MYELOID-DERIVED SUPPRESSOR CELL ACCUMULATION IN SECONDARY TARGET ORGANS PROMOTES METASTATIC GROWTH IN BREAST CANCER

By

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ABSTRACT

A role for bone marrow-derived cells (BMDCs) in promoting metastatic tumour growth is emerging. Previous work has shown accumulation of CD11b+ BMDCs in pre-metastatic niches in the lungs of mice bearing metastatic breast tumours, although questions remain about the precise identity of these cells and their potential long-term influence on metastatic growth. We studied the induction, identity, longevity, and function of CD11b+ BMDCs in tissues of mice bearing murine mammary tumours. Metastatic, but not non-metastatic, mammary tumours induced the accumulation of CD11b+Gr1+ cells, which we functionally identified as immunosuppressive myeloid-derived suppressor cells (MDSCs) using ex vivo assays. Unlike BMDCs associated with pre-metastatic niches in other breast tumour models, MDSCs were induced systemically, with levels increasing in metastatic target organs (lung, liver, bone-marrow) and in tissues that do not harbor metastases from these tumours (kidney, spleen). We also found that circulating MDSC levels can be used as a surrogate marker for monitoring MDSC accumulation in tissues.

Primary tumour resection caused decreased serum levels of granulocyte-colony stimulating factor and MDSCs, but functional MDSCs remained elevated in the lungs for several weeks after tumour resection. These MDSCs were associated with enhanced subsequent pulmonary metastatic growth, providing evidence that MDSC induction by metastatic primary tumours helps create a long-lasting metastasis-promoting environment in lung tissue. In addition to surgery, we utilized gemcitabine (GEM), 5-fluorouracil (5-FU) and tirapazamine (TPZ) to target MDSCs in the lungs and spleen of tumour bearing mice. Aside from its classification as a hypoxia-specific cytotoxic, we report TPZ as a novel MDSC cytotoxic, comparable in potency to other well characterized MDSC specific chemotherapeutics. We administered GEM to mice with resected tumours in order to target residual MDSCs. Our data demonstrates that GEM significantly eliminates residual MDSC levels in the lungs which resulted in a decreased ability of 4T1 tumour cells to colonize the metastatic target organ. While we were able to target the tumour-potentiating fraction of MDSCs, our data indicates that significant levels of residual MDSCs still remain in the lungs, whose function and phenotype remains to be characterized.

Taken together, our findings suggest that metastatic murine mammary carcinomas induce systemic elevation of MDSCs and highlight the potential importance of identifying patients with elevated MDSC levels. Additionally, our research provides support for therapeutic targeting of MDSCs in patients at risk of developing or re-developing metastatic disease.
PREFACE

I developed the hypothesis, the aims of this study and the experimental design with guidance from Dr. Kevin L. Bennewith. I performed the *in vivo* experiments (tissue harvests) with the help of Nancy E. LePard, Bryant T. Harbourne, Melisa J. Hamilton, and Ada Y. Kim. The *in vitro* experiments and flow cytometry data collection and analysis were performed by me. ELISA, immunofluorescent tissue staining and analysis, and all the chemotherapeutic work was done by me. Clonogenic assays were performed with help from Nancy E. LePard. Tumour implants were done by either me, Nancy E. LePard or Dr. Kevin L. Bennewith. Tumour excisions were performed by Dr. Kevin L. Bennewith and Nancy E. LePard. The T-cell proliferation assays were performed by Dr. Melisa J. Hamilton. I was responsible for thesis composition and edits under Dr. Kevin L. Bennewith’s guidance. All mouse work and methods were approved by the University of British Columbia’s Committee on Animal Care; project title: Promotion of Metastasis by Tumour Hypoxia and Myeloid Cells. Certificate #A13-0223 and A09-0251. I was funded by the Frederick Banting and Charles Best Canada Graduate Scholarships Master’s Award from the Canadian Institutes of Health Research (CIHR).
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>4T1</td>
<td>Murine mammary carcinoma; highly metastatic</td>
</tr>
<tr>
<td>4TO7</td>
<td>Murine mammary carcinoma; moderately metastatic</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-Fluorouracil</td>
</tr>
<tr>
<td>67NR</td>
<td>Murine mammary carcinoma; non-metastatic</td>
</tr>
<tr>
<td>ANV</td>
<td>Antigen negative variant</td>
</tr>
<tr>
<td>ATRA</td>
<td>All-trans retinoic acid</td>
</tr>
<tr>
<td>B16</td>
<td>B16 murine melanoma</td>
</tr>
<tr>
<td>BM</td>
<td>Bone-marrow</td>
</tr>
<tr>
<td>BMDC</td>
<td>Bone-marrow derived cell</td>
</tr>
<tr>
<td>C3</td>
<td>Cervical adenocarcinoma 3</td>
</tr>
<tr>
<td>CCL2</td>
<td>CC-chemokine ligand 2</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CT26</td>
<td>Murine colon carcinoma cell line 26</td>
</tr>
<tr>
<td>CTC</td>
<td>Circulatory tumour cell</td>
</tr>
<tr>
<td>CXCL12</td>
<td>CXC-chemokine ligand 12</td>
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<tr>
<td>CXCL2</td>
<td>CXC-chemokine ligand 2</td>
</tr>
<tr>
<td>DA3</td>
<td>Mammary adenocarcinoma 3</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECM</td>
<td>Extra-cellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EL4</td>
<td>Murine lymphoma cell line 4</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMT</td>
<td>Endothelial to mesenchymal transformation</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
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<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
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<tr>
<td>FISH</td>
<td>Fluorescence <em>in situ</em> hybridization</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>GEM</td>
<td>Gemcitabine</td>
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GM-CSF  Granulocyte-macrophage colony-stimulating factor
h  Hour
HEPES  4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER2/neu  Human epidermal growth factor 2
HIF-1α  Hypoxia-inducible factor 1 alpha
IDC NOS  Invasive ductal carcinoma not otherwise specified
IFNγ  Interferon γ
IL  Interleukin
IMC  Immature myeloid cell
iNOS  Nitric oxide synthase 2
IP or ip  Intraperitoneal
IV or iv  Intravenous
JAK3  Janus kinase 3
LLC  Lewis lung carcinoma
LOX  Lysyl oxidase
Ly6C  Lymphocyte antigen 6C
Ly6G  Lymphocyte antigen 6G
MC38  Murine colon carcinoma cell line 38
M-CSF  Macrophage colony-stimulating factor
MDSC  Myeloid-derived suppressor cell
MET  Mesenchymal to endothelial transformation
MethA  Murine sarcoma
MMP  Matrix metalloproteinase
MSC1  Immortalized myeloid suppressor cell type 1
MSC2  Immortalized myeloid suppressor cell type 2
NK  Natural killer cell
NO  Nitric oxide
O₂  Molecular oxygen
OCT  Optimum cutting temperature
OVA  Ovalbumin
PB  Peripheral blood
PBS  Phosphate-buffered saline
PDGF  Platelet-derived growth factor
PE  Phycoerythrin
<table>
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<tr>
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<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>PyMT</td>
<td>Polyoma middle T antigen spontaneous mammary carcinoma</td>
</tr>
<tr>
<td>RC</td>
<td>Responder control</td>
</tr>
<tr>
<td>RCC</td>
<td>Renal cell carcinoma</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem-cell factor</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>STAT5</td>
<td>Signal Transducer and Activator of Transcription 5</td>
</tr>
<tr>
<td>TDSF</td>
<td>Tumour derived secreted factor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNBC</td>
<td>Triple-negative breast cancer</td>
</tr>
<tr>
<td>TNM</td>
<td>Classification of malignant tumours: tumour, nodes, metastasis</td>
</tr>
<tr>
<td>TPZ</td>
<td>Tirapazamine</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T-cell</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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I would like to acknowledge all the members of the Bennewith lab who assisted me with much of this work and for advising me on many aspects of the research itself. I would like to thank Nancy E. LePard, Bryant T. Harbourne, Dr. Melisa J. Hamilton and Ada Y. Kim for assisting with the \textit{in vivo} and conceptual aspects of this project. In particular, I would like to thank Bryant for his help in many parts of my project, but I am mostly grateful for the laughs and important life talks. Ada, thank you for keeping my hands soft and putting up with my forgetfulness. Aside from research, you all have made working in the Bennewith lab a fun experience and one that I will always remember.

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I would also like to thank all my family and friends for helping make graduate school a less stressful experience and for providing me with countless laughs and good times.
DEDICATION

Most importantly, I would like to dedicate this work to my parents, Snjezana and Radoslav, and sister Jovana for their unyielding support during the entire process. Mom, thanks for the countless plastic containers packed with food and love. I also have to thank you for allowing me to win the Excellence Award for my tan. It’s tough for me to say this, but I think the award really belongs to you. Also, thanks for always making sure that I am doing fine, and always knowing when I am not. Dad, thanks for all the philosophical talks and especially for the lessons on true happiness near the end of my degree. Also, thanks for making me a curious person as my impulsivity and endless search for the most fulfilling hobby comes from you. Jovana, much like mom, thank you for feeding me and exercising your unmistakable sandwich making techniques on me. More importantly, thanks for being a truly authentic person and for being the best sister a brother can ask for. With you around, this boat’s well. Freja, thanks for being the laziest and most excitable dog, all at the same time.

Also, I want to dedicate this to the rest of my family, friends, and all the people I have met during my time at the BC Cancer Agency. All of you have shaped me in some way. I want to dedicate this work to Big Red also for getting me to work in record time. Additionally, I want to dedicate this to Liverpool FC and all LFC fans. You’ll never walk alone.

“There are only two tragedies in life: one is not getting what one wants, and the other is getting it.”
- Oscar Wilde
CHAPTER 1: INTRODUCTION

1.1 Cancer

Cancer is a general term used to refer to a group of diseases in which cells of the body begin to grow and reproduce in an uncontrollable way. The most common types of cancer, diagnosed in order of occurrence, are breast cancer, colorectal cancer, lung cancer and cervical cancer in females, and lung cancer, prostate cancer, colorectal cancer and stomach cancer in males [1]. The World Health Organization’s 2014 report states that in 2012, 14.1 million people were diagnosed with cancer, and that cancer was responsible for 8.2 million (14.6%) of all human deaths [1]. The Canadian Cancer Society reports breast cancer as the most prevalent cancer in Canadian women, making up 26% of new cases, and predicts that 1 in 9 women will likely develop breast cancer in their lifetime [2]. There are over 100 types of cancer that can affect the human body, and the risk of developing cancer increases significantly with age. Interestingly, cancers occur more commonly in developed countries with tobacco use, obesity, poor diet, lack of physical activity, alcohol consumption, viral infections, exposure to ionizing radiation and environmental pollutants being listed as the most common risk factors [1].

Malignancy refers to the ability of the primary tumour to disseminate or metastasize from its site of origin and colonize other organs of the body. It is metastasis and not the original tumour mass that is responsible for 90% of cancer related deaths [3]. While we are able to treat the primary tumour with surgery, curative chemotherapeutic targeting of metastatic tumour cells has not shown great results. Aside from the abnormal tissue mass being classified as malignant, tumours can also be benign, or pre-malignant. Benign tumours experience the same uncontrollable cell division as malignant tumours; however, they lack the ability to spread and grow in other parts of the body. It is specifically this ability to invade distant tissues that distinguishes a malignant tumour from a benign tumour [4]. Historically, much of cancer research has revolved around understanding and treating the primary tumour mass with surgery, chemotherapy and radiation and understanding the physical differences between benign and malignant tumours. As the field of cancer biology progressed, we have learned to appreciate the complexity of the metastatic process. The critical importance of understanding the formation of secondary tumours and discovering novel strategies for treating life threatening tumour metastases is an essential aspect of research in the current field of cancer biology.
Many questions relating to how cancers develop and the proper treatment of these malignancies still remain to be answered. Over the past 40 years, scientific literature has demonstrated the complexity of this disease that is almost beyond measure. However, in 2000, Hanahan and Weinberg published their paper “The Hallmarks of Cancer” which reduced the complexity of the disease down to 6 principles that they believed all cancers share. The 6 acquired capabilities of cancer they proposed aided in making cancer research a more comprehensible of science. The first 4 hallmarks deal with the ability of cancer to undergo uncontrollable growth: 1) self-sufficiency in growth signals (no need for external growth signals), 2) insensitivity to anti-growth signals (inhibitors have no effect on the cell cycle), 3) evading apoptosis (cells do not undergo programmed cell death), and 4) limitless replicative potential (cells do not undergo senescence and are therefore immortal) [5]. The fifth hallmark identified cancers as having the ability to undergo sustained angiogenesis (formation of new blood vessels) necessary for continual supply of oxygen and nutrients to the dividing cells [5]. The sixth and final proposed cancer hallmark is that of tissue invasion and metastasis [5]. This acquired characteristic allows cancer cells to break away from their site of origin and spread to distant organs creating life threatening metastases. In 10 years since its publication, “Hallmarks of Cancer” has been cited more than 15,000 times and became a highly influential article in the field of cancer biology; however, Hanahan and Weinberg were not without their critics. In 2010, Lazebnik pointed out that 5 of the 6 hallmarks were also characteristics of benign tumours and that the only hallmark of cancer is its ability to invade and metastasize [6].

Due to the technological advances in the cancer field and novel findings since the publication of the original article, Hanahan and Weinberg published another highly influential article in 2011 named “Hallmarks of Cancer: the next generation”. In the revised edition, they added two more hallmarks of cancer and two “enabling characteristics”. The two new hallmarks included the cancer cell’s ability to reprogram its energy metabolism through deregulation of cellular energetics, and avoidance of immune detection (cancer cells appear invisible to the immune system). In addition to avoiding the immune system, tumours are able to induce an immunosuppressive environment through the recruitment of cells such as MDSCs, regulatory T cells (Tregs) and macrophages. In concert, these immunosuppressive cell types allow for primary tumour survival and survival of disseminated cells, despite an immunological response [7]. The first enabling characteristic of cancer is genome instability and mutation. Cancer cells have unstable DNA and generally contain severe chromosomal abnormalities which worsen as the disease progresses [8]. Tumour-promoting inflammation is the second proposed enabling
characteristic which positively contributes to several of the previously mentioned hallmarks by providing pro-angiogenic factors and extracellular matrix (ECM) modifying enzymes to the tumour microenvironment [7]. A strong pipeline of recent discoveries has fortified this enabling characteristic and highlighted the importance of local chronic inflammation in inducing many types of cancer. Taken together, several decades of research has led to the consolidation of key findings and development of these hallmarks and enabling characteristics. While functionally distinct, the characteristics of cancer are complementary in their ability to ensure the survival of malignant tumours and the process of metastasis [7].

1.2 Breast Cancer

Breast cancer is a profoundly heterogeneous disease, varying in histology, molecular profile and clinical outcome. Cancer of the breast originates most commonly from the inner lining of milk ducts or the lobules that supply the ducts with breast milk. Ductal carcinomas are cancers originating from the ducts while lobular carcinomas originate from the lobules [9]. It is estimated that distant metastasis will develop within 3 years of primary tumour diagnosis in 10-15% of diagnosed patients [10]. The most common locations secondary tumours are found are the lung, liver and bone, and it is not atypical for these metastases to appear even 10 years after the initial diagnosis [10]. A 2013 study revealed the grim results that independent of race, ethnicity or location, the incidence of metastatic breast cancer is on the increase in women younger than 40 years of age [11]. Additionally, the 5-year survival for patients with metastatic breast cancer is a mere 31.4%. Such studies highlight the urgent importance of discovering novel therapies aimed at interrupting the metastatic cascade and preventing the dissemination of metastatic cells from the primary breast tumour.

There are several different ways that breast cancer can be characterized. Breast cancer is most widely classified by a combination of histological grading, TNM classification and gene-expression profiling [12]. Staging breast cancer by TNM classification is used to determine the type of treatment the patient should receive. TNM is defined by primary tumour size (T), whether or not the tumour has spread to axillary or other lymph nodes (N), and if the tumour cells have metastasized to distant parts of the body (M). With the accessibility of molecular analysis, hormone receptor (ER and PR) and human epidermal growth factor receptor 2 (HER2) status determinations are also used in conjunction with the traditional classification systems.
1.2.1 Breast cancer subtypes

Breast cancer is a tremendously heterogeneous disease. Based on histomorphological differences alone, breast cancer can be divided into 20 major and 18 minor subtypes [13]. As mentioned previously, the majority of breast cancers originate from epithelial ductal or lobular cells, however classification can often be difficult. The cancers that do not fall into one of the major or minor histopathological classes are termed “invasive ductal carcinoma not otherwise specified” or IDC NOS. A staggering 50-80% of cancers are diagnosed as IDC NOS, revealing the true heterogeneity and complexity of breast cancer. Patients diagnosed with IDC NOS have a predicted 35-50% 10-year survival rate [13].

Thanks to technological advances, breast cancer heterogeneity can now be classified based on molecular profiling of the tumour as well [14, 15]. Receptor status of a tumour can now be determined using DNA microarray technology, which together with traditional grading methods categorizes breast cancer into 7 conceptual classes [16], each differing in prognosis and ways of treatment [17]. These subtypes are separated primarily by the expression of 3 different receptors on their tumour cells: estrogen receptor (ER), progesterone receptor (PR), and HER2/neu status [18]. HER2/neu or ErbB2 is a member of the epidermal growth factor receptor family which can now also be analyzed using fluorescent in-situ hybridization (FISH) assays which some researchers and clinicians argue is superior to DNA microarrays [19]. The 7 molecular subtypes are luminal A, luminal B, luminal ER-/-AR+, normal breast-like, basal-like, HER2/neu+, and claudin-low [16, 20].

Luminal tumours express many of the genes normally expressed by breast luminal cells [14, 15]. Luminal A tumours are ER+ and low grade while luminal B tumours are ER+ and often high grade. Estrogen binds to the ER and stimulates cell proliferation leading to an increase in oncogenic mutations. Additionally to differing in grade, luminal A and B tumours differ in their expression of HER2/neu with HER2/neu being expressed more by luminal B tumours giving them a greater proliferative capacity [21]. Luminal ER-/-AR+ do not express ER, but have recently been identified to be androgen receptor (AR) responsive [22]. Overall, the luminal subtypes have the best clinical prognosis with the luminal A subtype having a lower relapse rate than the B subtype [21].

Breast tumours that have a high expression of genes characteristic of basal epithelia, adipose cells, and low expression of genes characteristic of luminal epithelia reflect a gene
expression pattern of a normal breast and are therefore labeled “normal-like” [14, 15]. Normal-like tumours are often small and have good prognosis [23].

The HER2/neu+ subtype represents tumours in which the HER2/neu gene is over expressed but are negative for the estrogen and progesterone receptors [21]. HER2/neu is amplified in 20-30% of all breast cancers [14, 15, 24], and is associated with poor prognosis, increased relapse, and metastasis [25]. Herceptin (Trastuzumab) is one of the first humanized antibodies raised against HER2/neu and benefits patients with HER2/neu over-expression by reducing proliferation of the tumour cells [26].

Basal-like tumours share a lot in common genetically with normal breast basal-like epithelial cells. The basal subtype is characterized by the absence of the estrogen, progesterone, HER2/neu receptors and the expression of high molecular weight cytokeratins 5 and 6 [27]. Triple negative breast cancer (TNBC) classification shares similarities to the basal-like subtype, and the two are often interchangeably classified [21, 27]. The basal-like and triple negative subgroups represent 15% of breast cancers and are the subgroup with the worst prognosis [12, 21, 27]. The poor prognosis has a lot to do with the fact that the tumours cannot be treated with hormone therapies or trastuzumab due to the absence of the key receptors. The final and most recently classified subtype is the claudin-low. These tumours are often triple-negative and display a low expression of certain cell-cell junction proteins including E-cadherin [20]. In addition, claudin-low tumours are frequently infiltrated with lymphocytes [28-30].

1.2.2 Diagnosis and personalized medicine

Screening for breast cancer is achieved by mammography which requires the use of low energy X-rays to examine the human breast. Mammography can be used as both a screening and diagnostic tool. Since its implementation in 1988, the incidence of breast cancer dramatically increased through the 1990s [2]. Once the breast tumour is detected, the standard practice is to determine the ER, PR, and HER2/neu status of the newly diagnosed breast tumour. Identification of receptor status is standard practice for management of breast cancer as it is a predictive marker for response to cytotoxic chemotherapy and endocrine therapy. For early stage and low risk patients, tumours are locally controlled by removing the primary tumour by mastectomy, or lumpectomy followed by radiation therapy [31]. As mentioned, metastasis is
the main cause of death in cancer patients, therefore chemotherapy or endocrine therapy is given after primary tumour resection to target cells that may have already disseminated [10]. Over 80% of patients receive adjuvant therapy (treatment given in addition to primary treatment) after primary tumour removal due to the inability to accurately predict the development of distant metastases.

Personalized medicine is a medical model that customizes healthcare for individuals using modern molecular analysis of tissue biopsies. Oncotype DX and pam50 are two of the more popular diagnostic tests that quantify the likelihood of cancer recurrence in patients with early-stage ER+ tumours. The tests have both prognostic and predictive significance as they can assess if a patient will benefit from certain types of chemotherapy. Based on a panel of genes, each patient receives a personal score that corresponds with the likelihood of breast cancer recurrence within 10 years of the initial diagnosis. In addition, patients should receive bilateral mammography at the time of their diagnosis since BRCA1/BRCA2 gene mutation carriers are at a risk of contralateral breast cancer [32]. Importance of personalized medicine is most evident for patients with TNBC where the tumours are insensitive to many of the available therapies. Knowing which chemotherapies will not benefit the patient is important as patients should not be subjected to harmful chemotherapeutics that will have no therapeutic value in their case.

1.2.3 Tumour models in the study

The tumour models used in our study are murine mammary carcinomas 4T1, 4TO7 and 67NR; however, the majority of research was carried out using the 4T1 model. These 3 sister cell lines were isolated from a spontaneous mammary tumour from a female Balb/cF3H mouse [33]. Several distinct populations of cells were obtained from the original morphologically heterogeneous culture and 5 major cell lines (4T1, 66c14, 4TO7, 168FARN, 67NR) are now utilized in many animal studies. 3D organotypic systems are used to characterize cell lines in vitro, and 67NR and 4TO7 cells form highly branched 3D structures, while 4T1 cells form spheroids lacking branching [34]. The 3 tumour sub-lines are not only heterogeneous in morphology but they also display varying degrees of metastatic potential [35].
When 4T1 cells are implanted in the mammary fat pad of mice, tumour cells metastasize primarily by a hematogenous route (blood) rather than by disseminating to draining lymph nodes. 4T1 cells are capable of metastasizing to the lungs, liver, bone, brain, lymph node and blood. After entering the circulation, these metastases are found earliest in the lungs, followed by the liver. In both of these tissues, 4T1 tumour cells form macrometastases which can visually be observed as metastatic nodules.

4TO7 cells spread hematogenously to the lungs, lymph nodes, and occasionally to the liver, but are unable to grow macrometastases in syngeneic Balb/c mice. 4TO7 cells are highly immunogenic in immune-competent mice (Balb/c) and therefore show a decrease in survival, effectively restricting metastatic growth. This is evident when 4TO7 cells are implanted in immune-compromised mice (nude mice) and develop metastatic nodules.

Finally, although 67NR tumours can grow to sizes exceeding that of 4T1 tumours, 67NR cells are unable to intravasate as demonstrated by the absence of tumour cells in the blood, lymph nodes, lungs or livers. Therefore, on the metastatic spectrum, 4T1 cells have the most aggressive metastatic behavior, 4TO7 cells are systemically invasive but unable to form macrometastases, and 67NR cells are noninvasive and therefore non-metastatic.

These cell lines are often referred to as isogenic cell lines meaning that they closely compare genetically. However, transcriptome analysis has shown that the same genes of the 3 cell lines show differential exon splicing [36]. 4T1 and 67NR cells differ in their exon expression of 257 genes (the cell lines are on the opposite side of the metastatic spectrum), whereas for 4TO7 and 4T1, exon differed for only 25 genes (both cell lines are metastatic). Not surprisingly, Eckhardt et al. have shown that the genes in which 4T1 (metastatic) and 67NR (non-metastatic) differed have functional roles in metastasis [37]. 4T1 cell line models stage IV TNBC in humans and expectedly has negative status for ER, PR, and HER2. It is of ductal differentiation shown by positive E-cadherin staining and cytokeratin 5/6 expression similar to basal-like molecular subtype [38]. Interestingly, the expression of E-cadherin (endothelial cell marker) by metastatic 4T1 tumour cells and N-cadherin (mesenchymal cell marker) by non-metastatic 67NR tumour cells demonstrates that metastatic ability of breast cancer does not strictly correlate with phenotypic properties of epithelial-mesenchymal transition (EMT) where mesenchymal cells are the ones that possess metastatic abilities [39]. EMT will be discussed further in section 1.3.2. The information regarding the 3 mammary carcinoma cell lines discussed in this section can be found in Table 1.
Table 1 Characteristics of murine mammary carcinoma cell lines used in our study.

<table>
<thead>
<tr>
<th></th>
<th>4T1</th>
<th>4T07</th>
<th>67NR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth in 3D media</td>
<td>Spheroids lacking branching</td>
<td>Highly branched</td>
<td>Highly branched</td>
</tr>
<tr>
<td>Orthotopic tumour growth</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Ability to invade</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Metastatic sites</td>
<td>Lung, liver, bone, brain</td>
<td>Lung and liver</td>
<td>Non-metastatic</td>
</tr>
<tr>
<td>Metastatic nodule form</td>
<td>Macrometastases</td>
<td>Micrometastases</td>
<td>Low</td>
</tr>
<tr>
<td>Aggressiveness</td>
<td>High</td>
<td>Moderate</td>
<td>Low</td>
</tr>
<tr>
<td>Phenotype</td>
<td>Epithelial</td>
<td>Mesenchymal</td>
<td>Mesenchymal</td>
</tr>
<tr>
<td>Cadherin expression</td>
<td>E-cadherin</td>
<td>N-cadherin</td>
<td>N-cadherin</td>
</tr>
</tbody>
</table>

1.3 Metastasis

Metastasis is the most poorly understood component of cancer pathogenesis and is responsible for 90% of cancer-associated mortality [3]. Advancements in surgical practices, radiotherapy and chemotherapy have only marginally improved patient survival. While surgical resection of the primary tumour and adjuvant therapy can cure well-confined tumours, metastatic disease is largely incurable due to its systemic nature and the resistance of disseminated cancer cells to respond to existing therapeutics. These poor clinical realities have been realised for decades and it is apparent that our ability to treat cancer is largely dependent on our capacity to interrupt or even reverse the process of metastasis.

The two popular models that hypothesize on the metastatic process are linear progression and parallel progression [40]. Linear progression model proposes that the primary tumour proceeds to full malignancy with the most malignant cells undergoing the metastatic process. This model argues that the primary tumour cells undergo successive rounds of mutation and selection, and only the malignant subset of clones has the potential to metastasize. The tumour suppressor gene TP53 exemplifies the linear progression model as T1
stage breast tumours (<2cm in diameter) rarely possess a mutation in the gene, while T3 stage (>5cm in diameter) have significantly more TP53 mutations. Therefore, it is argued that due to the clonal expansion of the primary tumour, TP53 mutations occur significantly more once tumours grow beyond 2 cm [41]. Additionally, the fact that larger metastatic tumours metastasize more as they grow provides evidence for the linear model. In contrast to the linear model, the parallel model suggests that genetic alterations can be found in metastatic cancer cells independent of modifications acquired in the primary tumour. Evidence for this model is provided by the changes in expression of certain miRNAs that regulate formation of metastases, such as miR-10b which is found only in metastasized cancer cells [42, 43]. This suggests that cancer cells acquire mutations late in tumour development which predispose them to metastasis. Regardless of which model is more representative of what actually occurs in the human body, invasion, intravasation, survival, arrest, extravasation, and colonization need to occur for successful the metastatic cascade to be complete (Figure 1.1).

Figure 1.1 The metastatic cascade. 
A, Cancer cells within the primary tumour acquire an invasive phenotype. B, Cancer cells invade into the surrounding matrix and intravasate to enter circulation. C, Cancer cells through the circulation. D, Circulating cancer cells exit the circulation and invade into the microenvironment of the target organ. E, At the foreign site, cancer cells must evade the innate immune response and survive as single cells or cluster of cells. F, In order to develop into macrometastatic deposits, cancer cells must adapt to the microenvironment and begin proliferating. Figure adapted from reference [3].
1.3.1 The metastatic process

After primary tumour formation, a cell must be able to locally invade surrounding tissue, intravasate into the circulatory system (or lymphatic system), survive transit in the circulatory system, arrest at a suitable secondary location, extravasate into the parenchyma, form micro-metastases able to establish a neo-vasculature system and successfully drive outgrowth of a secondary tumour [44]. It is interesting to note that the process is extremely inefficient and less than 0.02% of circulating tumour cells are capable of forming secondary tumours [45]. However, despite this inefficiency, a patient can concurrently present with significant metastatic disease at the time of primary tumour diagnosis. Additionally, metastatic disease can lay dormant for years after remission and cryptically re-emerge as a secondary tumour [46]. During tumour dormancy, breast cancer cells can evolve into a “differentiated” state by inhibiting signals such as β1-integrins and EGF, causing tumour cells to differentiate into non-proliferating acinar structures [47]. A switch in the dormancy can lead to micrometastatic growth and ultimately metastatic disease; however, the process by which this happens still remains poorly understood.

In the process outlined in Figure 1.1, the two limiting steps are local invasion (Figure 1.1B) and the successful establishment of a tumour at a secondary location (Figure 1.1F) [45, 48]. These two steps are critical determinants of whether the tumour cell is capable of spreading (4T1 murine mammary carcinoma) or remaining at the primary site (67NR murine mammary carcinoma). While these two steps allow for metastasis to occur, additional factors influence where the secondary metastases occur. Secondary locations are dependent upon the successful interaction between secreted factors from the primary tumour and the target site’s microenvironment made up of several factors, including host’s own immune cells.

1.3.2 Migration and invasion

The process of invasion and migration is not unique to cancer cells. Neuronal cells, immune cells and fibroblasts all exhibit these physiological abilities [7, 49, 50]. While immune cells maintain the ability to invade and migrate through the ECM, the majority of differentiated cells (ex. epithelial cells) do not. However, carcinomas, which derive from epithelial cells, regain the invasive and migratory phenotype indicating a switch from a non-migratory epithelial
phenotype has taken place. This switch represents an integral change from a dormant epithelial cell to a mesenchymal cell capable of invasion and migration [7].

Epithelial cells normally grow in an organized sheet interconnected with other epithelial cells through cell-cell junctions and adhesions [51]. This organization is easily appreciated by looking at hematoxylin and eosin stained slides of epidermal cells. Epithelial cells have a polarized morphology and express specific proteins, such as E-cadherin, which is common and prototypical among epithelial cells [51]. The EMT process induces changes in epithelial cell’s morphology, adhesion characteristics and migratory ability [51]. The most visually apparent shift is the morphological change from the uniform epithelial shape to a mesenchymal spindle shape. In order to allow for this change to occur, E-cadherin is down regulated while N-cadherin is increased and vimentin is expressed along with many other proteins and transcription factors [51]. In order to increase the migration speed of the transforming cells, the cytoskeleton undergoes re-organization, and the cells increase their production of matrix digestion enzymes, allowing for increased migration and invasion [52]. While the EMT process is required for the cancer cells to escape the primary tumour, once they reach a suitable metastatic site, the establishment of a new colony requires a reversion of the EMT process known as the mesenchymal to endothelial transformation (MET) [51].

As mentioned earlier, invasion and migration are key processes in metastasis and are intrinsically coupled together. The three major components of invasion are cell adhesion to the ECM (commonly the basement membrane), proteolysis of the ECM/basement membrane, and migration through the degraded matrix [50]. Migration enables cell movement, allowing contact between the cell and the ECM to occur as well as movement of the cell through the degraded basement membrane. However, the migratory path of a cancer cell is not simple. The disseminating cancer cell moves through a three dimensional substrate (stroma) and some degree of proteolysis is involved in order to allow the cell to spatially progress [50]. As a cell begins to migrate, actin rearrangements form pseudopods as the leading edge of the cell begins to protrude [50]. The extending pseudopods make contact with the ECM which binds adhesion molecules such as integrins on the cell membrane [50]. This ECM/integrin binding causes clustering of other cell membrane/matrix interactions and forms small focal complexes that can form stable focal adhesions [50]. Integrins are composed of an alpha and a beta chain which provide specificity to the kinds of ECM proteins that they can bind; for example, the α5β3 integrin can bind fibronectin among others [53]. Once stable focal adhesion has been established through integrin binding, matrix metalloproteinase (MMPs) are recruited to the site
As MMPs degrade the ECM around the focal adhesion sites, the cell contracts and the strong binding to the ECM by the integrins results in the cancer cell being pulled towards the sites of focal adhesion and attachment [50]. At the same time as new connections are being made on the leading edge, the trailing edge loses its connectivity with the ECM [50]. Taken together, invasion and migration along with EMT and MET processes provide key features to cancers, particularly the ability to invade surrounding local tissues and spread distantly throughout the body.

1.3.3 Tumour microenvironment

Under physiological conditions, the stroma functions as the main barrier against tumour progression; however, this environment can be converted to one that supports cancer progression by the presence of transformed tumour cells. The organization of such an environment requires the recruitment of fibroblasts, migration/invasion of immune cells, matrix remodeling and the development of vascular networks through angiogenesis (Figure 1.2) [54]. Tumour-associated stromal cells can arise from adjacent normal tissue, recruited bone marrow-derived stem and progenitor cells, and proliferation of existing stromal cells [7]. Virtually all tumour lesions contain immune cells at varying densities and they can be either tumour-promoting or tumour-suppressing [7, 55]. Immune cells present in the tumour microenvironment include those mediating adaptive immunity, T-lymphocytes, dendritic cells (DCs) and B-lymphocytes, as well as effectors of innate immunity including macrophages, polymorphonuclear leukocytes and natural killer (NK) cells [56]. In addition to fully differentiated immune cells, partially differentiated myeloid progenitors such as MDSCs infiltrate the tumour and suppress cytotoxic T-cells, aiding tumour cells in escaping immune destruction [47]. Immune cells are also major sources of various factors including matrix remodeling enzymes, pro-angiogenic factors, and growth factors, which enhance tumour progression.

We have already discussed the genetic and phenotypic variation that exists within tumours, but the important consideration lies in understanding how this variation, or intratumoural heterogeneity, influences tumour growth. The countless examples of genetic variability within the same tumour suggest complicated events of branched evolution are at play in setting the stage for a tumour promoting microenvironment. Aside from genetic differences among the tumour cells, regional differences in selective pressures such as hypoxia (lack of
oxygen), acidity and the presence of growth factors and cytokines exist within a tumour, all of which actively shape its development. It is conceivably the unstable environmental landscape within a given tumour that selects for mutations that support survival and expansion, thereby creating tumour cell heterogeneity within the microenvironment.

Figure 1.2 The tumour microenvironment. The tumour microenvironment is made up of tumour cells, immune cells, fibroblasts, vasculature, lymphatic vessels and other stromal cells. Figure adapted from reference [54].

1.3.4 Metastatic target-site tissue specificity

Before getting into the more complex concepts of metastatic target tissue determination, simple physical attributes of tumour cells should be discussed first. Tumour cells are generally larger than most cells found circulating in the blood. Smaller cells, like red blood cells and platelets, can easily pass through capillary beds. Even white blood cells which are larger than the diameter of capillaries stretch and contort in order to pass through the small diameter of the
vessel. Additionally, white blood cells can also enter and exit the circulation through the process of intra- and extravasation. Similar to white blood cells, cancer cells can also intra- and extravasate, however, they must first digest the extra-cellular matrix and basement membranes prior to successful invasion; however, due to their larger size, malignant cells can get trapped in capillaries. If we follow the logic that well perfused organs should be seeded by cancer cells more often than poorly perfused tissues, we would expect the lungs, liver and spleen to be very common secondary target sites. Interestingly, only two of these organs are common sites, with the spleen rarely growing secondary tumours. Observations like this make the argument that both blood flow and to other factors play a role in dictating where secondary tumours will form.

The topic of metastatic target tissue specificity was first mentioned 125 years ago by an English surgeon, Dr. Stephen Paget. In his famous article, he argued that if metastasis was a matter of chance, then the spread of tumours should be equal in all tissues and organs, proportional to the amount of blood flow within the tissue [57]. Knowing that metastasis was not merely a matter of chance he proposed an idea which gave rise to the “seed and soil” hypothesis of the metastatic process. Paget believed that the primary tumour had an influence on the body, predisposing certain sites for secondary tumour growth. Similar to how randomly spread plant seeds germinate only in soil where conditions are just right, he believed that the same is true for cancer metastases. The study of cancer metastasis has progressed significantly since Dr. Paget’s influential paper, and it’s now appreciated that for primary tumours, there are common organs or tissues in which metastasis typically occurs. For example, some primary tumours, like cervical cancer, do not metastasize to the brain, while breast cancer does. This disparity indicates that the primary tumour plays an important role in determining the metastatic target sites. The tumour may dictate tissue specificity through direct action of tumour secreted proteins at metastatic sites (ECM degrading enzymes) and indirect actions such as mobilization of host’s bone-marrow derived cells (BMDCs). The BMDCs can give rise to immunosuppressive MDSC, which together with tumour secreted factors create a favourable soil for tumour cells to seed [58].

1.3.5 MDSCs and the pre-metastatic niche

The pre-metastatic niche hypothesis is relatively novel, and its origins come from Dr. Paget’s “seed and soil” concept discussed in section 1.3.4. It is inherently a controversial theory
as it predicts the arrival of tumour cells in secondary target organs as a late event. Critics of the theory suggest that technical limitations of assays used in detecting single tumour cells in the metastatic target organs make it difficult to determine which event occurs first in the metastatic process: the development of the pre-metastatic niche, or the arrival of metastatic tumour cells. Additionally, the observation that the pre-metastatic niche can be formed in immune-compromised mice undermines the involvement of myeloid cells in the process [59]. However, based on our own research, the pre-metastatic niche is an attractive theory, but only under certain circumstances which are discussed in section 3.3 of the Results.

The pre-metastatic niche represents an area in a distant tissue or organ that has been prepared by the primary tumour to allow colonization by circulating tumour cells (CTCs). The tumour creates this niche through both direct and indirect mechanisms. The direct method relies on the action of tumour-derived secreted proteins at metastatic sites while the indirect method utilizes the host’s own cells, such as BMDC, to accumulate in the target site. Together, these actions create areas within a tissue in which tumour-derived secreted proteins have remodelled the ECM (MMP2 and MMP9 released from the tumour) allowing the accumulation of host BMDCs, as well the deposition of additional niche-associated secreted proteins such as VLA-4 and Id3 [58, 60, 61]. VLA-4 interacts with its ligand fibronectin, and has been shown to be essential in migration of BMDCs [62] and circulating leukocytes [63], while Id3 has been shown to be responsible for certain BMDC and stromal cell interactions, including motility and recruitment [64].

As discussed in section 1.4, BMDCs differentiate into MDSC under pathological conditions such as cancer. The architectural change in ECM and the presence of immunosuppressive cells and certain stromal cells creates the fertile soil that is permissive to the invasion, survival and growth of CTCs upon their arrest. The formation of pre-metastatic niches can be studied by observing the accumulation of tumour-derived secreted proteins (such as MMP9) and immunosuppressive cells like MDSCs in known metastatic target sites [60]. Additionally, Kaplan et al. showed this arrival of non-neoplastic cells in metastatic organs by looking at the accumulation of BMDCs expressing vascular endothelial growth factor receptor 1 (VEGFR1) [61]. The study elegantly showed that the VEGFR1+ cells arrived in the lungs of LLC tumour-bearing mice prior to the arrival of tumour cells [61]. As interest in the pre-metastatic theory grew, numerous reports showed that BMDC expressing the CD11b+Gr1+ phenotype routinely accumulate in the pre-metastatic niche [65].
Since it is proposed that the primary tumour secretes factors that contribute to the pre-metastatic niche directly and indirectly, researchers have utilized conditioned media (media that the tumour cells have been growing in) from tumour cells grown in vitro to induce a similar accumulation of BMDCs. Conditioned medium is generated in vitro which presumably contains similar secreted factors that an orthotopically implanted tumour would secrete in vivo. The difference in factors secreted between in vitro and in vivo models can be attributed to the presence of other cell types found in the solid tumour microenvironment (ex. macrophages, fibroblasts, other immune cells). A study in which mice injected intraperitoneally with conditioned medium derived from MDA-MD-231 cells (human breast cancer cell line) showed an accumulation of CD11b$^+$ cells in the lungs (a metastatic site of MDA-MD-231 cells) of naive immune-compromised mice [58]. Additionally, similar results were reported in the study which utilized conditioned medium from the spontaneously generating polyoma middle T antigen (PyMT) mammary carcinoma cell line. In this study, daily intraperitoneal injections of conditioned medium caused a significant increase in CD11b$^+$/Ly6G$^+$Ly6C$^{low}$ (granulocytic MDSC) in metastatic target organs [66]. Reports like these illustrate the interplay between tumour-derived secreted proteins and the host's cells in setting up the pre-metastatic niche. However, while the pre-metastatic niche theory does provide insight into the metastatic process, the exact mechanisms that guide tumour cells to specific sites are still largely unknown. Furthermore, questions like, 1) is the pre-metastatic niche required for metastatic growth? 2) are MDSCs necessary for metastatic growth? and 3) are MDSC always found in pre-metastatic niches, still remain to be answered.

1.4 Myeloid-Derived Suppressor Cells

Myeloid cells with suppressive capabilities were first described more than 25 years ago in cancer patients [67-69]. Accumulating evidence indicates that myeloid cells with immunosuppressive abilities (known as myeloid-derived suppressor cells or MDSCs) contribute to negative regulation of immune responses during cancer and other diseases [70]. MDSCs are a phenotypically heterogeneous cell population that includes mature myeloid cells (granulocytes, monocytes/macrophages, and DCs), as well as immature myeloid cells (IMCs) [70]. Under physiological conditions in healthy individuals, IMCs are generated in the bone-marrow and quickly differentiate into mature granulocytes, macrophages or DCs. However,
under pathological conditions such as cancer, trauma, sepsis, autoimmune diseases and various infectious diseases, IMC differentiation into mature myeloid cells is partially blocked which results in an expansion of this population. The pathological condition activates IMCs and causes an up-regulation of suppressive factors such as arginase-1 and inducible nitric oxide synthase (iNOS). Additionally, IMCs increase their production of nitric oxide (NO) and reactive oxygen species (ROS), resulting in an expansion of cells with a strong immunosuppressive activity collectively known as MDSCs. Interestingly, under the right conditions (presence of key cytokines), MDSCs can differentiate into mature macrophages and DCs in vitro and in vivo [71-73]. It is important to note that activated macrophages can be classified into M1-macrophages or M2-macrophages. Classically activated macrophages are of the M1 phenotype, and comprise immune effector cells, playing an integral role in acute inflammation (ex. against bacteria) [74]. On the other hand, M2-macrophages are anti-inflammatory and can have a variety of functions including immunosuppression in cancer [74]. The polarization of macrophages to an M2 phenotype strongly contributes to the immunosuppressive microenvironment present in tumour-bearing mice and cancer patients, and similarly to MDSC accumulation, an M2 macrophage skew correlates with poor patient outcome [75].

In mice, MDSCs are characterized by the expression of CD11b (αM-integrin) and Gr1 (myeloid-cell lineage differentiation antigen) cell surface markers [76]. CD11b is a common myeloid antigen also known as alpha M integrin or Mac-1α and complexes with the β2 integrin also known as CD18 [77]. The Gr1 antibody recognizes two epitopes, Ly6G and Ly6C, giving MDSCs additional heterogeneity. These epitopes allow for the division of MDSCs into the granulocytic MDSCs (CD11b⁺Ly6G⁺Ly6Clow) and monocytic MDSCs (CD11b⁺Ly6G⁺Ly6Chhi). These two subsets show different functions and distributions under inflammatory related conditions such as cancer [78, 79] with monocytic MDSC making up 20-30% of the total expanding CD11b⁺ cells, while 70-80% are granulocytic MDSCs. While granulocytic MDSCs expand to a much greater extent as compared to monocytic MDSCs, they are slightly less suppressive on a per cell basis [79]. Additionally, recent work from Gabrilovich’s group suggest CD115 and CD124 as additional markers for identification of MDSCs [79]. Taken together, perhaps the best way of thinking of MDSCs is not as a defined subset of cells but rather a group of phenotypically heterogeneous myeloid cells with a similar biological activity. As a result, CD11b⁺Gr⁺ cells must show ex vivo immunosuppressive ability, in a T-cell proliferation assay for example, in order to be labeled as MDSCs [28].
In humans, MDSCs are most commonly defined as CD14−CD11b+. Additional characteristics such as positive CD33 expression, the lack of mature myeloid and lymphoid markers and absence of MHC class II molecule HLA-DR, add more specificity to defining this cell population in humans [80, 81]. In healthy individuals, IMCs constitute only 0.5% of peripheral blood mononuclear cells, and with such a low proportion, any significant changes can be indicative of a pathological condition [81]. However, under pathological conditions such as breast cancer, studies show that MDSC levels correlate with clinical stage and metastatic breast tumour burden [82, 83]. In addition, a separate study showed that patients with higher than average blood MDSCs following palliative systemic therapy had shorter overall survival and peripheral MDCS levels corresponded to levels of circulatory tumour cells [84].

1.4.1 Expansion and activation of MDSCs

Under physiological conditions in mice, the bone marrow contains 20-30% of cells with CD11b+Gr1+ cellular markers, yet only 2-4% of spleen cells are CD11b+Gr1+. In our research, we find that tumour bearing mice will undergo CD11b+Gr1+ expansion, with up to 35% of splenocytes expressing these markers. Factors which contribute to MDSC expansion and activation can be divided into two main groups: primary tumour derived and T-cell derived [70].

An example of a primary tumour derived factor is STAT3 which is arguably one of the main transcription factors regulating MDSC expansion. It has been shown that MDSCs isolated from tumour bearing mice have a markedly increased level of phosphorylated STAT3 as compared to IMCs from naïve mice [85]. In addition, Kowanetz et al. show that G-CSF promotes lung metastasis through the mobilization of granulocytic MDSCs [65]. The group showed that 4T1 tumours over-express G-CSF which mobilizes MDSCs and facilitates their subsequent homing at distant organs before the arrival of tumour cells. While G-CSF may be responsible for MDSC expansion, GM-CSF has been implicated in being responsible for giving MDSCs their immunosuppressive ability [86]. Furthermore, Ferrera’s group showed that VEGF secreted by the primary tumour plays a key role in BMDC mobilization and pre-metastatic priming [87].

The second method of activating MDSCs is through T-cells released factors. These factors include IFNγ, TLR ligands, IL-4, IL-6, IL-10, IL-13 and TGFβ [88]. It is interesting to note that an increase in the generation or recruitment of IMCs in cancer does not necessarily mean
that an expansion of MDSCs has occurred. It is probable that two different mechanisms, expansion and activation, are responsible for the increase of MDSCs in pathological conditions. This system allows for flexibility in the regulation of these cells under pathological and physiological conditions, with activation occurring only under certain conditions. For a summary of factors responsible for expansion and activation of MDSCs discussed in this section, please refer to Table 2.

**Table 2 Factors implicated in the expansion and activation of MDSCs in cancer.**
Table adapted from reference [70].

<table>
<thead>
<tr>
<th>Factor</th>
<th>Tumour model</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>Breast cancer, sarcoma, melanoma, lymphoma and lung carcinoma</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Lewis lung carcinoma, colon carcinoma, mammary adenocarcinoma</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Lewis lung carcinoma, mammary carcinoma, methA sarcoma and melanoma</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Sarcoma and mammary carcinoma</td>
</tr>
<tr>
<td>Prostaglandins</td>
<td>Mammary carcinoma, lung cancer, renal cancer and colon cancer</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Mammary adenocarcinoma, fibrosarcoma, colon carcinoma and lymphoma</td>
</tr>
<tr>
<td>S100A8 and S100A9</td>
<td>Colon carcinoma, lymphoma, fibrosarcoma and mammary carcinoma</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Colon carcinoma, fibrosarcoma and mammary adenocarcinoma</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Fibrosarcoma and mammary carcinoma</td>
</tr>
<tr>
<td>IL-6</td>
<td>Mammary carcinoma</td>
</tr>
<tr>
<td>IL-10</td>
<td>Colon cancer, melanoma and mammary carcinoma</td>
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<tr>
<td>IL-13</td>
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</tr>
<tr>
<td>MMP9</td>
<td>Colon carcinoma, Lewis lung carcinoma and mammary carcinoma</td>
</tr>
<tr>
<td>CXCL5 and CXCL12</td>
<td>Mammary adenocarcinoma</td>
</tr>
</tbody>
</table>
1.4.2 Immunosuppression by MDSCs

A majority of studies concerned with the mechanism of suppression by MDSCs have shown that direct cell-cell contact is required for immunosuppression to occur. This suggests that MDSCs function through cell surface receptors, and/or through the release of quick-acting soluble mediators. As mentioned before, MDSCs are potent suppressors of T-cell functions and this section will discuss some of the best characterised mechanisms.

As discussed previously, MDSCs can be divided into two major subsets: monocytic and granulocytic. Under pathological conditions, approximately 20% of MDSCs are monocytic (CD11b⁺Ly6G⁻Ly6C_hi). Monocytic MDSCs express high levels of arginase-1 and iNOS, both of which are enzymes that utilize L-arginine as their substrate. The products of arginase-1 are urea and L-ornithine, while iNOS activity generates NO in a STAT1 dependent pathway (Figure 1.3). The activities of both of these enzymes have been show to inhibit T-cell functions [89]. NO is able to suppress T-cell functions through several different mechanisms which involve inhibition of JAK3 and STAT5 function in T-cells and the induction of T-cell apoptosis [28, 90, 91].

Granulocytic MDSCs (CD11b⁺Ly6G⁺Ly6C_lo) represent 80% of the expanded MDSC population due to pathological conditions. While these cells possess arginase-1, ROS is the other highly influential factor contributing to suppressive activity of granulocytic MDSCs (Figure 1.3). Increased production of ROS by MDSCs via the STAT3 pathway has emerged as a characteristic immunosuppression in both tumour-bearing mice and patients with cancer [28, 79, 92]. Moreover, inhibition of ROS in vitro completely abrogated the suppressive effects of MDSCs [79]. ROS production by MDSC can be further induced by tumour-derived factors such as TGFβ, IL-3, IL-6, IL-10, platelet-derived growth factor (PDGF) and GM-CSF [93].
**Figure 1.3 Suppressive mechanisms utilized by MDSCs in pathological conditions.**
Monocytic MDSCs make up approximately 20-30% of total MDSC under pathological conditions. 70-80% of MDSCs are granulocytic and are less immunosuppressive on a per cell basis. Both cell populations exert their immunosuppressive function on T-cells activities. Figure adapted from reference [70].

### 1.5 Targeting MDSCs

It is becoming increasingly clear that successful treatment of cancer will only be possible with a strategy that decreases or eliminates the immunosuppressive factors from the body in the pathological state. The understanding that suppression of the immune system has a critical role in promoting tumour progression, development of metastatic disease, and failure of cancer vaccines aimed at inducing an immune response against tumour cells has resulted in a considerable shift in how we approach immunotherapy. MDSCs are one of the main immunosuppressive factors (in addition to macrophages and Tregs) that contribute to poor prognosis in cancer and many other pathological conditions. As a result, several different therapeutic strategies aimed at targeting MDSCs have been developed (Table 3).
Table 3 Therapeutic strategies for targeting MDSCs.
Table adapted from reference [94].

<table>
<thead>
<tr>
<th>Differentiation</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) ATRA (all-trans-retinoic-acid)</td>
<td>Favours differentiation of myeloid progenitors to DCs and macrophages</td>
<td></td>
</tr>
<tr>
<td>(2) Vitamin D3</td>
<td>In humans, reduces the number of CD34+ cells, increases HLA-DR expression in PBMCs, together with IL-12, IFN-γ, improves T-cell blastogenesis</td>
<td></td>
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<table>
<thead>
<tr>
<th>Maturation</th>
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<tbody>
<tr>
<td>(3) STAT 3 Inhibitors</td>
<td>Inhibition of several pathways, among which, suppression of anti-apoptotic, pro-proliferative, pro-angiogenic genes and ROS production</td>
<td></td>
</tr>
<tr>
<td>(4) TK inhibitors</td>
<td>Sunitinib</td>
<td>Inhibits VEGFR, PDGFR, c-KIT, Flt-3 and CSF-1 receptor signal pathway Decreases Treg cells (decreased FoxP3 and IL-10) Increases Th1 response</td>
</tr>
<tr>
<td>(5) Bevacizumab</td>
<td></td>
<td>Decreases MDSC subsets (VEGFR+ cells)</td>
</tr>
<tr>
<td>(6) Anti-BV8 mAb</td>
<td></td>
<td>Reduces the number of tumor-associated CD11b+/Gr-1+ cells, regulating their mobilization and, potentially, their homing to the tumor site</td>
</tr>
<tr>
<td>(7) Amino-Biphosphonates</td>
<td></td>
<td>Reduce CD11b+/Gr-1+/F4/80+ cells in tumor stroma</td>
</tr>
<tr>
<td>(8) MMP9 Inhibitors</td>
<td></td>
<td>Reduce MDSC generation from HSC and their support to tumor stroma</td>
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<thead>
<tr>
<th>Accumulation</th>
<th></th>
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<tbody>
<tr>
<td>(9) Gemcitabine</td>
<td></td>
<td>Reduces the number of CD11b+/Gr-1+ cells</td>
</tr>
<tr>
<td>(10) 5-Flourouracil (5-FU)</td>
<td></td>
<td>Reduces the number of CD11b+/Gr-1+ cells</td>
</tr>
<tr>
<td>(11) CXCR2 and CXCR4 specific antagonists</td>
<td></td>
<td>Reduce MDSC accumulation</td>
</tr>
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<table>
<thead>
<tr>
<th>Function</th>
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</thead>
<tbody>
<tr>
<td>(12) ROS scavengers</td>
<td></td>
<td>Reduce nitration of proteins in tumor microenvironment</td>
</tr>
<tr>
<td>(13) Nitroaspirin</td>
<td></td>
<td>Reduces nitration of proteins in tumor microenvironment Inhibits ARG and NOS</td>
</tr>
<tr>
<td>(14) PDE-5 (phosphodiesterase-5)</td>
<td></td>
<td>Inhibits ARG and NOS Inhibits IL-4Rα expression</td>
</tr>
<tr>
<td>(15) COX2 inhibitors</td>
<td></td>
<td>Downregulate ARG and NOS expression</td>
</tr>
<tr>
<td>(16) Biotechnological drugs</td>
<td></td>
<td>Interfere with IL-13/IL-4Rα/STAT6 pathway</td>
</tr>
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</table>

Promoting MDSCs into mature myeloid cells that no longer possess suppressive activity is one of the promising approaches used at targeting this cell population. The idea behind this approach is that reducing the number of immature MDSCs will improve anti-tumoural immune responses and enhance the effects of cancer vaccines or adoptively transferred tumour-specific T-cells. ATRA, a vitamin A derivative, has been show to differentiate myeloid progenitors into DCs and macrophages in mice [95] and cancer patients [96]. However, recent work from our lab has shown that macrophages are more potent immunosuppressors than MDSC [28], suggesting that ATRA treatment may negatively affect breast cancer patients by inducing the production of potentially immunosuppressive macrophages.

Inhibition of MDSC maturation from hematopoietic precursors is another attractive strategy. Although this approach is promising, tumour-derived secreted factors which stimulate MDSC maturation are plenty. Neutralizing factors like prostaglandins, granulocyte-macrophage...
colony-stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), stem-cell factor (SCF), STAT3, VEGF, TGFβ, IL-1β, IL-6, IL-10, IL-12, CC-chemokine ligand 2 (CCL2), CXC-chemokine ligand 2 (CXCL2) and CXCL12 shows great potential for inhibiting MDSC maturation [94]. An example of this technique would be the use of a STAT3 inhibitor. The inhibitor specifically prevents the maturation of myeloid precursor cells in by blocking the STAT3/JAK2 pathway [97].

Reduction of MDSC accumulation in peripheral organs is a third way of depleting MDSC, and is the one we utilized in our studies. Utilizing chemotherapeutics such as GEM and 5-FU to directly kill MDSCs is an effective way of targeting this cell population [98, 99]. Removing the primary tumour by surgery decreases TDSFs such as G-CSF, which ultimately leads to a decrease in MDSCs in tissues and circulation. Please refer to section 4.1 for a more comprehensive explanation of this MDSC targeting strategy. Additionally, information on how primary tumour removal affects MDSC levels in tumour-bearing mice is thoroughly explained in section 4.1 and will not be discussed in this section as an MDSC reduction strategy any further.

The final approach affecting MDSCs interferes with pathways that directly control the immunoregulatory activity of these cells. As mentioned earlier, MDSC achieve immunosuppression primarily through the metabolism of L-arginine and the activity of two key enzymes; arginase-1 and iNOS. Several studies provided evidence that affecting MDSC immunoregulatory activity is an effective strategy by utilizing ROS scavengers or selective antagonists for arginase-1 and iNOS [100-102] and effectively restoring T-cell activities. The biggest downfall of this MDSC targeting strategy is that many of the compounds would have adverse effects if used as therapeutic approaches in the clinic. Arginase-1 inhibition blocks the essential urea cycle, and administration of high doses of iNOS inhibitor has very severe side effects (endotoxic shock). In order for this approach to gain favour, a deeper understanding of MDSC biology needs to be acquired. A study has shown that MDSCs most likely use both arginase-1 and iNOS at the same time to suppress T-cell mediated anti-tumour responses [103]. Therefore, drugs that interfere with both enzymes without relevant adverse side effects would be ideal candidates for therapeutic treatment of MDSC-induced tumour tolerance.
1.6 Hypothesis and Aims

Emerging evidence in the field of metastasis research implies MDSC as a key component of the immunosuppressive microenvironment found in secondary target organs. The induction of MDCSs by primary tumours and their accumulation in metastatic target sites correlates with decreased survival and increased cancer re-occurrence in mouse models and clinical studies. Therefore, decreasing MDSC levels is integral in improving survival of patients suffering from metastatic disease.

Decreasing MDSC levels in tumour-bearing mice can be accomplished using several different therapeutic strategies. It has been shown that surgically removing the primary tumour abolishes the TDSF required for MDSC expansion and accumulation in metastatic sites. In addition, MDSCs can be targeted in peripheral tissues using a chemotherapeutic approach. **We hypothesize that MDCS accumulation in secondary target organs of 4T1 tumour-bearing mice contributes to an increased metastatic potential of 4T1 circulating tumour cells and that surgical resection of the primary tumour followed by chemotherapeutic adjuvant therapy will decrease the metastatic potential of 4T1 tumour cells.**

The aims of our study are as follows:

**Aim 1:** Evaluate the ability of metastatic (4T1 and 4T07) and non-metastatic tumour (67NR) to recruit MDSC to the lungs (metastatic target site) and spleen (non-metastatic site).

- **Aim 1a:** Evaluate systemic accumulation of MDSC using the 4T1 model and the timing of MDSC vs. 4T1 tumour cell lung seeding.

**Aim 2:** Evaluate the ability of GEM, 5-FU and TPZ to target MDSCs *in vivo* and *in vitro*.

**Aim 3:** Evaluate the effects of primary tumour resection on MDSC tissue levels.

- **Aim 3a:** Evaluate if circulatory MDSC levels are a good tissue surrogate.

**Aim 4:** Chemotherapeutically target residual post-surgery MDSCs and evaluate effects on metastatic growth.
CHAPTER 2 : MATERIALS AND METHODS

2.1 Mice

Female Balb/cF3H mice, 8 to 10 weeks of age, were purchased from Simonsen Laboratories (Gilroy, CA). All mice were housed under specific-pathogen free conditions in the Animal Resource Centre at the BC Cancer Agency Research Centre. All animal experiments were performed in accordance with the Canadian Council on Animal Care Guidelines and the UBC Committee on Animal Care; Ethics Certificates A09-0251 and A13-0223. Where indicated, mice were given 90mg/kg 5-bromo-2'-deoxyuridine (BrdU; Sigma-Aldrich, Oakville, ON) and/or 100mg/kg pimonidazole (HPI Inc., Burlington, MA) intraperitoneally (ip) 90 minutes before tissue harvest; Hoechst 33342 (10mg/ml; Sigma-Aldrich) was iv injected in a 50µl volume 20 minutes before tissue harvest. For orthotopic tumour implantation, mice were injected with $10^5$ 4T1 cells in the fourth mammary fat pad. We have found that this cell number produces consistent tumour growth rates, with tumour volumes that approach ethical restrictions 4 weeks after implantation. For the intravenous (iv) studies, mice were injected with $2.5 \times 10^4$ 4T1 cells in a 200µl injection volume into the lateral tail vein. Euthanization was performed by CO$_2$ asphyxiation according to UBC guidelines.

2.2 Cell Lines and Media

4T1, 4TO7 and 67NR murine mammary carcinoma cells were provided by Dr. Fred Miller (Karmanos Cancer Institutes, Detroit, MI). MSC2 are an immortalized MDSC cell line obtained from BALB/C Gr1$^+$ splenocytes and were a kind gift from Dr. François Ghiringhelli (University of Burgundy, Dijon, France). For 4T1 and MSC2 cells, cell culture was performed using Roswell Park Memorial Institute (RPMI) 1640 (Life Technologies, Burlington, ON) supplemented with 10% fetal bovine serum (Fisherbrand, Ottawa, ON) as well as additional glucose, HEPES, sodium pyruvate and L-glutamine (standard media used). Routine passage was a 1:10 dilution performed every other day using 0.1% trypsin citrate. Cells were incubated at 37°C and 5% CO$_2$ with 21% O$_2$ used for normoxic conditions and 1% O$_2$ for hypoxic conditions.
2.3 Tissue Processing

Spleens and livers were mashed and rinsed through 100µm and 40µm mesh filters with PBS to create single cell suspensions. Lungs and kidneys were finely minced with crossed scalpels prior to agitation for 40 minutes at 37°C with an enzyme suspension containing 0.5% trypsin and 0.08% collagenase in PBS. After incubation, 0.06% DNase was added and the cell suspension was gently vortexed and filtered through 30µm nylon mesh to remove clumps. Cell suspensions were treated with NH₄Cl for erythrocyte lysis. For clonogenic assays, the single cell suspensions derived from lung tissue were washed in PBS, resuspended in medium, and aliquots of 3x10³ to 10⁶ cells were plated in triplicate in 60µM 6-thioguanine-containing medium with serum to allow for preferential outgrowth of tumour cell colonies (for GEM studies, 5x10³ to 10⁵ cells were plated). Excess cells were fixed in 70% EtOH and stored at -20°C for subsequent flow cytometry analysis. CD11b⁺Gr1⁺ cells were isolated from single cell suspensions derived from spleen or lung tissue via Gr1⁻PE positive selection using the EasySep system (StemCell Technologies, Vancouver, BC, Canada), according to the manufacturer’s instructions. CD11b⁺Gr1⁺ cell purity of the isolated cells was >95% as determined by subsequent flow cytometry analysis [28]. F4/80⁺ cells were isolated from single-cell lung or spleen suspension using EasySep magnetic bead-assisted isolation system (StemCell Technologies) using F4/80⁻PE⁺ selection according to the manufacturer’s instructions. Purity was >95% as determined by flow cytometry based on CD11b and F4/80 expression. Comparison of CD11b⁺Gr1⁺ cells isolated by either positive or negative Gr1 selection: % purity of CD11b⁺Gr1⁺ cells isolated from lungs or spleen of 4T1-bearing mice by Gr1 positive selection or negative selection with antibodies against CD4, CD5, CD11c, CD45R/B220, CD49b, CD117, TER119, and F4/80. For analysis of circulating MDSCs, 100 µl of peripheral mouse blood was collected once a week by bleeding the lateral tail vein (catheterization of tail vein). A 26G needle was inserted into the tail vein and blood was collected with a heparin treated capillary tube. Blood samples were transferred to K₂EDTA treated tubes (BD Microtainer, Franklin Lakes, NJ). The samples were centrifuged at 1000xg for 10 minutes at room temperature and the plasma was removed and stored at -80°C. The remaining sample was treated with NH₄Cl for red blood cell lysis prior to immunofluorescent staining.
2.4 Chemotherapeutics (\textit{in vivo})

2.4.1 Tirapazamine

For the TPZ kinetics study, a single dose of TPZ (2mg/ml in PBS; Toronto Research Chemicals, North York, ON, Canada) was injected ip (20 mg/kg or 40 mg/kg) into mice bearing 17 day old 4T1 tumours. Mice were euthanized and tissues were harvested at 24, 48, and 72 hours post drug administration.

\begin{center}
\includegraphics[width=0.2\textwidth]{tpz.png}
\end{center}

\textbf{Figure 2.1 TPZ chemical structure.}

2.4.2 Gemcitabine

A single dose of 60 mg/kg of GEM (Sandoz, Boucherville, QC) was injected ip into mice bearing 17 day old 4T1 tumours. GEM was diluted to a 15 mg/ml working solution in physiological saline. Mice were euthanized and tissues were harvested 24, 48, 72 and 96 hours post drug administration. Clonogenics were performed on resected mice treated with GEM as described in section 2.3.

\begin{center}
\includegraphics[width=0.2\textwidth]{gem.png}
\end{center}

\textbf{Figure 2.2 GEM chemical structure.}
2.4.3 5-Fluorouracil

A single dose of 50 mg/kg of 5-FU (Hospira, Saint-Laurent, QC) was injected ip into mice bearing 17 day old 4T1 tumours. 5-FU was diluted to a 5 mg/ml working solution in physiological saline. Mice were euthanized and tissues were harvested 1, 3, 5, 7 and 9 hours post drug administration.

\[ \text{Figure 2.3 5-FU chemical structure.} \]

2.5 Chemotherapeutics (in vitro)

2.5.1 Resazurin assay

The metabolic activity of cells was measured using the colorimetric resazurin assay. All resazurin assays were performed in 24-well tissue culture plates (VWR, Mississauga, ON). \(10^4\) cells (4T1 or MSC2) were seeded in 500 µl media/well in sextuplet for each drug concentration, and allowed to settle for 24-hours in media. Chemotherapeutics were added once the cells had settled and attached to the cell culture plates at the following concentrations: GEM and 5-FU at 10, 1, 0.5, 0.1, and 0.01 µM; TPZ at 80, 40, 20, 10 and 5 µM. Physiological saline was used as a vehicle control for all 3 chemotherapeutics. 24-well plates were incubated under normoxic or hypoxic conditions (section 2.2) for 24 or 48-hours as indicated. Resazurin sodium salt (Sigma, Oakville, ON) was made up in 0.9% NaCl saline to a concentration of 4.4 µM. The stock solution was diluted in a 1:1 ratio in PBS to make a 2.2 µM working solution before use. 55 µl of the working solution was added to each well of the 24-well plate containing 500 µl media and adherent cells. The assay was given 3-4 hours to develop, and the plates were read by a
TECAN GENios plate reader using a 535 nm excitation and 590 nm emission filter. Experimental values are reported as normalized to cells grown in physiological saline under normoxic or hypoxic conditions.

2.6 Flow Cytometry

Alcohol-fixed cells (5x10^5 to 1x10^6) were rehydrated and washed in PBS + 4% FCS prior to contact with the appropriate antibodies, including: CD11b-PE, Gr1-Alexa 488, unconjugated F4/80 (eBioscience), pan-cytokeratin (Dako, Markham, ON, Canada), and Alexa 488 or 594 secondary antibodies (Invitrogen, Burlington, ON, Canada). For BrdU analysis, cells were denatured with 2N HCl with 0.5% Triton-X prior to neutralization and anti-BrdU (Abcam, Toronto, ON, Canada) contact was done in PBS + 4% FCS + 0.1% Triton-X. 1 µg/ml DAPI was used to analyze DNA content in all samples; when analyzing lung samples that contain 4T1 tumour cells, the hyperdiploid DNA content of the tumour cells enabled their exclusion from analysis of the diploid normal cells. List mode files were collected using a dual laser Epics Elite-ESP flow cytometer (Coulter Corp., Hialeah, FL) and were subsequently reprocessed for analysis. Doublet correction and bitmap gating were used to select the cell populations of interest with the WINLIST software package (Verity Software House Inc., Topsham, ME).

Peripheral blood samples were stained with Fixable Viability Dye eFluor 780 (eBioscience, San Diego, CA) after NH₄Cl treatment. In addition to CD11b-PE and Gr1-Alexa 488, blood samples were stained with CD45-APC (eBioscience, San Diego, CA); samples were run on FACS Calibur (DxP 6-colour Upgrade) and events acquired/analyzed using FlowJo CE software. For cell cycle analysis data, ethanol fixed lung and spleen samples were rehydrated as described in section 2.5. 5x10^5 to 1x10^6 cells were stained with propidium iodide (PI) in order to generate a DNA profile for the samples. Using FlowJo CE software, we analyzed the cell cycle profiles by gating on specific cell populations in the G1, S, and G2 phase of the cell cycle.

2.7 Immunofluorescence

Tumours were frozen in Optimal Cutting Temperature (OCT) medium (Sakura Finetek, Torrance, CA) and 8-10µm serial sections were cut and stained in PBS + 4% FCS with
antibodies against: CD11b (eBioscience, San Diego, CA), Gr1 (eBioscience, San Diego, CA) or CD31 (PharMingen, Mississauga, ON) with Alexa 488 or 594 secondary antibodies. Slides stained for pimonidazole were processed in PBS + 4% FCS + 0.1% Triton-X with pimonidazole-FITC antibody (HPI Inc.). 1 μg/ml DAPI used for visualization of cell nuclei. Images were captured, processed and analyzed with a fluorescence microscope (Zeiss Imager Z1, Oberkochen, Germany), a cooled, monochrome CCD camera (Retiga 4000R, QImaging, Vancouver, BC, Canada), and Northern Eclipse software. ImageJ software (public domain program developed at the U.S. National Institutes of Health) was used for image analysis.

2.8 ELISA

G-CSF serum levels were quantified using a mouse G-CSF Quantikine ELISA (R&D Systems, Minneapolis, MN) as per manufacturer’s protocol. ELISA plates were read using a Tecan Safire² at 450 nm with wavelength correction at 540 nm. For ELISA samples, identical serum isolation protocol was used as outlined in section 2.3 except that blood was collected via cardiac puncture using a heparin-treated 1ml syringe with a 25G needle.

2.9 T-cell Proliferation Assays

Immunosuppression assays were performed using HL-1 medium (BioWhittaker; Basel, Switzerland), supplemented with 1% penicillin, 1% streptomycin, 1% Glutamax, and 50µM 2-mercaptoethanol. Serum was not used in these assays, as we have previously found that the use of serum in immunosuppression assays can mask the immunosuppressive function of CD11b⁺Gr1⁺ cells [104]. Erythrocyte-depleted splenocytes (an abundant source of T-cells) from naïve mice stimulated with 1µg/ml anti-CD3 + 5µg/ml anti-CD28 (eBioscience, San Diego, CA) were used as responder cells in the assay and cultured at 2x10⁵ cells/well ± isolated CD11b⁺Gr1⁺ cells that had been irradiated (2000 rads) to induce senescence. Co-cultured cells were incubated at 37°C for 72 hours and 1µCi/well ³H-thymidine (2 Ci/mM; PerkinElmer, Woodbridge, ON, Canada) was added for the last 18 hours of the assay. Cells were harvested onto filtermats and radioactivity was measured using a Betaplate liquid scintillation counter.
Data are expressed as mean ± SEM of the $^3$H counts per minute (cpm) from triplicate cultures, or as cell proliferation relative to control samples (stimulated splenocytes alone). These assays were performed by Dr. Melisa L. Hamilton.

### 2.10 Mouse Surgery

4T1 tumours were resected from anaesthetized (isoflurane) mice after 2 weeks of growth. A sterile environment was created inside a biosafety cabinet with a surgical tool bead sterilizer on hand. After the animals reached a surgical plane of anaesthesia in the gassing chamber, the mice were pulled out and placed on an isoflurane emitting nosecone. Before commencing with surgery, a total of 0.01 ml of a 0.5% lidocaine was injected in several locations around the planned incision site. A small incision was made using surgical scissors. Using a trochar, the primary tumour was liberated from surrounding adhesive tissues. Using sterile forceps, the tumour was pulled through the incision site and stored on ice. After surgery completion, 4-5 stitches were used to close the tumour excision site and 0.05 ml of a 0.5 mg/ml meloxicam solution was injected subcutaneously at the base of the neck. Mice were monitored on a regular basis to ensure proper healing. Mice that had tumour re-growth greater than 0.1 g were not used in our study. The surgery protocol was approved by the UBC Committee on Animal Care.

### 2.11 Statistics

Student’s t-tests were used for all comparisons, with p<0.05 being considered significant. p<0.05 is * or #; p<0.01 is ** or ##; p<0.001 is *** or ###; p<0.0001 is **** or ####. One-tailed and two-tailed T-tests were used as indicated.
CHAPTER 3: METASTATIC PRIMARY MAMMARY TUMOURS INDUCE SYSTEMIC ACCUMULATION OF MDSCs

3.1 Introduction

Myeloid cells represent the most abundant type of haematopoietic cells in the immune system and possess a large diversity of physiological and pathological functions [105]. Although the initial and most current information on MDSCs comes from the field of cancer research, studies carried out in different disease models have shown that MDSCs also regulate the immune system during parasitic and bacterial infections, acute and chronic inflammation, traumatic stress, tissue transplantation and sepsis [70]. What is common in both cancer and other pathological conditions is that the pathway of normal myeloid differentiation is partially blocked causing an expansion of cells with immunosuppressive functions, namely myeloid-derived suppressive cells [88]. This heterogeneous population of immature myeloid cells is capable of inhibiting both innate and adaptive immunity with an especially profound effect on suppressing T-cell responses and therefore playing a pro-metastatic role in cancer [106]. The presence of MDSCs in metastatic target organs is hypothesized to be one of the key early steps in priming the fertile ground for tumour cell colonization known as the pre-metastatic niche. These areas are believed to be highly permissive to circulating tumour cells and contribute significantly to the metastatic process.

In mice, MDSCs are characterized by the co-expression of myeloid lineage differentiation antigens Gr1 and CD11b [69, 107]. We utilized the 4T1, 4TO7 and 67NR orthotopically implanted murine mammary carcinoma models to study the recruitment and accumulation of MDSCs in metastatic and non-metastatic target organs of Balb/c mice. As will be detailed in this Chapter, in order to gain insight into MDSC involvement in metastasis, we took advantage of the fact that 4T1 and 4TO7 tumour cells form metastatic and hypoxic primary tumours while 67NR primary tumours do not metastasize and are very well oxygenated due to their well-developed vascular network. The oxygenation status of the primary tumours, or the involvement of hypoxia in metastasis is not investigated further in our study, and the data serve as a characterization tool only. Additionally, we explore the temporal and spatial involvement of MDSCs in the 4T1 tumour model specifically, and provide insight into the role of MDSCs in the pre-metastatic niche. Finally, we demonstrate that circulating G-CSF levels and MDSCs can be
used as a reliable and accurate surrogate for MDSC tissue accumulation in 4T1 tumour-bearing mice.

3.2 Results

3.2.1 Characterization of MDSCs in the 4T1 tumour model

The 4T1 mammary carcinoma cell line can be implanted orthotopically, or subcutaneously in order to generate solid primary tumours in Balb/c mice. In this study, 4T1 tumour cells were injected into the fourth mammary fat pad to allow for primary tumour growth in its orthotopic site. Upon implantation of tumour cells, the primary tumours grow in a very predictable way, with their weight steadily increasing with time (Figure 3.1).

![Figure 3.1 4T1 tumour weights over time. Tumour cells were implanted orthotopically into the fourth mammary fat pad (10^5 cells) and primary tumour weight was measured on tissue harvest days at one, two, three, and four weeks post implant. (n=15-21 mice per data point; bars are SEM).]
3.2.2 Association between primary tumour hypoxia and metastasis

While 4T1, 4TO7 and 67NR are sister cell lines that differ in their metastatic potential, they also differ in the hypoxic status of the primary tumours they form in Balb/c mice. We found that 4T1 and 4TO7 tumours, which induce MDSC expansion in the spleen and accumulation in the lungs (Figure 3.4C and Figure 3.5A), form poorly vascularized and poorly perfused primary tumours which contain substantial regions of hypoxia, while 67NR tumours of the same size have a high blood vessel density, exhibit high levels of perfusion and are not hypoxic (Figure 3.2A). Interestingly, only orthotopically implanted 4T1 and 4TO7 metastatic and hypoxic primary tumours are able to cause an accumulation of CD11b⁺ in the lungs of these animals, while the non-metastatic and non-hypoxic 67NR tumours do not induce pulmonary CD11b⁺ cell accumulation (Figure 3.2B). These data are consistent with reports describing the influence of hypoxia-induced secreted proteins in mediating BMDC accumulation in tissues and establishment of the pre-metastatic niche [58, 108].

Figure 3.2 Transplantable murine mammary tumours containing hypoxic tumour cells induce CD11b⁺ cell accumulation in the lungs.
A, Primary tumour and B, lung sections from Balb/c mice 2 weeks after orthotopic implantation of 4T1, 4TO7, or 67NR tumour cells. A, Tumour sections were stained for hypoxic tumour cells (pimonidazole; green), vasculature (CD31; red) and perfusion (iv Hoechst 33342; blue). B, Lung sections from the same mice were stained for CD11b⁺ cells (green) and DNA (DAPI; blue). Tumours were of comparable size at the time of harvest (~600 mg). Scale bars = 250µm.
We quantified the level of hypoxia and vascularization within each of the three tumour types by capturing random fields for analysis using ImageJ software. The greatest amount of intra-tumoural and inter-tumoural variability in hypoxia is seen for 4T1 tumours, followed by 4TO7 and 67NR tumours (Figure 3.3A-B). The variability in vascularization was greatest for 67NR primary tumours and most consistent for 4TO7 tumours. (Figure 3.3C-D). 4T1 primary tumours housed the largest hypoxic fraction (23.1% of tumour), followed by 4TO7 tumours (18.9% of tumour) and 67NR tumours (0.45% of tumour) which parallels their aggressiveness and metastatic potential (Figure 3.3E). As expected, 67NR primary tumours were the most vascularized (10.0% of tumour), followed by 4T1 (4.4% of tumour) and 4TO7 tumours (1.9% of tumour) (Figure 3.3F). It is interesting to note that while 4T1 tumours are more hypoxic than 4TO7 tumours, they are significantly more vascularized. As a result, the amount of vascularization within a tumour is not an accurate way of assessing the ability of substances to perfuse within the tissue. In Figure 3.2A’s left-top panel, we see that certain areas show positive CD31 staining (red) and are well perfused (blue), but actually reside in hypoxic zones (green). This indicates that these vessels are either permanently non-functional, or transiently collapsed. Both 4T1 and 4TO7 primary tumours are inferiorly vascularized as compared to 67NR which explains the absence of hypoxia in 67NR tumours. The reasons that 4T1 and 4TO7 but not 67NR tumours generate hypoxic zones is still poorly understood, but the aggressiveness and proliferative ability of the tumour cells may play a prominent role in affecting the formation of functional blood vessel networks. Taken together, these data demonstrate a positive correlation between the presence of hypoxia in primary mammary tumours and the accumulation of CD11b+ in the lungs of Balb/c mice.
Figure 3.3 Immunofluorescent image analysis of tumour sections for hypoxia and vasculature.

A, Hypoxic fraction (pimonidazole+ cells) within 5 different sectional planes of 4T1, 4TO7 and 67NR primary tumours (5 sections per tumour, 5 images per section). The random fields used for analysis were imported into ImageJ software and a digital mask was created to determine percent coverage in the visual field for a particular marker. B, Inter-tumoural hypoxia in 4T1, 4TO7 and 67NR tumours (n=3 per tumour type). C, Vascular coverage (CD31) within 5 different sectional planes of 4T1, 4TO7 and 67NR primary tumours (5 sections per tumour, 5 images per section). D, Inter-tumoural CD31 coverage in 4T1, 4TO7 and 67NR tumours (n=3 per tumour type). E, Quantification comparison of the hypoxic fractions between 4T1, 4TO7 and 67NR tumours. F, Quantification comparison of CD31+ vascular densities between 4T1, 4TO7, and 67NR tumours. Data are mean ± SEM for n=3 per tumour type.
It has previously been shown that CD11b+ BMDCs accumulate in the lungs of mice bearing hypoxic metastatic breast tumours such as the 4T1 murine mammary carcinoma [58]. Because CD11b cell marker is expressed on a variety of both myeloid and lymphoid cell types (MDSCs, macrophages, neutrophils, natural killer cells and to a lesser extent T-cells) flow cytometry was performed on tissue samples to more precisely characterize the identity of the tumour-induced CD11b+ cells. We found that the majority of CD11b+ cells in the lungs and spleens of Balb/c mice bearing syngeneic orthotopic 4T1 tumours co-express either Gr1 or F4/80. The proportion of CD11b+Gr1+ cells in both the lung and spleen were elevated relative to naïve Balb/c mice (Figure 3.4A), while the proportion of CD11b+F4/80+ showed no significant change. CD11b+Gr1+ cells represent a heterogeneous population of immature myeloid cells, while CD11b+F4/80+ cells are mature macrophages [28]. We observed a marked increase in the proportion of CD11b+Gr1+ cells in the lungs and spleens of 4T1 tumour-bearing mice (Figure 3.4B). The number of CD11b+Gr1+ cells in the lungs increased up to 90–94 weeks after tumour implantation, and this increase was concomitant with a 1400-fold increase in the number of CD11b+Gr1+ cells in the spleens (Figure 3.4C). The increased CD11b+Gr1+ cell content of the spleens in 4T1 tumour-bearing mice was associated with profound splenomegaly in proportion to primary tumour volume (Figure 3.4D), and a 42-fold increase in the number of proliferating cells in the spleen (Figure 3.4E). In contrast, the number of proliferating cells in the lungs of 4T1 tumour-bearing mice did not increase (Figure 3.4E), indicating that the elevated pulmonary CD11b+Gr1+ cell content in these mice was not due to expansion of CD11b+Gr1+ cells within the lung tissue. These data indicate that 4T1 tumours induce myelopoiesis and expansion of CD11b+Gr1+ cells in the spleens and accumulation of CD11b+Gr1+ cells in the lungs of tumour-bearing mice.

One important subset of CD11b+Gr1+ cells are MDSCs, a heterogeneous population of immature myeloid cells that accumulate and become activated in response to inflammatory stimuli, including tumour-derived factors [109]. Since the CD11b+Gr1+ cellular phenotype is not specific for MDSCs, identification of CD11b+Gr1+ cells as MDSCs requires ex vivo functional analyses of the immunosuppressive properties of CD11b+Gr1+ cells. We therefore isolated Gr1+ cells from the lungs and spleens of 4T1 tumour-bearing mice and established that >95% of the Gr1+ cells recovered from the lungs and spleen co-expressed CD11b indicating that we were assessing a relatively pure population of CD11b+Gr1+ cells. CD11b+Gr1+ cells recovered from the lungs (Figure 3.4F) of 4T1-tumour-bearing mice suppressed the proliferation of activated T-cells in a dose-dependent manner, indicating that these cells are immunosuppressive and
therefore can be defined as MDSCs. As we previously reported [28], 99.4% of Gr1\(^+\) cells exhibited a granulocytic morphology evident by the presence of cytoplasmic granules, high Ly6G and moderate Ly6C expression. Different from most other models [70], the monocytic MDSCs make up only 5-10% of the total MDSC population. These data indicate that immunosuppressive granulocytic CD11b\(^-\)Gr1\(^-\) MDSCs expand in the spleens and accumulate in the lungs of 4T1 tumour-bearing mice.
Figure 3.4 CD11b⁺Gr1⁺ myeloid-derived suppressor cells accumulate in the lungs and spleens of 4T1 tumour-bearing Balb/c mice. 
A, The proportion of CD11b⁺ cells in the lungs and spleens of naïve or 4T1 tumour-bearing Balb/c mice that are CD11b⁺Gr1⁺ immature myeloid cells or CD11b⁺F4/80⁺ macrophages. Data are mean ± SEM for 4 mice per group. B, The proportions of total cells recovered from the lungs and spleens of Balb/c mice that are CD11b⁺Gr1⁺ increase with time after orthotopic implantation of 4T1 tumour cells. C, The total number of CD11b⁺Gr1⁺ cells in tissues derived from the data in B normalized to the total cell yields from the tissues. Data are mean ± SEM for 4-15 mice per data point. D, Splenomegaly is induced in Balb/c mice in proportion to orthotopic 4T1 tumour size. Data shown are from individual mice. The circle is the average...
spleen weight of 6 naïve Balb/c mice and is not included in the linear regression. E, The number of proliferating (BrdU+) cells in the spleens and lung of naïve and 4T1 tumour-bearing mice. F, CD11b+Gr1+ cells isolated from the lungs of 4T1 tumour-bearing mice suppress T-cell proliferation in a dose-dependent manner and are immunosuppressive MDSCs. 'Ctrl' represents stimulated splenocytes alone. The inset is a representative graph showing typical flow cytometry gating for cells isolated from lungs by Gr1+ selection. Bars are mean ± SEM of triplicate wells; plots are representative of three independent experimental repeats. Significance compared to 'Ctrl'. The inset in F shows a typical flow cytometry plot of a sample rich in CD11b+Gr1+ cells (upper right quadrant). For this figure and every figure thereafter, significance will be symbolized as follows: * p<0.05, ** p<0.01, *** p<0.001, ns or nsd = no significant difference, if no comparison is made in bar graphs, no significant difference is seen.

3.2.3 Characterization of MDSCs in the 4TO7 and 67NR tumour models

We next assessed the ability of other mammary tumours to induce MDSCs. 4TO7 tumours are less aggressively metastatic (form small micrometastases) than 4T1 tumours (form larger macrometastases), while 67NR tumours do not metastasise. Similar to 4T1 tumours, we found that metastatic 4TO7 tumours induced accumulation of CD11b+Gr1+ cells in the lungs and spleen as a function of time after orthotopic tumour implant (Figure 3.5A), concomitant with splenomegaly seen in these animals (Figure 3.5B). Conversely, non-metastatic 67NR tumours did not induce CD11b+Gr1+ cell accumulation in the lungs or spleen (Figure 3.5A) and 67NR tumour-bearing mice did not exhibit splenomegaly (Figure 3.5B), even in mice with large 67NR tumours. As in 4T1 tumour-bearing mice, the CD11b+Gr1+ cells that accumulate in the lungs and spleens of mice with 4TO7 tumours are immunosuppressive MDSCs (Figure 3.5C). Interestingly, the CD11b+Gr1+ cells that were present in the lungs and spleens of 67NR tumour-bearing mice did not exhibit immunosuppressive function beyond that found in naïve Balb/c mice (Figure 3.5D-E), indicating that CD11b+Gr1+ cells in these mice are not MDSCs. These findings highlight the importance of functionally assessing the immune suppressive properties of CD11b+Gr1+ cells before concluding that the cells with the CD11b+Gr1+ surface marker phenotype are MDSCs. It is also interesting to note the link between CD11b+Gr1+ cell accumulation in tissues and acquisition of immune suppressive function in the CD11b+Gr1+ cells. Both 4T1 and 4TO7 tumours induced the splenic expansion of the CD11b+Gr1+ cell compartment and stimulated these cells to become immune suppressive MDSCs, which is consistent with reports that the same tumour-derived factors that induce the expansion and accumulation of CD11b+Gr1+ cells are responsible for the functional activation of these cells [109].
Figure 3.5 CD11b^+Gr1^+ cells isolated from Balb/c mice bearing 4TO7 tumours, but not 67NR tumours, are immunosuppressive MDSCs.

A, Y-axis represents the number of CD11b^+Gr1^+ cells in the lungs and spleens of Balb/c mice after orthotopic implantation of 4TO7 or 67NR tumour cells. B, Splenomegaly is induced in Balb/c mice in proportion to orthotopic 4TO7 tumour size, but not in Balb/c mice bearing 67NR tumours. Data are from individual mice. The circle is average spleen weight from 6 naïve Balb/c mice. CD11b^+Gr1^+ cells isolated from the lungs of Balb/c mice bearing C, metastatic 4TO7 tumours, D, non-metastatic 67NR tumours, or E, no tumours (naïve mice) were co-cultured at different ratios with stimulated splenocytes and T-cell proliferation was measured. CD11b^+Gr1^+ cells from 4TO7 tumour-bearing mice are immune suppressive MDSCs, while CD11b^+Gr1^+ cells from 67NR tumour-bearing or naïve Balb/c mice are minimally
immunosuppressive and therefore are not MDSCs. ‘Ctrl’ represents stimulated splenocytes alone. Bars are mean ± SEM of triplicate wells; plots are representative of three independent experimental repeats. Significance compared to Ctrl.

3.2.4 MDSC tissue accumulation is an early and systemic event

The putative existence of a pre-metastatic niche, or a localized microenvironment in secondary metastatic sites that is required for metastatic tumour seeding and/or growth, remains a topic of much debate. An outstanding question in the field is whether the tumour cells or primary tumour-induced MDSCs are the first to arrive to the metastatic target sites. To address this, we monitored accumulation of MDSCs and tumour cells in the lungs of 4T1 tumour-bearing mice over time after tumour implant. Day 0 CD11b⁺Gr1⁺ cells are non-suppressive myeloid cell, most likely neutrophils. At 7 days post-tumour implant, we found that while MDSCs were elevated, no metastatic tumour cells could be detected using the highly sensitive clonogenic assay (Figure 3.6). Worth noting however is that plating efficiency of 4T1 tumour cells isolated from lungs of tumour-bearing mice is 15-20% (data not shown). Tumour cells were detected in the lungs 9 days after tumour inoculation, at which time pulmonary MDSCs were 10.2 fold higher than in naïve mice. These findings suggest that, in the 4T1 model, MDSCs arrive in the lungs prior to metastatic tumour cells. The reason for this increase could be attributed to both and increase in MDSC accumulation and/or proliferation in the lungs. However, taken together with tissue proliferation data presented in Figure 3.4E, accumulation is the more likely source as the lungs are not a site of significant cell proliferation.
Figure 3.6 MDSCs arrive in lung tissue before 4T1 tumour cells.
Comparison between MDSC (left axis) and 4T1 tumour cell numbers (right axis) in lungs of 4T1 tumour bearing animals for different time points after implant. MDSC numbers were measured using flow cytometry and 4T1 tumour cells were detected in the lungs using the clonogenics assay. Data are mean ± SEM for a minimum of 4 mice per group.

We also found that MDSC accumulation was not limited to metastatic target organs. Rather, 4T1 tumours induced the systemic accumulation of functionally immune-suppressive MDSCs, in both metastatic (lungs, liver, bone-marrow) and non-metastatic tissues (kidneys and spleen) (Figure 3.7A-C). Systemic accumulation of the T-cell suppressing CD11b+Gr1+ MDSCs (Figure 3.7C) was verified by both immunofluorescent staining (Figure 3.7A) and flow-cytometric analysis of different tissue types (Figure 3.7B).
Figure 3.7 Primary 4T1 tumour growth induces a systemic accumulation of functional MDSCs in tissues.

A, Representative images of immunofluorescently stained frozen tissue sections of the brain, kidney and liver of a mouse bearing a 3 week old 4T1 tumour and a naïve mouse. B, Percentage of CD11b^+Gr1^+ cells in different tissues of naïve and mice bearing 3 week old 4T1 tumours as measured by flow cytometry. PB is peripheral blood and BM is bone-marrow. Data are mean ± SEM for 4 mice per tissue.
Significance measured between naïve and tumour bearing animal. C, CD11b^+Gr1^+ cells isolated from the spleen, lungs, liver and kidney of 4T1 tumour-bearing mice suppress T-cell proliferation in our immunosuppression assay. Data are mean ± SEM for 3 (spleen, lung, liver) or 4 (kidney and tumour) mice per group. Significance was measured compared to RC control (RC is responder control; activated T-cells alone).

These findings suggest that MDSC accumulation is not specific for sites of metastatic growth. They do not contradict the existence of the pre-metastatic niche; rather, our results suggest that the systemic induction of MDSCs by metastatic primary tumours is likely to be one of the first steps in the metastatic cascade. Consideration of other factors, such as the interplay between MDSCs and specific tumour secreted proteins at secondary target sites, is necessary to understand the metastatic tissue preference of circulating tumour cells.

We have shown that harvesting lung and spleen tissue and analyzing it using flow cytometry can provide us with a reliable number of CD11b^+Gr1^+ present in these tissues at various time points. Unfortunately, as the mice are sacrificed on harvest days, multiple measurements from the same animal are not possible. Multiple samples from the same animal allow for a more accurate read out (paired statistical analysis), and allow MDSC populations to be monitored over time in a single animal. As a result, we wanted to see if circulating MDSCs could act as an accurate and reproducible surrogate for tissue accumulation of these cells. The clinical implications are highly significant as it would prevent the need for multiple, highly invasive, tissue biopsies from cancer patients which could be replaced by taking peripheral blood samples at various time points during treatment to generate an MDSC biological profile that is specific to that patient.

Quality of the blood sample is paramount when analyzing samples with flow cytometry as different methods of blood collection vary in cell viability as well as the number of times the animal can be sampled [110]. The two methods we decided to test in our study were the cardiac puncture and the lateral tail bleed. The cardiac puncture is an endpoint method; therefore multiple samplings from the same animal are not possible. The tail bleed method allows multiple samples to be taken from the same animal and does not require sacrificing the animal. In our study using 4T1 orthotopically implanted tumours, we found that the tail bleed collection method is superior as it provides us with a sample with high viability and minimal cellular debris (Figure 3.8A). The two methods of collection produced similar proportions of MDSCs within the blood samples (Figure 3.8B). Ultimately, we decided to collect blood from the tail vein to determine if
circulating MDSCs can be used as a surrogate for tissue accumulation. We monitored mice over a four week period and saw a similar accumulation of MDSCs in the peripheral blood (Figure 3.9A) to what we saw in the lungs and spleen (Figure 3.4B-C).

![Figure 3.8 Blood collection via tail bleed and cardiac puncture in 4T1 tumour bearing mice. A, When blood is collected through the lateral tail vein, the viability of the cells in increased 1.6-fold as compared to blood collected by cardiac puncture. Difference between methods is significant, with the tail bleed being a superior method. B, A blood sample collected through a tail bleed or cardiac puncture produced very similar percentage of CD45+CD11b+Gr1+ cells. Samples analyzed using flow cytometry and in both A and B; Data are mean ± SEM with individual point plotted as well with n=6 in each group.](image)

Studies carried out in several mouse tumour models have shown that granulocyte-colony stimulating factor (G-CSF) is an important tumour derived factor capable of altering myelopoiesis and inducing an aberrant granulocytic MDSC expansion in the spleen and accumulation in tissues [65, 111, 112]. Additionally, G-CSF loss- and gain-of-function approaches have shown that abrogating G-CSF production significantly diminishes MDSC tissue accumulation and tumour growth, while ectopically over-expressing G-CSF has the opposite effect [113]. Furthermore, the study showed that treatment of naïve mice with recombinant G-CSF recapitulated the increased MDSC response seen in tumour-bearing animals. Consistent with previously published data, we found that for mice bearing metastatic 4T1 tumours, MDSCs expand dramatically in response to high levels of G-CSF secreted by the primary tumour (Figure 3.9B) and MDSC accumulation occurs in both metastatic and non-metastatic target sites via peripheral blood (Figure 3.9A, Figure 3.7B). As the primary tumour is
the main source of G-CSF in tumour bearing mice, we wanted to know how stable MDSCs are in the tissues after primary tumour resection and how targeting of MDSCs impacts metastatic growth. The results from these experiments are presented in Chapter 4 of our study.

Figure 3.9 Circulating MDSCs increase in proportion over time after 4T1 tumour implant which also corresponds with an increase in mouse G-CSF levels in the serum. A, MDSCs in peripheral blood were monitored from each of the mice by performing 8 tail bleeds (3, 7, 10, 14, 17, 21, 24 and 28 days post 4T1 tumour implant; naïve mice were bled only once and MDSC levels are show at 0 days after tumour implant). Blood samples were analyzed by flow cytometry. Data are mean ± SEM for an n=6. B, Mouse G-CSF serum levels were measured using a commercially available ELISA kit over a period of 3 weeks of 4T1 primary tumour growth. Data are mean ± SEM with n ranging from 4-6 mice per group.

3.3 Discussion

Aside from their differing metastatic potentials, 4T1, 4TO7 and 67NR differ in the oxygenation status of the primary tumours. It is well-established that patients with primary tumours that contain high proportions of poorly oxygenated (hypoxic) tumour cells experience decreased locoregional control and increased distant relapse rates after radiation or chemotherapy treatment. Furthermore, patients with hypoxic tumours also have decreased disease-free and overall survival rates after surgical resection of their primary tumour [114, 115] which are associated with the development of distant metastatic disease [116, 117]. Several hypoxia-induced genes are known to promote tumour cell invasion and migration, and previous data suggest that hypoxia-induced proteins such as LOX [118] can manipulate the environment within distant tissues to promote the development of tumour metastases. We show that 4T1 and 4TO7 primary tumours are hypoxic, poorly perfused, and contain an underdeveloped vascular
network made of, in part, of non-function blood vessels. On the other hand, 67NR primary tumours are well perfused, possess a well-developed vascular network and are not hypoxic. Our immunofluorescent image analysis illustrates the oxygenation heterogeneity that exists within the 4T1 tumours especially. Interestingly, only the hypoxic and metastatic primary tumours (4T1 and 4TO7) are able to induce CD11b+ accumulation in the lungs. While we do not continue with this research any further in our study, we use the oxygenation status of the primary tumours as another tool to characterize our models. Our research also encourages additional studies to be carried in order to determine the connection between primary tumour hypoxia, MDSC accumulation in metastatic target organs and its effects on the process of metastasis.

MDSCs are a heterogeneous population of immature myeloid cells phenotypically characterized in mice by their expression of CD11b and Gr1. Aside from expressing these cell surface markers, MDSCs possess a strong immunoregulatory activity, especially due to their ability to inhibit T-cell function [119, 120]. They do so by various mechanisms including depletion of arginine, production of reactive oxygen and nitrogen species, as well as production and secretion of inhibitory cytokines. It is their immunosuppressive ability that functionally classifies CD11b+Gr1+ cells as MDSCs [106]. In our study, we use 3 mammary carcinoma implantable models (4T1, 4TO7 and 67NR) to study the expansion and accumulation of MDSCs in metastatic and non-metastatic organs of Balb/c mice. Our work provides support for the pre-metastatic niche theory; however, it suggests that MDSC may not play a critical role in determining metastatic target organ specificity.

We orthotopically implanted 4T1, 4TO7 and 67NR cells in Balb/c mice and prefer it over other methods as the tumours are allowed to grow in the microenvironment from which they originally and spontaneously arose. The 4T1 and 4TO7 cell lines form metastatic primary tumours while 67NR tumours do not leave the primary site. We utilized this difference in metastatic ability between the 3 cell lines (4T1 tumour cell line being more metastatic than 4TO7) to gain insight into the metastatic process. We showed with the 4T1 model that the tumour growth over time is very predictable and provided us with a fairly controlled system to work with. While we characterize MDSCs in all 3 tumour models, we focused primarily on the 4T1 model in this chapter as it is the most aggressive variant, and closest in phenotype to human triple-negative breast tumours.
Emerging evidence suggests that CD11b⁺ bone-marrow derived cells (BMDCs) create an environment that is permissive to invasion and growth of tumour cells [121, 122]. We find that in the 4T1 model, the majority of the CD11b⁺ cells that increase in number and proportion in the lungs and spleens of tumour bearing mice also co-express the myeloid differentiation antigen Gr1 which is consistent with previous findings [123]. Both the proportion and number of CD11b⁺Gr1⁺ cells steadily increased with time after 4T1 tumour implant, with the proportion of CD11b⁺Gr1⁺ cells being higher in the lungs while the number of CD11b⁺Gr1⁺ cells was larger in the spleen. This finding makes sense in light of our data showing that in tumour-bearing animals, proliferation is significantly increased in the spleen while it remains non-significant in the lungs. Additionally, due to the high proliferation rate of cells in the spleen, mice develop splenomegaly which can result in a 15-fold enlargement of the animal’s spleen. As only functional assays can classify CD11b⁺Gr1⁺ cells as MDSCs, our T-cell proliferation assays show that CD11b⁺Gr1⁺ cells isolated from the lungs of tumour bearing animals possess strong immunosuppressive abilities and are therefore true MDSCs.

Similar to 4T1 tumour bearing mice, 4TO7 tumour bearing animals accumulated CD11b⁺Gr1⁺ cells in both the lungs and spleen. Additionally, the metastatic 4TO7 tumours induced a proliferation and accumulation of functional MDSCs as our T-cell proliferation assays show. Conversely, non-metastatic 67NR tumour bearing animals did not experience an increase in the number of CD11b⁺Gr1⁺ cells in the lungs or the spleen. As a result, 4TO7 tumour bearing animals developed splenomegaly, while 67NR tumours-bearing mice did not. Furthermore, CD11b⁺Gr1⁺ cells that were present in the lungs of 67NR tumour-bearing mice did not exhibit immunosuppressive function beyond that found in naïve Balb/c mice, indicating that the CD11b⁺Gr1⁺ cells in these mice are not MDSCs.

A controversial theory in the metastatic field that became popular over the past 10 years is that of the “pre-metastatic niche” [61]. The existence of the pre-metastatic niche is founded on the ability of the primary tumour to determine metastatic target organ specificity prior to dissemination [124]. It is believed that interplay between BMDCs and soluble factors released by the primary tumour play a crucial role in setting up the target tissue (soil) for the invading circulating tumour cells (seeds) [58]. Using the clonogenic assay to detect tumour cells in the lungs, in combination with detecting CD11b⁺Gr1⁺ cells in the lungs by flow cytometry, we are able to show that MDSCs are detectable in the lungs 9 days before tumour cells, providing evidence for the pre-metastatic niche theory.
Several groups grow tumors from fluorescently-tagged tumor cells and subsequently use flow cytometry to quantify fluorescent metastatic tumor cells in single cell suspensions derived from disaggregated tissues. While this is a reasonable approach for many tumor systems, several groups have shown that Balb/c mice can mount a potent immune response against tumor cells that express fluorescent proteins [125, 126], and we have observed decreased CD11b^+Gr1^+ cell accumulation and metastases in Balb/c mice implanted with GFP-labeled 4T1 cells [127]. Fluorescent tumor cells are therefore not recommended for use in Balb/c mice and as a result, we rely on the clonogenic assay to detect tumour cells in the lungs.

However, does the presence of MDSCs in a tissue imply that the tissue is a metastatic target organ? Our data suggest that this is not the case. 4T1 tumours metastasize to the lung, liver, bone-marrow and the brain and those are all places where MDSCs are found in increased numbers in tumour bearing animals. However, the kidney and spleen are not metastatic sites, and we report a significant increase in proportion of CD11b^+Gr1^+ cells in this tissue in 4T1 tumour bearing mice. The presence of MDSCs in the spleen can be explained by the fact that the spleen is a site of myelopoiesis; however, MDSC presence in the kidneys was not expected. Additionally, the CD11b^+Gr1^+ cells that accumulate in the kidney are in fact immunosuppressive to the same extent as the MDSCs that reside in the lungs which are well studied 4T1 metastatic target organ. Taken together with our own research and previously reported data, MDSC seeding seems to be an important and early event (occurs prior to tumour cell seeding) in the metastatic cascade, however, is not related to determining tissue specificity for tumour cell seeding.

Our experiments fortified the idea that MDSC accumulation is a systemic phenomenon where MDSCs proliferate in the spleen and arrest in a wide variety of organs via peripheral blood. Studies carried out in human breast cancer patients showed convincingly that elevated circulating MDSCs correlate with inferior overall survival, increased clinical cancer stage and metastatic tumour burden [82, 84]. With this in mind, we wanted to see if circulating MDSCs in our model can act as an accurate and dependable surrogate for tissue accumulation. The advantage of monitoring MDSCs in the blood (blood draw) versus tissue (tissue biopsy) is most apparent in the clinical setting where invasiveness of the procedure is taken into account. In 4T1 tumour bearing mice, we used the tail bleed method to collect blood samples as the cell samples were more viable and blood could be collected multiple times providing us with more reliable data. The alternate method tested was the cardiac puncture, which produced a viably inferior sample and only one blood draw could be done per animal as the method requires
euthanization prior to sample collection. Interestingly, we found that circulatory MDSC accumulation over time followed a very similar profile to that of the lungs and spleen in 4T1 tumour-bearing animals and could be used as an accurate surrogate for tissue accumulation. Additionally, we wanted to look at circulating levels of G-CSF in our model as Kowanetz et al elegantly showed in 2010 the importance of G-CSF in mobilizing MDSCs [65]. We also show that G-CSF serum levels are significantly elevated in 4T1 tumour-bearing mice, allowing for the recruitment of CD11b^+Gr1^+ cells from the bone-marrow, enhancing the robust proliferation of MDSCs in the spleen, and the subsequent accumulation of MDSCs in distant organs.

While the data presented in this chapter is mostly correlative, it serves the purpose of characterizing MDSCs in 3 of the most commonly used cell lines in murine mammary carcinoma research (4T1, 4TO7 and 67NR). The following chapter provides data that is less correlative, and answers conceptual questions that were alluded to in Chapter 3. As the stability of MDSCs in tissues after primary tumour resection is relatively unknown, we wanted to see how profound an effect tumour resection of 4T1 primary tumours had on CD11b^+Gr1^+ cell populations in the lungs and spleen. Additionally, we wanted to determine the effects that targeting MDSCs in metastatic target sites has on metastatic growth. The data presented in Chapter 4 provided us with answers to these important questions which lead to a deeper understanding of the role MDSCs play in the metastatic process.
CHAPTER 4: CHEMOTHERAPEUTIC TARGETING OF RESIDUAL POST-SURGERY MDSCs DECREASES METASTATIC GROWTH

4.1 Introduction

Historically, the field of cancer biology has almost entirely focused on malignant cells themselves, with limited merit given to non-neoplastic cellular components of the tumour microenvironment. However, emerging evidence indicating the importance of the network of non-transformed cells making up the tumour microenvironment brings forth a new understanding of cancer biology and shows promise for developing novel immunotherapeutic approaches. A plethora of current research has made it clear that the immune system is endowed with the ability to exert both tumour-promoting and tumour-suppressing effects [128]. Additionally, it has become apparent that primary tumours commonly avoid immune detection by creating an immunosuppressive environment which is permissive to the survival of immune-resistant cancer cell variants [129]. As a result of the cross talk that occurs between primary tumours and the immune system in cancer-bearing hosts, immune cells are phenotypically altered in ways that affect their functionality in executing proper anti-tumoural immune responses [7, 130].

While standard cancer treatments including surgery, chemotherapy and radiation are beneficial and lead to increased disease-free and overall survival, recurrence of the disease continues to affect a substantial proportion of patients. Primary tumour removal is an attractive strategy for decreasing MDSC levels; however, the longevity of MDSC in tissues, the magnitude of MDSC decrease, and the effect of this decrease on metastatic potential is still largely unknown. Another of the more promising strategies of combating metastatic disease is the stimulation of the patient’s own immune system to eliminate the disseminated tumour cells. However, the large arsenal of immunotherapeutics that showed promise in pre-clinical animal models has shown disappointing clinical results. In a 2006 review paper, Zitvogel et al summarize the multiple mechanisms that cancer cells utilize to evade the immune system [130]. Promotion of immunosuppressive cells, more specifically MDSCs, is one of the mechanisms shown to significantly compromise the efficacy of immune-based therapies [70]. Aside from the immunosuppressive abilities of MDSCs, they have been shown to play a role in tumour angiogenesis, proliferation, cancer cell survival and increasing metastatic potential [129]. As a
result of these findings, the elimination or inactivation of MDSCs has become a therapeutic objective in making progress in the field of metastasis research by altering the tumour microenvironment and further understanding anti-cancer immunity.

Work by several groups has shown that the conventional anti-cancer agents GEM and 5-FU, feature the ability to target MDSCs preferentially at doses low enough to not affect the growth of the primary tumour [98, 131-133]. Additionally, Ghochikyan et al very recently defined a temporal critical “window of opportunity” for immunotherapy that exists post primary tumour resection in which MDSC specific drugs would have the strongest efficacy [134]. One of the questions we wanted to address in our work was the longevity of MDSCs in metastatic target organs of mice post-resection of the primary tumour. Our findings show that residual MDSCs remain in metastatic target organs post-surgery; therefore, we designed a study aimed at eliminating residual MDSCs with chemotherapeutic post-surgery. Initially, we utilize the established MDSC targeting drugs GEM and 5-FU to demonstrate the ability of the drugs to deplete splenic and pulmonary MDSCs in our model. Furthermore, we propose TPZ as a novel MDSC chemotherapeutic as it showed MDSC specific cytotoxicity in our previous studies (data unpublished). Based on our initial characterization of the 3 chemotherapeutics, we decided to use GEM to target residual MDSCs in our surgery model and evaluate how depleting MDSCs in metastatic target sites affects the metastatic potential of 4T1 tumour cells.

**4.2 Results**

**4.2.1 Effects of 4T1 primary tumour resection on MDSCs and metastasis**

While metastatic primary tumours promote MDSC expansion in the spleen and accumulation in the lungs, the longevity of MDSCs in tissues in the absence of the primary tumour is unknown. This is an important clinical consideration, because the persistence of MDSCs in metastatic target organs after therapy would create a tissue microenvironment that promotes the secondary growth of disseminated tumour cells that survived therapy. To address this issue, we allowed 4T1 tumours to grow orthotopically for two weeks prior to surgical resection of the primary tumour. We found that the CD11b*Gr1* cell content of the spleen and lungs rapidly and significantly decreased within two days of primary tumour resection (Figure 4.1A-B). Importantly, CD11b*Gr1* cells remained significantly elevated by 5.4-fold in the lungs.
and 17.7-fold in the spleen (p<0.01) relative to naïve Balb/c mice for at least two weeks after tumour excision. The decrease in splenic CD11b+Gr1+ cells after surgery corresponded with a rapid reversal of splenomegaly (Figure 4.1C), indicating that MDSC expansion in the spleen is maintained by the primary tumour. Furthermore, the CD11b+Gr1+ cells that persisted in the spleen and lungs after surgery retained immune suppressive function in the absence of the primary tumour and are therefore MDSCs (Figure 4.1D).

Studies carried out in several mouse tumour models have shown that G-CSF is an important tumour derived factor capable of altering myelopoiesis and inducing aberrant granulocytic MDSC expansion in the spleen and accumulation in tissues [65, 111, 112]. Additionally, G-CSF loss- and gain-of-function approaches have shown that abrogating G-CSF production significantly diminishes MDSC tissue accumulation and tumour growth, while ectopically over-expressing G-CSF has the opposite effect [113]. Furthermore, Kowanetz et al. showed that treatment of naïve mice with recombinant G-CSF recapitulated the increased MDSC response seen in tumour-bearing animals [65]. In our study, we looked at the effect that primary tumour resection had on G-CSF levels in mouse sera. Similar to previous reports, we observed a steady increase in G-CSF serum levels with increasing 4T1 tumour burden. We found that serum G-CSF levels significantly decreased after primary tumour resection, suggesting that a major stimulus for MDSC expansion in the spleen and accumulation in metastatic and non-metastatic organs is arrested upon the removal of the primary tumour (Figure 4.1E).

While the levels of tissue MDSCs fluctuated dramatically in response to primary tumour growth and surgical removal (Figure 4.1A-B), we wanted to know if circulating MDSC levels could serve as a dependable surrogate to tissue sampling even after primary tumour resection. We observed a significant drop in circulating MDSCs after primary tumour resection yet the MDSC levels remained significantly higher for 1 week when compared to naïve mice (Figure 4.1F, open diamonds. Due to their immunosuppressive nature, we wanted to know if the residual MDSCs that remain in the lungs post-surgery affect metastatic tumour growth.
Figure 4.1 Functional MDSCs persist in the lungs after primary tumour resection.

A. The numbers of CD11b^+Gr1^+ cells in the spleen and B, lungs of Balb/c mice growing 4T1 orthotopic tumours or with 4T1 tumours excised two weeks after tumour implant. Levels rapidly decrease after primary tumour resection and high numbers of CD11b^+Gr1^+ cells remain in the tissues relative to naïve mice for at least 2 weeks after tumour resection. C, Primary tumour resection reverses splenomegaly. D, CD11b^+Gr1^+ cells isolated from the spleens or lungs of mice 2 or 10 days after 4T1 tumour excision retain immunosuppressive function (similarly to CD11b^+Gr1^+ cells isolated from the spleens or lungs of mice that did not undergo tumour resection. Data are expressed as the fraction of stimulated T cell proliferation in the absence of CD11b^+Gr1^+ cells (Ctrl). Bars are mean ± SEM of triplicate wells; data are mean ± SEM of two independent experimental repeats. Significance is compared to Ctrl. E, G-CSF levels in serum of naïve, tumour bearing, and tumour resected mice. Data are expressed as mean ± SEM with a minimum of 4 mice per group. F, Percentage of peripheral blood CD45^+CD11b^+Gr1^+ cells over time in mice with or without primary tumour resection at 2 weeks. Data are mean ± SEM with 6 Balb/c mice per time point except for primary tumour excision data points (4 Balb/c mice per time point); * used for statistical comparisons to control, # used for statistical comparison to naïve animals. Data in A-C are mean ± SEM of 4-8 mice per excision group; significance compared to mice 2 weeks after 4T1 tumour implant (n=14).

To assess the functional relevance of MDSCs that persist in the lungs after primary tumour resection, we allowed 4T1 tumours to grow for two weeks prior to surgical resection as above in order to “prime” the lungs with MDSCs. Two days after tumour resection, we iv injected 4T1 tumour cells to determine if the persistent MDSCs influenced the survival and proliferation of these tumour cells in the lungs over the next 8 days (Figure 4.2A, black line). We first verified that iv injection of 4T1 tumour cells did not affect the number of CD11b^+Gr1^+ cells in the lungs of mice 8 days later, regardless of whether the iv injection was administered to a naïve mouse or a mouse 2 days after primary 4T1 tumour resection (Figure 4.2B). We found a relatively small number of metastatic 4T1 tumour cells growing in the lungs 10 days after primary tumour excision (Figure 4.2A, C, dashed line/bar), and a small number of 4T1 tumour cells in the lungs of naïve mice 8 days after iv 4T1 tumour cell injection (Figure 4.2A, C, grey line/bar). However, we found that the presence of MDSCs in the lungs of mice after 4T1 tumour excision was associated with increased survival and growth of subsequently iv injected 4T1 tumour cells in the lungs by 13.6-fold relative to naïve mice iv injected with the same number of 4T1 cells (Figure 4.2A, C, black line/bar). Based on the numbers of 4T1 tumour cells in the lungs at the end of the experiment, the doubling time of 4T1 tumour cells in the lungs following tumour resection (Figure 4.2A, C, dashed line/bar) was 9.0 days (no 4T1 cells iv injected) and the doubling time of 4T1 tumour cells iv injected into naïve mice (Figure 4.2A, C, grey line/bar) was 10.4 days. In contrast, the doubling time of 4T1 tumour cells iv injected into mice 2 days after primary tumour resection (Figure 4.2 A, C, black line/bar) was only 0.92 days. The presence of MDSCs in the lungs after tumour resection was therefore associated with an 11.3-fold increase in the rate of 4T1 tumour cell growth in the lungs. Taken together, these data demonstrate that immunosuppressive MDSCs that persist in the lungs after surgical resection of primary tumours...
are associated with profoundly increased metastatic growth. The next step in our study was to target MDSCs in 4T1 tumour bearing mice using chemotherapeutics.

4.2.2 Targeting MDSCs with GEM in 4T1 tumour-bearing mice

GEM is known to target MDSCs in the 4T1 tumour model, and we wanted to determine the kinetics of GEM-mediated killing of MDSCs. Balb/c mice bearing 17 day old 4T1 tumours...
were injected with a single dose of GEM administered ip, while lung, spleen and tumour tissues were harvested at different time-points post drug administration. After the single 60 mg/kg dose of GEM, the number of CD11b*Gr1+ cells in the lungs and spleen of 4T1 tumour bearing mice dropped significantly. In the spleen, we saw a 4.3-fold decrease in the number of CD11b*Gr1+ 1 day after drug administration. CD11b*Gr1+ cells remained significantly low for the next 2 days, and increased back to pre-treatment levels after 4 days (Figure 4.3A). Due to the CD11b*Gr1+ cell targeting in the spleen, we saw a temporary reversion of splenomegaly (Figure 4.3C), with a significant change in tumour weight 2 days post GEM administration when compared to Day 0 (17 day old 4T1 tumours) (Figure 4.3D). However, when we compared age-matched tumours from mice that received GEM and the ones that did not, we did not see a significant difference in primary tumour weight (Figure 4.3E). In the lungs we saw a 7.3-fold decrease in the number of CD11b*Gr1+ cells 1 day after GEM treatment (Figure 4.3B). The number of cells in the lungs stayed significantly low for the next 2 days in the lungs, but started accumulating again 4 days after GEM treatment. As opposed to the number of CD11b*Gr1+ cells in the spleen, the cell levels were still significantly lower than that found in animals before GEM treatment.
Figure 4.3 Effects of GEM in vivo in 4T1 tumour-bearing mice over time after a single dose.

A, The number of CD11b^+Gr1^+ cells in the spleen after single GEM ip administration measured over time. Day 0 means untreated mice bearing 17 day old 4T1 tumours. B, The number of CD11b^+Gr1^+ cells in the lungs over time after GEM treatment; 0 days denotes start of treatment. Samples were analyzed using flow cytometry in A and B. C, Spleen weight and D, 4T1 tumour weight measured over time after GEM treatment. E, Comparison of 4T1 tumour weights with and without GEM treatment; the tumours are age matched (No GEM: 3 week old 4T1 tumours; GEM: 17 day old 4T1 tumours treated with GEM and allowed to grow for an additional 4 days). Data are mean ± SEM with n=4-6 for panels A-D and n=21 for “No GEM” and n=5 for “GEM in panel E. In panels A-D, significance is compared to “Day 0”. Significance in panel E is measured between the two groups (21 day old age-matched tumours: No GEM-21 days of GEM-free 4T1 tumour growth, GEM-17 days of GEM-free 4T1 tumour growth plus 4 days of growth post GEM administration).
4.2.3 Targeting MDSCs with 5-FU in 4T1 tumour-bearing mice

Similarly to GEM, studies have shown the efficacy of 5-FU at targeting MDSC in the same tumour model as in our study. We wanted to test the kinetics of 5-FU mediated MDSC killing and determine how long the effects of the drug last. The same experimental design used for GEM was also used for our 5-FU experiments. After a single 50 mg/kg dose of 5-FU, the number of CD11b\textsuperscript{+}Gr1\textsuperscript{+} cells in the lungs and spleen of 4T1 tumour bearing mice dropped significantly. In the spleen, we saw a 21.1-fold decrease in the number of CD11b\textsuperscript{+}Gr1\textsuperscript{+} cells 3 days after drug administration which is a faster response than the one seen with GEM (5-FU did not cause a significant decrease in the number of CD11b\textsuperscript{+}Gr1\textsuperscript{+} cells 1 day after administration while GEM did). The number of CD11b\textsuperscript{+}Gr1\textsuperscript{+} cells in the spleen stayed significantly low for 5 days post drug administration, and became significantly elevated 7 days after the single 5-FU dose (as compared to Day 0) (Figure 4.4A). Similar to what we saw in our GEM experiments, we report a temporary reversion of splenomegaly (Figure 4.4C), with a significant change in tumour weight 1 day post 5-FU administration when compared to Day 0 tumours (Figure 4.4D). However, when we compared age-matched tumours from mice that received 5-FU and untreated mice, we did not see a significant difference in primary tumour weight (Figure 4.4E). In the lungs we observed a 1.8-fold decrease in the number of CD11b\textsuperscript{+}Gr1\textsuperscript{+} cells 1 day after 5-FU treatment which increased to an 8.1-fold decrease 3 days post drug administration. The number of CD11b\textsuperscript{+}Gr1\textsuperscript{+} cells stayed significantly low for the next 2 days in the lungs, but started increasing again 5 days post 5-FU treatment. The number of CD11b\textsuperscript{+}Gr1\textsuperscript{+} cells exceeds the Day 0 value because we sacrificed the mice 9 days post drug administration whereas in the GEM experiment, euthanization was done 4 days after drug treatment (CD11b\textsuperscript{+}Gr1\textsuperscript{+} cell numbers did not return to Day 0 levels in the GEM experiment). The additional 5 days of 4T1 tumour growth in the 5-FU experiment allowed more time and stimulus for MDSCs to be produced.
Figure 4.4 Effects of 5-FU in vivo in 4T1 tumour-bearing mice over time after a single dose.

A, The number of CD11b^Gr1^ cells in the spleen after a single 5-FU ip. administration measured over time. B, The number of CD11b^Gr1^ cells in the lungs over time after 5-FU treatment; 0 days denotes start of treatment. Samples were analyzed using flow cytometry in A and B. C, Spleen weight and D, 4T1 tumour weight measured over time after 5-FU treatment. E, Comparison of 4T1 tumour weights with and without 5-FU treatment; the tumours are age matched (No 5-FU: 3 week old 4T1 tumours; 5-FU: 17 day old 4T1 tumours treated with 5-FU and allowed to grow for additional 4 days). Data are mean ± SEM with n=4-6 for panels A-D and n=21 for “No 5-FU” and n=6 for “5-FU” in panel E. In panels A-D, significance is compared to “Day 0”. Significance in panel E is measured between the two groups (age-matched tumours as close as possible: No 5-FU-21 days of 5-FU-free 4T1 tumour growth, 5-FU-17 days of 5-FU-free 4T1 tumour growth plus 3 days of growth post-5-FU administration).
4.2.4 Targeting MDSCs with TPZ in 4T1 tumour-bearing mice

While it has previously been reported that GEM and 5-FU can be used as a potent chemotherapeutics to target MDSCs, to our knowledge, TPZ’s ability to target CD11b*Gr1* cells in the mouse model has not been established. Originally, TPZ was developed as a hypoxia-specific cytotoxin; however, TPZ’s dose limiting toxicity in clinic was myeloablation. As a result, we were curious to see whether TPZ is able to kill tumour induced MDSCs. We repeated the same experimental design as we used for our GEM and 5-FU experiments except that we tried out two different doses of TPZ (20 mg/kg and 40 mg/kg). The single dose of 20 mg/kg TPZ did not have any significant effect on the number of CD11b*Gr1* cells in the spleen (Figure 4.5A) and lungs (Figure 4.5B) of the tumour bearing animals, yet we saw a transient reversion of splenomegaly at this dose (Figure 4.5C). We did see a significant decrease in the number of CD11b*Gr1* cells in both the spleen and the lungs at the higher 40 mg/kg TPZ dose 3 days post drug administration. We report a 2.8-fold decrease in MDSC numbers in the spleen (Figure 4.5A) and a 2.2-fold decrease in the lungs (Figure 4.5B). The higher TPZ dose had a more profound effect on the spleen size as we saw a significant reversion of splenomegaly even 3 days post drug administration (Figure 4.5C). Similarly to what we saw in our 5-FU experiments, we report a significant change in tumour weight 1 day post TPZ administration when compared to day 0 tumours for both the high and low dose (Figure 4.5D). However, when we compared age-matched tumours from mice that received either the high dose or low dose of TPZ, and untreated mice, we did not see a significant difference in primary tumour weight (Figure 4.5E).
Figure 4.5 Effects of TPZ in vivo in 4T1 tumour-bearing mice over time after a single dose.

A, The number of CD11b^Gr1^ cells in the spleen after a single dose of TPZ measured over time; 0 days denotes the start of drug treatment. B, The number of CD11b^Gr1^ cells in the lungs over time after TPZ treatment. C, Spleen weight and D, 4T1 tumour weight measured over time after TPZ treatment. Samples were analyzed using flow cytometry in panels A and B. E, Comparison of 4T1 tumour weights with and without TPZ treatment; the tumours are age matched. (No TPZ: 3 week 4T1 tumours; TPZ: 10 day old 4T1 tumours treated with TPZ for 10 days) Data are mean ± SEM with n=3-4 for panels A-D and n=21 for “No TPZ” and n=4 for “20 mg/kg TPZ” and n=4 for “40 mg/kg TPZ” in panel E. In panels A-D, significance is compared to “Day 0”; * denotes significance for 20 mg/kg TPZ treated mice and # for 40 mg/kg TPZ. Significance in panel E is measured between the control (No TPZ) and the two treatment groups (21 day old age-matched tumours: No TPZ-21 days of TPZ-free 4T1 tumour growth, TPZ-17 days of TPZ-free 4T1 tumour growth plus 4 days of growth post TPZ administration).
In Figure 4.6, we compare GEM, 5-FU and TPZ over a 3 day period post injection to compare these different parameters. We are more interested in targeting pulmonary MDSCs as the lungs are a metastatic target site. Therefore, we decided to base our drug preference for future experiments on how well each chemotherapeutic performed in the lung tissue. GEM had the fastest effect in both the lungs (Figure 4.6A) and the spleen (Figure 4.6B) as compared to the other two drugs. 5-FU was just as potent as GEM in targeting pulmonary MDSCs 3 days after administration, and its effects in the spleen were nearly 5-fold more potent. However, as our future experiments required a fast acting MDSC chemotherapeutic in the pulmonary setting, we decided to choose GEM as our top candidate for further studies. The reason for wanting a fast but short acting MDSC specific chemotherapeutic is because our future work required a time-specific depletion of MDSCs with fast clearance in order to reduce the off-target effects of the drug.

Both GEM and 5-FU fall into the class of chemotherapeutics known as antimetabolites. More specifically, these drugs act as nucleoside analogs and disrupt the normal proliferation of the cells undergoing replication. Due to their mode of action, we wanted to know if the decrease in the number of CD11b^+Gr1^+ cells we see in the spleen and lungs after drug administration is
due to a cell cycle block of proliferating CD11b*Gr1+ cells or death of these cells. In order to answer that question, we analyzed the cell cycle profiles of our lung and spleen samples using flow cytometry. While TPZ is not a classical antimetabolite, we decided to see what effects the single dose had on the cell cycle profiles of our lung and spleen samples. We found that TPZ did not have any significant cell cycle blocking properties in either of the tissues tested (Figure 4.7A-B). The spleens of mice treated with 5-FU showed a definite G1 block which subsided 5 days post drug treatment (Figure 4.7D). The lungs of 5-FU treated mice did not show a G1 block which is not surprising as the lungs are not a site of immense cell proliferation in tumour-bearing mice (Figure 3.4E). The increase in the G1 proportion was concomitant with a decrease in the S and G2 phases of the cell cycle profile. Similarly, GEM caused a G1 cell cycle block in the spleen (Figure 4.7F) but not in the lungs (Figure 4.7E). Taken together with the data presented in Figure 3.4 and the 7.3-fold decrease of CD11b*Gr1+ cells we saw in the lungs 1 day post GEM treatment, we believe that the decrease in the number of pulmonary CD11b*Gr1+ cells is most likely not due to a cell cycle block but due to direct cell kill. Our data suggest that the same holds true for both 5-FU and TPZ. However, it is also important to note that it is possible that a decrease in the number of CD11b*Gr1+ cells could be due to the effects the drug treatment has on the proliferation of lung and spleen cells.
Chemotherapeutics elimination of MDSCs is through direct cell kill and not due to a cell cycle block.

Cell cycle analysis of fixed lung and spleen cells analyzed using flow cytometry after drug treatment (TPZ, 5-FU and GEM lungs are found in panels A, C and E respectively; TPZ, 5-FU and GEM spleen data are found in panels B, D, F respectively). Data are mean ± SEM with n=5-6 per group per day. Significance comparisons are between “Control” and subsequent days. “Control” is “Day 0” data presented in Figures 4.3, 4.4 and 4.5.
4.2.5 Effects of GEM, 5-FU and TPZ in vitro

Since most of our work to this point has been done in vivo, we were interested to see how the drugs we used in mice affect primary cell lines in vitro. The aims of this part of the study were to determine the relative toxicity of our drugs in tumour cells versus myeloid cells, and to determine whether the decrease in CD11b^+Gr1^+ cells is due to direct cell kill or due to the killing of G-CSF producing tumour cells. After several failed attempts at growing MDSCs ex vivo using a variety of growing conditions, we decided to utilize the immortalized MDSC cell line MSC2 for our in vitro studies. Additionally, a report showing that MDSCs do not survive well ex vivo [98] (50% MDSC death 24 hours post isolation), further justified our use of the immortalized MSC2 cell line.

For our in vitro experiments, we utilized the resazurin assay which measures the metabolic activity of cultured cells using a colorimetric quantification method. Surprisingly, GEM did not show a big difference in preferential targeting of MDS2 cells over 4T1 tumour cells (Figure 4.8A). There are several explanations for this phenomenon which we will examine in the discussion section of this Chapter. More unexpectedly, 5-FU was more effective at killing MDS2 cells preferentially as compared to GEM (Figure 4.8B). Lastly, we tested the ability of TPZ to affect the metabolic activity of the two cell lines. Since TPZ is a hypoxia activated drug, it was important for us to test its efficacy under both normoxic and hypoxic conditions. Using the resazurin assay for all of our in vitro studies, we found that TPZ was 8-fold more efficient at killing hypoxic (1% O_2) 4T1 tumour cells as opposed to cells grown at 21% O_2 (Figure 4.8C-same metabolic activity decrease seen (95% survival) with 5 µM for hypoxic 4T1 cells while it required 40 µM of TPZ for the normoxic 4T1 cells). Interestingly, the oxygenation status of the immortalized MDSC cell line, MSC2, did not have an effect on TPZ cytotoxicity, and TPZ was actually more efficient at killing MSC2 cells as compared to 4T1 tumour cells (Figure 4.8C).

The 48-hours post-drug treatment plots (Figure 4.8D-F) follow a similar trend to the 24-hour drug graph, with more overall cell death occurring due to the longer exposure to the chemotherapeutic agent. When we plotted panels A-C of Figure 4.8 on the same graph with the x-axis showing µM of drug used, we see that GEM’s effects on both 4T1 tumour cells and MSC2 cells are the most potent on a concentration basis (Figure 4.8G-H respectively). Our data, taken together with previously published work showing GEM as having preferential specificity towards
myeloid cells [98], convinced us to pursue GEM as our drug of choice in targeting residual MDSCs in tumour resected mice.

Figure 4.8 Effects of chemotherapeutics on 4T1 and MSC2 cells *in vitro.*

**A**, Using the resazurin assay to measure metabolic activity of cells after drug treatment, we saw that a 24-hour incubation of cells with GEM did not have a drastic differential effect on the 4T1 and MSC2 cell lines. **B**, Unlike GEM, 5-FU’s cytotoxic effects were mainly exerted on MSC2 cells and not 4T1 tumour cells. **C**, TPZ was better at killing 4T1 cells grown in the 1% O₂ hypoxia chamber as compared to 4T1 cells grown at 21% O₂. MSC2 cell line was even more susceptible to the cytotoxic effects of TPZ than 4T1 cells grown in hypoxia. **A**, **B** and **C** panels show data for 24-hour drug exposure, while panels **D**, **E**, and **F** show the 48-hour drug exposure data. Data are mean ± SEM with each drug dose being done in sextuplicate. **G**, Comparison of all three drugs in 4T1 cell line or **H**, MSC2 cell line after 24 hours of drug exposure. Data shown in **G** and **H** is derived from graphs shown in panels **A-C**.
4.2.6 Targeting residual lung MDSCs with GEM

The final experiment of our study was aimed at targeting the residual MDSC that remain in the lungs after the removal of the primary tumour. We wanted to perform this experiment as our data led us to believe that residual MDSCs are potentiators of metastatic growth. Directly targeting residual MDSCs with GEM would decrease their numbers in the lungs and potentially affect the metastatic niche. The experimental outline (Figure 4.9A) describes the method we used for their targeting. When deciding on which MDSC specific chemotherapeutic to use for this study, we took into account the time required for the MDSCs to become depleted by the drug, before 4T1 tumour cells could be injected iv. We decided to use GEM in these experiments as we wanted to replicate the experimental design in Figure 4.2A, and GEM was able to drastically decrease the numbers of MDSCs in the lungs 24-hours after the administration of the single dose (Figure 4.3B). The fast action of GEM allowed us to inject 4T1 tumour cells iv 24-hours after the drug administration and still keep the experimental timing of the two experiments constant (no GEM in Figure 4.2A; with GEM in Figure 4.9A).

Our data demonstrates that the single dose of GEM given 24-hours after tumour excision did not have a significant effect on the number of CD11b+Gr1+ cells in the spleen after the 4T1 tumour cell iv injection (Figure 4.9B). The CD11b+Gr1+ cell levels in the spleen of naïve mice receiving 4T1 tumour cells iv alone were significantly less than the levels in both the GEM treated and untreated groups (Figure 4.9B). On the other hand, GEM had a modest, but significant effect on the number of CD11b+Gr1+ cells in the lungs which in contrast to the spleen is a metastatic target site of the 4T1 tumour cells (Figure 4.9C). When compared to the “4T1 iv” group (naïve mice receiving 4T1 tumour cells iv alone), GEM administration did not reduce the CD11b*Gr1+ cell number down to these control levels (Figure 4.9C). What panel F in figure 4.8 shows is our ability to target CD11b*Gr1+ using both the surgical and chemotherapeutic method, and bringing the MDSC levels down significantly in the lungs of these mice. Finally, we asked the question whether the statistically significant targeting of the residual pulmonary CD11b*Gr1+ cells using GEM after primary tumour resection has an effect on metastatic potential of the 4T1 tumour cells? We found that even though GEM was not able to reduce the CD11b*Gr1+ cell number down to control levels (4T1 iv; naïve mouse injected with 4T1 tumour cells iv alone), the significant reduction had a profound effect on the metastatic potential of the 4T1 tumour cells injected iv (Figure 4.9D). Although we were able to target only a fraction of the residual MDSCs in the lungs, their removal from this metastatic target site correlated with a significant decrease
in the metastatic ability of the 4T1 tumour cells in our clonogenics experiment (Figure 4.9D). Not only did we decrease the number of 4T1 tumour cells arresting and surviving in the lungs 8 days post iv injection, the number of 4T1 tumour cells was decreased to control levels (4T1 iv; naïve mouse injected with 4T1 tumour cells iv alone) (Figure 4.9D).

**Figure 4.9 MDSCs promote pulmonary metastatic tumour growth in 4T1 tumour-bearing mice.**
A, Experimental outline of panels B-D; B, Comparison of the number of splenic CD11b^+Gr1^+ cells in the 3 different treatment groups. GEM did not have a significant effect on CD11b^+Gr1^+ cell numbers in the spleen. C, Comparison of the number of pulmonary CD11b^+Gr1^+ cells in the 3 different treatment groups. GEM had a significant effect on CD11b^+Gr1^+ cell numbers in the lungs; the first bar in the graph shows what CD11b^+Gr1^+ cell numbers are in an untreated animal undergoing normal 4T1 tumour progression. Both surgery and GEM treatment significantly decrease the number of CD11b^+Gr1^+ cells in the lungs. D, Clonogenics assay showing the effect of GEM treatment post-surgery on the ability of 4T1 cells injected iv to survive in the lungs. Targeting residual MDSCs with GEM significantly decreased the metastatic potential of 4T1 cells. Significance comparisons are indicated by lines above the bar graphs.
4.3 Discussion

In this chapter, our studies focus primarily on the 4T1 tumour model as several groups, in addition to ours, believe that out of the available tumourigenic murine mammary carcinoma cell lines, 4T1 is the most clinically relevant to aggressive triple-negative human breast cancers [35, 99, 135-137]. Moreover, Ostrand-Resenberg’s group showed that 4T1 tumours very closely models human breast cancer in their immunogenicity, metastatic properties, and growth characteristics. Additionally, their group was largely responsible for showing that surgery alone does not have a prolonged effect on the prevention of metastatic disease [138, 139]. Much like the patients who develop metastatic disease, mice bearing 4T1 tumours that either do or do not undergo surgery have a very low rate of survival post treatment with immunotherapies. It is believed that the failure of immunotherapies in the 4T1 model can be attributed to the cell line’s poor immunogenicity and tumour associated immunosuppression [134]. Whether or not the low survival rate due to metastatic disease is correlated with MDSC levels in mice and patients post-surgery are questions that need to be addressed. MDSCs along with macrophages are the major populations of cells connected with tumour-associated immunosuppression and are not limited to the 4T1 model [28]. MDSC expansion and accumulation has been reported in a wide array of tumours including EL4, DA3, CT26, MC38, C3, ANV, LLC, B16, MethA and spontaneous tumour models [79].

While primary tumours promote MDSC expansion in the spleen and accumulation in the lungs, the longevity of MDSCs in tissues in the absence of the primary tumour remains to be elucidated. As mentioned earlier, data showing that surgery alone (excising the source of G-CSF) does not have a prolonged effect on the prevention of metastatic disease made us wonder how MDSC tissue levels respond to primary tumour resection [138, 139]. We were interested in knowing if tumour resection restores MDSC levels back to naive mouse levels or if residual MDSCs persist in metastatic organs. Previous reports have underlined the importance of G-CSF in inducing an aberrant granulocytic MDSC expansion in the spleen and accumulation in the tissues [65, 111-113]. Consistent with these findings, we observed a steady increase in G-CSF serum levels with increasing 4T1 tumour growth which significantly decreases after primary tumour removal, bringing serum G-CSF down to naïve mouse levels. While data demonstrates may suggest that removal of the stimulus for MDSC expansion and accumulation (i.e. primary tumour) would return MDSC levels to that of a naïve mouse, we found that in both the spleen
and the lungs, residual MDSCs remained after tumour resection. The majority of the MDSC decrease in the lungs and spleen is seen 2 days post tumour excision with a significant reversal of splenomegaly. Residual MDSC stay at elevated levels for at least the next 2 weeks post-surgery (the length of the experiment) despite G-CSF serum levels returning to naïve levels, which suggests that the survival of MDSCs in the lungs and spleen is G-CSF independent at this stage. Additionally to seeing a drop in the lungs and spleen post-surgery, we observed a very similar decrease in circulating MDSCs. Data demonstrates implies that removing the primary tumour causes a systemic decrease in MDSCs and more interestingly, it suggests that monitoring MDSC in the blood is a viable method for monitoring MDSCs levels in animals and potentially in patients as it closely mirrors MDSC tissue levels. Importantly, we confirmed that the residual CD11b*Gr1* cells that remained in tissues after tumour resection are immunosuppressive and therefore are MDSCs. What this suggested to us is that the lungs, a metastatic target site of 4T1 tumour cells, contain a significant number of immunosuppressive cells after surgery. Surgery done on early stage cancer patients has a good outcome; however, cancer of a later stage poses the deadly thread of poorly treatable metastatic disease. Our experimental design outline in Figure 4.8A was aimed at determining if residual MDSCs are potentiators of the metastatic process. By injecting 4T1 tumour cells iv into mice, we are able to replicate tumour seeding as majority of the cells injected seed directly to the lungs. We report that the presence of residual MDSCs in the lungs of mice post 4T1 tumour excision increased the number of 4T1 tumour cells surviving in the lungs by 13.6-fold relative to naïve mice injected with the same number of 4T1 cells. It is important to note that the 4T1 iv tumour cell injection did not have an effect on the numbers of MDSC in the spleen or lungs. Taken together, our data demonstrates that MDSCs that persist in the lungs after surgical removal of the primary tumour are associated with significantly increased metastatic growth.

A number of studies have shown that targeting MDSCs leads to improved anti-tumour immunity, mainly through a recovery of CD8+ T-cells. Restored T-cell anti-tumour activity leads to tumour suppression and as a result, multiple modes of targeting MDSCs are in clinical development. One example of such a study is the use of ATRA in patients with metastatic renal cell carcinoma (RCC). Researchers found that all-trans retinoic acid (ATRA) increased MDSC differentiation (effectively decreasing the immature MDSC cell pool) which enhanced T-cell antigen specific immune responses [70]. One aspect of our study focuses on the ability of the anti-metabolic drugs GEM and 5-FU and hypoxia activated pro-drug TPZ to target MDSCs in the spleen and lungs of 4T1 tumour bearing animals. The hypoxia targeting abilities of TPZ are
being explored in a different project in our lab. However, we observed that TPZ could directly target MDSC in the spleen and lungs of tumour-bearing animals. To our knowledge, this is a novel characteristic of TPZ and consequently, we wanted to pursue the idea further in this study.

Based on the work done by Suzuki et al and Vincent et al, we deemed GEM and 5-FU as well characterised MDSC-specific cytotoxics when used in a single injection at a low enough dose [98, 132, 133]. The reason we decided to use a single dose versus a weekly dosing schedule is because multiple doses of anti-metabolic and anti-proliferative drugs like GEM and 5-FU have tumouricidal effects in addition to MDSC cytotoxicity, which complicates studying the process of tumour metastasis. As a result, we wanted to see if a single dose of GEM or 5-FU was able to reduce MDSC numbers in the spleen and lungs of 4T1 tumour-bearing animals, and determine how long the effects last. We did the same experiment with TPZ and based our doses on our preliminary unpublished studies. We found that GEM was the fastest acting of the 3 chemotherapeutics, significantly decreasing MDSC numbers in both the spleen and lungs 24-hours after administration. 5-FU had a slightly stronger effect on decreasing MDSC numbers; however the effect was not seen until 3 days post administration. Due to the previously reported slower drug kinetics of 5-FU over GEM, we decided to allow the experiment to go 9 days post drug treatment whereas GEM treated mice were euthanized 4 days post drug administration. Similarly to 5-FU, TPZ did not have significant effects on MDSC numbers until 3 days post administration and only at the higher dose. Our GEM data nicely illustrate the difference in MDSC re-population dynamics of spleen and lung tissue. We show that 4 days post drug treatment, MDSC levels were back to pre-treatment levels in the spleen, yet in the lungs they remained significantly reduced in number. This can be explained by the observation that MDSC proliferate and expand in the spleen and accumulate in the lungs through peripheral blood. All 3 drugs caused a reversion of splenomegaly in the tumour bearing animals without having a significant effect on age-matched tumour weights.

As mentioned earlier, using anti-proliferative drugs to target a specific cell population can be challenging as the drugs have the potential to affect all proliferating cells. Studies have theorized on why GEM and 5-FU show MDSC specificity [98, 132] but the question of how the MDSC reduction is attained remained to be answered; the theories mainly deal with differential expression of certain enzymes (ex. deoxycytidine kinase) in MDSCs versus other lymphoid and tumour cells [140]. Additionally, Vincent et al postulate that MDSCs express a lower level of thymidylate synthase as compared to tumour cells and other immune cells. This enzyme is a
critical enzyme in cell replication and MDSCs low expression of it makes it more susceptible to antimitabolites. With GEM and 5-FU being nucleoside analogues, we needed to determine if MDSC reduction in the spleen was due to direct cell kill or simply a cell cycle block. Based on the cell cycle analysis of our drug treated spleen and lung samples, we demonstrate that in the lungs, MDSC reduction is due to direct cell kill for all 3 drugs. On the other hand, GEM and 5-FU cause a G1 cell cycle block in the spleens of the drug treated tumour-bearing animals whereas TPZ does not. Data demonstrates can be rationalized by the observation that myeloid cell proliferation occurs in the spleen and any effect of an anti-proliferative drug will be exaggerated in this tissue.

In our in vitro experiments, we wanted to determine how the 3 drugs affect cell cultures of 4T1 tumour cells and MDSCs. The best way to model the in vivo effects we observed in vitro is by utilizing ex vivo MDSCs obtained from tumour bearing mice. Ex vivo MDSCs are notoriously difficult to culture, and even under the most favourable conditions, 50% cell death 24-hour post isolation is expected [98]. After several failed attempts at growing MDSCs ex vivo we decided to utilize an immortalized MDSC cell line (MSC2) to carry out our in vitro studies. Our in vitro data demonstrates the preferential targeting of MSC2 cells over 4T1 tumour cells with 5-FU. This effect is lessened for GEM, with little difference existing between the drugs ability to target MSC2 cells over 4T1 cells. The results could partially be explained as MSC2 cell line is an immortalized MDSC cell line and could behave differently from ex vivo MDSCs. Most interestingly, the hypoxia activated drug TPZ showed stronger toxicity towards MSC2 cells that 4T1 tumour cells grown in 1% oxygen (hypoxia). Furthermore, TPZ’s ability to kill MSC2 cells was independent of the environmental oxygenation status, suggesting that its cytotoxic effects occur through an oxygen independent pathway. The TPZ in vitro data provide further evidence that TPZ targeting of MDSCs we observed in vivo is in fact specific and that TPZ needs to be considered as novel MDSC targeting agent.

The next step in our research led us to make a decision on which drug to choose to target the residual MDSCs in our surgery model (GEM, 5-FU or TPZ). Our findings that MDSCs remain in the lungs (metastatic target site) for at least 2 weeks after primary tumour removal (Figure 4.1B) led us to the idea of targeting the residual MDSC with a chemotherapeutic that is fast acting, specific, effective and fairly well characterized in the literature. Based on our in vivo, in vitro, and published data from other labs, we decided that GEM was the best candidate for our study as it has a half life of 16.8 minutes in mice. We required effective targeting of MDSCs to occur 24-hours post administration of a single dose as our experimental design allowed for
only 24-hours of recovery time before 4T1 tumour cell iv injection (Figure 4.9A). The reasoning behind using a fast acting drug is that we wanted to minimize the negative effects of the drug on 4T1 tumour cells being injected iv into the animals. We report that in the spleen, a single dose of GEM did not have a significant effect on targeting the residual splenic MDSCs after primary tumour resection. However, when we looked at the effect the single dose of GEM had on residual MDSC in the lungs, we saw a significant decrease in their number. It is important to note here that the lack of targeting of MDSCs in the spleen, and the modest but significant decrease of MDSCs in the lungs (when compared to MDSC depletion in Figure 4.3A-B) is not surprising considering the single dose of GEM is administered post- surgery when MDSC are already significantly depleted. What data demonstrates suggests, especially in the spleen, is the putative existence of multiple MDSC populations with varying sensitivities to chemotherapeutic and surgical approaches of depletion. Certain MDSC sub-types may be more sensitive to chemotherapeutic cell kill, while others may require the removal of the primary tumour to induce their demise. As previously discussed, granulocytic MDSCs are the variety that undergo extreme expansion under pathological conditions, however they are slightly less immunosuppressive on a per cell basis as compared to monocytic MDSC variety [79]. As our drug therapies are aimed at targeting the granulocytic MDSC population, monocytic MDSCs may still remain in the metastatic niche after treatment, still maintaining a certain level of immunosuppression. Our most promising and novel finding is that combining surgery with chemotherapeutic targeting of lung MDSC has a profound effect on the metastatic potential of 4T1 tumour cells. The decrease in MDSCs we report in Figure 4.9C between mice receiving or not receiving GEM (1.6-fold decrease in MDSC if GEM administered) is sufficient to bring about a 4.8-fold decrease in the metastatic potential of the iv injected 4T1 tumour cells in the clonogenics assay (Figure 4.9D). More importantly, targeting the GEM sensitive residual MDSC population in the lungs after surgery, we were able to decrease the metastatic potential of the 4T1 tumour cells down to control levels (control: injecting 4T1 cells into a naïve animal). It is important to note that residual MDSC (potentially monocytic MDSCs) have not completely been eradicated with GEM as can be seen in Figure 4.9D. We are very interested in further characterizing the CD11b*Gr1+ cells that escaped our bi-modal therapy and determine if they have the potential to re-generate the MDSC population that contributes to potentiating metastatic tumour growth.

Even though we report a significant decrease in metastasis after GEM treatment in tumour resected mice, it is difficult to draw a conclusion if the decrease is due to targeting of
MDSCs. GEM significantly reduced pulmonary MDSCs in resected mice, however, the decrease is disproportional to the effect we observed on metastasis. As mentioned earlier, one explanation could be that we successfully targeted the slightly less immunosuppressive subset of MDSCs with GEM which are responsible for modulating the immunosuppressive environment due to the profound accumulation of these cells. Due to the fact that we observed MDSCs in metastatic target organs and non-metastatic organs undermines the importance of MDSCs in the metastatic process. Based on our data, we can argue that pulmonary MDSCs contribute, at least in part, to a pre-metastatic niche. However, in other organs, such as the kidneys, MDSCs may not play a pro-metastatic role. Our data on targeting residual MDSCs with GEM also suggests that perhaps GEM has a direct effect on 4T1 cells ability to metastasize. As GEM is an antimetabolite, it is possible that GEM affected the ability of 4T1 cells that colonized the lungs (via iv injection) to proliferate and survive. It is difficult to say whether or not MDSCs are a good therapeutic target or if they are a red herring. The metastatic process is a complicated cascade which requires multiple players to interact in intricate way to create the right conditions for metastases. How this pre-metastatic niche forms and what the important factors are still remains to be elucidated. MDSCs correlation with poor outcome in mice and cancer patients does provide evidence for their monitoring and targeting. However, other cell types that have been shown to be more immunosuppressive (macrophages) or Tregs may be just as good at monitoring disease progression and evaluating the chance or metastatic reoccurrence. It is also important to note that the effects we observed in our study could be specific to the 4T1 model. In order for this to be validated, additional studies need to be carried out in other cell lines and mice of different background to make sure that the effects we report in our work is not model specific. Potential future directions are discussed in the following section.
CHAPTER 5: SUMMARY AND FUTURE DIRECTIONS

5.1 Summary of Research

Collectively, our studies demonstrate that metastatic primary tumours (4T1 and 4TO7) promote splenic expansion of MDSCs and systemic accumulation of MDSCs (via peripheral blood) in both metastatic and non-metastatic target tissues prior to tumour cell arrival. Conversely, non-metastatic 67NR primary tumours do not induce myeloid cell recruitment from the bone-marrow, indicating that tumour burden alone is not sufficient to induce myelopoiesis. The presence of MDSCs in non-metastatic organs indicates that MDSCs alone are not solely responsible for creating a microenvironment more permissive to metastatic disease. Factors such as tumour-secreted proteins, other myeloid cell types and lymphoid cells may play a critical role in determining metastatic tissue specificity along with MDSCs. The immunosuppressive environment that MDSCs create in metastatic target organs inhibits important T-cell mediated anti-tumoural responses leading to an altered immunological response. Additionally, we found that circulating MDSCs are an accurate and dependable surrogate for monitoring MDSC tissue levels.

Our surgery model shows that functional MDSCs remain associated with spleen and lung tissue after primary tumour resection, and are associated with enhanced growth of metastatic tumour cell foci. Additionally, removal of the primary 4T1 tumour decreases levels of G-CSF to naïve levels, yet the reduction in this MDSC stimulatory and stabilizing cytokine was not enough to cause complete MDSC depletion in the lungs and spleen. We utilized GEM, 5-FU and for the first time, TPZ, to target MDSCs in tumour-bearing mice. All 3 drugs showed significant cytotoxic effects towards MDSCs in the lungs and the spleen, however, as GEM was the fastest acting drug, it was the most suited chemotherapeutic for our research. Our study found that targeting of residual pulmonary MDSCs with GEM post primary tumour resection significantly decreases the metastatic potential of 4T1 tumour cells in this metastatic target site. The effect targeting residual MDSCs had on metastasis was larger in magnitude than the level of MDSC depletion achieved in the lungs. We believe that in our study, we targeted the granulocytic MDSC population that is more susceptible to GEM, leaving behind a sub-population of MDSCs that is still potentially pro-metastatic.
Given the critical need for novel therapies to treat breast cancer metastasis, these findings highlight both granulocytic and monocytic MDSCs as a potential target for combination therapies. Specifically, the development of strategies that combine reduction of MDSCs in metastatic target organs and continual monitoring of MDSC populations in circulation before and after primary tumour removal shows great promise. Furthermore, therapeutic MDSC targeting is likely to enhance the effectiveness of immune-based therapies and lead to improved treatment of metastatic disease in the clinic.

5.2 Future Directions

Although immune evasion is emerging as a hallmark of cancer [7], there is a distinct lack of immunological biomarkers associated with prognosis or clinical outcome for breast cancer. Given our findings that the induction and accumulation of MDSCs are associated with enhanced metastatic growth, increased MDSC levels in breast cancer patients may represent useful prognostic indicators of metastatic disease. Moreover, circulating MDSC levels may be useful for screening and monitoring purposes, both before and after treatment. Indeed, elevated MDSC levels are observable in the peripheral blood of patients with metastatic cancer [82, 141], and increased MDSCs in the circulation of breast cancer patients correlates with clinical stage and decreased survival [142]. These studies could be carried out using GEM in the 4T1 model with circulatory MDSCs providing information on MDSC tissue accumulation and drug targeting efficiency.

Our findings indicate that primary tumours capable of metastasis induce MDSC expansion and accumulation, and support the current interest in developing novel strategies to target MDSC accumulation and/or function. Targeting residual MDSCs with GEM showed that reducing MDSCs in the metastatic site can decrease the metastatic potential of 4T1 tumour cells injected iv. In order to fortify our findings, this study needs to be repeated with a larger sample size in the control and experimental groups. Additionally, administering GEM into a non-tumour bearing animal, followed by 4T1 iv tumour cell injection will shine additional light on the specificity of GEM as an MDSC preferential chemotherapeutic. Additionally, anti-G-CSF antibodies could be utilized to reduce MDSC expansion in 4T1 tumour bearing mice and the
effects of GEM in this model could be evaluated. Gr-1 neutralizing antibody could also be administered to animals to determine if MDSCs in the lungs correlate with pulmonary metastatic growth. Furthermore, MDSC targeting effects of GEM, 5-FU and TPZ observed in our study need to be validated in other mouse strains and different tumour models.

Clinical studies have demonstrated the efficacy of pharmacological strategies to reduce MDSC number (i.e. sunitinib) [143], to inhibit MDSC suppressive function (i.e. sildenafil) [144], or to differentiate MDSCs into mature myeloid cells (i.e. ATRA or 25-hydroxyvitamin D₃) [96, 145, 146] in a variety of human cancers. In addition to our work, mouse models show that directly targeting MDSCs with 5-FU [132] or GEM [140] significantly enhances T-cell dependent anti-tumour immunity. However, it is not yet clear whether reducing MDSC accumulation and/or function improves patient outcome [146]. In particular, therapies that differentiate immature MDSCs may need to be used with caution as mature myeloid cells (i.e. dendritic cells, granulocytes, and macrophages) also possess pro-tumourigenic properties [105], including potent immune suppressive function [28]. The ability of TPZ, a hypoxia specific cytotoxic, to target MDSCs is a novel finding in our study, and its characterization as a myeloid specific chemotherapeutic requires further studies (experimenting in other myeloid cell lines, utilizing ex vivo MDCS for in vitro studies, performing mechanistic studies to determine how TPZ kills MSC2 cells equally well in 21% O₂ and 1% O₂. Taken together, these studies suggest that successful therapies will likely target multiple myeloid cell populations, and that further work is needed to address the clinical implications of targeting MDSCs in the treatment of metastatic disease.

A topic that our research only slightly touched on is that of primary tumour hypoxia. The link between myeloid cell recruitment from the bone-marrow and the hypoxic status of the primary tumour is a well-established correlation and one that deserves more attention. In our study, we utilize hypoxia markers to characterize the 3 sister, mammary carcinoma generating cell lines. While TPZ has fallen out of favour as a hypoxia specific cytotoxic for quite some time now (phase III trial found no evidence that addition of TPZ to treatment in patients with advanced head and neck cancer improved overall survival – patients were not selected for the presence of hypoxia in their tumours [147]) there are several next-generation agents available to preferentially kill hypoxic tumour cells, many of which (including PR-104 [148] and TH-302 [149]) have shown promising results in pre-clinical studies and in Phase I/II trials. Effective
targeting of primary tumour hypoxia and evaluation of its effect on MDSC recruitment, accumulation in tissues and effect on metastatic potential is a potentially exciting future area of research. Additionally, there are also a variety of clinically-tested methods to detect hypoxic cells in solid tumours, including physical probes that measure tumour $pO_2$ (oxygen content), immunohistochemical detection of endogenous hypoxia-induced proteins, or quantification of the uptake of compounds that bind in hypoxic cells and are detectable by immunohistochemistry, flow cytometry, or positron emission tomography [150]. Thus, several options are available to identify patients with hypoxic cells in their primary tumour prior to treatment which, when combined with quantification of MDSC levels in the peripheral circulation, could be potentially useful to identify patients at increased risk for developing metastatic disease. Based on the positive correlation between primary tumour hypoxia, MDSC recruitment, and potentiation of metastasis by MDSCs, studies aimed at determining the efficacy of hypoxic cell-specific cytotoxins to reduce MDSC levels and metastatic tumour growth still needs to be evaluated. In order to gain a better understanding of the involvement of primary tumour hypoxia and MDSCs in the metastatic cascade, exciting research remains to be carried out in hypoxic pre-clinical tumour models or in patient populations selected based on the presence of hypoxic cells in their primary tumours.

Our work utilizes murine mammary carcinoma cell lines that are often used in metastatic research. We identify a clear induction of MDSCs in mice bearing metastatic tumours, which is consistent with many previously published studies. However, our work shows that MDSC accumulation is systemic and does not support the idea the MDSCs are required for the formation of the pre-metastatic niche. While in some organs, such as the lungs, MDSC play a tumour potentiating role, in other organs, such as the kidneys, they may simply act as spectator cells. This finding undermines the importance of MDSCs but does not discredit their tumour potentiating ability in certain tumour models, like the 4T1 model used in our study. We believe that interplay between TDSF, MDSCs, macrophages, Tregs and stromal cells is required for the development of the pre-metastatic niche. While our work demonstrates that circulating MDSCs are an accurate surrogate for tissue accumulation, its usefulness in the clinic remains to be determined. In order to gain a broader understanding of MDSCs as potentiators of metastatic tumour growth, additional studies need to be carried out, with the understanding that MDSCs play only one part in the cryptic metastatic cascade.
REFERENCES


