until 8 weeks of age for use in this experiment. All procedures were approved by the University of South Carolina Institutional Animal Care and Use Committee. Tumor cell implantation was performed as described previously. Briefly, 4T1-luc2-RFP cells (PerkinElmer) were resuspended in PBS at a density of 10×10^6 cells/mL. On Day 0, 15 female Balb/c mice were bilaterally injected with 2×10^5 4T1-luc2-RFP tumor cells in the 4th pair of mammary fat pads. Tumor volume was calculated as stated before and was measured every 3 days beginning at Day 11. Mice were divided into three groups of five mice each with equal average tumor volume on the day that tumors were to be removed from the first group of mice. This first resection took place, as described previously, when the average volume reached 500 mm^3. Tumors were removed from the second group at 1000 mm^3 average volume and the third group at 1500 mm^3 average volume. These volumes were reached on Days 18, 22, and 26, respectively, since tumor cell implantation. Mice were imaged using the IVIS Spectrum (PerkinElmer) prior to tumor
removal and weekly thereafter until the conclusion of the study. When mice died during the course of this study or exhibited significant moribundity and were sacrificed, their lungs and other metastatic sites identified on IVIS Spectrum imaging were taken for gross determination of metastatic burden. On Day 52 the lungs of surviving mice were removed for gross determination of metastatic burden.

**IVIS Imaging**

BALB/c mice bearing 4T1-luc2-RFP tumors were imaged using the IVIS Spectrum (PerkinElmer) prior to tumor removal, and each cohort of mice was imaged after tumors were resected in order to gage the completeness of resection and presence of metastasis at the time of tumor removal. Mice were then imaged weekly thereafter. Mice were allowed to recover for 10 min in order for the D-luciferin to fully perfuse the mouse and were then re-anesthetized and imaged at 600nm wavelength. Photons/second was used to determine the luminous flux from the tumor cells and adjust for differences in exposure time.

**Statistical Analysis**

Data were shown as mean ± standard error of mean (SEM) whenever the mean was the primary value representative of a sample group’s behavior. Three group comparison of means was accomplished using a two tailed ANOVA followed by Tukey multiple comparisons test, while two group comparison was accomplished using a two tailed Student’s t test. All such comparisons were performed using GraphPad Prism 5 software (Graphpad Software Inc.). Differences between proportions were determined using a two tailed N-1 Chi-Square test. $p \leq 0.05$ was considered statistically significant for all tests.
2.3.2 RESULTS

This study was performed to determine the amount of time required between 4T1-luc2-RFP cell inoculation and tumor resection in order to achieve ≥80% metastatic recurrence rate under control/untreated conditions. The secondary purpose of this study was to again observe the metastatic tropism of the 4T1-luc2-RFP cells and determine whether lung metastasis alone could be used as a measure of metastatic recurrence. If the metastatic pattern is too erratic, then quantification of total body metastases and observation of the effect any treatment may have on this would be much more difficult.

Our previous study had shown that 10 days of tumor growth, producing a 200 mm$^3$ tumor prior to resection, was too little time for the tumor to metastasize in a majority of the mice, and many of the metastases that did occur formed outside of the lungs.

*The metastatic tropism of 4T1-luc2-RFP cells following primary tumor resection is not lung-specific*

The average tumor volume of all tumor-bearing groups was the same at the time of each tumor surgery, when multiple groups were still bearing tumors, as intended (Figure 2.5B). The average tumor radiances were also the same at each of these time-points (Figure 2.5C). The increasing average tumor weights at each of the sequential resections mirrors the increasing volume measurements, and proves to be a much less variable indicator of tumor size than the radiance data collected from the IVIS Spectrum (Figure 2.5D).

Upon tumor resection, mice in each group were imaged using an IVIS Spectrum to determine whether or not the entire tumor had been resected and the locations of any pre-surgical metastases. After the first group of tumors had been removed on Day 18
(Group 1), one of the mice presented with residual local tumor tissue upon Day 19 IVIS imaging (Figure 2.5E). After the second resection on Day 22 (Group 2), one of the mice in Group 1 seemed to exhibit an established lung metastasis, but none of the Group 2 mice appeared to have any residual tumor or metastases on Day 23 IVIS imaging (Figure 2.5E). Upon resection of the third group of tumors (Group 3) on Day 26, three out of the five mice were found to already possess distant metastases, two of which were in the lung fields on Day 27 IVIS imaging (Figure 2.5E). The third was located in the shoulder/jaw region as observed previously when testing this model. One of these lung metastasis-bearing mice also presented with substantial residual tumor tissue at one of its excision sites. One of the mice in Group 2 presented with a non-lung distant metastasis, and three mice in Group 1 appeared to exhibit some rapidly growing local recurrence. One of these mice also still seemed to show the previously noted lung metastasis (Figure 2.5E).

Before the next IVIS Spectrum imaging on Day 38 post tumor implantation, two mice in Group 3 died, necropsy showed that these deaths were likely due to metastatic spread of the tumor. One of these mice had significant residual tumor and lung metastases obvious on IVIS Spectrum imaging, while the other was found to have some relatively small lung metastases on necropsy as well as a substantial tumor in the jaw/neck region (Figure 2.5E). Day 45 IVIS Spectrum imaging revealed that of the five mice in Group 1, one appeared to still have a relatively stagnant local recurrence, while another of the mice exhibited bilateral local recurrence and lung and other metastases. One of the mice in Group 2 showed a potential local recurrence and a large shoulder metastasis. Of the three remaining mice in Group 3, one still exhibited a large lung field metastasis (Figure 2.5E).
Gross lung metastases only developed after 26 days of tumor growth before resection

The final IVIS Spectrum imaging was done on Day 52 since tumor cell implantation. On this date, another mouse in Group 3 died, and though it was not apparent that lung metastases were present in previous images, many large metastases were present on necropsy (Figure 2.6A). Another mouse in Group 3 that had exhibited a lung metastasis for the past several weeks was also moribund and would have required sacrifice even if this had not been the final day of the experiment. Two of the mice in Group 1 were sacrificed due to impairment brought on by their metastases prior to Day 52 imaging, both exhibited what appeared to be metastases upon necropsy, yet none were present on the lungs. All of the metastases visualized on IVIS Spectrum imaging of Group 1 mice were located, and even those appearing to be in the region of the lung fields were either outside of or originating from the ribcage. Mouse #1 in this group appeared to have a local recurrence from IVIS Spectrum imaging, but necropsy showed that this was found to be associated with the muscle/bone of the hind limb, and did not appear to originate from the primary tumor site. All of the mice in Group 2 survived until termination of the experiment, though two appeared to bear tumor cells in various locations on IVIS spectrum imaging. Interestingly, when all of mice were sacrificed, it was found that none of the surviving mice from Group 2 had any lung metastases, and only one metastasis was present on the lungs of one of the Group 1 mice, yet all three of the Group 3 mice examined did exhibit lung metastases (Figures 2.6A&B). Thus, there were only 3 mice in Group 1, 5 mice in Group 2, and 3 mice in Group 3 that survived until the end of the study.
2.3.3 DISCUSSION

The results of this study were at times difficult to interpret during its course, and the most important finding was only apparent once all mice had been sacrificed. Only the 26-day tumor resection time-point yielded ≥80% lung metastases. Only a single metastatic nodule was observed on the lungs of mice with tumors removed at either of the earlier time-points. This was the case even with the single instance of local recurrence in Group 1, which still exhibited no lung metastases. These findings indicate both that the tumors must be allowed to grow for a great deal more time that originally suspected in order to reliably (≥80%) yield lung metastases and that the 4T1-luc2-RFP cell line continues to produce a substantial proportion of extra-pulmonary distant metastases. Allowing the tumors to grow to the substantial volume of 1500 mm$^3$ made clean resection much more difficult and caused there to be less skin available to properly close the incisions from which the tumors had been removed.

Both of these findings pose their own problems and the following potential solutions are proposed. First, to attempt to solve the difficulties in resecting such large tumors, only a single tumor injection into the right, 4$^{th}$ (9$^{th}$) mammary fat pad will be performed in subsequent studies. This should allow enough tissue to remain to effectively close the excision site even if more tissue is taken to leave clean margins on a larger tumor. Second, to attempt to both shorten the time to micrometastasis establishment and identify a tumor cell line with more specific lung metastatic tropism, 4T1-luc2 cells will be used. Studies comparing these two cell lines and others expressing one or multiple tagging proteins have shown that adding more of these “useless” proteins can adversely affect tumor progression and ever serve as immunogenic antigens [119].
shown that not all of the 4T1-luc2-RFP cells express high enough levels of RFP to
distinguish them from the native tissue, and thus it is unlikely that they can fulfill their
originally intended purpose as an easy target to determine lung metastatic burden.
Additionally, other more widely accepted measures of metastatic burden are not difficult
to perform in future studies. Thus, future studies will be performed to test the usefulness
of a unilateral 4T1-luc2 tumor cell implantation followed by a 20+ day tumor progression
period followed by resection model as a method of reliable induction of lung metastases
with a micrometastatic/circulating tumor cell treatment window.

2.4 9th MAMMARY FAT PAD 4T1-LUC2 METASTASIS MODEL

2.4.1 DETAILED METHODS

*Tumor Model*

8-12-week-old female BALB/c mice were injected with 2x10⁵ 4T1-luc2 cells
suspended in 20 µL PBS into only the right, 4th (9th) mammary fat pads. The target tumor
size for metastasis determined in the previous study was 1500 mm³. Tumors were
measured every 3 days beginning at Day 10 until they reached this size. Once the target
tumor volume was reached (Day 28), IVIS images were obtained and mice were
separated into two groups based on equal average tumor size and metastatic burden while
also maintaining equal average ages between groups. Tumors were resected on Day 28 as
described previously, and i.p. injections of PBS, 2% DMSO by volume containing either
0 mg/kg or 40 mg/kg emodin were administered daily beginning on Day 29. Volume of
injection was calculated based on mouse weight, and ranged from 0.8 mL to 1 mL.
Injections were given until mice died or were sacrificed due to moribun
proportions were tracked until the last mouse was sacrificed (Day 41 since tumor injection).

*IVIS Imaging*

BALB/c mice bearing 4T1-luc2 tumors were imaged using the IVIS Spectrum (PerkinElmer) as described previously in Subsection 2.3 prior to tumor removal, and all mice were imaged after tumors were resected, to gage the completeness of resection and presence of metastasis at the time of tumor removal. Photons/second was used to determine the luminous flux from the tumor cells and adjust for differences in exposure time.

*Statistical Analysis*

Data were shown as mean ± standard error of mean (SEM) whenever the mean was the primary value representative of a sample group’s behavior. Two group comparison was accomplished using a two tailed Student’s *t* test. All such comparisons were performed using GraphPad Prism 5 software (Graphpad Software Inc., San Diego, CA). Survival data were analyzed using the Mantel-Cox log-rank test. p≤0.05 was considered statistically significant for all tests.

2.4.2 RESULTS

*4T1-luc2 cells metastasize almost exclusively to the lungs*

The 4T1-luc2 cells, surprisingly, took two days longer to reach the target volume for removal (1500 mm³) than the 4T1-luc2-RFP cells did. However, the 4T1-luc2 cells exhibited earlier metastatic dissemination. ~64% of the mice were found to have overt lung metastases on IVIS Spectrum imaging 24 h after primary tumor removal, and these metastases were visible prior to tumor excision when the primary tumor was covered
during imaging to prevent the much brighter tumor tissue from saturating the detector (Figure 2.7B).

*Emodin increased overall survival in lung metastasis bearing mice*

Mice were divided evenly between 40 mg/kg emodin treatment and vehicle control groups based on tumor volume/radiance and lung field radiance, indicative of metastatic dissemination prior to tumor resection (Figure 2.8A). There was also no difference between lung field radiance 48 h after primary tumor removal (Figure 2.8B). Several of the mice also appeared to have residual primary tumor tissue after surgery. Emodin and vehicle i.p. injections were given daily until the conclusion of the study, which was only 13 days after tumor resection. The health of the mice deteriorated much more rapidly than in previous studies due the presence of advanced disease at the time treatment was initiated. We found that even this abbreviated course of emodin treatment led to a statistically significant increase in the median overall survival of the mice by 19% (Figure 2.8C). Additionally, the final 3 emodin-treated mice were sacrificed once statistical significance was reached due to the fact that they were exhibiting some signs of labored breathing due to the presence of lung metastases. It is likely, however, that these mice may have survived a few more days before they each died or became impaired to the extent requiring sacrifice. All of the mice in both groups were found to have extensive lung metastasis on necropsy or at sacrifice. This was likely due to their being established prior to tumor resection and adjuvant therapy.

### 2.4.3 DISCUSSION

This experiment demonstrated that the 4T1-luc2 cells implanted only into the 9th mammary fat pad had a more rapid onset of metastasis and exhibited a much more
specific lung tropism than the 4T1-luc2-RFP cells implanted bilaterally. It is not clear from this study whether this improvement is due solely to the change in cell line or in injection location or is affected by both, and knowing this is not crucial for the design of our final study.

In this study, the 4T1-luc2 cells had already established observable metastases in a majority of the mice before tumor removal, and several of the mice bore residual tumor tissue post-surgery. Our goal is to apply emodin treatment to mice bearing only micrometastases and circulating tumor cells with no local recurrence and no lung metastases visible on post resection IVIS Spectrum imaging. In order to accomplish this, mice will be imaged frequently during the course of tumor growth with the tumors obscured. This should allow for the observation of the first detectable lung metastases and initiation of emodin treatment when the majority of the mice have either only very small metastases or circulating tumor cells. More tissue will also be removed around the primary tumor to ensure clean margins.

We also found that even mortality due to the progression of established lung metastases can be delayed by emodin treatment at a dose that we have used previously and have shown to not be directly tumoricidal [103, 118].

2.5 FINAL 4T1-LUC2 METASTATIC RECURRENCE MODEL TEST

2.5.1 DETAILED METHODS

Tumor Model

27 7-week-old, female BALB/c mice were purchased from Jackson Labs, and were housed at the University of South Carolina Animal Research facility for one week prior to use in experiments. At 8 weeks of age, all mice were injected with $2 \times 10^5$ 4T1-
luc2 cells (PerkinElmer) suspended in 20 µL PBS into only the 9th mammary fat pad. The target date for tumor resection was between 20 and 25 days after tumor cell implantation. Tumors were measured every 3 days beginning at Day 11. Mice were separated into three groups based on equal average tumor volume measured on the day of tumor resection. Tumors were resected on Day 23 as described previously, and i.p. injections of PBS, 2% DMSO by volume containing either 0 mg/kg, 20 mg/kg, or 40 mg/kg emodin were administered daily beginning on Day 24. Injection volume was calculated based on mouse weight, and ranged from 0.8 mL to 1 mL. Injections were given daily until the termination of the study. Three mice from each group with obvious lung metastases one week after surgery were sacrificed. The remaining mice were sacrificed at 2 weeks post tumor resection.

**IVIS Imaging**

BALB/c mice bearing 4T1-luc2 tumors were full-body imaged using the IVIS Spectrum (PerkinElmer) prior to, 48 h after, and one week after tumor resection as described in Subsection 2.3. Photons/second was used to determine the luminous flux from the tumor cells and adjust for differences in exposure time.

The lungs of mice were also imaged *ex vivo* using the IVIS Spectrum. To do this, two more solutions of D-luciferin were prepared at 150 µg/mL and 300 µg/mL in addition to the 15 mg/mL used for i.p. injection. All luciferin solutions were sterile filtered using 22-µm syringe filters. Mice were injected i.p. with the same volume of 15 mg/mL D-luciferin solution as in full body imaging, but were only allowed 5 min to recover before being sacrificed. Lungs of the mice were then perfused with the 150 µg/mL D-luciferin solution by cutting the left atrium and injecting the D-luciferin
solution into the right ventricle. The lungs were then excised and placed in the wells of a 12-well plate containing 1 mL of 300 µg/mL D-luciferin solution and imaged immediately on the IVIS Spectrum.

**Statistical Analysis**

Data were shown as mean ± standard error of mean (SEM) whenever the mean was the primary value representing a sample group’s behavior. Two group comparison of means was accomplished using a 2-tailed Student’s t test, and three group comparisons of means was accomplished using a two tailed ANOVA followed by Tukey multiple comparisons test. Survival data were analyzed using the Mantel-Cox log-rank test. All such comparisons were performed using GraphPad Prism 5 software (Graphpad Software Inc., San Diego, CA). p≤0.05 was considered statistically significant for all tests.

2.5.2 RESULTS

*4T1-luc2 cells implanted in the 9th mammary fat pad metastasize efficiently to the lungs*

The growth of 4T1-luc2 tumors was very uniform across all mice and across the three groups prior to resection (**Figure 2.9B**). Tumor radiance, collected from full body IVIS Spectrum imaging, and the weights of resected tumors were also very similar between groups prior to the onset of treatment (**Figure 2.9C**). Full body IVIS Spectrum imaging 48 h after tumor resection shows only three, faint metastases in the cohort of 27 mice, two of which appear to be in the lungs (**Figure 2.9D**). Similar imaging of the mice at one week post resection shows 11 of the mice now bearing metastases, 10 of which appear to lie in the lung fields (**Figure 2.9E**). At this point, none of the mice exhibit local recurrence. Between all of the groups at the conclusion of the study, 21 out of the 28 mice were found to be bearing lung metastases detectable on *ex vivo* IVIS or visible at
necropsy. In the vehicle control group, eight of the nine mice developed lung metastases, fulfilling the goal of achieving ≥80% metastasis formation in the absence of adjuvant treatment (Figure 2.9F).

Two weeks of emodin treatment decreased both metastasis number and metastatic burden

After one week of daily emodin or vehicle treatment, the three mice with the highest lung field luminous flux from each group on IVIS Spectrum imaging were sacrificed, and their lungs were imaged *ex vivo* (Figure 2.10A). Both the full body and *ex vivo* lung imaging showed that there were no differences in total radiance between the selected mice from the three treatment groups (Figure 2.10B). Lungs were then fixed in 4% paraformaldehyde (PFA) for 48 h, imbedded in paraffin, and sectioned. Sections at 200 µm intervals were stained with hematoxylin and eosin (H&E) and metastases were counted and quantified using ImageJ software. The total metastatic volume per total lung volume was measured and termed the “metastatic index”. The number of metastases per lung section area was also quantified. It was found that while there was no statistically significant difference in the metastatic index or number of metastases per mm² between these three groups, there was a trend toward a decreasing number of metastases per unit lung area with increasing emodin dosage (Figure 2.10C).

The mice receiving two weeks of emodin treatment exhibited a strong decreasing trend in lung field luminous flux with increasing emodin dose (Figure 2.10D&E). Lung section metastasis quantification yielded evidence of a significant decrease in both metastatic index and number of metastases per unit lung section area for the 40 mg/kg emodin treated group versus vehicle control (Figure 2.10F).
2.5.3 DISCUSSION

These data show that, though the trials described above, we have developed a reliable method to mimic the clinically observed phenomenon of metastatic recurrence from disseminated micrometastases and circulating tumor cells after primary surgical removal of the tumor. This model allows for the testing of adjuvant therapies for their effectiveness in slowing or preventing recurrence, which is a much more clinically relevant therapeutic goal than simply reducing the growth of a primary tumor or limiting the metastatic spread of a tumor that is allowed to remain in the body long after it would have been diagnosed and removed. Here this model was used to test emodin, but any number of adjuvant or neoadjuvant therapies can be screened using this model. It could also be adapted to humanized mouse models in the future to further increase the clinical relevance of tested treatment paradigms. The availability of a murine model that addresses goals relevant to human breast cancer treatment is necessary for effective pre-clinical screening of proposed therapies.

This final iteration of the model has also demonstrated that emodin can effectively reduce both metastatic burden and number when administered as an adjuvant therapy. This is likely due to both the direct effects of emodin on TAMs observed previously in our lab as well as the interference with tumor-promoting TAM-tumor cell crosstalk [118]. It is likely that other mechanisms may also be at play, and further studies of such phenomena are ongoing.
Table 2.1 Antibodies for flow cytometry.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Product #</th>
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<tr>
<td>CD45 (PE/Cy7)</td>
<td>103113</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD3 (APC/Cy7)</td>
<td>100221</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD8 (FITC)</td>
<td>100803</td>
<td>Biolegend</td>
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Figure 2.1 10 days of tumor growth was insufficient to achieve an appropriately high metastatic rate with 4T1-luc2-RFP cells. A, Timeline of this experiment. 7-week-old, female BALB/c mice received 2x10^5 4T1-luc2-RFP cells, bilaterally, in the 4th pair of mammary fat pads. Tumors were resected as indicated on the timeline, and treatment with either 2% DMSO in PBS (vehicle), 20 mg/kg emodin, or 40 mg/kg emodin was administered i.p. daily for two weeks post surgery, then every other day for two more weeks. B, Tumor growth was monitored until tumor resection, there were no differences in measured tumor volume between groups. C, IVIS imaging of mice prior to tumor removal and lung field radiance. D, IVIS imaging of mice after tumor removal and lung field radiance. E, IVIS imaging of mice on Day 26 since tumor cell implantation; lung metastases are visible. F, Percentage of mice in each treatment group to ever develop metastases. Results are shown as means ± SE (n=9).
Figure 2.2 Metastases did not occur preferentially in the lungs. A, Percentages of metastases by location in all groups of mice determined from IVIS imaging.

Figure 2.3 Mice were able to recover from surgery and metastases appeared quickly. A, Prevalence of metastases (all locations) across all groups.
Figure 2.4 Emodin treatment showed a decreasing trend in metastatic burden and an increasing trend in overall survival. A, Lung field IVIS data from Days 9-26 since tumor cell implantation. Day 21 where mice began to die from their disease is indicated. B, Percentage of mice bearing metastasis in each treatment group based on IVIS data. C, Overall survival of each treatment group for the duration of the experiment. D, The final IVIS image obtained, 48 days since tumor cell implantation. E, Lung CD8+ T cell flow cytometry result. Results are shown as means ± SE (n=9).
Figure 2.5 The metastatic tropism of 4T1-luc2-RFP cells following primary tumor resection is not lung-specific. A, Timeline of this experiment. 8 week-old, female BALB/c mice received 2x10^5 4T1-luc2-RFP cells, bilaterally, in the 4th pair of mammary fat pads. Tumors were resected as indicated on the timeline at 3 time-points in order to determine the duration of tumor growth necessary for metastatic dissemination. B, Average tumor volume per group; there were no differences in measured tumor volume between groups. C, Tumor radiance from IVIS imaging by resection group. D, Average tumor weights at each removal date. E, IVIS images showing tumor removal and recurrence over the course of the study. Results are shown as means ± SE (n=5).
Figure 2.6 Gross lung metastases only developed after 26 days of tumor growth before resection. A, Lungs of mice remaining alive at the study endpoint (Day 53) with surface metastases indicated. B, Quantification of lung surface metastases per mouse. Results are shown as means ± SE (n=3–5).
Figure 2.7 4T1-luc2 cells metastasize almost exclusively to the lungs. A, Timeline of this experiment. 8-12 week-old, female BALB/c mice received $2 \times 10^5$ 4T1-luc2 cells in the 4th right (9th) mammary fat pad. Tumors were resected as indicated on the timeline, and treatment with either 2% DMSO in PBS (vehicle) or 40 mg/kg emodin was administered i.p. daily for the duration of the study. B, Pre-surgery tumor IVIS image, and pre- and post-surgery lung field IVIS imaging.
Figure 2.8 Emodin increased overall survival in lung metastasis bearing mice. A, Tumor and lung field radiance prior to resection (Day 28); no difference is observed between treatment groups in either parameter. B, Lung field radiance after tumor removal, again showing no difference between treatment groups. C, Overall survival of mice until the termination of the study. Results are shown as means ± SE (n=6-7). * p<0.05.
Figure 2.9 4T1-luc2 cells implanted in the 9th mammary fat pad metastasize efficiently to the lungs. A, Timeline of this experiment. 7 week-old, female BALB/c mice received $2 \times 10^5$ 4T1-luc2 cells in the 4th right (9th) mammary fat pad. Tumors were resected as indicated on the timeline, and treatment with either 2% DMSO in PBS (vehicle), 20 mg/kg emodin, or 40 mg/kg emodin was administered i.p. daily for the duration of the study. B, Average tumor volume per group; there were no differences in measured tumor volume between groups prior to treatment. C, Pre-surgery IVIS image, tumor weight, and tumor radiance by group. D, Post-surgery lung field radiance, Day 25. E, Post-surgery lung field radiance, Day 30. F, Proportions of mice with lung metastases visible on IVIS at Day 30 since tumor cell implantation. Results are shown as means ± SE (n=9).
Figure 2.10 Two weeks of emodin treatment decreased both metastasis number and metastatic burden. A, *Ex vivo* IVIS image of lungs from 3 mice per group with the largest metastases after 1 week of treatment. B, *In vivo* and *ex vivo* IVIS data showing no differences between groups. Results are shown as means ± SE (n=3). C, Quantification of metastases from serial lung sections from these 3 mice. D, *Ex vivo* IVIS image of lungs from remaining mice after 2 weeks of treatment. E, *Ex vivo* IVIS data from these mice. F,
Quantification of metastases from the final 6 mice and representative lung section images. Results are shown as means ± SE (n=6). *p<0.05, **p<0.01.
CHAPTER 3
EMODIN AS AN AGENT TO PREVENT BREAST CANCER POST-SURGERY
METASTATIC RECURRENCE

3.1 BACKGROUND

Breast cancer is still the second leading cause of cancer-related death among women worldwide. Triple-negative breast cancer has remained difficult to treat, and clinical outcomes are very poor, especially if the cancer has metastasized [3]. Our lab has established that emodin, an anthraquinone derived from herbs used in traditional Chinese medicine, is capable of modulating macrophage activation and interfering with the tumor-promoting feedback loop between tumor cells and macrophages, leading to decreased metastatic dissemination and decreased tumor growth [103, 118, 120]. Emodin, like many other naturally derived compounds, is inexpensive and capable of affecting multiple pathways simultaneously [121-123]. It has also demonstrated very low toxicity [104]. There are currently no cancer treatments specifically targeting macrophages or innate immunity. Thus emodin may prove complementary to other immune therapeutic strategies, the majority of which are intended to boost adaptive immunity. The generally immune suppressive tumor microenvironment fostered by M2-like tumor associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), and regulatory T cells (Tregs) hampers existing antitumor immunity and decreases the effectiveness of immune therapeutic strategies. Some therapies meant to address this general immune suppression
have proven effective in the laboratory setting, but none have yet progressed to clinical use [124-126].

In order to help bridge this gap, we have performed a study investigating emodin’s effectiveness as an adjuvant chemotherapy for triple negative breast cancer. The relative pattern of human breast cancer treatment was mimicked in this murine model with surgical resection before macrometastases were visible, followed by emodin treatment and recurrence monitoring. In this model, as is often the case with human cancer treatment, the primary tumor is removed prior to the initiation of chemotherapy, therefore the therapy is meant to only affect any residual tumor tissue, either at the primary sites or disseminated to the circulation or distant organs. However, the immune suppressive environment engineered by the tumor for its own protection is diminished for a brief period, but still present following resection, and is still capable of aiding in the survival of any established or circulating tumor cells and reducing the success of immune therapeutic strategies [127]. Here we show that through emodin’s direct effects on these remaining tumor cells and its effects on macrophages, metastatic recurrence of triple negative breast cancer is significantly decreased, and overall and metastasis-free survival are both increased. The mechanism for this is at least in part related to the direct and indirect modulation of cancer cell epithelial to mesenchymal transition (EMT) by emodin. We show that emodin can directly decrease transforming growth factor-β (TGF-β) production by TAMs which in turn reduces tumor cell EMT. Acting in this way, emodin can both reduce the ability of circulating and newly arrived cancer cells to effectively establish metastases and help to counteract the systemic immune suppression imposed by the primary tumor.
3.2 DETAILED METHODS

Tumor cell culture and conditioned medium collection

EO771 tumor cells were obtained in 2012 and 4T1 cells were obtained from the American Type Culture Collection (ATCC) in 2013 and were expanded in high-glucose Dulbecco’s modified eagle medium DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin (Sigma-Aldrich Corp.), and 100 µg/mL streptomycin (Sigma-Aldrich Corp.) in a 37°C, humidified, 5% CO₂ incubator. Tumor conditioned medium was collected by first culturing tumor cells expanded to 70% or 90% confluence for EO771 and 4T1 cells, respectively, in T-75 bottles (Corning Inc., Corning, NY). Medium was then removed, plates were gently rinsed once with phosphate-buffered saline (PBS), and 10 mL of serum-free DMEM containing penicillin and streptomycin (P/S) was added. Cells were cultured for 48 h in this medium, which was then collected, centrifuged at 350 x g for 5 min to remove large cellular debris, and filtered through a 45-µm vacuum filter (Corning Inc.). This medium was then concentrated using 3-kD molecular weight cutoff centrifuge concentrators (Sigma-Aldrich Corp.) to 25X. Aliquots were stored at -80°C for later use. To treat macrophages, conditioned medium was diluted 1:5 in fresh serum-free DMEM and termed tumor-conditioned medium (TCM).

Peritoneal macrophage harvesting and conditioned medium collection

Mice were injected intraperitoneally (i.p.) with 4% Brewer thioglycollate at a volume of 100 µL/g of mouse body weight. Three days later mice were sacrificed and macrophages were collected by peritoneal lavage using 20 mL ice-cold PBS. Peritoneal cells were plated in DMEM + 10% FBS + P/S and cultured in a 37°C, humidified, 5%
CO₂ incubator for 1 h, at which time non-adherent cells were rinsed away. The adherent macrophages were then cultured overnight in serum-free DMEM before treatment.

Treatment consisted of 24 h in 4T1 or EO771 tumor cell-conditioned medium (depending on whether macrophages were obtained from BALB/c or C57BL/6 mice, respectively). This medium was either supplemented with emodin at 25 µM or DMSO as a vehicle control. After this 24 h treatment, macrophages were rinsed and cultured in serum-free DMEM for an additional 48 h. This macrophage-conditioned medium was then collected and used immediately to treat the tumor cell line of the corresponding background.

*Non-contact macrophage/tumor cell co-culture*

1x10⁵ 4T1 or EO771 tumor cells were seeded in 24-well plates in serum-free DMEM. 1x10⁵ syngenic murine macrophages, previously treated for 24 h with TCM with or without 25 µM emodin, were seeded in trans-well inserts with 8-µm-pore polyethylene terephthalate membranes (Corning Inc.). This non-contact co-culture setup was placed in a 37°C, 5% CO₂ incubator for 48 h. At the end of this incubation period, the tumor cells in the bottom chamber were rinsed with PBS and lysed with Qiazol (Qiagen, Hilden, Germany) for RNA extraction and qPCR.

*Trans-well invasion assay*

Tumor cell invasion capacity was tested by determining their ability to migrate through Matrigel® Basement Membrane Matrix (Corning Inc.). To do this, 100 µL ice-cold Matrigel was added to the inside of trans-well inserts with 8-µm polyethylene terephthalate membranes which fit into the wells of 24-well plates (Corning). Matrigel-containing trans-well inserts were then incubated at 37°C for 30 min to allow the Matrigel
to solidify. 300 µL of DMEM + with 10% FBS, as a chemoattractant, or serum-free DMEM was added to wells of 24-well plates followed by the Matrigel-containing trans-well inserts. 1x10^5 4T1 or MDA-MB-231 breast tumor cells were seeded on top of the Matrigel inside the trans-well inserts in serum-free DMEM. Prior to this seeding, tumor cells had been pretreated with either 0.05% DMSO (as vehicle control), 6 ng/mL TGF-β1, 25 µM emodin, 6 ng/mL TGF-β1 + 25 µM emodin, or, for 4T1 cells, conditioned medium from macrophages treated with either 4T1-conditioned medium, 25 µM emodin, or both. The base medium for all pretreatments was serum-free DMEM.

Migration assay

4T1 or MDA-MB-231 cells were cultured in DMEM 10% FBS+P/S to confluency in 12-well plates. A scratch was then made through the layer of cells with a sterile pipette tip. Various treatment media were applied for 16 h, at which time the amount that the tumor cells had migrated in to fill the gap was measured using ImageJ software. The treatment media consisted of serum-free DMEM supplemented with either 0.06% DMSO (as vehicle control, also included in all other treatments in this list), 25 µM emodin, 6 ng/mL TGFβ1, and 6 ng/mL TGFβ1 + 25 µM emodin. Tumor cells were also treated with macrophage-conditioned medium from macrophages treated with either 4T1-conditioned medium or 4T1-conditioned medium + 25 µM emodin as described previously.

RNA extraction and quantitative real-time PCR

For RNA extraction, cells were lysed with TRIzol reagent (Thermo Fisher Scientific, Waltham, MA). RNA was then extracted by precipitation according to the manufacturer’s protocol. Briefly, 0.5 mL isopropanol was added to the 1 mL TRIzol, incubated at RT for 10 min, and centrifuged at 12,000 x g at 4°C for 10 min. Supernatant
was discarded, and the resulting pellet was washed in 1 mL 75% ethanol, then centrifuged for 5 min at 7,500 x g at 4˚C. Supernatant was again discarded and the pellet was allowed to air dry. RNA pellets were resuspended in 20 µL of RNase-free water and heated at 60˚C for 10 min. RNA concentration was measured using an Evolution 60 spectrophotometer (Thermo Fisher Scientific). cDNA was then synthesized using 1 µg RNA, or the maximum amount possible if less than 1 µg, using iScript cDNA Synthesis Kits (Bio-Rad Laboratories, Hercules, CA). qPCR was performed using iQ SYBR Green Supermix (Bio-Rad Laboratories). All cDNA stocks were diluted 1:25, and 10 µL of the resulting solution was used for each qPCR reaction. Primers are listed in table 3.1. qPCR run conditions were 95˚C for 5 min followed by a repeated cycling of 95˚C for 10 sec, 58˚C for 15 sec, and 72˚C for 20 sec. A melt curve was then collected through the following temperature fluctuations: 95˚C for 10 sec, 65˚C for 5 sec, and terminating again at 95˚C. All samples were run in duplicate using a Bio-Rad CFX Real Time thermocycler. The ΔΔCt method was used to determine relative expression.

**Western Blotting**

For protein extraction, cultured tumor cells were lysed in RIPA buffer (Pierce, Rockford, IL) supplemented with 1:100 protease inhibitor cocktail and 1:100 phosphatase inhibitor cocktail (Sigma-Aldrich Corp.). Protein was quantified using the Lowry method, and loaded into 10% SDS-PAGE precast gels (Bio-Rad Laboratories). Protein was then transferred onto nitrocellulose membranes (Sigma-Aldrich Corp.). Membranes were probed with indicated primary antibodies (Cell Signaling Tech. Danvers, MA), then the corresponding secondary antibodies (Santa Cruz Biotech. Dallas, TX) conjugated to horseradish peroxidase. Protein was detected using ECL kits (Pierce) and signal
intensities were quantified using Image-Pro Plus analysis software (Media Cybernetics, Rockville, MD)

**Flow cytometry**

Flow cytometry was performed for both *in vitro* and *in vivo* experiments. For *in vitro* experiments, peritoneal macrophages were removed from plates using Accutase (Biolegend). Single cell suspensions of $1 \times 10^6$ cells in 100 µL PBS were blocked with CD16/32 blocking antibodies (Biolegend) at a concentration of 2.5 µg/mL for 10 min at 4˚C. Cells were then stained with anti-CD206 PE for 30 min at 4˚C.

Lymph node cells were dissociated using 70-µm cell strainers (Corning), rinsed, counted, and $1 \times 10^6$ cells were suspended in 100 µL PBS for staining. Lymph node cells were blocked with anti-CD16/32 antibodies as described and surface stained with the following markers for 30 min at 4˚C: anti-CD45 PE-Cy7, anti-CD11c PE, anti-CD3 APC-Cy7, anti-CD4 APC, anti-CD8 FITC, anti-CD69 PE, and anti-CD103 FITC. Internal staining was performed using a BD Cytofix/Cytoperm Fixation Permeabilization Kit (BD Biosciences) according to the manufacturer’s instructions. The antibodies used to stain intracellular antigens were anti-IFNγ PE and anti-FOXP3 PE, each at a concentration of 4 µg/mL in the manufacturer specified buffer.

All samples were washed twice with PBS after staining and resuspended in 500 µL PBS for flow cytometry. Samples were analyzed on a BD FACS Aria II using FACS Diva software (BD Biosciences). 10,000 to 100,000 events were collected for each sample. All antibodies mentioned here were purchased from Biolegend.
Orthotopic tumor model and surgical resection

7-week-old, female BALB/c mice were housed at the University of South Carolina Animal Research Facility; all procedures were approved by the Institutional Animal Care and Use Committee. At 8 weeks of age, 4T1-luc2 cells (PerkinElmer) were suspended in PBS at a concentration of 1x10^7 cells/mL and 2x10^5 cells were implanted into the 9th mammary fat pad of each mouse as described previously. Tumor size was monitored and measured every 3-4 days beginning on Day 10 after implantation. The following equation was used to calculate tumor volume: $(\text{short axis})^2 \times (\text{long axis})/2 \approx \text{volume}$. The tumors were removed according to the following procedure on Day 21: each mouse was anesthetized using an isoflurane vaporizer, and maintained under anesthesia for the duration of the surgery with the vaporizer set to deliver 2% isoflurane. The surgical field was cleaned liberally with 70% ethanol and betadine swabs. A 1-2 cm incision was made medial to the tumor, and the tumor, with as much surrounding fat pad as possible, was resected. An electrocautery unit was on hand to stop excess bleeding if necessary. Incisions were closed with stainless steel clips and suture. Mice were allowed to recover in a cage heated to 30°C. Immediately following surgery, the three groups of mice received i.p. injections of PBS with 2% DMSO by volume containing either 0 mg/kg, 20 mg/kg or 40 mg/kg emodin. The volume of these injections was ~800 µL per mouse. These i.p. injections were administered daily for the next 17 days. On the 17th day after tumor resection (38th day since tumor cell implantation), the mice were anesthetized with isoflurane and sacrificed by cervical dislocation. Lungs and lung-draining lymph nodes were collected for further analysis.
**In vivo IVIS imaging**

BALB/c mice bearing 4T1-luc2 tumors were imaged using an IVIS Spectrum (PerkinElmer). D-luciferin (Sigma-Aldrich Corp.) was dissolved in PBS at a concentration of 15 mg/mL. Mice received 10 µL of this solution per gram body weight intraperitoneally (i.p.), were anesthetized using an isoflurane vaporizer, and images were obtained at 600 nm wavelength 10 min after receiving the D-luciferin injection. Metastatic recurrence and primary tumor size were monitored using the IVIS Spectrum to image the luciferase activity in the 4T1-luc2 cells using the same imaging procedure described here. *In vivo* IVIS imaging was performed on Days 20, 21, 24, and 30 since tumor cell implantation.

**Ex vivo IVIS imaging**

*Ex vivo* IVIS imaging was intended to generate a more accurate estimate of the mice with lung macro-metastases than the *in vivo* imaging of disseminated tumor in which the mouse’s tissue blocked a great deal of the signal and could render relatively small metastases undetectable. In order to do this, mice were first injected i.p. with D-luciferin in PBS in the same manner as with *in vivo* imaging already described. However, mice were allowed 5 min to recover and ensure D-luciferin perfusion following i.p. injection. Mice were then anesthetized with isoflurane and sacrificed by cervical dislocation. Lungs of mice were immediately perfused with a 150 µM solution of D-luciferin in PBS, removed, and placed in a solution of 300 µM D-luciferin for imaging. All of these solutions were maintained at RT. Lungs were imaged at 600 nm emission wavelength for 5 sec, then washed with PBS and fixed in 8 mL 4% paraformaldehyde (PFA).
**Metastasis quantification**

Following PFA fixation for 24-48 h at RT, mouse lungs were paraffin imbedded and sections were cut 5 µm thick at 150 µm intervals. Five such sections were obtained for each lung pair. These were deparaffinized and stained with hematoxylin and eosin. Stained slides were imaged at 10X magnification using an EVOS FL Auto 2 System (Thermo Fisher Scientific). Images were tiled in order to view the entire lung using the EVOS System software. Size of images was limited such that they did not require compression to be saved by the EVOS System software, ensuring that pixels represented equivalent real dimensions on every section. Pixel areas of lung tissue and metastases were measured for each image by hand using ImageJ software. These pixel values were converted to mm² using the conversion factor 1.142 pixels/µm determined by the 10X objective. The number of metastatic nodules per section was also counted. These values were used to calculate the following parameters for each lung pair: Metastasis Index = (metastatic volume)/(total lung volume), and Metastasis Number Index = (number of metastases per section)/(lung section area).

**Statistical analysis**

Data were shown as mean ± standard error of mean (SEM) whenever the mean was the primary value representative of a sample group’s behavior. Three group comparison of means was accomplished using a one-way ANOVA followed by Tukey multiple comparisons test, while two group comparison was accomplished using a two tailed Student’s t test. All such comparisons are performed using GraphPad Prism 5 software (Graphpad Software Inc.). Differences between proportions were determined.
using a two tailed N-1 Chi-Square test. \( p \leq 0.05 \) was considered statistically significant for all tests.

3.3 RESULTS

*Adjuvant emodin treatment decreases metastatic recurrence of TNBC after surgical resection*

Given our previous results demonstrating the effects of emodin on macrophage-induced tumor growth and metastatic dissemination, we sought to determine its relevance in a model of post-surgical breast cancer treatment. Our model of metastatic recurrence involved implanting 4T1-luc2 cells into the 9\(^{th}\) mammary fat pads of mice, then removing these tumors at a time-point experimentally determined to yield \( >80\% \) metastatic recurrence, yet no local recurrence in untreated mice. The metastases occur almost exclusively in the lungs and develop to a detectable extent within 3 weeks after tumor resection, again in untreated mice. The timeline and treatment schedule of this model is intended to biologically parallel that implemented in human breast cancer treatment, where diagnosis is often quickly followed by surgical resection and adjuvant chemotherapy (Figure 3.1A). After the implantation of 4T1-luc2 cells, tumors were allowed to grow for 21 days, and were imaged using an IVIS Spectrum prior to resection (Figure 3.1B). Tumors were surgically removed on Day 21, immediately followed by IVIS imaging to confirm that all local tumor tissue had been removed and check for detectable metastases (Figure 3.1C). There was one detectable lung metastasis in the DMSO only group, and this mouse was excluded from later quantitative analyses. Treatment was initiated about 8 h post-surgery and consisted of i.p. injections of PBS + 2\% DMSO, 20 mg/kg emodin, or 40 mg/kg emodin. Emodin was suspended in PBS +
2% DMSO for these injections. Mice were divided into three treatment groups based on equal average tumor size at resection. This was determined both by tumor radiance detected on IVIS and resected tumor weight (Figure 3.1D). There were no differences in calculated tumor volume at any point from when tumor size monitoring began on Day 10 through resection (Figure 3.1D). Metastatic recurrence was tracked by IVIS imaging on Days 3 and 9 after surgery (Days 24 and 30 since tumor implantation) (Figure 3.1E&F). From the post-surgery IVIS data, it was apparent that tumor cells were present to a lesser extent in the lung fields of mice that received emodin treatment. Mice were sacrificed 17 days after tumor resection (38 days since tumor implantation) for analysis of the metastatic microenvironment and the measurement of lung metastatic burden. First, lungs were perfused and quickly imaged ex vivo using the IVIS Spectrum, yielding a more accurate estimate of metastatic burden than similar imaging through the chest wall (Figure 3.1E&G). Lungs were then fixed and 5 sections were taken at 150 µm intervals though each lung pair. These were H&E-stained in order to render tumor nodules more visible, and both the number and size of metastatic nodules were quantified for each section. These values, as well as the total lung area from each section, were used to calculate the metastatic index (metastasis volume/total lung volume), the number of metastases per mm² of total lung section area, and the average size of each metastasis. The 40 mg/kg emodin dose decreased the metastatic index, the number of metastases, and the average size of each metastasis (Figures 3.1H&I). Previous studies of the effect of emodin had only focused on the primary tumor microenvironment, but here for the first time, we have shown that emodin, when administered only after the primary tumor had been surgically removed, is still able to affect the establishment and growth of distant
metastases. Based on IVIS imaging, emodin treatment during this post-surgery period could reduce metastatic recurrence at the endpoint of the study to ~64.3% from about ~84.6% in the control-treated group (Figure 3.1J). These results lend further credence to the possibility that emodin has potential as a therapy for both the prevention and arrested development of triple negative breast cancer metastasis.

Emodin modulates the immune microenvironment of the metastatic site

To gain a more complete understanding of emodin’s effect on the immune microenvironment of metastases, lung-draining lymph nodes were collected from the mice involved in the adjuvant emodin therapy experiment and analyzed via flow cytometry. This revealed some key differences between mice that received emodin and those that did not. Chiefly, in both emodin-treated groups (20 mg/kg and 40 mg/kg), there was a significant decrease in CD4+FOXP3+ regulatory T cells (Figure 3.2A). It has been shown that M2 macrophage-conditioned medium is capable of inducing FOXP3 expression in naïve human CD4+ T cells [128]. It was further shown that of all the cytokines released by the M2 macrophages, TGF-β was found to be the one on which CD4+ T cell FOXP3 induction was crucially dependent. A second key finding is that there appear to be less migratory DCs in the tumor draining lymph nodes of untreated mice than those treated with 40 mg/kg emodin (Figure 3.2B). Here migratory DCs are identified as CD11c+CD103+ cells [129]. Again, a likely contributor to this phenomenon is TGF-β, as it has been demonstrated that TGF-β1 has been shown to arrest DCs within tumor tissue and prevent migration to lymph nodes where an adaptive immune response could be more effectively fostered [130]. Lung sections containing metastases were also stained for markers of macrophage presence (CD68) and activation phenotype (Ym1).
Less macrophages were present in and around metastatic nodules in the lung tissue of mice treated with 40 mg/kg emodin (Figure 3.2C). Ym1+ cells, were also fewer in the regions around metastatic nodules in both the 20 mg/kg and 40 mg/kg emodin-treated mice compared to the control, indicating a less M2-like phenotype of macrophage polarization (Figure 3.2D) [131]. It was also interesting to note the obvious ring of macrophages surrounding many developing metastasis (Figure 3.2E).

These data show that post-surgical emodin treatment is capable of creating a less immune suppressed, pro-tumor environment in potential metastatic sites, which in turn may account for the reduction in metastatic burden. It is likely that this effect is at least in part due to modulation of macrophage activation at sites of metastasis formation resulting in a change in the release of cytokines from these macrophages, such as TGF-β, known to increase the metastatic success rate of tumor cells [132].

Emodin inhibits breast tumor cell EMT through modulating macrophage phenotype and TGFβ secretion.

Previous work by our lab and others has shown that tumor associated macrophages (TAMs) secrete TGF-β1, and that this can increase the metastatic and invasive capabilities of tumor cells through affecting tumor cell epithelial-to-mesenchymal transition (EMT) [133, 134]. Based on previous observations from our lab that emodin suppresses tumor-cell induced M2-like macrophage polarization, an activation phenotype associated with increased TGF-β1 secretion, we hypothesized that this may be a mechanism by which emodin was able to decrease the establishment of distant metastases in our murine breast cancer studies [103, 118]. One of our first steps was to determine whether or not emodin affected TAM TGF-β1 secretion. In order to do
this, peritoneal macrophages from BALB/c mice were treated with 4T1-conditioned medium with or without 25 µM emodin for 24 h. Our previous work has indicated that this concentration is similar to the serum emodin levels in mice treated with 40 mg/kg emodin administered i.p. [118] qPCR showed that tumor-conditioned medium treatment increased TGF-β1 expression, and this was abolished when emodin was added. Emodin alone had little or no effect on macrophage TGF-β1 expression (Figure 3.3A). To further confirm this relationship in vitro, conditioned medium was collected from macrophages treated in the same way and ELISA was performed for TGF-β1. Emodin treatment suppressed the tumor-conditioned medium-induced increase in TGF-β1 protein release (Figure 3.3B). It is important to note that this increase in TGF-β1 transcription coincided with a transcriptional profile indicative of M2-like macrophage polarization.

After demonstrating that emodin has the ability to interfere with TAM TGF-β1 production at physiologically achievable concentrations, it is important to also determine whether or not this interference could lead to changes in tumor cells which affect metastatic potential, such as the EMT status of tumor cells. To do this, peritoneal macrophages from BALB/c and C57BL/6 mice were pretreated with serum-free DMEM supplemented with tumor-conditioned medium from syngenic tumors with or without 25 µM emodin. Macrophage treatment with 0.05% DMSO in serum-free DMEM was used as a vehicle control. The macrophages were then co-cultured with syngenic tumor cells using a trans-well setup such that the two cell populations could not directly contact one another but shared the same medium (Figure 3.3C). After 24 h of co-culture with macrophages, qPCR on the tumor cells showed that multiple indicators of EMT, such as N-cadherin and vimentin, exhibited increased expression in the tumor cells co-cultured
with macrophages that had been exposed to syngenic tumor conditioned medium only (Figures 3.3D&E). The addition of emodin to this tumor-conditioned medium restored tumor cell EMT marker expression back to levels similar to co-culture with control-treated macrophages (Figures 3.3D&E). Other genes indicating a shift from an epithelial-like to a mesenchymal-like phenotype also exhibited a similar pattern, including matrix metalloproteinases 2 and 9 (MMP2 and MMP9) and fibronectin (Figures 3.3D&E). Macrophage pretreatment with emodin alone had varying effects on the levels of these EMT markers (Figures 3.3D&E). Taken together, these results show that while tumor cells can condition macrophages to exhibit a more M2-like phenotype and secrete increased levels of EMT-promoting TGF-β1, the addition of emodin can counteract both M2-like polarization and TGF-β1 secretion in macrophages. This in turn leads to an inhibition in TAM-induced EMT in breast tumor cells.

Emodin inhibits TGF-β and tumor-associated macrophage-induced migration and invasion of tumor cells

In our adjuvant therapy model, the primary tumor is never exposed to emodin, so the observed differences in metastatic burden must be due to either effects on circulating tumor cells’ ability to initiate metastases, newly established micrometastases, or the microenvironment at the metastatic site. An important, enabling trait for the formation of distant metastases is tumor cell invasion/migration. In order for tumor cells to both escape the primary tumor, enter the vasculature, and to extravasate once at the metastatic site, they must have sufficient invasive and migratory potential. This motility is enhanced by EMT, a prime driver of which is TGF-β1 [135]. As expected, TGF-β1 treatment drastically increased the migratory capacity of 4T1 cells in a scratch migration assay
(Figure 3.4A&B). When 25 µM emodin was added to the TGF-β1 treatment, migration ability was reduced back to control-treated levels. Again, emodin alone had little to no effect on the tumor cells’ ability to migrate. This experiment was also performed with the human breast cancer cell line, MDA-MB-231. Emodin again reduced the TGF-β1-induced tumor cell migration, but in this case, emodin alone also seemed to reduce the migration ability of the cells (Figure 3.4B).

In order to more firmly establish the connection between macrophage-released cytokines and these changes in migration, a similar scratch migration assay was performed on 4T1 cells treated with various macrophage-conditioned media. Macrophages were pretreated with either DMSO as vehicle control, 25 µM emodin in DMSO, 4T1-conditioned medium + DMSO, or 25 µM emodin in DMSO + 4T1-conditioned medium. After this pretreatment, macrophages were washed to remove emodin and 4T1-conditioned medium then cultured in serum-free DMEM, which was collected as macrophage-conditioned medium. This was an attempt to isolate to effects of emodin on the cytokines secreted by the macrophages from the direct effects of emodin on the tumor cells. The results of this assay showed that the conditioned medium from macrophages treated with 4T1-conditioned medium drastically increased the migration of tumor cells, and the addition of emodin decreased migration back to control-treated levels (Figure 3.4C&D). Pretreatment of macrophages with emodin alone had little effect on subsequent tumor cell migration.

To further test this signaling loop in another model of tumor cell motility, Matrigel® invasion assays were performed. These were intended to test the hypothesis that emodin could affect tumor cells’ ability to move through a network of ECM proteins,
an attribute closely linked to EMT and crucial for both tumor cells leaving the primary tumor and establishing themselves as distant metastases. Our results from these experiments were similar to those from the scratch-migration assays, and indeed were performed under similar conditions. Again TGF-β1 directly increased tumor cell invasion, and 25 µM emodin was able to directly decrease this effect back to baseline in both murine and human breast cancer cell lines (Figure 3.4E&F). Treating tumor cells with conditioned medium from macrophages that had been exposed to 4T1-conditioned medium also increased their invasive capacity, and this effect was again reversed by the addition of emodin to the macrophage pretreatment (Figure 3.4G&H). Taken together these results show that not only is emodin capable of skewing macrophage activation phenotype such that less EMT-inducing TGF-β1 is produced, it is also capable of reducing the EMT-promoting effects of TGF-β1 on tumor cells.

The intent of these in vitro studies is to focus on the potential effects that emodin may be exhibiting in vivo on the ability of tumor cells to extravasate and establish themselves in the lungs. Previous intravital imaging studies have shown that one of the first steps of extravasation involves the extension of structures termed invadopodia through the endothelium into the extravascular space [136]. These invadopodia are enriched for proteins such as cortactin, Tks4, and Tks5, and proteases such as MMP9 and MMP2 [136, 137]. Inhibition of invadopodia-related proteins, such as Tks5 has been shown to reduce breast tumor progression and metastasis [136, 137]. Given our observations that emodin is able to both decrease metastatic recurrence when administered as an adjuvant therapy, in the absence of the primary tumor, and decrease migration and invasion of tumor cells in response to cytokines including TGF-β1 released
by tumor-exposed macrophages, it is likely that emodin is affecting tumor cells’ ability to extravasate and establish metastases in vivo. We have already demonstrated the ability of emodin to reduce the ability of tumor-exposed macrophages to initiate MMP2 and MMP9 expression in tumor cells (Figure 3.3E&F). We further show that both cortactin and Tks5 expression are reduced in EO771 tumor cells when exposed to conditioned medium from macrophages pretreated with both emodin and EO771-conditioned medium versus the tumor-conditioned medium alone, indicating decreased formation of invadopodia-like structures (Figure 3.3I). In addition to this finding, Emodin was also capable of significantly decreasing Notch1 expression in EO771 tumor cell/macrophage non-contact co-culture (Figure 3.3I). It has been shown that macrophages induce the formation of invadopodia and extravasation of tumor cells via Notch1 signaling [138].

*Emodin interferes with both TGF-β canonical and non-canonical signaling in tumor cells*

TGF-β binding to its receptors largely results in signaling through two branching cascades referred to as the canonical and non-canonical signaling pathways. The canonical pathway occurs largely downstream of a phosphorylated Smad2/3 complex, while the non-canonical pathways are more broadly branching and have been defined as all non-Smad TGF-β signaling pathways, including but not limited to MAP kinase pathways and PI3K/Akt pathways [139]. A number of these non-canonical functions have been linked to EMT, and we examine several in our efforts to determine where emodin is interfering in the complex web of TGF-β signaling.

We have shown that emodin decreases TGF-β1 secretion from macrophages exposed to tumor-conditioned medium and that emodin can interfere with TGF-β1’s induction of a pro-metastatic tumor cell phenotype. Others have shown that emodin is
capable of interfering with TGF-β canonical signaling in human cervical cancer cells [140], but neither its effects on the canonical nor the non-canonical pathways of TGF-β signaling in breast cancer have been demonstrated. In order to assess canonical TGF-β signaling, we determined the level of Smad2/3 phosphorylation, which should increase in response to TGF-β. We found that the ratio of phosphorylated to total (p/t) Smad2/3 is increased by TGF-β, as expected, and is decreased by the addition of 25 µM emodin in both mouse and human breast cancer cell lines (Figure 3.5A-C). Akt is phosphorylated as a component of non-canonical TGF-β signaling leading to induction of EMT in tumor cells [141]. The addition of emodin to TGF-β treatment decreased its phosphorylation, though not to a statistically significant degree (Figure 3.5D). STAT3 signaling has previously been shown to be required for TGF-β-induced EMT in other tumor models [142]. Here we show that its phosphorylation in 4T1 cells is increased in response to TGF-β and decreased back to control levels by the addition of emodin (Figure 3.5D). Finally the transcription factors Twist and Zeb1, both of which are induced by TGF-β signaling and are important for EMT in various tumors including breast cancer [143, 144], exhibit decreased expression upon the addition of 25 µM emodin to TGF-β treatment in 4T1 cells (Figure 3.5E). Thus emodin interferes with many aspects of both canonical and non-canonical TGF-β signaling pathways in breast tumor cells.

3.4 DISCUSSION

Our data show that adjuvant emodin is capable of preventing metastatic recurrence and slowing the progression of existing metastatic disease in a murine model of triple-negative breast cancer. Emodin reduced both the size and number of metastases and increased overall and recurrence-free survival when administered shortly after tumor
resection in mice not yet presenting with detectable lung metastases. It was also capable of eliciting changes in the frequency of Tregs (CD4^+FOXP3^+) and migratory DCs (CD11c^+CD103^+) in lung-draining lymph nodes, both of which have been linked to decreased TGF-β1 signaling. Further investigation showed that indeed emodin treatment was capable of reducing TAM TGF-β1 production, due at least in part to its ability to decrease macrophage M2-like polarization. Macrophages pulsed with tumor-conditioned medium increased tumor cell migration and invasion as well as markers of EMT: all parameters linked to improvements in the ability of tumor cells to establish metastases. When emodin was added to this macrophage pretreatment, all three of these phenotypic markers were significantly decreased, indicating that the changes elicited by emodin treatment in macrophage cytokine release were capable of imparting less metastatic tumor cells. In addition to acting through macrophages, emodin also exhibited direct effects on tumor cell response to TGF-β1. Treatment of tumor cells with TGF-β1 increased marker of EMT, migration, and extracellular matrix invasion as expected, and addition of emodin again restored tumor cell phenotype to near baseline. It has recently been discovered that emodin is capable of down-regulating TGF-β signaling in pancreatic and cervical cancer cells, but its effect on this pathway in breast tumor cells has not been investigated [140, 145]. Further investigation showed that emodin was able to interfere with both the canonical and non-canonical pathways of TGF-β1 signaling in several breast tumor cell lines. Thus emodin is capable of not only reducing TGF-β1 secretion from TAMs, it also reduces tumor cell response to TGF-β1 by inhibiting its signaling cascades. These effects combine to render tumor cells much less capable of establishing distant metastases.
The majority of literature focused on the effects of emodin on cancer describes only its direct effects on tumor cell viability or phenotype [98, 146]. Our lab has previously detailed emodin’s interference with the cross-talk between tumor cells and macrophages. This feedback loop leads to the promotion of tumor growth and immune suppression [118]. Furthermore, the concentrations of emodin found to be tumoricidal were not achievable in serum in our murine models [103]. There have also been a number of studies in recent years showing that emodin is capable of interfering with TGF-β1 signaling in several cell types, including a few tumors, though results of these studies are mixed [7, 140, 145]. Some show that emodin has no effect on TGF-β signaling [147]. A single study has shown that emodin is capable of decreasing tumor cell migration and invasion as well as MMP-2 and MMP-9 secretion in MDA-MB-231 human breast cancer cells. This study also demonstrated that emodin treatment decreased lung metastasis in athymic mice implanted with MDA-MB-231 cells [148]. By employing athymic mice, this study was able to determine the in vivo effects of emodin on a model of human breast cancer, but the resulting picture is incomplete as immune cell interaction with tumor cells is crucial for tumor growth and metastasis, and is certainly important for human cancer patients. Our findings that emodin affects the production of TGF-β by TAMs, thus also indirectly reduces metastatic capacity, are novel. An in vitro-only study has shown that emodin can interfere with TGF-β signaling from the fibroblasts collected from patients with triple-negative breast cancer and a human breast cancer cell line, BT20 [132]. This study found that pretreating the isolated fibroblasts with emodin inhibited BT-20 cell EMT in a direct co-culture system. This direct co-culture model, however, does not limit
the interactions between fibroblasts and tumor cells to only soluble mediators. Our indirect co-culture model simply and effectively enforces this limitation.

The major novelty of this study is the implementation of emodin as an adjuvant therapy in a model biologically mimicking the pattern of human breast cancer treatment. Showing that emodin is capable of decreasing metastatic recurrence when the primary tumor has been removed prior to treatment is valuable knowledge that bolsters emodin’s translational potential.
### Table 3.1 Antibodies for flow cytometry

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### Table 3.2 Antibodies for Western Blot/Immunohistochemistry

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### Table 3.3 Primers for RT-qPCR

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A

Day 0

0.2x10^6 4T1-luc2 cells
Right 4th mammary fat pad

Day 20
Day 21
Day 24
Day 30
Day 38

IVIS
IVIS

Resect tumor,
IVIS, & begin
treatment

IVIS

Sacrifice,
resect lungs,
ex vivo IVIS

B

Pre-surgery tumor: Day 20

DMSO

40mg/kg Emodin

40mg/kg Emodin

C

Post-surgery tumor: Day 21

DMSO

40mg/kg Emodin

40mg/kg Emodin
Figure 3.1 Adjuvant emodin treatment decreases metastatic recurrence of TNBC after surgical resection. A, Timeline of this experiment. 7 week-old, female BALB/c mice received 2x10^5 4T1-luc2 cells in the 4th right (9th) mammary fat pad. Tumors were resected as indicated on the timeline, and treatment with either 2% DMSO in PBS (vehicle), 20 mg/kg emodin, or 40 mg/kg emodin was administered i.p. daily for the duration of the study beginning on the same day that tumors were resected. B, IVIS image of tumors prior to resection. C, IVIS image of mice immediately following tumor resection. D, Tumor weight at resection, tumor radiance from IVIS image in B, and tumor growth until resection; no differences between groups are apparent. E, Day 24 and Day 30 lung field radiance from IVIS imaging and Day 38 IVIS radiance from lungs imaged ex vivo. F, IVIS image from Day 30 showing lung metastases from lungs imaged ex vivo. G, Ex vivo lung IVIS image on Day 38. H, Representative lung sections with metastases identified. I, Quantification of lung metastases from serial sections. J, Prevalence of gross lung metastases from IVIS data at the conclusion of the experiment. Results are shown as means ± SE (n=13-14). *p<0.05, **p<0.01, ***p<0.005.
Figure 3.2 Emodin modulates the immune microenvironment of the metastatic site.

A, Representative flow cytometry result and quantification of Tregs in lung-draining lymph nodes.  
B, Representative flow cytometry result and quantification of migratory DCs in lung-draining lymph nodes.  
C, Quantification and representative immunofluorescence staining of Ym1^+ cells in lung metastases; scale bar = 50 µm.  
D, Ym1 staining showing M2 macrophages aggregating in the peripheral area of the lung metastases; scale bar = 100 µm.  
E, Quantification of activated CD8^+ T cells in lung-draining lymph nodes from flow cytometry. Results are shown as means ± SE (n=13-14). *p<0.05. **p<0.01.
Figure 3.3 Emodin inhibits breast tumor cell EMT through modulating macrophage phenotype and TGFβ secretion. A, qPCR of TGF-β1 in macrophages treated with EO771-conditioned medium, emodin (25 µM), or both. B, ELISA for TGF-β1 in supernatants from macrophages treated as in A. C, Non-contact macrophage/tumor cell co-culture schematic. D, qPCR of 4T1 cells after 24 h of non-contact co-culture with macrophages pretreated as shown for EMT-related genes. E, qPCR of EO771 cells after 24 h of non-contact co-culture with macrophages pretreated as shown for EMT-related genes. Results are shown as means ± SE (n=3). *p<0.05, **p<0.01, ***p<0.005.
A

DMSO  Emodin  TGF-β1  TGF-β1+Emodin
0h
16h

B

4T1 Migration  MDA-MB-231 Migration

C

DMSO  Emodin  4T1CM  4T1CM+Emodin

D

4T1 Migration
Figure 3.4 Emodin inhibits TGF-β and tumor-associated macrophage-induced migration and invasion of tumor cells. A, Representative images of 4T1 cell migration during treatment with TGF-β1 (5 ng/mL), emodin (25 µM), or both. B, Quantification of 4T1 and MDA-MB-231 cell migration when treated as in A. Migration index = (width before migration-width after migration)/width before migration, normalized to vehicle control (DMSO). C, Representative images of 4T1 cell migration during treatment with conditioned medium from macrophages pretreated as shown. D, Quantification of 4T1 cell migration treated as in C. E, Representative images of 4T1 cell Matrigel® invasion during treatment with TGF-β1 (5 ng/mL), emodin (25 µM), or both. F, Quantification of 4T1 and MDA-MB-231 cell migration treated as in E. G, Representative images of 4T1 cell Matrigel® invasion during treatment with conditioned medium from macrophages pretreated as shown. H, Quantification of G. I, qPCR of invadopodia-related genes from EO771 tumor cells treated with conditioned media from macrophages pretreated as shown. Results are shown as means ± SE (n=3). *p<0.05, **p<0.01, ***p<0.005.
Figure 3.5 Emodin interferes with both TGF-β canonical and non-canonical signaling in tumor cells. 4T1 (A), EO771 (B), and MDA-MB-231 cells (C) were treated with TGF-β1 (5 ng/mL) with or without emodin (25 μM) for 1 h; Smad2/3 and p-Smad2/3 protein levels were detected by western blot. D, 4T1 cells were treated as in A-C; protein levels of STAT3, p-STAT3, Akt, and p-Akt were detected by western blot. E, 4T1 cells were treated with TGF-β1 (5 ng/mL) with or without emodin (25 μM) for 24 h; protein levels of Twist and Zeb1 were detected by western blot. Results are shown as means ± SE (n=3). *p<0.05, **p<0.01, ***p<0.005.
CHAPTER 4

GENERATION OF A MICRORNA-155 TRANSGENIC MOUSE MODEL

4.1 BACKGROUND

MicroRNA-155 (miR155) is processed into its active form from its primary transcript, the B cell Integration Cluster (BIC), located on chromosome 21 in humans [149]. BIC is so named due to its initial discovery as a transcript present in chicken B cell lymphomas induced by integration of the avian leukosis virus [150]. Sometime later it was appreciated that pre-miR155 was transcribed from the BIC region in chickens and was similarly expressed in humans. In fact, miR155 is highly conserved between species and orthologs have been identified in many species including the evolutionarily ancient Ciona intestinalis. This early appearance and relatively unaltered maintenance of miR155 in the evolutionary tree also seem to coincide with the development of innate and adaptive immunity. C. intestinalis possesses a complex innate immune system and some degree of adaptive immunity through antigen receptor rearrangement [151, 152]. Tied to this and the initial discovery of the BIC locus in relation to abnormal, virally-induced B cell expansion, a number of studies have demonstrated the importance of miR155 for many immune processes and for antitumor immunity, specifically, in several cell types [153]. BIC, and subsequently miR155, overexpression has been observed in human Hodgkin’s and diffuse large B cell lymphomas [154-157]. Transgenic mice, engineered to overexpress miR155 specifically in B cells, exhibit abnormally enhanced pre-B cell proliferation which develops into B cell lymphoma [158]. It is not clear where enhanced
miR155 expression falls in the pathogenesis of human B cell lymphomas, but this murine study at least lends some credence to the possibility of a causative relationship. In addition to mounting evidence demonstrating a connection between B cell development and proliferation and miR155, it has also been shown that miR155 deficiency in B cells reduced both IgM and IgG antibody production in response to Salmonella infection [159, 160]. In addition to this finding, other studies have shown decreased number and size of lymph node germinal centers in miR155-deficient mice [161-163].

miR155 transcription is physiologically induced in both B and T cell activation [157, 164, 165]. There have been many studies detailing mechanisms of miR155 activity in T cell populations. Tumor-infiltrating CD8+ and CD4+ T cells have been shown to require miR155 for proper IFNγ expression and tumoricidal activity [166]. Overexpression of miR155 in CD4+ T cells has also been shown to promote in vitro Th1 cell differentiation [167], but proliferation was not found to be different between wild-type and miR155-deficient CD4+ T cells in an in vivo murine model of antigen-dependent T cell activation [160]. Notably, miR155 can directly target SOCS1, a JAK/STAT inhibitor, Ptpn2, a STAT5 inhibitor, and SHIP1, a PI3K/AKT inhibitor, thus increasing the activity of these three pathways [168-170]. Due to these regulatory pathways, increased T cell miR155 expression led to enhanced T cell survival and antitumor activity in murine models of melanoma. The role of miR155 in Tregs has been shown to involve similar pathways, but to a different immunologic effect. It was observed that miR155 was inhibited by the STAT5 pathway in response to CD69 deficiency, which in turn led to increased SOCS1 expression and impairment of Treg development [171]. Interestingly this study places STAT5 upstream of miR155 in Tregs, while the previously described
studies place it downstream [168-170]. It may be the case that both of these relationships
are present simultaneously in both cell types, creating a negative feedback loop on
miR155 activity. Other studies have also reported Treg dependence on miR155
expression for proliferative capacity, but also that the miR155-dependent reduction in
total Treg burden did not correspond to a measurable decrease in their immune
suppressive activity [172, 173]. All of the studies described thus far were performed in
mice, but it has also been found that miR155 in human dermal lymphatic endothelial cells
(HDLECs) suppresses PD-L1 expression induced by TNFα and IFNγ [174]. Indeed
miR155 is also the most highly up-regulated microRNA in HDLECs responding to TNFα
and IFNγ; in this case, naturally dampening the increase in PD-L1 expression [174].

In cells of monocytic lineage, miR155 has been shown to play both positive and
negative roles in controlling the mammalian immune response [165, 175-177]. The
immunosuppressive functions of myeloid-derived suppressor cells (MDSCs) have been
shown to be both increased and decreased by miR155 in murine models of lung cancer
and melanoma[178, 179]. miR155 signaling has also been implicated in the activities of
monocytes, macrophages, and DCs [180-183]. Direct targeting of arginase-2 (Arg2) by
miR155 in DCs has been linked to increasing the extracellular availability of arginase,
facilitating proper DC-induced T cell activation [184]. Our lab and others have
previously shown that miR155 is a key regulator of DC function, and that its decreased
expression in DCs leads to decreased migration and chemotaxis as well as generalized
depression of maturation and subsequent T cell activation [7, 184-186]. Our studies
uncovered a correlation between CCR7 expression, important for DC migration, and
miR155 in a murine orthotopic breast tumor model. We demonstrated that a potential
mechanism for miR155’s regulation of CCR7 expression is through directly targeting Jarid2, which in turn recruits PRC2, a component protein of a complex with histone methyltransferase activity. PRC2 represses genes primarily by trimethylation of histone H3 at lysine 27 (H3K27me3), and we found enriched H3K27 at the CCR7 promoter in miR155−/− BMDCs [105]. Unsurprisingly, SHIP1, also shown to be an important target of miR155 in T cells, has been revealed to be similarly influenced in DCs. In a model of DC-induced autoimmunity, miR155−/− DCs pulsed with self-antigen and matured with toll-like receptor (TLR) ligands ex vivo did not bring about a break in self-tolerance, while wild-type DCs treated in the same manner did, and DCs overexpressing miR155 did so even without TLR-ligand assisted maturation. SHIP1 expression was increased in miR155−/− DCs, and SHIP1 knockout in DCs allowed for the induction of autoimmunity in the absence of TLR ligand stimulation, similar to what was achieved with miR155-overexpressed DCs [187]. Studies of human DCs differentiated from peripheral blood monocytes have also focused on the role of miR155. IL-12p70 has been shown to correlate with miR155 expression level, and maturation of monocyte-derived DCs has been associated with increased miR155 expression [173, 185].

Only one of these studies used a transgenic miR155 overexpression model [187]. The miR155-transgenic mice in this study were generated by crossing mice containing a Rosa26 knock-in of the miR155/bic gene preceded by a stop codon with mice expressing Cre recombinase under the control of the CD11c promoter. Generating transgenic mice using this method requires continued crossing and genotyping to verify that both the transgene and Cre recombinase are expressed in the same mouse. Given that these mice were used to generate bone marrow-derived dendritic cells and not directly for in vivo
experiments, this CD11c specificity does not seem necessary. Our model globally overexpresses miR155 at a stable level, therefore removing the additional steps of crossing and genotyping when generating BMDCs for in vitro studies or adoptive transfer.

However, the majority of studies seeking to investigate the effects of increased miR155 expression achieve this by viral transfection. DCs are notoriously difficult to successfully transfect, and successful infection rates are often low [188]. To reliably study the effects of augmented miR155 expression, a stable, transgenic overexpression model could prove very useful. We have developed and validated such a model and demonstrated the effects of total immune system miR155 overexpression in a murine orthotopic breast tumor model. Detection of the presence of the transgene as well as increased expression of mature miR155 in bone marrow, bone marrow-derived DCs (BMDCs), peritoneal macrophages, and genotyping tail clips confirm that the transgene is actively transcribed and processed. Transplantation of miR155 transgenic (miR155tg) bone marrow into wild-type mice later implanted with breast tumor cells confirmed that the net effect of increased global immune system miR155 leads to more effective antitumor immunity and prevention of metastatic dissemination.

4.2 DETAILED METHODS

*Generation and validation of a miR155 transgenic mouse model*

A DNA fragment consisting of a CMV promoter, the mouse Bic sequence, and a SV40 late polyadenylation signal sequence was used for generation of miR155 transgenic mice by microinjection. Of the 90 pups implanted with the DNA fragment, four were found to be positive for the transgene following PCR screening. qPCR of tail clips from
these four mice further showed that only one of them was obviously producing mature miR155. Every pup from this single founder’s line was genotyped using PCR and assessed for overexpression of mature miR155 using qPCR until six generations of mice were produced.

Genotyping PCR

3 mm tail clips were obtained from 4 week-old pups. 6 wild-type tail clips were also obtained as age-matched controls for genotyping and qPCR; 3 male, 3 female. Half of each piece of tissue was used for end-point PCR genotyping and half was used for qPCR. Tail clips were incubated in 150 µL DirectPCR (Tail) solution (Viagen Biotech Inc., Los Angeles, CA) containing 2 µL Proteinase K (Viagen Biotech Inc.) at 55°C in a shaking water bath overnight. The digestion was terminated by 1 h incubation at 85°C. Resulting lysate was then centrifuged at 17,000 x g for 1 min. 3 µL of the supernatant was taken and mixed with 10 µL 2X green master mix (Promega Corp., Madison, WI), 0.1 µL of both the forward and reverse primer, and 6.8 µL of water to bring the final reaction volume to 20 µL. PCR amplification was done as follows: 5 min at 94°C followed by 36 cycles of 30 s at 94°C for denaturation, 1 min at 60°C for annealing, and 1 min at 72°C for extension. The last cycle was followed by 3 min at 72°C additional extension. Primers are listed in table 4.1. 10 µL of each resulting solution and a 100 bp ladder (Bioline, London, England) were loaded into separate wells of a 1% agarose gel containing 6 µL ethidium bromide per gram agarose and run for 50 min at 120V, after which the gel was imaged using a GelDoc XR+ Imaging System (Bio-Rad Laboratories) for a band at ~215bp.
Total RNA extraction and quantitative real-time PCR

Tissue/cells were lysed in 700 µL qiazol lysis reagent (Qiagen) and tissue samples were homogenized. Samples were stored at -80°C if RNA extraction was not performed immediately. RNA was extracted using miRNeasy Mini Kits (Qiagen) to also ensure collection of micro-RNAs. cDNA was then synthesized using 1 µg RNA, or the maximum amount possible if less than 1 µg, using miScript II RT Kits (Qiagen). RNA concentration was measured using an Evolution 60 spectrophotometer (Thermo Fisher Scientific). qPCR was performed using miScript SYBR® Green PCR Kits (Qiagen). All cDNA stocks were diluted 1:50, and 2 µL of the resulting solution was used for each qPCR reaction. Primers are listed in table 4.1. qPCR run conditions were 95°C for 15 min followed by a repeated cycling of 94°C for 15 s, 55°C for 30 s, and 70°C for 30 s. A melt curve was then collected through the following temperature fluctuations: 95°C for 10 s, 65°C for 5 s, and terminating again at 95°C. All samples were run in duplicate using a Bio-Rad CFX Real Time thermocycler. The ΔΔCt method was used to determine relative expression.

Peritoneal macrophage collection

Brewer’s thioglycollate was prepared at a concentration of 4% and injected i.p. into mice at a volume of 100 µL per gram mouse weight. Three days after thioglycollate injection mice were sacrificed and macrophages were obtained by peritoneal lavage with 20 mL PBS. Cells were resuspended and plated in DMEM 10% FBS P/S, cultured for 1 h. Non-adherent cells were then rinsed away with serum-free DMEM; adherent cells were cultured overnight in serum-free DMEM before being rinsed twice with PBS and lysed for RNA extraction and qPCR.
Bone marrow derived dendritic cell differentiation

4-12 week old mice (wild-type C57Bl/6 or miR155tg) were anaesthetized with isoflurane and sacrificed by cervical dislocation. Hind legs were removed, dipped in 70% ethanol, and placed in PBS on ice. Femurs and tibiae were cleaned and disarticulated in a cell culture hood on ice. Epiphyses were removed from diaphyses and rinsed with complete RPMI (RPMI-1640, 10% heat-inactivated FBS, P/S, and BME) to remove the bone marrow. Bone marrow was then resuspended in 1 mL RBC Lysing Buffer (Sigma-Aldrich Corp.) per mouse and gently disrupted by pipetting for 1 min. Lysing buffer was then diluted with 20 mL RT PBS, centrifuged to pellet and resuspended in complete RPMI for counting. Bone marrow cells were plated in 10-cm tissue culture-treated, polystyrene dishes at a density of 3x10^6 cells/mL in 10 mL complete RPMI supplemented with 20 ng/mL recombinant murine GM-CSF (Peprotech, Rocky Hill, NJ) and 20 ng/mL recombinant murine IL-4 (Peprotech) (DC medium) and cultured at 37°C, 5% CO2 incubator. 10 mL additional DC medium was added on Day 3, and immature, differentiated DCs were collected on Day 7.

Bone marrow transplantation

6-week-old, female C57Bl/6 wild-type mice were purchased from Jackson Labs and housed at the University of South Carolina Animal Research Facility. Procedures were approved by the Institutional Animal Care and Use Committee. Mice were treated with Neomycin and polymyxin B, administered in drinking water at concentrations of 100 mg/L and 5x10^5 U/L, respectively, for two weeks prior to irradiation and bone marrow transplantation. Mice were exposed to a dose of 950 rad to ablate existing bone marrow cells. Donor bone marrow cells (wild-type or miR155tg) were prepared at a
concentration of 10x10^6 cells/mL in PBS by rinsing femurs and tibiae followed by RBC lysing buffer as described previously. 100 µL of appropriate genotype donor bone marrow cell suspension was injected retro-orbitally into each mouse 6 h after irradiation. Mice were then maintained on neomycin/polymyxin B-containing water for two additional weeks after bone marrow transplantation. At sacrifice bone marrow was collected from all mice and both end-point PCR and qPCR were performed as described previously to confirm that the transgene was present in the bone marrow and that miR155 was overexpressed.

**Tumor model**

Four weeks after bone marrow transplantation, the recipient mice were inoculated with breast cancer cells. 0.2x10^6 EO771 cells were implanted into the 4th and 9th mammary fat pads. Tumors were measured using calipers every 3-5 days beginning on Day 14 when the majority of tumors were obviously palpable. Tumor volume was calculated using the following formula: (short axis)^2 x (long axis)/2 ≈ volume. Mice were sacrificed 37 days after tumor implantation, and tumors, lungs, blood, spleens, and tumor-draining lymph nodes were collected.

**Lung metastatic burden determination**

Lungs were perfused with PBS, then fixed for 24 to 48 h in 10 mL 4% paraformaldehyde. Fixed lung were imbedded in paraffin and sectioned at 5 µm thickness at 150 µm intervals. 5 levels were obtained at this spacing from each pair of lungs. Sections were mounted, de-paraffinized, and H&E stained. Stained sections were then imaged at 10X magnification using an EVOS FL Auto 2 System (Thermo Fisher Scientific). Images were tiled in order to view the entire lung using the EVOS System
software. Size of images were limited such that they did not require compression to be saved by the EVOS System software, ensuring that pixels represented equivalent real dimensions on every section. Pixel areas of lung tissue and metastases were measured for each image by hand using ImageJ software. These pixel values were converted to mm$^2$ using the conversion factor 1.142 pixels/µm determined by the 10X objective. The number of metastatic nodules per section was also counted. These values were used to calculate the following parameters for each lung pair: 1) metastatic burden = (metastatic volume)/(total lung volume), 2) (number of metastases per section)/(lung section area).

Flow cytometry

Tumor tissue, spleens, and tumor-draining lymph nodes were collected from mice and single cell suspensions of these tissues were analyzed using flow cytometry. Tumors were chopped into <1 mm$^3$ fragments and enzymatically digested in the following mixture: 20 mg Collagenase IV (Worthington Biochemical Corp.), 10 µg Hyaluronidase (Sigma-Aldrich Corp.), and 100 µg DNase 1 (Sigma-Aldrich Corp.) in 5 mL RPMI-1640 medium supplemented with P/S. Digestion took place in a 37°C water bath for 1 h during. During this time, the mixture was agitated every 15 min by pipetting or vigorous shaking. The mixture was then further disrupted using an 18 gauge needle and 10-mL syringe until most visible fragments were broken up. The mixture was then placed back at 37°C for 15 more min of digestion. After this final incubation, add 20 mL RT or ice-cold PBS to each tube to stop digestion. Cells were thoroughly rinsed to remove all digestion enzyme. Red blood cells were lysed using RBC lysing buffer (Sigma-Aldrich Corp.), 3 mL for 1 min at RT with gentle pipetting. This was then diluted with 20 mL PBS and passed through a 70-µm strainer, then suspended in PBS and counted. Lymph nodes and spleens were
mechanically dissociated and passed through a 70-µm strainer. Spleen cells were then treated with 1 mL RBC lysing buffer for 1 min at RT with gentle pipetting. Both splenocytes and lymphocytes were then rinsed, resuspended in PBS and counted. Blood was collected in 1.5 mL Eppendorf tubes containing 12 µL of 0.5-M EDTA to prevent clotting. 100 µL of each blood sample was collected for treatment with 1 mL RBC lysing buffer as described previously, rinsed with PBS and counted.

Fc receptors were blocked with anti-CD16/32 antibodies (BD Biosciences, San Jose, CA) for 10 min at 4˚C. Cells from the various tissues collected were then stained for the following surface markers: CD45, CD3, CD4, CD8, CD11c, MHCII, CD40, CD80, CD86, CCR7, CD11b, Ly6C, and CXCL1 (BD Biosciences, San Jose, CA) in 100 µL PBS for 30 min at 4˚C. Internal staining was performed for IFNγ (BD Biosciences) using eBioscience Cell Stimulation Cocktail (Thermo Fisher Scientific) followed by BD fixation and permeabilization treatment per manufacturer protocols. All samples were rinsed with PBS twice before being analyzed using a BD FACS Aria II flow cytometer and CXP software version 2.2 (BD Biosciences). Data were collected for 1x10^4 to 1x10^6 cells per sample depending on the rarity of the population being analyzed.

Statistical analysis

Data were shown as mean ± standard error of mean (SEM) whenever the mean was the primary value representative of a sample group’s behavior. Two group comparison of means was accomplished using a 2-tailed Student’s t test. All such comparisons were performed using GraphPad Prism 5 software (Graphpad Software Inc.). p≤0.05 was considered statistically significant for all tests.
4.3 RESULTS

_The miR155-containing transgene is present, expressed, and functional in one founder_

Previous studies in our lab have shown that total body miR155−/− increased breast tumor growth and metastasis, largely though hampering the immune stimulatory abilities of DCs [105]. Administration of a miR155−/− DC-based vaccine was also carried out in an orthotopic model of murine breast cancer, which was less effective that a wild-type DC-based vaccine at stimulating an antitumor immune response. We next aim to investigate whether increasing immune cell miR155 expression could represent a potential therapeutic strategy for breast cancer, setting the stage for possible future testing of a miR155-overexpressed DC-based vaccine. In order to produce a model stably overexpressing miR155, whole-body transgenic mice were generated. The transgene we designed is under the control of the cytomegalovirus (CMV) promoter for constitutive expression, though the microRNA will likely be processed into its active form to varying degrees in different cell populations. Four founder mice (3 male, 1 female) successfully integrated the transgene into their DNA. Through qPCR on tissue samples from these founders as well as transgene-positive offspring, it was determined that only one of the male founders functionally overexpressed miR155, so this line was bred and expanded. All transgene-positive offspring from this line were found to overexpress miR155 (Figure 4.1A). One of the transgene-positive F1 mice was sacrificed; peritoneal macrophages and bone marrow-derived DCs were obtained from this mouse as well as an age and gender-matched C57BL/6 wild-type control mouse (Figure 4.1B-D). It was revealed that miR155 was also overexpressed in these two immune cell types, and, unsurprisingly, that baseline miR155 expression was higher in both of these cell types.
than it was in the ear tissue obtained for genotyping. The expression levels of a few other
genesis were also determined in order to more convincingly establish that more functional
miR155 was indeed present in the transgenic DCs. SOCS1 and CEBPβ are known to be
direct targets of miR155, and thus their expression levels should be reduced in the
miR155tg cells. Our previous work detailed a regulatory pathway by which CCR7
expression is indirectly increased by miR155, thus it would be expected to be more
highly expressed in the miR155tg DCs. These three genes do appear to reflect a pattern
indicative of the effect of increased functional miR155 (Figure 4.1E). These results
suggest that our model would yield stable, functional miR155 overexpression in immune
cells that could be used in subsequent studies.

*Bone marrow cell miR155 overexpression reduces breast tumor growth and lung metastatic burden*

In order to further test both the stable presence and the effects of transgenic
overexpression of miR155, a bone marrow transplant study was performed. There is an
ever-increasing body of work demonstrating that manipulation of miR155 expression
levels leads to altered activity in many immune cell populations. Our previous work using
whole-body miR155−/− mice showed that miR155 was important for a number of
pathways governing the antitumor immune response in DCs, which led to increased
breast tumor growth and progression. Bone marrow transplantation using either C57BL/6
wild-type or miR155tg donor tissue and wild type recipients was performed and mice
were allowed four weeks after transplantation for immune system reconstitution before
orthotopic implantation of EO771 tumor cells. This resulted in reduced EO771 tumor
growth and tumor weight at sacrifice after 35 days of tumor growth (Figure 4.2B). There
was little difference between the average spleen weights between the two groups (Figure 4.2C). However, it was found that both spleens and tumor-draining lymph nodes contained significantly more cells in the miR155tg bone marrow transplant recipient (BM-miR155tg) mice (Figure 4.2D). These data suggest that there was likely a more robust antitumor immune response in the BM-miR155tg mice, both locally and, to some degree, systemically. The final gross phenotypic observation in this study was the difference in metastatic dissemination. Lung metastases were drastically reduced in overall burden (metastasis index = metastasis volume/total lung volume) (Figure 4.2E). The average number of metastases was lower, but did not reach statistical significance, though the average size of each metastasis was decreased in the BM-miR155tg recipient mice (Figure 4.2E). Taken together, these results indicate that bone marrow miR155 overexpression resulted in a stronger antitumor immune response against orthotopic breast tumors.

Further analysis of the immune response in the tumor microenvironment as well as in other tissues relevant to the development of local and systemic antitumor immunity is necessary to determine the impact of bone marrow-derived cell miR155 overexpression. Bone marrow was also collected from the right femur and tibia of every mouse in this experiment in order to roughly determine the efficacy of the transplant. qPCR showed that miR155 expression was on average ~10-fold higher in the miR155tgBMT mice than those that received wild-type bone marrow, though there was some variability (Figure 4.2F). Interestingly, though the number of bone marrow cells collected from each mouse was not counted, we found that the total amount of mRNA collected from the BM-miR155tg mice bone marrow was significantly higher than that of
the BM-wild-type mice (Figure 4.2G). This most likely indicates that more bone marrow cells were present in the BM-miR155tg mice, though the same number of cells were originally transplanted into each mouse. It is likely that the miR155 imparts some increased proliferative capacity to these progenitor cells [189].

*Bone marrow cell miR155 overexpression increases dendritic cell frequency, maturation, and T cell activation in tumor tissue and tumor draining lymph nodes*

Based on results previously reported by our lab and others, the observed differences in EO771 tumor progression between BM-miR155tg and BM-wild-type mice may be due to the activities of any number of immune cell populations. Our previous studies using total body miR155−/− mice indicated that, of the immune cell types analyzed in tumor draining lymph nodes (B cells, T cells, macrophages, and DCs), DCs were most significantly decreased in number, and likely contribute to the observed T and B cell deficits in addition to the miR155−/+ in both of these cell types. In order to generate a more complete picture of the role of miR155 in the immune response to breast cancer, we investigated several immune cell populations in the tumors, tumor-draining lymph nodes, spleens, and blood of BM-miR155tg and BM-wild-type mice. These tissues were chosen to collectively represent both systemic and local tumor immunity, and determine the effect of miR155 overexpression on the balance between antitumor immunity and tumor-induced immune suppression.

In the blood, it was found that there was no difference in total T cell percentage, but there were more CD8+ T cells and less CD4+ T cells in the BM-miR155tg mice (Figure 4.3A). CD69 staining was used to determine the activation of circulating T cells. Interestingly, there were no differences in the percentages of CD69+ cells among either
the CD4\(^+\) or CD8\(^+\) T cells, though as a percentage of total circulating T cells, there were more CD8\(^+\)CD69\(^+\) and less CD4\(^+\)CD69\(^+\) cells in the BM-miR155tg mice due to the aforementioned differences in CD4\(^+\) and CD8\(^+\) T cell populations (Figure 4.3B). The percentage of Ly6C\(^+\) blood monocytes was not different between the two groups, and neither was the overall presence of CX3CR1 or CCR2 on these Ly6C\(^+\) monocytes different between the two groups Figure 4.3C). These markers have been shown to identify monocytes preferentially infiltrating non-inflamed and inflamed sites, respectively [190-192].

Spleen tissue was mechanically dissociated and processed for flow cytometry. When total numbers of cells were considered, there were more CD11c\(^+\) DCs present in the spleens of BM-miR155tg mice (Figure 4.3D). Of the maturation markers MHCII, CD40, CD80, and CD86, all exhibited slightly higher expression on the splenic DCs of the BM-miR155tg mice (Figure 4.3E). The overall percentages of T and B cells (identified using CD3 and CD19, respectively) were not different between the BM-miR155tg and BM-wild-type mice, but the percentage of splenic CD8\(^+\) T cells also expressing IFN\(\gamma\) was significantly higher in the miR155tgBMT mice (Figure 4.3F&G). Taken together, these blood and spleen flow cytometry data indicate that there is a mild systemic increase in cytotoxic adaptive immunity. The splenic DCs, while present in greater numbers, do not appear to be drastically more activated in the BM-miR155tg mice. The examination of relative B cell burden was meant to address concerns that miR155 has been observed to be overexpressed in B cell lymphomas and is thought to be causative in some cases. In fact, B cell-specific miR155 overexpression in mice has been observed to result in B cell lymphoma formation, though we observed no aberrant
relative expansion of B cells when miR155 was overexpressed in our bone marrow transplant model. This could be due to the adult bone marrow contributing more to myeloid than lymphoid populations.

Tumor draining lymph nodes were also analyzed by flow cytometry and were found to contain many more CD11c+ DCs in the BM-miR155tg mice, and these DCs also expressed much higher levels of the maturation marker CD80. CD40, MHCII, and CD86 expression was not found to be significantly different between the two groups (Figure 4.3H&I). CCR7 expression was found to be much higher on the BM-miR155tg lymph node DCs, a result supportive of our previous finding that miR155 indirectly upregulates CCR7 transcription in DCs (Figure 4.3J). BM-miR155tg mouse lymph nodes also contained slightly more CD8+ T cells, but many more of these cells expressed IFNγ than those of BM-wild-type mice (Figure 4.3K).

Primary tumors in BM-miR155tg mice were found to contain more CD11c+ DCs which also expressed more MHCII, CD80, and CD86 than tumor DCs from BM-wild-type mice (Figure 4.3L&M). Also the DCs in the BM-miR155tg group’s tumors again expressed more CCR7 than those of BM-wild-type mice (Figure 4.3N). Interestingly, significantly more macrophages (F4/80+ cells) were present in the tumors of BM-miR155tg mice, but these macrophages expressed less CD206, a marker of tumor-promoting M2-like macrophages (Figure 4.3O). Macrophage populations were not analyzed in any of the other tissues, but these data appear to demonstrate that miR155 may also affect macrophage polarization and the innate antitumor immune response. Though with only this one piece of data, we cannot be sure if this is a direct effect of
elevated miR155 expression in the macrophages, or an indirect effect brought about by the other alterations observed in the tumor microenvironment.

4.4 DISCUSSION

Previous work by our lab and others has pointed to an immune stimulatory role for miR155, and several studies have been performed using knockout, knockdown, and cell-specific overexpression models to demonstrate this. However, our current study is the first time that a total-body miR155 overexpression model has been generated. The plan for this model is to generate DCs that constitutively express higher levels of miR155 and to test their function compared to wild-type cells in response to the breast tumor microenvironment, but it is possible that this mouse model can be used to investigate the effects of constitutive miR155 overexpression in other immune cell types and tissues. When generating a transgenic mouse it is important to not only determine the presence of the transgene in the genome, but to ensure that it is expressed to a relevant degree and that this expression occurs throughout the animal. Due to random insertion, successful integration of the transgene into a region that is not heavily repressed can be challenging and require a large number of attempts to insure the successful generation of a transgenic animal [193]. In order to produce a single transgenic founder, 90 pronuclei were injected with the transgene. We have also confirmed that the transgene is expressed in a variety of tissues and that this expression has been maintained over five generations. The miR155 overexpression was also shown to regulate a sampling of genes, SOCS1, CEBPβ, and CCR7 in a pattern consistent with the literature in order to further confirm that the overexpressed microRNA was also able to carry out its biological functions. This allowed
us to proceed with using these mice for further studies with some degree of confidence that stable, constitutive overexpression of miR155 had been achieved.

The first study that we performed with these mice was transplantation of bone marrow from miR155tg mice into wild-type recipient mice, followed by EO771 breast tumor implantation. Based on previous work, this would likely affect multiple populations of immune cells into which these bone marrow progenitors would differentiate. Cellular components of adaptive as well as innate immunity were analyzed in a diverse group of tissues related to the antitumor immune response. Gross assessment of the progression of the cancer was also quantified. In the tumor, spleen, blood, and tumor-draining lymph nodes, the most strongly affected populations of cells were the DCs and CD8+ T cells. These two populations exhibited both increased activation and relative abundance in response to miR155 overexpression. This finding is consistent with a number of previously performed studies [105, 169]. Interestingly, in the tissues analyzed, there did not appear to be a relative expansion of B cells, pathological or otherwise, as had been observed by others in models of B cell-specific miR155 overexpression. The development of B cell lymphoma was reported to progress as the mice aged. We have not observed this phenomenon in the miR155tg mouse line even at relatively advanced ages of greater than 18 months.

Increased DC and cytotoxic T cell activation, as well as the observed decrease in the pro-tumor tumor-associated macrophage (TAM) phenotype likely all play roles in the observed inhibition of primary tumor growth and metastatic dissemination in the BM-miR155tg mice. The reduction in metastasis was particularly striking and is the more clinically relevant of the two measures of breast cancer progression monitored in this
experiment. Through these experiments we have generated and validated a murine model of global miR155 overexpression, and can now move forward with studies of specific immune cell types, and their impacts on breast cancer progression.
### Table 4.1 Antibodies for flow cytometry

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<tr>
<th>Antibody</th>
<th>Product #</th>
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<td>CD45 (PE/Cy7)</td>
<td>103113</td>
<td>Biolegend</td>
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<tr>
<td>CD3 (APC/Cy7)</td>
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### Table 4.2 Primers for RT-qPCR

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Figure 4.1 The miR155-containing transgene is present, expressed, and functional in one founder line. A, miR155 qPCR results from 4 wild-type control and all F1 miR155tg mice from the viable line determined to carry the transgene; all mice were tested at 4 weeks old. B-D, miR155 expression in ear tissue, macrophages, and DCs of 5 wild-type vs. one miR155tg mouse at 4 weeks of age. E, The differential expression of genes known to be influenced by miR155 between wild-type and miR155tg mice determined by qPCR. Results are shown as means ± SE (n=1-5). *p<0.05.
Figure 4.2 Total immune system miR155 overexpression reduces breast tumor growth and lung metastatic burden. A, Timeline of this experiment. 7 week-old, female C57Bl/6 mice received oral antibiotics for 2 weeks both prior to and after irradiation and bone marrow transplantation with either wild-type or miR155tg bone marrow. 4 weeks after bone marrow transplant, 2x10^5 EO771 tumor cells were implanted into each of the 4th pair of mammary fat pads. B, Tumor growth over 35 days and tumor weights at sacrifice. C, Spleen weights at sacrifice. D, Total cells in spleens and tumor-draining lymph nodes. E, Manual counting of lung surface metastases; lung metastatic index, number, and average size determined from serial lung sections. F, miR155 expression in bone marrow of all mice at the conclusion of the study to confirm successful bone marrow transplant. G, RNA quantities obtained from right femurs and tibiae of all mice. Results are shown as means ± SE (n=10). *p<0.05, **p<0.01, ***p<0.005.
Figure 4.3 Total immune system miR155 overexpression increases dendritic cell frequency, maturation, and T cell activation in tumor tissue and tumor draining lymph nodes. Blood cells were stained with CD3, CD8, CD4, CD69, Ly6C, CXC3CR1, and CCR2 antibodies and analyzed using flow cytometry. A, Total circulating T cells (CD3+) and relative percentages of CD4+ and CD8+ cells within this population. B, CD69 expression on CD8+ and CD4+ T cells was used to identify activated T cells. C, Circulating monocytes were identified using Ly6C, and CXC3CR1+ and CCR2+ populations of monocytes were quantified. Spleen tissue was dissociated to a single-cell suspension; stained with CD11c, MHCII, CD40, CD80, CD86, CD19, CD3, CD8, and IFNγ antibodies; and analyzed using flow cytometry. D, Splenic DCs (CD11c+). E, Splenic DC maturation was determined by MHCII, CD40, CD80, and CD86 staining. F, Splenic B cells (CD19+). G, Total splenic T cells (CD3+) and active cytotoxic T cells (CD8+IFNγ+). Tumor-draining lymph nodes were dissociated to a single-cell suspension; stained with CD11c, MHCII, CD40, CD80, CD86, CCR7, CD3, CD8, and IFNγ antibodies; and analyzed using flow cytometry. H, Lymph node DCs (CD11c+). I, Lymph node DC maturation was determined by MHCII, CD40, CD80, and CD86 staining. J, CCR7 expression on DCs. K, Total lymph node T cells (CD3+) and active cytotoxic T cells (CD8+IFNγ+). Tumor tissue was digested to a single cell suspension; stained with CD11c, MHCII, CD40, CD80, CD86, CCR7, F4/80, and CD206 antibodies; and analyzed using flow cytometry. L, Tumor DCs (CD11c+). M, Tumor DC maturation was determined by MHCII, CD40, CD80, and CD86 staining. N, CCR7 expression on DCs. O, Total tumor macrophages (F4/80+), M2-like macrophages (F4/80+CD206hi). Results are shown as means ± SE (n=10). *p<0.05, **p<0.01, ***p<0.005.
CHAPTER 5
MIR155 OVEREXPRESSION BOOSTS DENDRITIC CELL VACCINE EFFICACY FOR BREAST CANCER

5.1 BACKGROUND

DCs take up, process and present antigen to adaptive immune cells to foster an effective tumoricidal response [194-196]. In fact they are considered the most effective antigen presenting cells (APCs) [197]. It is for this reason that DCs are considered a bridge between innate and adaptive immunity and are key to eliciting an effective T cell response [198]. However, the immunosuppressive tumor microenvironment often renders DCs dysfunctional [194, 199-202]. This is one of the most important mechanisms of tumor escape from immune surveillance [203-205]. Considerable effort has been put into developing therapies targeting or consisting of DCs to attempt to overcome or circumvent this immune suppression, respectively [206]. One of the strategies to circumvent this tumor-induced immune suppression is to take the patient’s own precursor cells, often peripheral blood monocytes, and generate DCs from these for \textit{ex vivo} manipulation [207]. These cells are pulsed with tumor peptide, then often treated with activating agents such as TLR ligands or TNF-\(\alpha\) to increase their immune stimulatory capacity [208]. This artificial maturation and antigen acquisition process helps to focus the DCs’ activity on the tumor while removing them from the immune suppressive environment of the tumor-affected tissue. Clinical success of these vaccines, however, has been limited for a number of reasons, including the lack of appropriate antigens, treatment of only high
stage disease, and re-introduction of these DCs into an immune suppressive environment [209]. Further enhancement of antigen presentation through regulatory manipulation may prove to be clinically valuable and help to overcome the immune suppression of the environment into which the DCs are introduced.

It is known that several targets of microRNA-155 are downregulated in order to facilitate adequate expression of MHCII and costimulatory molecules on DCs [184-186]. Our lab has identified microRNA-155 (miR155) as a key regulator of DC function through studies involving miR155−/− mice and virally-induced DC overexpression in orthotopic models of breast cancer [105]. These studies showed that a lack of miR155 results in defective antigen presentation and dampened costimulatory molecule expression, and as a result, reduced T cell activation. It was also determined that miR155 exhibits control over CCR7 expression by decreasing H3K27me3 at the CCR7 locus through direct targeting of Jarid2. In order to test the potential therapeutic ramifications of increased miR155 expression in DCs for breast cancer treatment, a robust model of miR155 transgenic overexpression was generated, and cells from these mice were used to demonstrate the effects of increasing DC miR155 expression on antitumor immunity in a murine model of breast cancer. The goals of this study were to investigate the phenotypic changes resulting from miR155 overexpression in DCs and to test the efficacy of a miR155-overexpressing DC vaccine for breast cancer.

5.2 DETAILED METHODS

*Tumor cell culture, conditioned medium, and lysate collection*

EO771 tumor cells were expanded in high-glucose Dulbecco’s modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL
penicillin (Sigma-Aldrich Corp.), and 100 µg/mL streptomycin (Sigma-Aldrich Corp.) in a 37°C, humidified, 5% CO₂ incubator. Tumor conditioned medium was collected by first expanding EO771 cells to 70% confluence for in T-75 bottles (Corning). Medium was then removed, plates were gently rinsed once with phosphate-buffered saline (PBS), and 10 mL of serum-free DMEM containing penicillin and streptomycin (P/S) was added. Cells were cultured for 48 h in this medium, which was then collected, centrifuged at 350 x g for 5 min to remove large cellular debris, and filtered through a 45-µm vacuum filter (Corning). This medium was then concentrated using 3000D molecular weight cutoff centrifuge concentrators (Merck Millipore, Burlington, MA) to 25X. Aliquots were stored at -80°C for later use. To treat immature DCs, conditioned medium was diluted 1:5 in fresh RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin (Sigma-Aldrich Corp.), 100 µg/mL streptomycin (Sigma-Aldrich Corp.), and 50 µM beta mercaptoethanol.

Tumor cell lysate for DC antigen loading was obtained by culturing EO771 cells in 10 cm cell culture-treated plates to 70% confluency in DMEM+10% FBS+P/S. About 30 plates of cells were processed for each batch of lysate. In preparation for lysate collection, plates were washed with PBS, then replenished with 10 mL serum-free DMEM and cultured for 48 h in a humidified, 5% CO₂ atmosphere. At the end of this incubation period, EO771 cells were rinsed with PBS and removed from plates using 5 mL of 3-mM EDTA in PBS for 10 min at RT. Cells were pelleted by centrifugation, rinsed with PBS, and again pelleted. The cell pellet was then subjected to four freeze/thaw cycles alternating from 5 min in liquid nitrogen to 15 min in a 37°C water bath. After the final freeze/thaw cycle, the pellet was vigorously resuspended in 5 mL
PBS, then centrifuged at 1x10⁴ g for 10 min to remove cell fragments. The supernatant was collected and protein concentration was determined using the Lowry method. Single-use aliquots of known protein concentration were stored at -80°C. This material was used as a source of soluble tumor antigen.

**Bone marrow-derived dendritic cell differentiation and antigen pulsing**

Entire hind limbs were removed from mice and dipped in 70% ethanol then placed in ice-cold PBS. Femurs and tibiae were cleaned on ice in a laminar flow hood. Sterile mortar and pestle (Thermo Fisher Scientific) were used to crush bones in 4 mL RPMI-1640 medium+10% heat-inactivated FBS+P/S+BME (complete RPMI). Crushed bone fragments were rinsed with complete RPMI until all bone marrow was removed and collected. Bone marrow was depleted of erythrocytes using 1 mL red blood cell lysing buffer (Sigma-Aldrich Corp.) for 1 min with gentle pipetting at RT. The resulting cells were counted and cultured in 10cm cell culture-treated dishes at a density of 1x10⁶ cells/mL (10 mL per dish) in complete RPMI supplemented with 20 ng/mL recombinant GM-CSF (rGM-CSF) and 20 ng/mL rIL-4 (Peprotech) in a 37°C, humidified incubator. 10 mL fresh complete RPMI supplemented in the same way was added on Day 3 of culture. After eight days of culture, loosely adherent cells were collected by gentle pipetting. Cells were confirmed >80% CD11c⁺, >90% CD11b⁺, and <5% Gr1⁺. These resulting cells were suspended in either control or treatment medium at densities ranging from 0.2x10⁶ cells/mL to 1x10⁶ cells/mL for various experiments, then cultured for 48 h. Control medium consisted of complete RPMI+20% serum-free DMEM. Treatment medium was made up of complete RPMI+20% concentrated EO771-conditioned medium+100 µg/mL tumor lysate. After this tumor antigen pulsing treatment, DCs were
collected using 3-mM EDTA in PBS for 10 min at RT, washed, and used for various purposes.

**In vitro dendritic cell migration assay**

100 ng/mL CCL19 (Biolegend) in serum-free RPMI-1640 medium was added to 24 well plates and allowed to equilibrate overnight in a 37°C humidified, 5% CO₂ incubator. 0.2x10⁶ control and tumor antigen-pulsed DCs were seeded into 8-µm pore size polystyrene trans-well inserts (Corning) in serum-free RPMI-1640 and allowed to migrate for 3 h at 37°C. Trans-well inserts were then removed from these inserts and fixed in 4% paraformaldehyde for 20 min on ice. Cells on the upper surface of the inserts were wiped away with cotton swabs. Cells that had migrated to the underside of the insert were stained with DAPI and counted. Each sample was run in triplicate, and 10 fields were collected at 10X magnification for each membrane using an EVOS FL Auto 2 System. DAPI-stained cells were counted in each field using Image-Pro Plus analysis software (Media Cybernetics, Rockville, MD).

**In vivo dendritic cell migration assay**

Female C57BL/6 mice were housed at the University of South Carolina Animal Research Facility until they were 8 weeks old; all procedures were approved by the Institutional Animal Care and Use Committee. 0.2x10⁶ EO771 tumor cells suspended in 20 µL PBS were implanted into the 4th pair of mammary fat pads. The following day control and tumor antigen-pulsed DCs, treated as described, were stained with CFSE (Biolegend) per the manufacturer’s protocol and injected subcutaneously into the groins of tumor-bearing mice. 48 h later, mice were sacrificed, lymph nodes were removed and processed for flow cytometry to identify CFSE-labeled cells.
In vitro T cell activation and proliferation

Day 8 bone marrow-derived DCs were plated in 24-well plates at a density of 0.1x10^6 cells in 0.5 mL of either control or treatment medium and cultured for 48 h in a 37°C, 5% CO₂, humidified incubator. T cells were obtained from the spleens of C57BL/6 wild-type mice 12-20 weeks of age. This involved mechanical dissociation using a Seward Stomacher followed by passage through a 70-µm cell strainer (Corning) and treatment with 3 mL red blood cell lysing buffer for 1 min at RT. T cells were isolated from mixed splenocytes using EasySep™ Mouse T cell Isolation Kits (Stem Cell Technologies, Vancouver, Canada). Isolated T cells were then co-cultured with DCs to assess DC-induced naïve T cell activation and proliferation.

To assess DC-induced T cell activation, DCs were rinsed with PBS very gently twice to remove residual treatment medium, then 1-2x10^6 splenic naïve T cells were added in 1 mL complete RPMI-1640 medium. The co-culture was maintained in a 37°C, 5% CO₂, humidified incubator for 48 h, then T cells were collected by rinsing the wells and stained to determine cell surface expression of CD69 on all T cells and intracellular expression of IFNγ on CD8⁺ T cells for flow cytometry.

T cell proliferation was determined by staining naïve splenic T cells with CFSE, then co-culturing these cells with matured DCs for 5 days in complete RPMI-1640 medium supplemented with 20 U/mL recombinant mouse IL-2 (Biolegend). CFSE dilution through division was determined by flow cytometry. CFSE-stained samples of T cells alone and unstained T cells were used as representative negative and positive proliferation controls, respectively.
**In vivo T cell activation**

7 week-old, female C57BL/6 mice were housed at the University of South Carolina Animal Research Facility; all procedures were approved by the Institutional Animal Care and Use Committee. At 8 weeks of age, 0.2x10^6 EO771 breast tumor cells were implanted into each of the 4th pair of mammary fat pads in all mice. 24 h after tumor implantation, EO771 tumor-conditioned medium and lysate-matured wild-type or miR155tg bone marrow-derived DCs were collected and 1x10^6 cells were injected subcutaneously into the groins of tumor-bearing mice (0.5x10^6 DCs injected into the left groin area, and the same for the right). A second DC injection was administered in the same manner four days later. Mice were sacrificed seven days after tumor cell implantation. Spleens and lymph nodes were collected and processed for flow cytometric analysis of T cell activation and a few other immunologically relevant parameters.

**Tumor model and dendritic cell vaccination**

Female C57BL/6 mice were purchased at 7 weeks of age (Jackson Labs) and housed at the University of South Carolina Animal Research Facility; all procedures were approved by the Institutional Animal Care and Use Committee. At 8 weeks old, 0.2x10^6 EO771 breast tumor cells suspended in 20 µL PBS were implanted into the 4th pair of mammary fat pads in all mice. 24 h after tumor implantation, EO771 tumor-conditioned medium and lysate-matured wild-type or miR155tg bone marrow-derived DCs were removed from culture dishes by incubating in 3-mM EDTA for 10 min at RT. These DCs were then resuspended in PBS and 1x10^6 cells were injected subcutaneously into the groins of tumor-bearing mice (0.5x10^6 DCs injected into the left groin area, and the same for the right). This DC harvesting and injection procedure was repeated every three to
four days for a total of six DC injections. Tumor volume was monitored until sacrifice and was calculated according to the following formula: \((\text{short axis})^2 \times \text{(long axis)}/2 \approx \text{volume}\). Mice were sacrificed 38 days after tumor cell implantation (19 days after the final vaccine injection). Tumors, tumor-draining lymph nodes, spleens, and lungs were collected for various analyses.

**Flow cytometry**

Several tissues were collected and dissociated into single cell suspensions for flow cytometric analysis. Blood was collected in 1.5 mL Eppendorf tubes containing 10 µL 0.5-M EDTA as an anticoagulant. 100 µL of this mixture was then treated with 3 mL red blood cell lysing buffer for 1 min at RT with gentle pipetting, rinsed counted, and 1\times10^6 cells were suspended in 100 µL PBS for staining. Blood cells were blocked with anti-CD16/32 antibodies as described and stained with the following markers for 30 min at 4°C: anti-CD45 PE-Cy7, anti-CD3 APC-Cy7, anti-CD4 APC, anti-CD8 FITC, anti-CD69 PE, anti-Ly6C APC, anti-CCR2 PE, and anti-CX3CR1 FITC.

Spleens were mechanically dissociated using a Seward Stomacher in 10 mL RPMI-1640 medium supplemented with P/S. This mixture was then passed through a 70-µm cell strainer (Corning) and treated for 1 min at RT with 3 mL red blood cell lysing buffer (Sigma-Aldrich Corp.), rinsed with PBS, counted, and 1\times10^6 cells were suspended in 100 µL PBS for staining. Splenocytes were blocked with anti-CD16/32 antibodies as described and stained with the following markers for 30 min at 4°C: anti-CD45 PE-Cy7, anti-CD11c APC, anti-CD11c FITC, anti-CD3 APC-Cy7, anti-CD19-APC, anti-CD4 APC, anti-CD8 FITC, anti-IFNγ PE, anti-CD40 PE, anti-IA/IE(MHCII) APC-Cy7, anti-CD80 FITC, anti-CD86 PE.
Lymph node cells were dissociated using 70-µm cell strainers (Corning), rinsed, counted, and 1x10^6 cells were suspended in 100 µL PBS for staining. Lymph node cells were blocked with anti-CD16/32 antibodies as described and surface stained with the following markers for 30 min at 4°C: anti-CD45 PE-Cy7, anti-CD11c APC, anti-CD11c FITC, anti-CD3 APC-Cy7, anti-CD4 APC, anti-CD8 FITC, anti-CD69 PE, anti-CD40 PE, anti-CD80 FITC, anti-CD86 PE, anti-IA/IE(MHCII) APC-Cy7, anti-CCR7 PE, anti-F4/80 PE, and anti-CD206 APC. Internal staining was performed using a BD Cytofix/Cytoperm Fixation Permeabilization Kit (BD Biosciences) according to the manufacturer’s instructions. The antibodies used to stain intracellular antigens were anti-IFNγ PE and anti-FOXP3 PE, each at a concentration of 4 µg/mL in the specified buffer.

Tumor tissue was chopped into <1 mm³ fragments and enzymatically digested in the following mixture: 20 mg Collagenase IV (Worthington Biochemical Corporation, Lakewood, NJ), 10 µg Hyaluronidase (Sigma-Aldrich Corp.), and 100 µg DNase I (Sigma-Aldrich Corp.) in 5 mL RPMI-1640 medium supplemented with P/S. Digestion took place in a 37°C water bath for 1 h during. During this time, the mixture was agitated every 15 min by pipetting or vigorous shaking. The mixture was then further disrupted using an 18 gage needle and 10 mL syringe until most visible fragments were broken up. The mixture was then placed back at 37°C for 15 more min of digestion. After this final incubation, add 20 mL RT or ice-cold PBS to each tube to stop digestion. Cells were thoroughly rinsed to remove all digestion enzyme. Red blood cells were lysed using RBC lysing buffer (Sigma-Aldrich Corp.), 3 mL for 1 min at RT with gentle pipetting. This was then diluted with 20 mL PBS and passed through a 70-µm strainer, then suspended in PBS and counted. Lymph nodes and spleens were mechanically dissociated and passed
through a 70-µm strainer. Spleen cells were then treated with 1 mL RBC lysing buffer for 1 min at RT with gentle pipetting. Both splenocytes and lymphocytes were then rinsed, resuspended in PBS and counted. Blood was collected in 1.5 mL Eppendorf tubes containing 12 µL 0.5-M EDTA to prevent clotting. 100 µL of each blood sample was collected for treatment with 1 mL RBC lysing buffer as described previously, rinsed with PBS and counted.

All samples were washed twice with PBS after staining and resuspended in 500 µL PBS for flow cytometry. Samples were analyzed on a BD FACS Aria II using FACS Diva software (BD Biosciences). 100,000 events were collected for each sample. All antibodies mentioned here were purchased from Biolegend.

**Total RNA extraction and quantitative real-time PCR**

Tissue/cells were lysed in 700 µL qiazol lysis reagent (Qiagen) and tissue samples were homogenized. Samples were stored at -80°C if RNA extraction was not performed immediately. RNA was extracted using Qiagen miRNeasy Mini Kits to also ensure collection of micro-RNAs. cDNA was then synthesized using 1 µg RNA, or the maximum amount possible if less than 1 µg, using Qiagen miScript II RT kits. Primers are listed in table 5.1. qPCR run conditions were 95°C for 15 min followed by a repeated cycling of 94°C for 15 s, 55°C for 30 sec, and 70°C for 30 s. A melt curve was then collected through the following temperature fluctuations: 95°C for 10 s, 65°C for 5 s, and terminating again at 95°C. All samples were run in duplicate using a Bio-Rad CFX Real Time thermocycler. The ΔΔCt method was used to determine relative expression.
**Metastasis quantification**

Following PFA fixation for 24-48 h at RT, mouse lungs were paraffin imbedded and sections were cut 5 µm thick at 150 µm intervals. Five such sections were obtained for each lung pair. These were deparaffinized and stained with hematoxylin and eosin. Stained slides were imaged at 10X magnification using an EVOS FL Auto 2 System (Thermo Fisher Scientific). Images were tiled in order to view the entire lung using the EVOS System software. Size of images were limited such that they did not require compression to be saved by the EVOS System software, ensuring that pixels represented equivalent real dimensions on every section. Pixel areas of lung tissue and metastases were measured for each image by hand using ImageJ software. These pixel values were converted to mm² using the conversion factor 1.142 pixels/µm determined by the 10X objective. The number of metastatic nodules per section was also counted. These values were used to calculate the following parameters for each lung pair: 1) metastatic burden = (metastatic volume)/(total lung volume), 2) (number of metastases per section)/(lung section area).

**Statistical analysis**

Data were shown as mean ± standard error of mean (SEM) whenever the mean was the primary value representative of a sample group’s behavior. Three group comparison of means is accomplished using a two tailed ANOVA followed by Tukey multiple comparisons test, while two group comparison was accomplished using a two tailed Student’s t test. All such comparisons are performed using GraphPad Prism 5 software (Graphpad Software Inc.). Differences between proportions were determined
using a two tailed N-1 Chi-Square test. $p \leq 0.05$ was considered statistically significant for all tests.

5.3 RESULTS

*Dendritic cell maturation is increased by miR155 overexpression*

DCs constantly sample their environments, take up antigen, and mature. This maturation process involves the up-regulation of MHCII, on which antigen is presented, as well as co-stimulatory molecules such as CD40, CD80, and CD86. Upon interaction with the corresponding stimulatory surface receptors of T cells, including CD40 ligand (CD154), T cell receptor, and CD28, T cell activation is induced. DC maturation is also accompanied by an increase in CCR7 expression, which enables DCs to respond to chemoattractant molecules such as CCL19 and CCL20 and migrate to draining lymph nodes where their chance of interacting with a receptive T cell is greater. Our previous results in miR155−/− mice showed that all of these molecules associated with DC maturation and migration were decreased compared to wild-type in response to tumor material, and their induction of a T cell response was much weaker. For our desired therapeutic outcome of increasing dendritic cell-mediated antitumor immunity, augmented miR155 expression is the goal. Thus a total body transgenic model of miR155 overexpression was generated on a C57BL/6 background and was confirmed to express increased levels of functional miR155. Bone marrow cells from these transgenic mice (miR155tg) and wild-type mice were harvested and differentiated into DCs by treating with GM-CSF and IL-4 for 8 days. At the end of this differentiation period, flow cytometry confirmed that >80% of the cells were CD11c+, >95% of the cells were CD11b+, and <5% of the cells were Gr1+ (Figure 5.1A). These cells were considered...
immature DCs. In order to mature them and gage the effects of their exposure to tumor antigen, immature wild-type and miR155tg DCs were each divided into two groups. One of which was treated with EO771 tumor-conditioned medium (ECM) and EO771 tumor cell lysate, the other was treated only with the corresponding medium as a control. Our results show that miR155tg DCs pulsed with tumor material in this way exhibit higher expression of MHCII, CD80, and CD86 than wild-type cells (Figure 5.1B). Interestingly, CD40 appeared to be expressed at a lower level in miR155tg DCs. CCR7 expression, which our lab has shown is linked to miR155, is drastically increased in both tumor antigen-pulsed and naïve miR155tg DCs (Figure 5.1C). Overall, these results point to a likely increase in miR155tg DCs’ capacity to both reach lymph nodes, and once there to activate T cells. In vivo assessment of DC maturation in the spleens, lymph nodes, and tumors of mice transplanted with either miR155tg or wild-type bone marrow yielded similar results (Figure 4.3). Thus over-expression of miR155 in DCs does appear to increase maturation upon exposure to breast cancer antigens both in vitro and in vivo.

miR155tg dendritic cells exhibit increased migration in vitro and in vivo

The observation of increased CCR7 expression on miR155tg DCs is a strong indication that their migratory ability may be increased. A trans-well migration assay was performed using a gradient of CCL19 on control and tumor-pulsed wild-type and miR155tg DCs. Wells containing no CCL19 were used to gage the baseline levels of migration for each genotype and treatment condition. The results of this in vitro experiment mirrored the CCR7 expression results (Figures 5.2A). Transgenic DCs migrated to a much greater extent than wild-type under both treatment conditions. In fact, the migration of control-treated miR155tg DCs was greater than wild-type cells pulsed
with tumor antigen. Interestingly, miR155tg DCs exhibited less baseline, random migration but were much more responsive to CCL19 (Figure 5.2B).

In *vivo* DC migration was also tested by introducing wild-type and miR155tg tumor antigen-pulsed DCs, which had been labeled with CFSE dye, into the groins of wild-type mice bearing EO771 tumors. DCs were given two days to migrate, at which time the draining lymph nodes were removed. Mice receiving no DCs served as negative controls for identifying CFSE+ cells. Results showed that CFSE+ DCs of both genotypes arrived at the draining lymph nodes, and there was a trend toward miR155tg DCs having migrated to a greater extent, but the difference was not statistically significant (Figure 5.2C). The small number of mice per group (3) may have contributed to this null result. However, taken together, these data suggest that miR155 expression levels directly correlate with the ability of DCs to migrate in response to lymph node chemoattractants.

*miR155tg dendritic cells elicit increased T cell activation in vitro and in vivo*

In response to the observed increase in maturation marker expression on miR155tg DCs, it should follow that T cell activation and proliferation would also increase. To determine if this was indeed the case, splenic T cells from healthy, wild-type mice were co-cultured with miR155tg and wild-type DCs pulsed with tumor antigen. By analyzing T cell surface expression of CD69 and internal expression of IFNγ, we determined that miR155 overexpression led to significant increases in CD4+ and CD8+ T cell activation with IFNγ expression also increased in CD8+ T cells (Figures 5.3A&B).

T cell proliferation in response to this DC co-culture was also tested by staining the naïve T cells with CFSE and determining the decrease in fluorescence due to division. Our results show that T cells proliferate significantly more in response to DCs pulsed
with tumor antigen for both genotypes, and that miR155tg DCs induce more T cell proliferation than wild-type DCs, whether naïve or antigen pulsed (Figure 5.3C).

It is important to determine whether these T cell activation results can be replicated in vivo for the sake of therapeutic relevance. This was done by administering tumor antigen-pulsed wild-type and miR155tg DCs subcutaneously into the groins of wild-type, EO771 tumor-bearing mice. CD69 expression was used to define both CD4+ and CD8+ activated T cells. T cell activation in tumors, spleens, and tumor-draining lymph nodes was determined to be increased in mice that received miR155tg DCs compared to wild type and no DC controls (Figures 5.3D&E). This increased activation was present in both CD4+ and CD8+ T cells in most tissues. It was also noted that spleen and tumor-draining lymph node cellularity was increased in mice receiving miR155tg DCs (Figure 5.3F). Given the increased T cell activation in vivo and in vitro, this change in the numbers of lymph node and spleen cells is likely due to an augmented antitumor immune response initiated by the introduction of mature miR155tg DCs.

miR155tg dendritic cells reduce systemic immune suppression

Apart from assessing T cell activation, several other immune cell populations were analyzed. These were intended to provide a broader picture of the immune phenotype brought about by the introduction of miR155tg and wild-type DCs to tumor-bearing mice. Spleen DC maturation, which was determined by MHCII expression, was significantly higher in mice inoculated with miR155tg DCs than both wild-type DC and no DC groups (Figure 5.4A). Tumor draining lymph node DCs also expressed more MHCII in miR155tg vaccinated mice than wild-type or no DC control mice (Figure 5.4B). It is likely that a portion of these DCs are those that were injected, but given
previous tracking of DCs implanted in the same way, it is more likely that the vast majority of these cells were the mice’s own. Macrophages were more abundant in the tumors of mice that received no DCs than either of the DC-inoculated groups (Figure 5.4C). Macrophage abundance is an important and generally negative prognostic indicator in breast cancer [210]. There was also an abundance of myeloid-derived suppressor cells (MDSCs) in the tumors of non-DC injected mice, here identified as CD11b+Gr1+ (Figure 5.4D). Significant increases in these two cell populations brought about an overall increase in tumor CD45+ cells (Figure 5.4E). These data suggest that, in this case, only two injections of tumor antigen pulsed DCs can bring about a robust and systemic increase in immunologic tone, and this is significantly augmented by miR155 overexpression in the implanted DCs.

miR155 overexpression increased the efficacy of a dendritic cell-based vaccine for breast cancer

To investigate the potential therapeutic efficacy of the miR155-overexpressing DC-based cancer immune therapy, a therapeutic vaccine strategy was implemented in mice bearing EO771 breast tumors. These mice received tumor antigen-pulsed wild-type or miR155tg DCs twice per week for 3 weeks. Another group of mice received injections of only PBS on the same schedule as a vehicle control. Tumor growth was monitored during this vaccination period and for 19 days after (Figure 5.5A). Tumor growth was significantly inhibited in both DC vaccine groups, but miR155tg DC-vaccinated mice exhibited more suppressed tumor growth than those receiving wild-type DC vaccination (Figure 5.5B). It was clear from the examination of mice shortly after receiving DC injections, that DC miR155 overexpression could induce a significant increase in T cell
activation. In this study we wanted to see if this gain in immune function could be maintained for a significant period after the full course of the vaccine was administered. With the intent of such therapeutic vaccines being to boost specific and systemic antitumor immunity, it would be advantageous for the treatment to bring about a sustained immune response focused on the primary tumor as well as distant metastases. At the time of sacrifice, 19 days after the administration of the last vaccine injection, miR155tg DC-vaccinated mice exhibited lower average tumor weight than mice that had received no DCs, while wild-type vaccinated mice did not maintain this difference (Figure 5.5C). Tumor draining lymph node weight and cellularity were also higher in miR155tg DC-vaccinated mice (Figure 5.5D). Cytotoxic T cells (CD8+IFNγ+) were also more abundant in the tumors, spleens, and tumor-draining lymph nodes of miR155tg DC-vaccinated mice than non-vaccinated mice (Figure 5.5E). Cytotoxic T cell frequency at least trended higher in miR155tg DC-vaccinated mice than mice that received wild-type DCs (Figure 5.5E). Tumor DC MHCII also remained higher in miR155tg vaccinated mice. It is somewhat possible, but unlikely that any of these DCs were the ones introduced in the vaccine injections after 19 days (Figure 5.5F). Taken together, these data show that a systemic elevation in immunologic tone is maintained for an extended period after vaccination has ended. This immune response as well as the contemporaneous effects of the vaccine leading to decreased metastasis are important indicators of its therapeutic potential.

5.4 DISCUSSION

Our data revealed that overexpression of miR155 in DCs increased maturation in response to tumor-produced cytokines and antigen. CCR7 expression was also increased
in agreement with our previous results in miR155^{-/} animals. This increase in CCR7 expression yielded an increase in the migratory response of DCs to CCL19, a key cytokine produced by high endothelial venules and T cell zones in lymph nodes to attract antigen-loaded DCs [211]. DC-induced T cell activation and proliferation were also significantly increased by DC overexpression of miR155. The injection of miR155tg DCs into EO771 tumor-bearing mice resulted in a more pronounced decrease in systemic tumor-induced immune suppression than the injection of wild-type DCs. This was observed as an increase in tumor, spleen, and tumor-draining lymph node DC activation, as well as dramatic decreases in tumor MDSCs and macrophages. Finally, implementation of a basic DC vaccination regimen in an orthotopic model of metastatic murine breast cancer showed that DC miR155 overexpression was able to improve the vaccine’s capability to slow primary tumor growth and maintain antitumor immunity long after the last vaccine injection had been administered. Our DC vaccine regimen does not employ any additional maturation stimulants, such as TLR4 ligands or TNF-α due to the fact that our objective here is not to implement the maximally matured DC vaccine, it was to study the effects of miR155 overexpression on DC maturation and resulting antitumor immunity. Taken together, these data support the conclusion that overexpression of miR155 significantly increases the efficacy of DC vaccines targeting breast cancer, and that such a regulatory manipulation may improve the prospects of developing effective DC-based immune therapies for human breast cancer.

A number of previous studies have been focused on the role of miR155 in a wide variety of DC functions from development to apoptosis to immune activation [161, 186]. Depending on the focus of the study, miR155 has been linked to everything from
hampering immune function to improving it to a beneficial or even pathological degree [180, 212, 213]. Previous models of miR155 overexpression have been based on either Cre-Lox recombination or viral overexpression. Some such models have shown that increased DC miR155 led to increased apoptosis upon activation, though based purely on viability we did not observe this, and even if apoptosis was increased to some degree, this did not seem to effect the outcomes of increased T cell activation or antitumor immunity induction [186]. Many studies, however have demonstrated that miR155 is upregulated upon treatment with various maturation-inducing ligands, most commonly LPS [185, 186, 214]. However, some studies have reported no difference in LPS-induced DC maturation (expression of CD40, CD80, CD86, and MHCII) when miR155 is knocked down or even completely knocked out [161, 186]. Even without this difference in maturation markers, impaired T cell activation was observed [185]. Our previous work focused on inducing DC maturation using only tumor-derived material, not LPS, and we found that lack of miR155 resulted in both significantly impaired maturation and subsequent T cell activation as well as augmented tumor growth.

One way in which increasing DC miR155 expression has been found to have a potentially deleterious effect on proper antigen presentation is through indirectly decreasing the expression of the C-type lectin DC-SIGN, a molecule capable of binding high mannose-containing glycoproteins present on viruses, bacteria, fungi, and tumor cells [212, 215-217]. It has been shown that APCs such as DCs interact with tumor glycoproteins such as MUC-1 [215]. The status of DC-SIGN as a Pattern-Associated Molecular Pattern receptor (PAMP) has also led to its targeting in some cocktails intended to stimulate DCs for therapeutic and preventative vaccination [218-222]. This
decrease in DC-SIGN expression occurs physiologically over the course of DC maturation, during which antigen uptake decreases and mir155 expression increases [212]. However, our results show that DC-SIGN is actually expressed to a higher degree on miR155tg DCs than wild-type (Figure 5.6A), and the net effects of increasing DC miR155 generally increased the ability of DCs to foster an antitumor immune response. It has even been shown that miR155 suppresses IL-1 expression in DCs during maturation [180]. It is possible that this is to prevent potentially damaging level of inflammatory cytokine production while still inducing an effective immune response. Our data show that miR155tg DCs do indeed express much less IL-1β in response to tumor material than wild-type DCs (Figure 5.6B).

Overall, the balance between immune activation and suppression by miR155 was tipped in the direction of increased DC maturation and T cell activation due to its multiple directly immune stimulatory effects, some of which we have confirmed in our model. Suppressor of cytokine signaling 1 (SOCS-1) is a direct target of miR155 and has been shown to regulate the production of IL-12, a key cytokine necessary for the complete activation of T cells and other immune cells with which DCs interact [186]. Overexpression of miR155 in DCs increased IL-12p70 expression, and thus increased IFNγ production in co-cultured NK cells [186]. We found that SOCS-1 expression was decreased in our miR155tg DCs, consistent with these previous results (Figure 4.1E), and that IL-12β subunit expression was increased compared to wild-type DCs upon tumor exposure (Figure 5.6B). The transcription factor component c-Fos has also been shown to be a direct target of miR155 in DCs [185]. Transcription factor complexes containing c-Fos were shown to suppress DC maturation, thus miR155 blocks this
suppression and facilitates DC activation. Our miR155tg DCs also exhibited decreased c-Fos expression and increased maturation in response to tumor material (Figure 5.6B).

Though there is still debate regarding the complex roles of miR155 in DC development, maturation, and immune activation, it is clear that miR155 is an important regulator of DC biology. It has been shown that miR155 directly influences a network of other microRNAs in DCs in addition to the many functions described previously [223]. In our model of miR155 transgenic overexpression, the net result was increased DC maturation, T cell activation and proliferation, and increased migration in response to lymph node chemoattractants. Use of these DCs in a breast cancer therapeutic vaccination resulted in decreased tumor growth and tumor-induced immune suppression. These results, as well as the regulatory activities of miR155 described in previous literature, suggest that forced overexpression of miR155 may enhance the therapeutic efficacy of DC-based vaccines for solid tumors such as breast cancer.
Table 5.1 Antibodies for flow cytometry

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<th>Antibody</th>
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<td>CD11b (FITC)</td>
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Table 5.2 Primers for RT-qPCR

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<td>Reverse</td>
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<td>CCR7</td>
<td>GCTTCTGCAAGATGAGGTC</td>
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<tr>
<td>IL-12β</td>
<td>GAGAAGGTCACACTGGACCA</td>
<td>TGACCTCCACCTGTAGTGC</td>
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<td>cFos</td>
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Figure 5.1 Dendritic cell maturation is promoted by miR155 overexpression. A, Representative flow cytometry dot plots showing the results of bone marrow DC differentiation with 20 ng/mL GM-CSF and 20 ng/mL IL-4. B, miR155tg and wild-type DCs were pulsed with tumor material, stained for maturation markers (MHCII, CD40, CD80, and CD86), and analyzed with flow cytometry. C, CCR7 expression on tumor-pulsed DCs was also analyzed. Results are shown as means ± SE (n=3). *p<0.05, **p<0.01, ***p<0.005.
Figure 5.2 miR155tg dendritic cells exhibit increased migration in vitro and in vivo. A, Wild-type and miR155tg DCs were pulsed with tumor material, then exposed to a CCL19 gradient. Migration in response to CCL19 through a trans-well insert was quantified and representative images are shown. B, This experiment was also performed without the CCL19 gradient. Results are shown as means ± SE (n=3). **p<0.01. C, Migration of CFSE-stained, tumor-pulsed DCs in vivo to tumor-draining lymph nodes. Results are shown as means ± SE (n=4).
Figure 5.3 miR155tg dendritic cells elicit increased T cell activation in vitro and in vivo. Wild-type and miR155tg DCs were pulsed with tumor antigen, then cocultured with naïve wild-type T cells. A and B, After 48 h of co-culture, T cells were stained for CD3, CD4, CD8, CD69, and IFNγ, then analyzed using flow cytometry. C, Some naïve T cells were stained with CFSE prior to co-culture; after 120 h of co-culture, T cell CFSE fluorescence was analyzed using flow cytometry. Results are shown as means ± SE (n=3). *p<0.05, **p<0.01, ***p<0.005. D and E, Tumor-bearing wild-type mice were s.c. injected twice with wild-type or miR155tg tumor-pulsed DCs. Tumors, spleens, and tumor draining lymph nodes were then harvested, dissociated to a single cell suspension, and stained with CD45, CD3, CD4, CD8, and CD69 antibodies. Stained cells were analyzed using flow cytometry. F, Spleen and tumor-draining lymph node cellularity after 2 tumor pulsed DC s.c. injections. Results are shown as means ± SE (n=6-7). *p<0.05, **p<0.01, ***p<0.005.
Figure 5.4 miR155tg dendritic cells reduce systemic immune suppression. Tissues collected for in vivo T cell activation analysis shown in Figure 5.4 were also stained for other immune cell populations; spleens and lymph nodes were stained with CD11c and MHCII antibodies; tumors were stained with CD11b, F4/80, and Gr1 antibodies. A, Spleen DC maturation represented by the extent of MHCII staining on CD11c⁺ cells. B, Lymph node DC maturation represented by the extent of MHCII staining on CD11c⁺ cells. C, Tumor macrophages (CD11b⁺F4/80⁺). D, Tumor MDSCs (CD11b⁺Gr1⁺). E, Total immune cells in tumors (CD45⁺). Results are shown as means ± SE (n=6-7). *p<0.05, **p<0.01, ***p<0.005.
A

Day 0
- Inject 0.5x10^6 DC subcutaneously
- 0.2x10^6 EO771 cells 4th pair mammary fat pads

Day 1
- Inject 0.5x10^6 DC subcutaneously

Day 5

Day 8
- Inject 0.5x10^6 DC subcutaneously

Day 12
- Inject 0.5x10^6 DC subcutaneously

Day 15

Day 19
- Inject 0.5x10^6 DC subcutaneously

Day 38
- Sacrifice

B

Tumor Volume [mm^3]

Day

0 10 15 20 25 30 35

No DC
WT DC
TG DC

**

C

Tumor Weight [g]

No DC
WT DC
TG DC

*

D

Lymph Node Weight [mg]

Lymph Node Cells [x10^6]

No DC
WT DC
TG DC

*

**
**Figure 5.5 miR155 overexpression increased the efficacy of a dendritic cell-based vaccine for breast cancer.** A, Timeline of this experiment. 7 week-old, female C57Bl/6 mice were implanted with 2x10^5 EO771 cells in each of the 4th pair of mammary fat pads. Beginning the following day and continuing for 3 weeks, mice received twice-weekly s.c. injections of tumor-pulsed wild-type or miR155tg DCs; mice were sacrificed 38 days after tumor implantation. B, Tumor growth. C, Tumor eight at sacrifice. D, Lymph node weight and total cells at sacrifice. E, T cell activation in tumors, spleens and tumor-draining lymph nodes was determined by staining for CD45, CD3, CD4, CD8, CD69, and IFNγ on single cell suspensions and performing flow cytometry. F, Tumor and lymph node dendritic cell maturation was determined by staining for CD11c and MHCII on single cell suspensions and performing flow cytometry. G, Percentages of original 20
mice in each group that developed tumors after EO771 cell implantation. Results are shown as means ± SE (n=8-17). *p<0.05, **p<0.01, ***p<0.005.
Figure 5.6 Effects of miR155 transgenic overexpression on molecules relevant to dendritic cell function. A, Wild-type and miR155tg DCs were pulsed with EO771 tumor lysate and conditioned medium, then stained for CD11c and CD209 (DC-SIGN) and analyzed using flow cytometry. B, Wild-type and mir155tg DCs were treated as in A; RNA was then extracted and qPCR performed for mir155 and genes relevant to DC biology that have been linked to miR155 by previous studies. Results are shown as means ± SE (n=3). **p<0.01, ***p<0.005.
CHAPTER 6

CONCLUSIONS AND FUTURE WORK

The potential benefits of immune therapies for breast cancer are now beginning to be realized for patients. Stand-alone therapies focusing on well-defined tumor antigens, such as HER2, have been combined with more traditional therapeutic strategies such as systemic chemotherapy, other immune stimulatory agents, and even radiation [224-226]. These combination therapies are having increased success in clinical trials and will be important for the future of breast cancer therapy. The development and testing of new treatments to target tumors from different angles and a comprehensive understanding of the effects that these treatments have on the tumor itself, as well as the immune microenvironment, are important for the design of combination therapies with additive or synergistic effects. The major focus of cancer immune therapy has been on targeting therapies to the tumor via unique or overexpressed antigens or increasing the activity of adaptive immunity, specifically T cells. The two immune therapies described here focus on macrophages and DCs in the TME. These therapies may prove to be even more beneficial in combination with other immune or traditional therapies than on their own. Each of these therapies will be discussed individually, then some future studies to progress each toward clinical implementation will be discussed.

6.1 EMODIN ADJUVANT THERAPY CONCLUSIONS

Macrophages in the TME and in metastatic sites exhibit a high degree of plasticity both over the course of cancer progression as well as in different regions of an individual
tumor [227, 228]. During late stage disease, M2-like macrophages generally predominate, but this activation state remains plastic and is the result of a complex network of pathways [229, 230]. Innate immune therapies designed to address this regulatory network must exert change while not shifting the balance too far in a pro-inflammatory direction. Herb-derived compounds such as emodin often exert complex yet measured effects, and thus are promising to address the TME and complement existing therapies [231-233]. The specific mechanisms by which emodin exerts these effects are still not known, but it has been shown to affect a number of important proteins relevant for both inflammation and cancer including HER2/neu, NFκB, TNFα, PPARγ, IL-4, and TGF-β, just to name a few [98, 121, 234-236]. Emodin’s effects on TGF-β signaling in a variety of cancer cell types, including breast cancer, have been reported. Our lab, however, has attempted to characterize emodin’s effects on macrophages in the context of breast cancer, and has shown that it is capable of modulating macrophage M2-like polarization [103, 118]. This resulted in reduced lung metastases as well as tumor volume. The studies described here go further in both describing a new avenue by which emodin disrupts macrophage-tumor cell crosstalk and demonstrating that emodin is effective as an adjuvant chemotherapy. We show that emodin is capable of reducing macrophage TGF-β production and reducing the effects of TGF-β (and macrophage-conditioned medium containing secreted TGF-β) on tumor cell EMT. Such a relationship between macrophage-secreted TGF-β and tumor cell EMT has been established in a number of tumor models [237, 238]. As a result of these effects on tumor cells, and importantly macrophages subjected to tumor-secreted cytokines, emodin was able to reduce metastatic recurrence when administered as an adjuvant therapy following tumor
resection. In this adjuvant therapy model, migratory DCs were increased and Tregs were decreased in metastatic site-draining lymph nodes of mice treated with emodin. Both of these components of the tumor-bearing host’s immune phenotype have been linked with reduced TGF-β signaling. This is the first time that emodin has been shown to be an effective adjuvant cancer immunotherapy, increasing its potential effectiveness in similar regimens of human breast cancer treatment.

6.2 EMODIN ADJUVANT THERAPY FUTURE WORK

Though these data are promising, there are some questions that remain unanswered which may be beneficial to address and some additional work that remains to be done. Pertaining to the connection between adjuvant emodin therapy and increased recurrence-free survival, while we have demonstrated a mechanistic link between the metastatic site immune phenotype and the known effects of TGF-β, this connection should be more firmly established. If our hypotheses are correct, knockdown of TGF-β should diminish emodin’s effect in our orthotopic tumor resection model. This knockdown in vivo could be achieved through crossing TGF-β-flox mice with a relatively macrophage-specific Cre model such as the readily available LysM-cre or murine CD68-cre mice, the latter of which may prove to be more specific [239]. In vitro studies employing the addition of a TGF-β-neutralizing antibody to macrophage-conditioned medium or non-contact co-cultures with tumor cells could demonstrate the same effect [240].

In addition to more firmly establishing this connection, moving beyond demonstrating broadly that emodin inhibits metastatic recurrence when treatment is initiated after surgery and determining whether emodin has a more significant effect on
tumor cells or cells in the lung pre-metastatic niche. This could be accomplished by performing a group of experiments based on some form of intravenous injection of breast cancer cells and monitoring lung metastasis formation with emodin treatment of either tumor cells, mice prior to tumor cell implantation, or tumor-bearing mice.

An additional relationship between lung macrophages, extravasating tumor cells, TGF-β, and emodin involves macrophage CXCR4 expression. It has been known that perivascular macrophages play a significant role in local tumor recurrence, and that this is at least in part due to M2-like TAMs, which express high levels of CXCR4 [241]. CXCR4 expression is also dependent on TGF-β, which is produced at relatively high levels by tumor-exposed macrophages and tumor cells [242, 243]. CXCR4 interacts with CXCL12, which is produced by perivascular fibroblasts at especially high levels in metastatic sites such as lung, liver, and bone [244]. It has recently been shown that CXCR4+ macrophages interact with motile tumor cells and facilitate their extravasation into the surrounding tissue [242]. Given that we demonstrate here that emodin is capable of interfering with tumor cell-macrophage TGF-β crosstalk on several levels and reducing M2 polarization of macrophages, which is closely linked to CXCR4 expression, it is likely that this mechanism is at play in our model of adjuvant emodin therapy to prevent metastatic recurrence.

6.3 MIR155TG DENDRITIC CELL VACCINE CONCLUSIONS

The second immune therapy tested focuses on DCs and their potential implementation in therapeutic vaccines for breast cancer. Though they seemed to show great promise only recently, there have been many obstacles to the development of effective DC vaccines. Said difficulties have often been attributed to a few crucial
factors. First, many such therapeutic vaccine trials only involved patients with very advanced disease. The second major issue with DC vaccines centers on the immune suppression induced by the tumor for purposes of self-preservation. Additional and lasting immune activation of DCs is necessary to overcome this when reintroduced into the patient. TLR agonists such as LPS are effective maturing agents for DCs prepared \textit{ex vivo} as therapeutic vaccines. However, maturation with ligands such as these is more vulnerable to anti-inflammatory cytokines, such as IL-10, present upon re-introduction to the patient than strategies involving costimulatory receptor ligation or genetically forced upregulation of maturation [245, 246]. It is for this reason that we have focused on increasing the maturation of DCs via miR155. Our previous results have shown that miR155 deficiency impairs DC maturation and leads to increased breast cancer progression. The next step was to develop a stable miR155 overexpression model, then test the effects of miR155 overexpression on DCs and use these cells in a DC therapeutic vaccine model for breast cancer. We generated a novel, total body miR155 transgenic overexpression mouse line and used the bone marrow from these mice for both bone marrow transplantation and to generate DCs for \textit{in vitro} testing and breast cancer vaccination. miR155 has been shown to generally increase the activation of a variety of pro-inflammatory immune cells while at the same time reducing the activity of tolerogenic cell populations [247]. Bone marrow transplantation followed by orthotopic tumor implantation allowed for the comparison of several of these cell types’ responses between wild-type and miR155tg immune systems. The results largely pointed to an increase in systemic immune activation, especially DC activation and presence, as well as an increase CD8$^+$ T cell activity. Further testing of miR155tg DCs \textit{in vitro} revealed
increased CD86, MHCII, and CD80 expression upon exposure to tumor material. Co-culture of these DCs with T cells increased T cell activation and proliferation. miR155tg DCs also exhibited higher CCR7 expression and a corresponding increase in migration in response to CCL19. Introduction of ex vivo matured miR155tg DCs into breast tumor-bearing mice reduced tumor growth and augmented antitumor immunity to a greater extent than wild-type DCs. This effect persisted for many days after the last DC inoculation was administered and resulted in a lower lung metastatic burden. These data show that at least some method of delivering miR155 to DCs of immune cells more broadly in the TME would be a beneficial strategy to pursue.

6.4 MIR155 DENDRITIC CELL VACCINE FUTURE WORK

The project focusing on the effects of miR155 in DCs and the development of a miR155-overexpressed DC vaccine requires less additional work, but the development of the miR155tg mouse model opens up many possibilities. Not only could a much more detailed understanding of the regulatory changes brought about by stably increasing miR155 expression in DCs be gained, but also in many other cell types, both immune and otherwise. The majority of what is known about the many roles or miR155 has been gained by making use of miR155−/− mice. Virally introduced overexpression often ends up affecting only a small percentage of certain cells and is a more invasive method than constitutive overexpression. This model could provide the corresponding overexpression side to the many miR155−/− experiments, which is necessary to more firmly establish the previously described roles of miR155 and those yet to be uncovered.

Both of these potential therapies still require further testing in order to develop delivery methods that are more readily translatable to patients. It also may prove fruitful
to combine these methods with other immune therapies or more traditional chemotherapies. The added focus on treating the immune component of diseases is being seen as more valuable, and this is particularly the case with cancers. The results of these studies point to both emodin and miR155 as important potential additions to the developing arsenal of immune therapies for breast cancer.
REFERENCES


160


