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The Role of Myeloid Cells in Breast Cancer Metastasis

Agnieszka Swierczak

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Department of Medicine (RMH/WH)
The University of Melbourne

Abstract

Metastasis is the leading cause of death in breast cancer patients. The current methods for predicting likelihood of metastasis are insufficient and treatment options are limited once distant metastasis occurs. Emerging data implicate myeloid cells as active participants in breast cancer metastasis. Myeloid cells in cancer are often studied as Gr-1^{+}CD11b^{+} myeloid derived suppressor cells. However, this group of cells is comprised of many different cell types, including macrophages and neutrophils. Tumour-associated macrophages (TAMs) can play key roles in metastatic events but little is known about the roles of monocytes, i.e. macrophage precursors, in metastasis, and it has been suggested that monocyte/macrophage subpopulations may have different functions in breast tumour metastasis. Recent data link tumour-associated neutrophils (TANs) to the progression of malignancy in other cancers, but the link between TANs and breast cancer metastasis has not been well studied.

This thesis is the first body of work to include a comprehensive analysis of monocytes/macrophages/TAMs and neutrophils/TANs during various stages of mammary tumour metastasis. Utilising three isogenic murine mammary tumour variants, namely 4T1.2 (highly metastatic), 66cl4 (weakly metastatic) and 67NR (non-metastatic), as well as the myeloid cell markers Ly6C (a marker for monocytes and neutrophils) and Ly6G (a marker for neutrophils), it was found that increases in both primary tumour Ly6C^{low} (non-classical, mature) TAM and TAN numbers, as well as that of circulating Ly6C^{high} (classical, immature) monocytes and neutrophils were associated with metastasis. Furthermore, increases in these cells occurred early during tumour progression. Pro-MMP-9 was increased in the serum around this time; hence a model is proposed where increased production of MMP-9, either from the tumour or from the surrounding stroma, enhances mobilisation of monocytes and neutrophils leading to increased TAM and TAN numbers that promote metastasis. Furthermore, preliminary data indicate that co-injection of either TAMs and TANs isolated from highly metastatic tumours may promote the growth of less metastatic tumours.

Colony stimulating factor-1 (CSF-1/M-CSF), a key macrophage growth factor, has been implicated in advanced breast cancer. Since inhibition of CSF-1 receptor (CSF-1R) signalling depletes TAMs and reduces metastasis in some mammary tumour models, CSF-1 and CSF-1R are considered targets for the treatment of metastatic disease. It is shown in this thesis, for the first time, that treatment of 4T1.2 tumour-bearing mice with neutralising anti-CSF-1R antibody can increase metastasis to lung and bone, without altering primary tumour growth. Furthermore, CSF-1R blockade leads to increased neutrophils in the primary tumour, metastasis-associated lung and peripheral blood, as well as to increased 'classical' Ly6C^{high} blood monocytes.

These data indicate that neutrophils can also play a critical role in 4T1.2 tumour metastasis. In support of this, it was found that increased expression of a key neutrophil CSF, G-CSF, in weakly metastatic primary tumours enhances circulating neutrophil and Ly6C^{high} monocytes and is associated with increased metastasis. Thus, cellular changes in neutrophils and Ly6C^{high} monocytes may be linked to metastasis and their subsequent increase upon anti-CSF-1R treatment may be related to the enhanced metastasis observed.

The above data indicate that both macrophages and neutrophils may be involved during the early stages of breast tumour metastasis. Further analysis of monocyte/macrophage/TAM subpopulations, as well as neutrophils/TANs, may lead to improved treatment of aggressive breast tumours. Targeting both macrophages and neutrophils may be required for successful treatment of certain breast tumour subtypes. It may also be important to target monocytes and neutrophils both in the circulation and at metastatic sites and, if possible, during early stages of metastasis. Stratification of patients based on primary tumour TAM or TAN numbers may better predict the likelihood of metastasis and also guide treatment strategies.

Declaration

This is to certify that:

(i) the thesis comprises only my original work towards the PhD except where indicated in the Preface,

(ii) due acknowledgement has been made in the text to all other material used,

(iii) the thesis is fewer than 100,000 words in length, exclusive of tables, maps, bibliographies

Signed _____ June 2012

Preface

The following experiments were contributed by collaborators:

Dr. Yuan Cao (Peter MacCallum Cancer Centre): Generation of G-CSF over-expressing 66cl4 and E0771 tumours and all the associated animal work as described in Chapter 5.

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Abbreviations

αMEM	Alpha-modified minimum essential medium
Ab(s)	Antibody(ies)
ANOVA	Analysis of variance
APC	Allophycocyanin (flow cytometry)
APC(s)	Antigen presenting cell(s)
APOC/E	Apolipoprotein C/E
ARG	Arginase
ATP	Adenosine triphosphate
bFGF	Basic fibroblast growth factor
BMDC(s)	Bone marrow derived cell(s)
BRCA 1/2	Breast cancer type 1/2 susceptibility gene
BSA	Bovine serum albumin
CCL	Chemokine (C-C motif) ligand
CCR	Chemokine (C-C motif) receptor
CD	Cluster of differentiation
cDNA	Complementary DNA
cm	Centimetre(s)
CSF-1	Colony stimulating factor-1
CSF-1R	Colony stimulating factor-1 receptor
Ct	Threshold cycle
ΔCt	Delta Ct
CX_3CR	Chemokine (CX_3C motif) receptor
CXCL	Chemokine (C-X-C motif) ligand
CXCR	Chemokine (C-X-C motif) receptor
DC(s)	Dendritic cell(s)
DCIS	Ductal carcinoma in situ
DMEM	Dulbecco’s modified eagle medium
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor

ER	Oestrogen receptor
FACS	Fluorescence-activated cell sorting
FITC	Fluorescein isothiocyanate (flow cytometry)
FSC	Forward scatter
g	Gram(s)
G-CSF	Granulocyte-colony stimulating factor
G-CSFR	Granulocyte-colony stimulating factor receptor
GM-CSF	Granulocyte-macrophage-colony stimulating factor
Gr-1	Granulocyte differentiation antigen-1
h	Hour(s)
H_2O_2	Hydrogen peroxide
HER2	Human epidermal growth factor receptor 2
HGFR	Hepatocyte growth factor receptor
HIF-1/2	Hypoxia-inducible factor 1/2
HNSCC	Head and neck squamous cell carcinoma
HPRT	Hypoxanthine phosphoribosyltransferase
HRP	Horseradish peroxidase
ICAM-1	Intercellular adhesion molecule-1
IFN α/β/γ	Interferon alpha/beta/gamma
Ig	Immunoglobulin(s)
IL	Interleukin
IL-(x)R	Interleukin (x) receptor
iNOS 1/2	Inducible nitric oxide synthase 1/2
int	Intermediate
KC	Keratinocyte-derived chemokine
LCIS	Lobular carcinoma in situ
lin	Lineage
LIX	LPS-induced CXC chemokine
LLC	Lewis lung carcinoma
LPS	Lipopolysaccharide
M	Molar
mAbs	Monoclonal antibodies
MCP-1	Monocyte chemotactic protein-1

M-CSF	Macrophage-colony stimulating factor
MDSC(s)	Myeloid derived suppressor cell(s)
MHC	Major histocompatibility complex
min	Minute(s)
miRNA	MicroRNA
mL	Millilitre(s)
mM	Millimolar
mm	Millimetre(s)
MMP	Matrixmetalloproteinase
MMTV	Mouse mammary tumour virus
MPO	Myeloperoxidase
NE	Neutrophil elastase
neg	Negative
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK cell(s)	Natural killer cell(s)
nmol	Nanomole(s)
NO	Nitric oxide
op/op	Osteopetrotic
PBMC(s)	Peripheral blood mononuclear cell(s)
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PE	R-phycoerythrin (flow cytometry)
PerCP	Peridinin-chlorophyll-protein complex (flow cytometry)
PR	Progesterone receptor
PyMT	Polyoma middle T-antigen
RANTES	Regulated upon activation, normal T-cell expressed, and secreted
RNA	Ribonucleic acid
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
ROS	Reactive oxygen species
rpm	Revolutions per minute
RT-qPCR	Real-time quantitative PCR

SDF	Stromal cell-derived factor
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
siRNA	Small interfering RNA
SNT	Supernatant
SSC	Side scatter
TAM	Tumour-associated macrophage
TAN	Tumour-associated neutrophil
TEN	Tumour-entrained neutrophil
TGF α/β	Transforming growth factor alpha/beta
TIE 1/2	Tyrosine kinase with Ig and EGF homology domains 1/2
TIMP	Tissue inhibitor of metalloproteinases
TN	Triple negative
TNFα	Tumour necrosis factor alpha
TP	Thymidine phosphorylase
Treg	Regulatory T cell
uPA	Urokinase-type plasminogen activator
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WT	Wild type
w/v	Weight per volume
zymo	Zymography
µg	Microgram(s)
µL	Microliter(s)
µM	Micromolar

1 Introduction: The role of myeloid cells in breast cancer metastasis

1.1 Introductory statement

Breast cancer is the most common invasive cancer and the foremost cause of cancer death in females [1]. Our inability to treat advanced disease effectively is the primary reason for this low survival rate. This is, in part, due to a poor understanding of the cellular and genetic components involved in the metastatic process. A more comprehensive understanding of these components, and the pathways in which they are involved, would greatly increase our ability treat advanced breast cancer.

The link between inflammation and cancer initiation is well established, but the link between inflammation and tumour metastasis is not yet clear. Myeloid cells have been implicated in tumour progression. In murine models of breast cancer, they are identified as Gr-1^{+}CD11b^{+} cells, and have been associated with early metastatic events [2, 3]. However, Gr-1 cells can be both macrophages and neutrophils. Thus, it remains unclear which cell types are important for metastasis. Tumour-associated macrophage (TAM)-mediated processes have been implicated in metastasis [4]. Hence, monocytes (macrophage precursors) may also contribute to disease progression. Recent studies also implicate neutrophils in tumour metastasis [5-8], although little is known about neutrophils in breast tumours. This project aims to investigate the contributions of macrophages and neutrophils to mammary tumour metastasis.

1.2 Breast cancer

Worldwide, breast cancer is the fifth most common cause of cancer death [9]. Metastasis is the main cause of mortality, accounting for 90% of fatalities [10]. The aetiology of breast cancer is not yet completely established; obesity, alcohol consumption, and cigarette smoking have been identified as risk factors. However, factors that most strongly associate with breast cancer risk include nulliparity, age and breast density [11]. The dominantly inherited BRCA 1 and BRCA 2 genes have long been established as susceptibility genes for breast cancer and contribute to familial breast cancer [12].

Breast cancer is an adenocarcinoma that occurs as either ductal carcinoma or lobular carcinoma. The TNM system defined by the American Joint Committee on Cancer forms the basis for breast cancer staging. This system takes into account the tumour size (T), the extent of regional lymph node involvement (N), and the presence or absence of metastasis beyond the regional lymph nodes (M) [13].There are five stages that are summarised in Table 1.1.

Table 1.1: Staging of breast tumours according to the American Joint Committee on Cancer TNM classification

Stage	**Tumour size (T)**	**Regional lymph node status (N)**	**Distant metastasis (M)**
0	Carcinoma in situ (DCIS, LCIS or Pagets)	No involvement	No
I	Tumour ≤ 2 cm	No involvement	No
IIA	No primary tumour	1-3 nodes involved	No
	Tumour ≤ 2 cm	1-3 nodes involved	No
	Tumour > 2 cm but ≤ 5 cm	No involvement	No
IIB	Tumour > 2 cm but ≤ 5 cm	1-3 nodes involved	No
	Tumour > 5 cm	No involvement	No
IIIA	No primary tumour	4-9 nodes involved	No
	Tumour ≤ 2 cm	4-9 nodes involved	No
	Tumour > 2 cm but ≤ 5 cm	4-9 nodes involved	No
	Tumour > 5 cm	1-3 nodes involved	No
	Tumour > 5 cm	4-10 nodes involved	No
IIIB	Any size with invasion into chest wall or skin	No involvement	No
	Any size with invasion into chest wall or skin	1-3 nodes involved	No
	Any size with invasion into chest wall or skin	4-10 nodes involved	No
IIIC	Any designation	> 10 nodes involved	No
IV	Any designation	Any designation	Yes

1.2.1 Breast cancer subtypes

Tumour-intrinsic features, including classical histology, genetic alterations and molecular changes have been used to define breast tumour subgroups. Breast cancers are more classically subdivided by oestrogen receptor (ER), progesterone receptor (PR) and human epidermal receptor 2 (HER2) status, as determined by immunohistochemistry. Combinations of these markers allow for consignment of individual cases to different categories, specifically: ER^+ ($ER^+/HER2^{neg}$), $HER2^+$ ($ER^{neg}HER2^+$), triple negative (TN; $ER^{neg}PR^{neg}HER2^{neg}$) and triple positive ($ER^+PR^+HER2^+$) [14]. ER^+ tumours are generally associated with good clinical outcome [15-17]. HER2 gene amplification and protein overexpression are predictors of poor outcome in breast cancer [18-20] and often indicate resistance to treatment [21, 22], although new therapies have improved prognosis for $HER2^+$ tumours [23-27]. TN tumours have the worst prognosis of the breast tumour subtypes and provide the greatest treatment challenge [28-30]. More recent markers used in classifying breast tumour subtypes include Ki-67 [31, 32], topoisomerase II alpha (TOP2A) [33, 34], B-cell lymphoma 2 (Bcl2) [35, 36] and protein/tumour protein 53 (p53) [37].

The introduction of high-throughput platforms for gene expression analysis has led to the opinion that breast cancer should be classified at the genetic level [38-41]. Studies have revealed that breast cancer is made up of an assortment of diseases that have different risk factors, clinical presentation, histopathological features, outcome and response to treatment [14, 42]. Seminal studies by Perou-Sorlie et al. subclassified breast cancers based on gene expression profiling into luminal A, luminal B, triple negative/basal like and HER2 subtypes [14]. However, the authors then went on to show that the luminal epithelial/ER^+ group could be divided into at least two subgroups, each with a distinctive gene expression profile [40]. Other studies have also shown that breast tumours may overlap between subtype classifications [43, 44] and it has been proposed that a broader subclassification is required to better diagnose and treat this disease [45].

Although genetic profiling of breast cancer has yielded a considerable amount of information, the prognostic and therapeutic implications are limited. Tumour microenvironmental factors, such as immunopathology and stromal content, have also

been suggested to be useful markers in breast tumour classification [46-48]. Tumour stromal heterogeneity, such as differences in the extracellular matrix (ECM) and differences in immune cells, fibroblasts and endothelial cells, also plays an important role in tumour progression [49]. Identification of new markers expressed in tumour-associated stromal and immune cells may help to better predict which tumours are likely to respond to which treatments, and possibly lead to improved breast cancer therapies.

1.3 Breast cancer metastasis

Metastasis is the foremost cause of death in cancer patients [50-52]. Established metastasis at diagnosis occurs in 15-20% of breast cancer patients and drastically affects survival rates. If the cancer is contained in the breast there is a 90% chance of survival. However, this falls to 20% if the cancer has spread [1]. Metastasis occurs via a sequential, multi-step process. First the tumour cell must detach from the surrounding ECM and intravasate from the primary site. Once in the circulation, the cancer must survive shear forces and destruction by circulating immune cells, extravasate into the secondary organ, and finally survive and grow in the new microenvironment [50, 53]. Each step of metastasis can be rate-determining [54, 55], for example, apoptosis of cancer cells in the circulation or senescence at metastatic sites inhibits subsequent growth of the majority of tumour cells that successfully lodge in a tissue [56, 57]. Furthermore, tumour cells can lie dormant for up to twenty years in breast cancer before growing into clinically detectable tumours [58].

The mechanisms of metastatic lesion development from primary tumours are under dispute [59]. Two main doctrines have been proposed. The linear model suggests that metastasis occurs late in disease progression. Tumour cells undergo consecutive rounds of mutation and selection [60] that result in a heterogeneous population of tumour cells containing a subset of clones with the necessary alterations required for metastasis [61]. Seminal studies by Fidler et al. showed that only a subset of malignant cells within a heterogeneous primary tumour was capable of metastasis [62]. Recently, genomic sequencing of a primary basal-like breast tumour revealed that a subset of primary tumour cells were enriched in the resulting brain metastases [63].

The parallel progression model suggests that malignant mutations are acquired primarily at secondary sites. In this model, tumour cells disseminate early during tumour progression to metastasis and acquire the necessary mutations required for survival and growth as a result of microenvironmental pressures at metastatic sites. Thus, they adapt specifically to the secondary environment [64]. In support of this, genomic analyses show discrepancies not only between the primary tumour and metastases, but also between different metastases from one patient [65, 66]. Comparison of primary tumour and metastatic lesion growth rates indicates that metastases are too large to be occurring in later stages of progression to malignancy [67, 68] and early systemic spread has been reported in transgenic murine mammary tumour models [69]. It is now evident that seeding of metastatic sites can occur before detection of primary tumours [58]. However, it remains unclear how metastases relate to primary tumours as there are data to support both the linear and parallel models of progression.

Seeding can occur in multiple organs, but metastatic growth occurs only in a few sites [69] and different tumours display characteristic patterns of secondary organ metastasis [50, 70]. In advanced breast cancer, tumours metastasise to bone, lung, lymph node, brain and liver [57, 70]. It was originally believed that metastasising tumour cells were directed by the circulatory and lymphatic systems and would lodge upon reaching smaller vessels [71, 72]. However, Paget's 'seed and soil' hypothesis dramatically changed how pathway of metastatic dissemination is viewed. Paget's hypothesis states that the tumour cells, or 'seeds', are systemically distributed but only grow in permissive environments, or 'soils'[73]. Fidler's research was the first to demonstrate that tumour cells disseminate to many organs, but only grow in some [74]. Since then, the pre-metastatic niche theory has been proposed. It states that the primary tumour influences sites of future metastasis to create a permissive growth environment prior to the arrival of circulating tumour cells [75].

Epithelial to mesenchymal transition (EMT) is a biological process that typically occurs during embryogenesis, organ development and wound healing [76]. However, EMT has also been proposed to be a key mechanism in the acquisition of an invasive phenotype by epithelial cancer cells, such as breast tumour cells [77]. Such 'invasiveness' precedes, and is essential for, metastatic dissemination of tumour cells [77]. During EMT, a polarised epithelial cell undergoes biochemical changes resulting in the

transition to a mesenchymal phenotype. This includes increased migratory capacity, increased invasiveness, resistance to apoptosis, and increased production of ECM components [78]. The final step of the EMT process is the degradation of the underlying basement membrane and migration of the cell away from the epithelial layer from which it originated.

EMT allows for tumour cell phenotypic plasticity which is emphasised by the occurrence of the reverse process; mesenchymal to epithelial transition (MET). Circulating tumour cells must revert from their mesenchymal phenotype to that of an epithelial one for successful metastatic growth to occur [79]. In carcinomas EMT is thought to be induced via signals originating from the surrounding stroma. For example, HGF, EGF, PDGF and TGF-β have been implicated in the induction and/or activation of a number of EMT-inducing transcription factors, namely Snail, Slug, Twist, Goosecoid and FOXC2 [77, 80-84]. MET is also thought to be induced by the local environment at the metastatic site [77].

The microenvironment of the primary tumour has been better characterised than that of metastatic lesions. However, both contain non-malignant stromal and immune cells, soluble factors, blood vessels and the ECM [85-87]. Inflammation has been associated with metastasis [88-90] and inflammatory chemokines have been implicated in recruiting circulating tumour cells and myeloid cells to metastatic sites [91]. A growing body of evidence implicates myeloid cells in metastatic events. In murine models of Lewis lung carcinoma and melanoma, tumour-derived vascular endothelial growth factor (VEGF)-A, transforming growth factor (TGF)-β and tumour necrosis factor alpha (TNFα) recruited myeloid cells to lungs, but not other organs [91]. Bone marrow derived cells (BMDCs) expressing VEGFR1 have been shown to cluster in the lungs prior to the arrival of tumour cells in mouse models of Lewis lung carcinoma and melanoma [92]. These myeloid cells, in conjunction with stromal cells, secrete chemokines and cytokines, growth factors, matrix degrading enzymes and adhesion molecules, all of which contribute to metastatic tumour growth [91-93]. Furthermore, tumour cells appear to localise to clusters of myeloid cells in metastasis-associated lungs [92, 94]. However, there is still much we do not know about the individual myeloid cell populations that are involved in metastasis.

1.4 Tumour microenvironment

Random mutations alone cannot account for the heterogeneity of tumours. Tumour progression is influenced immensely by extracellular signals from the surrounding environment [95]. The tumour microenvironment is highly complex, varies between tumour types and disease stages, and consists of many cell types, factors and signalling pathways, some of which can have opposing roles on tumour progression. Hanahan and Weinberg proposed that cancer cells must acquire six essential adaptations to progress to malignancy; 1) self-sufficiency in growth signals, 2) insensitivity to anti-growth signals, 3) the ability to evade apoptosis, 4) limitless replicative potential, 5) the ability to induce and sustain angiogenesis and 6) the ability to invade and survive at secondary sites [96], although it is the ability to survive and invade at secondary sites that ultimately distinguishes malignant from benign tumours. Recently, these authors have described tumour-promoting inflammation as an 'enabling characteristic' that supports the acquisition of these six hallmarks [51].

The extracellular signals that influence tumour progression include cytokines [97], chemokines [98], ECM molecules [99], and adhesion molecules [100]. These factors form a signalling network that can either promote or arrest tumour development. These signalling networks affect cellular processes such as angiogenesis, inflammation and recruitment of circulating cells, as well as the type of immune response generated. Solid tumours are thus comprised of a unique microenvironment made up of malignant, stromal and inflammatory cells. Although stromal and inflammatory cells have been overlooked in the past, they are now known to be important in tumour progression [101].

1.4.1 Stromal cells in the tumour microenvironment

Stromal cells associated with tumours include adipocytes, fibroblasts and endothelial cells, as well as inflammatory cells. Just as these non-malignant cells can influence the neoplastic phenotype of the tumour, they too can be influenced by the tumour. Stromal cells are involved in a number of steps in tumour progression. Tumours recruit endothelial cells to facilitate vascularisation [102]. This allows tumours access to unlimited nutrient and oxygen supplies. Tumour cells also induce fibroblasts to express

angiogenic factors that promote growth of new blood vessels [103]. Cancer-associated fibroblasts can also degrade the ECM, releasing sequestered growth and angiogenic factors that promote tumour progression [104, 105]. Adipocytes induce anti-apoptotic transcriptional programs in tumour cells [106], and increase tumour cell invasion [107]. Recent work has focused on the role of inflammatory cells in breast tumours as it has emerged that macrophages are key for progression to malignancy. Furthermore, neutrophils have now also been implicated as active participants in breast tumour growth and metastasis.

1.4.2 Inflammation and tumours

In 1863, Rudolf Virchow first proposed a link between inflammation and cancer after noticing infiltrating leukocytes in malignant tissues [108]. Since then, studies have shown that chronic inflammation predisposes to many cancers, such as gastric [109], colon [110], oesophageal [111], pancreatic [112] and cervical cancer [113]. Inflammation is present in the microenvironment of most animal and human tumours, generally from the earliest stages of development. Cancer-associated inflammation encompasses a number of components and processes that include production of cytokines and chemokines, tissue remodelling, angiogenesis and infiltration of leukocytes.

1.5 Tumour-associated myeloid cells

Tumour-associated leukocytes include macrophages, dendritic cells (DCs), natural killer (NK) cells, neutrophils and mast cells. Tumours manipulate the host immune system to evade destruction [114], and then utilise this immune system to facilitate their own growth and progression to malignancy [115]. Macrophages, neutrophils and mast cells are the most prominent inflammatory cell populations found in tumours. Mast cells have been shown to play dual roles in cancer, affecting tumour growth and progression both positively and negatively [116]. In breast cancer, the presence of mast cells tends to be associated with improved prognosis [117, 118]. DCs, on the other hand, are modified by breast tumours to suppress anti-tumour immunity [119, 120]. In an experimental model of metastasis, DCs were implicated in promoting 4T1 mammary tumour growth by

skewing the immune response from Th1 to Th2 [121]. In human breast cancer DCs are thought to stimulate regulatory T cell (Treg) activation and expansion resulting in suppression of anti-tumour responses [122, 123]. Much research now focuses on developing DC-based therapies that aim to enhance anti-tumour immunity [124-128]. Breast tumours also suppress NK cell cytotoxic functions [129-131] and, as for DCs, emerging studies are looking at simulating NK cell anti-tumour activity as an adjuvant therapy in combination with existing treatments, in particular at enhancing trastuzumab-mediated antibody dependent cell mediated cytotoxicity (ADCC) [132, 133]. Conversely, TAMs can comprise up to 50% of the tumour mass [134] and have been shown to play a number of key roles in breast tumour progression, with many therapies now focusing on inhibition of their functions [135-137]. Furthermore, emerging data also implicate neutrophils as key players in tumour growth and development [8, 138-141].

1.5.1 Macrophages

Macrophages are phagocytic leukocytes that regulate innate immune responses. Macrophages are made up of a number of subpopulations that are defined by anatomical location and phenotype, including specialised macrophages such as osteoclasts (bone), alveolar macrophages (lung), Kupffer cells (liver), marginal zone macrophages (spleen) and microglia (brain) [142]. Due to their anatomical diversity and their multiplicity of roles during host responses to insults, macrophages display a phenotypic spectrum of which ‘classically activated’ macrophages (M1-like) and ‘alternatively activated’ macrophages (M2-like) are the extremes [143-146]. Macrophages are phenotypically flexible and can switch from one functional phenotype to another in response to different microenvironmental signals [147-149].

Given this phenotypic plasticity, macrophages can be indicators of either a positive or negative prognosis in cancer. High macrophage infiltration in stomach cancer results in a favourable prognosis [150], whereas there is conflicting evidence for their role in prostate, lung and brain tumours [151]. In bladder, oesophageal and breast cancer, high numbers of macrophages correlate with a poor prognosis [152].

1.5.1.a Macrophage phenotypes

The different macrophage phenotypes are associated with the secretion of a variety of factors that can control cell recruitment, survival, differentiation and the vast majority of cellular functions. These factors include cytokines and chemokines, proteases and growth factors. A summary of key macrophage factors, their functions and their roles in breast cancer can be found in Table 1.2.

Macrophage phenotypes *in vitro* have been classified as M1 and M2, although these two phenotypes represent the extremes of a phenotypic spectrum. *In vivo* M1-like macrophages are the first macrophages to appear following tissue injury or infection. They arise from monocytes exposed to bacterial products, such as lipopolysaccharide (LPS), granulocyte-macrophage-colony stimulating factor (GM-CSF) and interferon gamma (IFNγ) [143, 153, 154], and are typically 'pro-inflammatory.' M1-like macrophages are characterised by high secretion of IL-12 and IL-23 pro-inflammatory cytokines that activate a polarised type I T cell response [145, 155]. They also produce reactive oxygen and nitrogen intermediates, such as nitric oxide (NO) and superoxide, are cytotoxic, and have a high capacity to present antigens [143].

M2-like macrophages arise from monocytes exposed to colony stimulating factor-1 (CSF-1), IL-4, IL-13 and IL-10 [153, 154, 156]. These macrophages suppress inflammation elicited by M1-like macrophages and initiate wound repair [157, 158] which is critical for the restoration of homeostasis [159, 160]. In accordance with their trophic functions, M2-like macrophages display high levels of scavenger, mannose and galactose type receptors [154]. They suppress immune responses by secretion of anti-inflammatory mediators such as IL-10 and arginase (ARG)-1 [161-163], and by induction of regulatory T cells (Tregs) [164]. M2-like macrophages phagocytose dead cells, debris and various ECM components, and are involved in scavenging [165], immune suppression [164], matrix remodelling [166] and angiogenesis [167]. The M1 and M2-like macrophage phenotypes are summarised in Figure 1.1. It has been suggested that M2 macrophages can be further subdivided into M2a (after exposure to IL-4 or -13), M2b (after exposure to immune complexes along with IL-1β or LPS) and M2c (after exposure to IL-10, TGFβ or glucocorticoids) [168] phenotypes.

1.5.1 Neutrophils

Neutrophils are short-lived leukocytes derived from bone marrow myeloid precursors. They are the most abundant immune cell type in the circulation and are typically the first effector cells to arrive at sites of inflammation. Neutrophils contain a number of granules that house a battery of proteins, proteases, antibiotics and reactive oxygen species. These include antimicrobial proteins, (e.g. lactoferrin and lysozyme), matrixmetalloproteinases (e.g. MMP-8,-9 and -25), serine proteases (e.g. cathepsin G and neutrophil elastase; NE) and myeloperoxidase (MPO). All of these factors, and others, allow neutrophils to migrate to sites of inflammation, destroy invading pathogens, stimulate angiogenesis and modulate inflammatory responses [169, 170]. When stimulated, they attach to the endothelium, secrete proteolytic enzymes, degrade and migrate through the basement membrane [171]. Once they arrive at sites of inflammation, neutrophils release a battery of cytokines and chemokines [172]. Any of these processes can be hijacked by the tumour to promote growth and invasion. A summary of key factors, their functions and their roles in breast cancer can be found in Table 1.2.

Table 1.2: Key macrophage and neutrophil factors, their function and role in breast cancer

Factor	Function	Role in breast cancer
IL-12	Pro-inflammatory cytokine	-Stimulates T and NK cell cytotoxicity [173, 174] -Suppresses metastasis [175]
IL-10	Anti-inflammatory cytokine	-Suppresses T cell responses [176]. -Increased serum levels associate with poor prognosis [177]
IL-23	Pro-inflammatory cytokine	-Increased serum levels associate with poor prognosis [178]
IL-1	Pro-inflammatory cytokine	- Promotes angiogenesis and tumour cell invasion [179] - Recruits pro-angiogenic neutrophils and macrophages to tumours [180]

Table 1.2 continued on next page

Table 1.2 continued

IL-6	Recruits leukocytes to inflammatory sites	-Can block DC differentiation [181] -Increased serum levels associate with poor prognosis [182]
TNFα	Regulates inflammation	-Promotes tumour invasion [183] -Increased serum levels associate with poor prognosis [184]
TGFβ	Regulates immune cell functions	-Can suppresses [185], or promote [186] metastasis
IFNγ	Regulates inflammation	-Suppresses tumour cell proliferation [187] -Suppresses metastasis [188]
Nitric oxide	Cytotoxic	-Suppresses tumour growth [189] -Suppresses metastasis [190]
Arginase (I and II)	Convert L-Arg to urea and L-Orn	-Inhibit anti-tumour T cell responses [191]
ROS	Reactive oxygen species	-Increase DNA mutations [192] -Inhibit anti-tumour T cell responses [193]
VEGF	Pro-angiogenic factor	-Promotes new blood vessel growth in primary tumours [194] and metastases [92]
EGF	Growth factor	-Promotes tumour cell growth/survival [195] -Promotes co-migration of TAMs and tumour cells [196]
CXCL12 and CXCR4	Chemokines for myeloid cells	-Promote directional metastasis [197]
CSF-1	Macrophage factor	-Recruits and polarises TAMs [198] -Promotes metastasis [136, 199]
G-CSF	Granulocyte-stimulating factor	-Recruits neutrophils [8, 200] -Can promote [8] or suppress [201] metastasis
CCL2 (MCP-1)	Monocyte chemoattractant	Promotes metastasis [202]

Table 1.2 continued on next page

Table 1.2 continued

MMP-9 and MMP-2	Degrade the basement membrane	-Promote tumour growth, angiogenesis and invasion [203] -Promote metastasis [204]
NE	Neutrophil-derived protease	-Promotes tumour cell invasion [205]

1.6 Myeloid derived suppressor cells and Gr-1

Studies investigating the role of inflammatory cells in cancer have been impeded by confusion regarding the heterogeneity of the myeloid-derived suppressor cells (MDSCs). The term 'myeloid-derived suppressor cell' was proposed by Gabrilovich [206] and describes a heterogeneous population composed of immature macrophages, granulocytes, DCs and other myeloid cells at earlier stages of differentiation [206-208]. MDSCs have been reported to promote tumour growth and progression primarily through suppression of anti-tumour T cell responses and Treg expansion [191, 209]. However, these functions have also been described individually for tumour-associated neutrophils (TANs), TAMs and even monocytes [193, 210, 211]. In fact, two studies that have reported MDSC-mediated expansion of Tregs were in fact analysing monocyte/macrophage-mediated expansion of Tregs instead of that due to MDSCs [212, 213].

Murine MDSCs can be identified by their expression of Gr-1 and CD11b and lack of, or reduced expression, of mature myeloid cell markers [206]. Human MDSCs are not well characterised, but are thought to be MHC class II^{low} $CD33^{+}CD11b^{+}CD14^{neg}CD15^{+}/lin^{neg}$ [214]. $Gr\text{-}1^{+}CD11b^{+}$ cells form clusters in the lung prior to the arrival of tumour cells [3] and increases in $Gr\text{-}1^{+}CD11b^{+}$ cells have been reported in the spleen [215], bone marrow [216] and circulation [217] of tumour-bearing mice. $Gr\text{-}1^{+}CD11b^{+}$ cells, when isolated from tumours and spleens of metastatic tumour-bearing mice and co-injected with 4T1 tumour cells, increase metastasis to lung, unlike $Gr\text{-}1^{+}CD11b^{+}$ cells from normal spleens co-injected with 4T1 tumour cells [185]. The authors found that MMP-14, MMP-13 and MMP-2 gene expression and activity were elevated in $Gr\text{-}1^{+}CD11b^{+}$ cells isolated from tumours compared to $Gr\text{-}1^{+}CD11b^{+}$ cells isolated from the spleens of non-tumour bearing mice. Furthermore 4T1 cells seeded with tumour-derived Gr-

1^+CD11b$^+$ cells were more invasive *in vitro* and this increased invasion could be reduced by a broad spectrum MMP inhibitor. [185]. Thus, the authors concluded that the Gr-1^+CD11b$^+$ cells were influenced by the tumour microenvironment to increase MMP production and consequently promote tumour cell invasion and metastasis [185]. However, Gr-1^+ cells are not always considered MDSCs and are often described as neutrophils [218, 219] or monocytes [220]. In fact, Gr-1 does not distinguish between neutrophils and monocytes/macrophages.

The use of Gr-1 has led to the investigation of MDSCs as a discrete cell 'population' when in reality the MDSC classification clusters a number of different cell types together. In tumours, three distinct cell populations have been found within Gr-1^+CD11b$^+$ cells, namely, SSC^{high}CD11b$^+$Gr-$1^{neg/int}$ cells eosinophil-like cells, SSC^{low}CD11b$^+$Gr-1^{high} cells with neutrophil-like morphology and SSC^{low}CD11b$^+$Gr-$1^{int/low}$ cells which also expressed F4/80 and Ly6C, indicating that they are macrophages [218]. MDSCs are also highly plastic and can become adherent, F4/80$^+$, macrophage-like cells [215, 221], or, alternatively, differentiate into DCs when exposed to GM-CSF and IL-4 [208]. They have even been shown to differentiate into endothelial cells in tumours [222]. Hence tumour-driven functional changes in the diverse cell types that make up this 'population' may have been confused and/or overlooked.

Given the large number of different cell types that make up MDSCs, the different roles that they play in immunity, and the plasticity they can display phenotypically, it is important for future cancer therapies that we begin to distinguish between the cells that make up this 'population.' Clarification of how monocytes/macrophages and neutrophils are involved in tumour progression independently of each other, as well as how they are related in terms of phenotype and function is required. More recently, analyses of myeloid cells in murine cancer models have utilised antibodies against Ly6C and Ly6G, which allows for separation of neutrophils from monocytes/macrophages [8, 201, 223-225]. These markers have proven to be more specific in analysing monocytes/macrophages and neutrophils compared to Gr-1 [226], and recent studies using anti-Ly6G antibodies have shown that previously reported neutrophil functions in studies using Gr-1 antibodies need to be re-considered [227, 228].

1.7 Tumour-associated macrophages (TAMs)

Until the 1970s it was presumed that TAMs indicated a favourable prognosis in breast cancer [229]. However, it has since been shown that TAMs promote breast tumour growth [230-232], and high numbers of TAMs in breast tumours are now known to associate with poor prognosis [233-235]. Furthermore, genes associated with macrophage infiltration, such as CD68, have been shown to contribute to molecular signatures associated with worse disease outcome in breast cancer [236].

Tumour microenvironmental factors, such as CSF-1, TGFβ, IL-6 and IL-10, polarise TAMs towards an M2-like phenotype [231]. As a result of their trophic phenotype, TAMs are involved in a number of processes that promote tumour growth, such as ECM remodelling, angiogenesis and suppression of the immune response. A summary of TAM functions in the primary tumour microenvironment can be found in Figure 1.2.

1.7.1 TAM-derived growth factors

TAMs secrete various growth factors, such as platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), TGFα and β and EGF, all of which play a role in tumour cell survival and proliferation. bFGF and PDGF increase human mammary carcinoma cell proliferation *in vitro* [237, 238]. EGF is a key growth factor in breast cancer, not only promoting tumour cell proliferation, but also acting as a powerful chemoattractant of breast cancer cells [239] and TAMs are reported to be the major source of EGF in breast tumours [240].

1.7.2 TAM-mediated angiogenesis

Once primary tumours grow to 1-2 mm^2 in size, development of new blood vessels is required for acquisition of sufficient nutrient and oxygen supplies, without which the tumour becomes necrotic and dies [241]. An imbalance between positive and negative regulators of angiogenesis results in the induction of tumour vascularisation, a process termed the angiogenic switch [242].

The concept that TAMs are involved in angiogenesis was first proposed by Sunderkotter in 1991 [243]. Since then, TAMs have been shown to have potent angiogenic activity in tumours, secreting a number of pro-angiogenic factors such as VEGF [234], CCL2 [244], MMP-9 [137], and thymidine phosphorylase (TP) [245].

TAMs migrate into poorly vascularised, necrotic and hypoxic areas of the tumour where they become trapped [246]. This triggers upregulation of angiogenic factors by TAMs resulting in new blood vessel formation [247, 248]. This is thought to occur under the influence of the hypoxia inducible factor 2α (HIF-2α) [249]. In the MMTV-PyMT transgenic model, TAMs can mediate the angiogenic switch [250] and are recruited early in tumour development [199]. Depletion of TAMs reduces vascular density, whereas enhanced recruitment of TAMs accelerates tumour vascularisation [199].

1.7.3 TAM-mediated ECM degradation, migration and invasion

ECM degradation in cancer is a critical step that releases sequestered growth and pro-angiogenic factors [251], and allows for tumour cell escape into the vasculature [96]. TAMs produce a number of enzymes that regulate matrix digestion such as MMPs and urokinase-type plasminogen activator (uPA). In MMTV-PyMT mammary tumours, TAMs have been found in areas of basement membrane breakdown [199]. Co-culture of TAMs with breast cancer cell lines increases invasiveness of tumour cells *in vitro* and this is associated with upregulation of MMP-9 in TAMs. Blockade of MMP-9 abrogates this increased tumour cell invasion [252].

1.7.4 TAM-mediated suppression of anti-tumour immune responses

Through the secretion of anti-inflammatory cytokines, TAMs are able to suppress adaptive immunity, which allows tumours to escape immune-mediated destruction. TAMs secrete the anti-inflammatory cytokine, IL-10, that inhibits cytokine production by T cells and antigen presentation by macrophages and DCs [253]. IL-10 knock-out mice display an increased ability to reject bladder tumours and had a higher survival rate [254]. Furthermore, IL-10 has been shown to promote the differentiation of monocytes into TAMs and block their differentiation into DCs [255]. TAM expression of HIF-1α has also been implicated in suppression of anti-tumour T cell responses.

Deletion of HIF-1α in TAMs in a murine model of breast cancer reduced primary tumour growth. HIF-1α was shown to enhance suppression of anti-tumour T cell responses, and the absence of HIF-1α allowed for tumour cell destruction. [256].

1.7.5 TAM phenotypes differ between tumour regions

Recent gene profiling data have shown that, although TAMs express many genes associated with the M2 macrophage phenotype [225, 257, 258], they also express M1 phenotype-associated genes [225, 259]. This raises the possibility that there are distinct subpopulations of TAMs, with distinct functions [260]. In MMTV-PyMT tumours, higher numbers of TAMs were found at the margins of the tumour and numbers decreased throughout the stroma deeper in the tumour. In the centre of the tumour, TAMs were found in association with blood vessels [261]. This indicates that TAMs reside within different tumour regions, and may have specialised functions depending on the microenvironmental influences of each region. TAMs capable of promoting tumour cell invasion show different gene expression signatures to the general TAM population [262], suggesting that these 'invasive' TAMs may have a unique phenotype as a result of their role in promoting tumour cell escape into the vasculature.

TAMs from different regions of murine TS/A breast tumours also differed phenotypically [225]. The TS/A tumour cell line was derived from a spontaneously arising mammary adenocarcinoma and is metastatic to lung [263]. In this study two TAM subpopulations were identified based on MHC II expression: an MHC II^{high} subpopulation, that were more M1-like in phenotype, and an MHC II^{low} subpopulation, that were more M2-like in phenotype. Furthermore, MHC II^{low} TAMs were found to reside preferentially in hypoxic regions of tumours and had a higher pro-angiogenic activity, supporting the theory that TAM subpopulations within different tumour regions have specialised roles.

Two distinct TAM subpopulations were also found in a mouse model of hepatocellular carcinoma [264]. During the early phase of tumour development, an MHC II^{high} TAM subpopulation was associated with tumour suppression. However, an MHC II^{low} tumour-promoting TAM subpopulation became increasingly prominent later. Thus, it is possible that combinations of M1-like and M2-like phenotypes in TAM subpopulations are

required for tumour growth. This requires further investigation as it may be possible to individually target TAM subpopulations, without affecting other macrophage populations, for treatment of advanced breast cancer.

1.7.6 CSF-1

The colony stimulating factors (CSFs) are a group of haematopoietic growth factors that include CSF-1 (M-CSF), CSF-2 (GM-CSF) and CSF-3 (G-CSF) [265]. GM-CSF acts at earlier stages of cell lineage progression and is considered a factor in both macrophage and neutrophil differentiation. G-CSF acts predominantly as a neutrophil lineage factor and CSF-1 is a key factor in macrophage survival, proliferation, differentiation and recruitment [266, 267].

CSF-1 is constitutively produced by several cell types including fibroblasts, endothelial cells, stromal cells, smooth muscle cells, osteoblasts and macrophages [265]. CSF-1 exists in three major isoforms [268]: a circulating secreted glycoprotein, a circulating secreted proteoglycan and a membrane-spanning cell surface protein. The effects of CSF-1 are mediated by a high-affinity receptor tyrosine kinase [269, 270] encoded by the *c-fms* proto-oncogene [271, 272] (CSF-1 receptor; CSF-1R). CSF-1R is expressed primarily on mononuclear phagocytic cells and their precursors [273]. Binding of soluble CSF-1 to the CSF-1R activates downstream cytoplasmic signalling leading to survival, proliferation, differentiation and activation of macrophages [274, 275]. CSF-1 is also chemotactic for macrophages [276]. Recently, a new and independent ligand for the CSF-1R, interleukin-34 (IL-34), has been identified that can stimulate CSF-1R phosphorylation [277] but displays a different expression pattern to that of CSF-1 [278].

Csf1^{op}*/Csf1*^{op} mice have an inactivating mutation in the coding region of the CSF-1 gene and therefore lack CSF-1 activity [279]. These mice are toothless, have a low growth rate and body weight, and are deficient in certain populations of tissue macrophages [279-282]. Reconstitution of circulating CSF-1 levels in *Csf1*^{op}*/Csf1*^{op} mice corrected F4/80^{+} macrophage deficiencies in liver, bladder, salivary gland, kidney, dermis and synovial membrane. Macrophage deficiencies in the peritoneal cavity, adrenal gland, spleen and large intestine were only partially restored [282, 283], indicating that CSF-1 does not regulate all macrophage populations.

1.7.6.a CSF-1 in breast tumours

In breast tumours, both tumour cells and TAMs express CSF-1 [284]. Increased CSF-1R expression has been correlated with breast tumour grade and clinical stage [285], and circulating CSF-1 levels were found to be elevated in patients with aggressive or metastatic tumours [286]. High expression of CSF-1 in breast cancer correlates with poor prognosis [287] and increased numbers of TAMs [198].

The effect of CSF-1 on tumour growth and progression appears to be governed by which isoform of CSF-1 is expressed by tumour cells. *In vitro* studies have shown that glioma cells transfected with the membrane isoform of CSF-1 were killed upon co-culture with macrophages, whereas glioma cells transfected with the secreted isoform were not [288, 289]. Similar results have been seen with the MADB106 breast cancer cell line, where transfection with membrane CSF-1 resulted in rejection of tumours [290]. Thus, destruction of tumour cells appears to require cell-to-cell contact with macrophages mediated by the membrane isoform of CSF-1. The anti-tumour effects of the membrane form of CSF-1 can be abolished by saturation with recombinant CSF-1 [288]. Breast tumours primarily express the soluble isoform of CSF-1 [291].

Small molecule inhibitors of CSF-1R block receptor autophosphorylation and, as a result, CSF-1R downstream signals that control macrophage function. Treatment of human mammary xenografts (immunocompromised mice) with CSF-1 antisense oligonucleotides [292], anti-CSF-1 antibodies [293], or small interfering RNAs (siRNAs) [294] against CSF-1 or CSF-1R suppressed tumour growth. This effect on tumour growth was attributed to depletion of TAMs. In MMTV-PyMT mice crossed with $Csf1^{op}/Csf1^{op}$ mice, TAMs were unable to accumulate in the primary tumour, highlighting the importance of CSF-1 in recruiting TAMs to primary tumours [199]. Although tumour growth was not affected in $Csf1^{op}/Csf1^{op}$ mice, metastasis to lung was reduced. The role of CSF-1 in metastasis will be discussed later in this chapter (Section 1.10.3).

1.8 Monocytes

Monocytes are a heterogeneous population that represent 5-10% of peripheral blood mononuclear cells (PBMCs). They arise in the bone marrow, are released into the blood stream and enter tissues where they can give rise to macrophages or DCs [142, 295, 296]. The relationship between monocytes and DCs is beyond the scope of this chapter, but is reviewed thoroughly by Geissmann et al. [297].

1.8.1 Monocyte subpopulations

It was initially proposed by Geissmann et al. that monocytes can be grouped into two main subpopulations, an 'inflammatory' subpopulation, containing short-lived $CX_3CR1^{low}Gr\text{-}1^{+}$ monocytes that homed to inflamed tissue, and a 'resident' subpopulation, containing $CX_3CR1^{high}Gr\text{-}1^{neg}$ cells with a longer half-life that gave rise to resident myeloid cells in non-inflamed tissues [298]. Similar populations have been described in human monocytes: $CD14^{low}CD16^{+}CX_3CR1^{high}CCR2^{neg}CD62L^{neg}$ cells are morphologically similar to the 'resident' $CX_3CR1^{high}Gr\text{-}1^{neg}$ murine subpopulation, whereas $CD14^{+}CD16^{neg}CX_3CR1^{low}CCR2^{+}CD62L^{+}$ monocytes more closely resemble the 'inflammatory' $CX_3CR1^{low}Gr\text{-}1^{+}$ murine subpopulation [298]. $CD14^{low}CD16^{+}$ monocytes increase in number during infections [299, 300] and are considered pro-inflammatory in phenotype [301].

Subsequent studies have shown that the 'inflammatory' and 'resident' designations for monocyte subpopulations do not take into account the heterogeneity and phenotypic complexity of monocytes. The inflammatory subpopulation is now called 'classical' ($CD14^{high}CD16^{+}$ or $CX_3CR1^{low}Gr\text{-}1^{+}$), and the resident subpopulation is now called 'non-classical' ($CD14^{low}CD16^{+}$ or $CX_3CR1^{high}Gr\text{-}1^{neg}$) [302]. Murine monocytes express CD115 (CSF-1R) and CD11b and, as previously described, can be distinguished by levels of CX_3CR1 expression; however, they can also be distinguished by expression of the murine monocyte differentiation marker, Ly6C [303]. $Ly6C^{high}$ monocytes comprise the 'classical' subpopulation and $Ly6C^{low}$ monocytes the 'non-classical.'

1.8.2 Monocyte differentiation

Monocytes are thought to arise in the bone marrow as immature Ly6C^{high} cells and are released into the blood where it is thought that, as they mature, they downregulate Ly6C expression, becoming Ly6C^{low} monocytes. In support of this, a third subpopulation of monocytes with intermediate expression of Gr-1 exists, and in human monocytes an 'intermediate' CD14^{+}CD16^{+} population has also been identified [304] indicating a transition from Ly6C^{high} to Ly6C^{low} in the mouse and from CD14^{high} to CD14^{low} in humans. It has been suggested that Ly6C^{high} monocytes can move bi-directionally between blood and bone marrow [296, 305].

Adoptively transferred Gr-1^{high} (Ly6C^{high}) disappear from the blood in the absence of inflammation [298] indicating that they may be converting to Gr-1^{low}. Following depletion of all blood monocytes, the returning circulating monocytes were predominantly Gr-1^{high} and after 5 days converted to Gr-1^{low} [304]. In another study, monocytes initially labelled as Gr-1^{high} had fully converted to Gr-1^{low} after 7 days during steady state [306]. Injection of Gr-1^{high}/Ly6C^{high} monocytes into the femoral bone cavity resulted in the isolation of graft-derived Gr-1^{low}/Ly6C^{low} monocytes in both the bone and the blood of the recipient mice, implying that Ly6C^{high} monocytes act as precursors for Ly6C^{low} monocytes [305]. However, the conversion of Ly6C^{high} to Ly6C^{low} monocytes has been questioned [297]. Reduction of Gr-1^{+}/Ly6C^{high} monocytes does not always result in reduction of Gr-1$^{neg/low}$/Ly6C^{low} monocytes [307, 308] indicating that Ly6C^{high} do not always convert into Ly6C^{low}.

Ly6C^{high} monocytes are more migratory and typically recruited first to sites of tissue damage and inflammation, whereas Ly6C^{low} monocytes generally arrive later [298, 309]. Gr-1/Ly6C^{high} monocytes have been shown to be pro-inflammatory in nature [298, 304, 310-312]. However, increasing evidence indicates that Ly6C^{low} monocytes also have inflammatory properties and can secrete inflammatory cytokines [313]. A fraction of Ly6C^{low} monocytes has recently been shown to patrol blood vessel walls during the resting state and infiltrated inflammatory sites before Ly6C^{high} monocytes, where they initiated a macrophage differentiation program [313]. Ly6C^{high} monocytes infiltrated later and gave rise to inflammatory DCs [313].

In humans $CD14^{low}CD16^{+}$ monocytes were found to patrol the blood vessel endothelium after adoptive transfer into mice, and gene expression analysis revealed similarities with patrolling murine $Ly6C^{low}$ monocytes [314].

Conversely, in atherosclerotic lesions and liver injury, Gr-1/$Ly6C^{high}$ monocytes were recruited early and displayed pro-inflammatory functions [315, 316]. This was also seen in a model of myocardial infarction, where $Ly6C^{high}$ monocytes dominated the early phase of recruitment and displayed inflammatory, phagocytic and proteolytic functions, while $Ly6C^{low}$ monocytes infiltrated later and expressed VEGF and initiated wound healing [317]. In skeletal muscle injury, $Ly6C^{high}$ monocytes were recruited first but declined rapidly, whereas $Ly6C^{low}$ monocytes appeared later but steadily increased in number [318]. In this study, $Ly6C^{high}$ monocytes expressed pro-inflammatory cytokines, such as IL-1β and TNFα, whereas $Ly6C^{low}$ monocytes expressed anti-inflammatory cytokines, such as IL-10 and TGFβ [318]. Similar findings were also seen in a murine model of kidney injury, where it was proposed that $Ly6C^{high}$ monocytes converted into trophic $Ly6C^{low}$ monocytes [319].

$Ly6C^{high}$ monocytes were found to accumulate in the central nervous system prior to the onset of experimental autoimmune encephalomyelitis (EAE) and West Nile virus encephalitis [320, 321]. In the EAE model, these monocytes were pro-inflammatory in phenotype, secreting TNFα and producing inducible nitric oxide synthase (iNOS) [320]. $Ly6C^{low}$ monocytes upregulated IL-12, IL-23 and IL-6 gene expression in this model, indicating that they were also somewhat pro-inflammatory in phenotype, although they were far less numerous and not as thoroughly examined [320]. However, in another study, a subset of $Ly6C^{high}$ monocytes was shown to suppress T cell responses in EAE [322]. In spinal cord injury, adoptively transferred $Ly6C^{high}$ monocytes contributed to resolution of inflammation and initiation of tissue repair [323], whereas in this case, $Ly6C^{low}$ monocytes appeared to be pro-inflammatory in phenotype [324]. During hindlimb ischemia it was the $7/4^{high}$ cells (a marker of neutrophils and monocytes), most likely representing $Ly6C^{high}$ monocytes, that contributed to angiogenesis [325]. A summary of the reported $Ly6C^{high}$ and $Ly6C^{low}$ monocyte functions in various models of inflammation can be found in Table 1.3.

Given the above results, it is possible that Ly6C^{high} and Ly6C^{low} monocyte subpopulations are both capable of initiating and inhibiting inflammatory responses. A model of monocyte differentiation based on published studies is shown in Figure 1.3.

Table 1.3: Summary of monocyte subpopulation recruitment and function

Model of Inflammation	**Ly6C^{high} monocytes**		**Ly6C^{low} monocytes**	
	Recruitment	Function	Recruitment	Function
Atherosclerotic lesions [315]	Arrive first	Inflammatory	Not investigated	
Liver injury [316]	Arrive first	Inflammatory	Not investigated	
West Nile virus encephalitis [321]	Arrive first	Inflammatory	Not investigated	
Bacterial infection [313]	Arrive second	Inflammatory	Arrive first	Inflammatory
Myocardial infarction [317]	Arrive first	Inflammatory	Arrive second	Trophic
Skeletal muscle injury [318]	Arrive first	Inflammatory	Arrive second	Trophic
Kidney injury [319]	Arrive first	Inflammatory	Arrive second	Trophic
EAE [320]	Possibly arrive first	Trophic and inflammatory	Not investigated	Inflammatory
Spinal cord injury [323]	Not investigated	Trophic	Not investigated	Inflammatory
Hindlimb ischemia [325]	Arrive first	Trophic	Arrive second	Not investigated

1.8.3 Monocytes in cancer

Blood monocytes increase in patients with metastatic tumours compared to normal individuals [326] and, in particular, the CD14^{low}CD16^{+} (non-classical) subpopulation is elevated in gastrointestinal carcinoma patients [327]. However, it is not clear how monocytes or monocyte subpopulations are involved in breast tumour development.

CCL2 is a key factor involved in monocyte recruitment [304]. CCL2-producing tumours recruit increased numbers of monocytes into the tumour mass, whereas in tumours that do not produce CCL2, monocytes are found mainly in the peritumoural region [328]. It has been proposed that recruitment of monocytes via CCL2 can have dual effects. In a melanoma model, low-level secretion of CCL2 promoted tumour formation, whereas high-level CCL2 secretion resulted in considerable TAM infiltration and tumour destruction [329]. However, given that TAMs can make up a substantial proportion of the breast tumour mass, and increased TAM numbers have been correlated with poor prognosis in breast cancer, it is less likely that this same effect would be seen in breast tumours. In fact, increased CCL2 expression correlates with poor prognosis in breast cancer [330]. RANTES (CCL5) is another monocyte chemoattractant whose levels correlate with more advanced disease in breast tumours [331, 332]. Thus, monocytes may be involved in breast tumour progression.

Monocytes in cancer have frequently been interpreted to be MDSCs (see Section 1.6). Little is known about how monocytes are influenced by breast tumours. Monocytes will ultimately differentiate into TAMs [225], and in this way can promote tumour progression. However, a unique population of monocytes, termed TIE2-expressing monocytes, can suppress $CD8^+$ T cells and expand Treg numbers [211] and have also been implicated in promoting tumour angiogenesis [333]. Murine $TIE2^+$ monocytes have been shown to express CD45 (a hematopoietic cell marker) and CD11b (a myeloid cell marker) and are Gr-$1^{low/neg}$ [334]. The Ly6C and Ly6G expression status of $TIE2^+$ monocytes is not known. In addition to differentiating into TAMs, circulating monocytes are also likely to encounter tumour cells in the blood and may directly influence tumour progression in this way.

Tumour cells attached to cells with a monocytic morphology have been observed in the lung vasculature [335] and incubation of breast cancer cells with monocytes increases tumour cell invasiveness *in vitro* [336, 337]. It has been proposed that stromal cells may create a ‘pathway’ that tumour cells can follow. For example, carcinoma cells move within tracks in the extracellular matrix left behind by fibroblasts [338]. These fibroblasts have been called ‘leading cells.’ In light of these data, a model has been

proposed for multicellular migration of tumour cells [339]. Given their migratory nature, it is possible that monocytes may encounter and attach to tumour cells in the circulation and possibly protect them against destruction by shear forces and other immune cells, or even act as 'leading cells' in this setting, directing tumour cell homing to distant organs and/or extravasation at these sites.

1.9 Tumour-associated neutrophils (TANs)

The majority of the literature on immune cells in tumours focuses on the role of macrophages [4, 230, 232, 233, 340], lymphocytes [341-343] and natural killer (NK) cells [344, 345]. However, neutrophils are also commonly found in tumours and in many cases tumour progression is associated with neutrophilia [101, 346-349].
The relationship between neutrophil infiltration and prognosis in human cancer has not been examined comprehensively [87] but evidence exists for a role for neutrophils in enhancement of disease progression in some human tumours [139, 348].

Elevated serum neutrophil numbers, neutrophil factors or neutrophil to lymphocyte ratios generally predict poor prognosis in a number of human cancers, including gastric [350, 351], colon [352, 353], lung [354], oesophageal [355], pancreatic [356], cervical [357] and ovarian [358, 359]. In breast cancer, increased levels of the neutrophil specific protease, NE, correlate with poor prognosis [360]. In patients with renal cell carcinoma [361], bronchioloalveolar carcinoma [348], head and neck squamous cell carcinoma (HNSCC) [362] and melanoma [363], the presence of infiltrating neutrophils in the tumour or in the tumour microenvironment correlates with poor prognosis. The role of neutrophils in tumour growth remains controversial and, given that immune cells have been shown to play dual roles in cancer, it is likely that neutrophils will promote tumour progression in some, but not all, cancers. Little is known about neutrophils in breast cancer; hence neutrophils in cancer in general will be reviewed here.

Early studies have found neutrophils to be cytotoxic to human tumours *in vitro* [364, 365] and *in vivo* [366-368]. However, in most cases this cytotoxicity was elicited after neutrophils were primed with bacterial products or inflammatory agents [369], or alternatively, mice were 'immunised' via axillary lymph node injections of tumour cells prior to intraperitoneal challenge with the same tumour cells [370]. It was also reported

that high expression of G-CSF [371, 372], IL-2 [89, 372] or TNFα [89] in murine mammary tumours led to TAN-mediated rejection of tumours. However, most of these studies involved non-physiological levels of cytokine expression. Only one study showed that G-CSF expression at physiological levels led to suppression of tumours in a murine model of adenocarcinoma [373].

Conversely, Aeed et al. showed that rat adenocarcinomas are resistant to lysis by neutrophils [374] and that circulating neutrophil numbers increase with increasing metastatic capacity [5]. In human HNSCC, more aggressive tumours displayed increased TAN infiltration compared to less invasive tumours [362]. Increased numbers of TANs in lung tumour spheroids increased spheroid growth rates and inhibition of TAN infiltration reduced tumour growth [375]. Aeed et al. also showed that co-injection of neutrophils with mammary adenocarcinoma clones of varying metastatic capacity increased primary tumour growth compared to the same clones injected alone [5].

1.9.1 IL-8, CXCR1 and CXCR2

Neutrophil recruitment is driven predominantly by members of the CXCL chemokine family that bind to the CXCR1 and CXCR2 receptors on neutrophils [376]. In particular, CXCL1 and CXCL2 tend to be highly expressed in cancers [377-379]. Neutrophil migration can be inhibited by blocking CXCR1 and CXCR2 *in vitro* [7], and in both CXCR2-deficient mice or mice treated with a CXCR2 antagonist, a reduction in TAN numbers is seen [375]. In humans, IL-8 is a potent neutrophil chemoattractant [380] that is expressed in many cancers including breast cancer [381], cervical cancer [382], melanoma [383], and bronchioloalveolar carcinoma [349]. Tumours expressing increasing levels of IL-8 show increased TAN numbers [349, 384].

1.9.2 TAN-mediated angiogenesis

TANs secrete various pro-angiogenic factors [172, 385, 386] and can also mediate the angiogenic switch in cancer [387, 388]. Recent studies have indicated that neutrophils are often the predominant cell to produce MMP-9, and MMP-9-mediated ECM degradation releases sequestered angiogenic factors [389]. In pancreatic lesions Gr-1^+CD11b$^+$ cells were the primary cell type to express the *MMP-9* gene [387]. These Gr-

1^+CD11b^+ cells stained positive for 7/4 and negative for F4/80 (a macrophage marker) and displayed polymorphic nuclei, confirming them as neutrophils [387]. Furthermore, neutrophils express tissue inhibitor of metalloproteinases (TIMP)-free pro-MMP-9 [138, 390], making MMP-9 more freely available for the induction of angiogenesis [390]. Hence neutrophils are important contributors to tumour angiogenesis in a number of cancers and possibly also in breast cancer.

1.9.3 TAN-mediated ECM degradation, migration and invasion

In a normal inflammatory response, neutrophils need to extravasate from the bloodstream. They do so via a four step mechanism that involves: 1) rolling along the vascular endothelium, 2) activation and upregulation of leukocyte integrins, 3) immobilisation on the vascular endothelium via integrin-mediated adhesion, and 4) migration into the target organ [391]. It has been proposed that, whilst migrating through the ECM to reach tumours, neutrophils create 'channels' through which tumour cells can escape into the vasculature [392].

As previously described, neutrophils contain four types of granules that house a number of proteinases capable of degrading a wide range of cytokines, chemokines and their receptors, as well as remodelling the ECM [393, 394]. Human fibrosarcomas and prostate tumours recruit MMP-9-expressing TANs and display increased tumour cell intravasation that is reduced upon inhibition of TAN recruitment [138]. TAN-derived NE can also promote tumour cell invasion [205]. In breast tumours, co-culture of human neutrophils with breast tumour cells increases neutrophil expression of oncostatin M that subsequently increases breast tumour cell detachment and invasiveness *in vitro* [395].

1.9.4 TAN-mediated suppression of anti-tumour immune responses

Activation of T cells is a necessary step in the development of specific anti-tumour immune responses and to survive, tumours need to suppress T cell activity [96]. Neutrophils can inhibit T cells in cancer [193], they produce arginase and hydrogen peroxide (H_2O_2) that are known to suppress anti-tumour T cell responses [191, 193].

In tumours, NK cell anti-tumour cytotoxicity is also suppressed [129-131]. Gr-1^+ cells and neutrophils have been shown to be required for NK cell function and activation during homeostasis, infection and inflammation. Under homeostatic conditions, NK cell survival, maturation and function were impaired in mice depleted of Gr-1^+ cells and a similar phenomenon was seen in neutropenic patients [396]. In a murine model of *Legionella pneumophila* infection, neutrophils were found to activate caspase-1 and produce IL-18, both of which are key factors in NK cell activation [397]. However, Gr-1^+ cell-derived arginase suppresses NK cell activation and has been reported to impair human NK cell proliferation *in vitro* [398]. Conversely, NK cells can regulate neutrophil survival and function. NK cells have been shown to induce neutrophil apoptosis [399] and in mice infected with *Mycobacterium tuberculosis* and *Escherichia coli* depletion of NK cells impaired neutrophil trafficking and activation [400]. However, NK cells can also recruit Gr-1^+ cells [401] and inhibit apoptosis of neutrophils [402]. Constantini et al have suggested that NK cells can modulate neutrophil activation and survival both positively and negatively [403]. The regulation of NK cells by neutrophils, and vice versa, is likely to depend on the nature of the tissue insult and the surrounding microenvironment. Thus it appears that a signalling network exists between neutrophils and NK cells, and it is possible that in the tumour microenvironment TANs suppress the anti-tumour functions of NK cells and NK cells prolong TAN survival.

1.9.5 TAN-derived reactive oxygen species (ROS)

The previously reported tumouricidal activity of neutrophils has often been associated with the generation of oxygen free radicals [366, 404]. Neutrophils generate various oxidants as part of the 'respiratory burst' [170, 405], which is defined as the increased consumption of oxygen to create ROS. Neutrophils also express iNOS [406, 407] and are the primary producers of iNOS in murine fibrosarcomas [408]. Although neutrophil ROS have been reported to have cytotoxic effects, they have also been shown to initiate DNA damage and increase mutations [384, 408]. These genetic mutations can instigate tumour initiation and enable metastatic progression [409, 410]. Spontaneous mutations were seen to occur more frequently in tumours with higher numbers of infiltrating TANs [408] and TAN numbers correlated with ROS activity and mutation frequency [384, 408].

1.9.6 TAN phenotypes

Recently it has been suggested that the same pro-tumour/anti-tumour phenotypes seen in TAMs (M1/M2) and T cells (Th1/Th2) may also occur in TANs [411]. Fridlender and colleagues found that TGFβ stimulates neutrophils towards a pro-tumoural (N2) phenotype. Inhibition of TGFβ in two different murine cancer models generated a population of TANs that significantly suppressed tumour growth [412]. TANs isolated from mice treated with a TGFβ inhibitor were hyper-segmented, expressed higher levels of pro-inflammatory cytokines and were more cytotoxic towards tumour cells, indicating a pro-inflammatory, M1-like phenotype. TANs isolated from untreated mice expressed higher levels of arginase, VEGF and MMP-9 mRNA [412], factors associated with the M2-like trophic phenotype in macrophages [154].

Conversely, IFNβ inhibits neutrophil angiogenic activity in tumours [141]. In the absence of IFNβ, melanomas and fibrosarcomas displayed increased numbers of TANs, better developed blood vessels and faster growth. These data indicate that some factors may promote a pro-inflammatory or N1 phenotype in TANs, while others induce an anti-inflammatory, trophic or N2 phenotype. However, it remains to be clarified whether N1 TANs are merely highly activated neutrophils or if this N1/N2 polarisation does in fact represent distinct neutrophil phenotypes. Further investigation of neutrophil phenotypes in tumours is thus warranted. A more comprehensive analysis of the factors within the tumour microenvironment that influence neutrophil phenotypes may yield further insight into how tumours manipulate TANs, and whether these processes can be targeted for cancer treatment.

1.10 Macrophages and neutrophils in metastasis

Different tumours display characteristic patterns of secondary organ metastasis. In advanced breast cancer, tumours metastasise to bone, lung, lymph node, brain and liver [57, 70]. Metastasis occurs through a series of sequential steps [52] that can be rate limiting [54]. Studies analysing myeloid cells in metastasis often use experimental models of metastasis, where the tumour cells are injected directly into the circulation, but these models do not encompass all the steps of metastasis and may not accurately reflect the roles of myeloid cells in spontaneous metastasis. Therefore, meaningful analysis of macrophages and neutrophils in the context of all the stages of metastasis is still lacking.

1.10.1 Myeloid cells in metastasis

Inflammation is important at the primary tumour site [87], as well as systemically [191] and at the metastatic site [88]. The pre-metastatic niche theory states that immune cells are recruited to metastatic sites where they create a permissive growth environment prior to the arrival of tumour cells [413]. In support of this, BMDCs and Gr-1^+ cells have been shown to be recruited to metastatic sites prior to the arrival of tumour cells [3, 92]. Furthermore these BMDC clusters occur only in organs typical of the characteristic metastatic pattern displayed by the particular tumour [8, 92]. Once at the pre-metastatic site, BMDCs secrete factors that facilitate tumour cell survival and growth [8, 92]. Increases in Gr-1^+CD11b$^+$ cells have been reported in the spleen [215], bone marrow [216] and circulation [217] of tumour-bearing mice, and their human counterparts are increased in cancer patients [214].

Increased TAM numbers in primary tumours correlate with establishment of metastases [235], and associate with tumour progression to a metastatic phenotype [414]. High tumour expression of macrophage chemoattractants and growth factors, such as CCL2 and CSF-1, has also been correlated with advanced disease [330]. In ovarian tumours, inflammation was shown to enhance metastasis and TAMs were implicated as the main effector cell in this process [415].

Tumours that secrete IL-8 have an increased metastatic potential, and it has been proposed that this is due in part to their ability to attract TANs [416]. TANs have also been implicated in the progression to metastasis in murine fibrosarcoma and melanoma, where it was proposed that increased TAN numbers support acquisition of a metastatic phenotype in benign tumours [417, 418].

1.10.2 TAMs and TANs co-migrate with tumour cells

It has been suggested that tumour cells and TAMs co-migrate during breast tumour cell invasion into the circulation [419]. TAM-derived EGF promotes directional motility and invasion of tumour cells, and this is dependent on reciprocal tumour cell CSF-1 production. CSF-1 expression by tumour cells promotes EGF production by macrophages and a paracrine loop is formed. This loop can be blocked by inhibition of the epidermal growth factor receptor (EGFR) or CSF-1R, leading to inhibition of macrophage-tumour co-migration and invasion [196]. Furthermore, blocking either CSF-1 or EGF, or depleting macrophages, disrupts invasion of tumour cells towards other chemotactic factors such as CXCL12 [420].

Using multiphoton imaging, Wyckoff et al. showed that motile tumour cells intravasated only when adjacent to perivascular TAMs. [261]. Thus, it appears that TAMs provide chemotactic signals that recruit tumour cells to blood vessels and subsequently allow tumour cells to escape into the vasculature [261]. It has even been hypothesised that tumour cells ‘piggy-back’ onto TAMs whilst entering the circulation [421]. It is also highly possible that tumour cells can commandeer monocytes to help them migrate through the circulation to metastatic sites.

A similar suggestion has been made for neutrophils. Melanoma cell arrest and extravasation from the circulation was increased in the presence of neutrophils [422]. Increased numbers of blood neutrophils have been reported in a model of fibrosarcoma [418]. When these ‘granulocytosis-positive’ fibrosarcoma-bearing mice were injected intravenously with melanoma cells, the latter were retained for longer in the lung, and the number of lung colonies was increased [418].

For tumour cells to escape into the vasculature, they must detach from the primary tumour site. This process involves downregulation of adhesion molecules that are also required for transendothelial migration. For example, MDA-MB-231 breast tumour cells express high levels of intercellular adhesion molecule-1 (ICAM-1) but do not express CD11a, CD11b or CD18 that are required for transendothelial migration [423]. These proteins and integrins are upregulated on TANs [7, 140, 423]. Thus it is possible that, at both the primary and metastatic sites, tumour cells attract and attach to neutrophils, which then promote tumour cell migration through the basement membrane via expression of adhesion receptors and proteases lacking in tumour cells.

1.10.3 Macrophages in metastasis

In an experimental model of 4T1 tumour metastasis, depletion of $CD11b^+$ macrophages, but not resident lung $CD11c^+$ macrophages, inhibited metastatic growth in lungs [335]. Thus, it was suggested by the authors that a distinct pro-tumour macrophage population is recruited from the blood to the lungs, where it associates with tumour cells and promotes tumour cell growth [335].

A number of studies have implicated CSF-1 in breast cancer metastasis [4, 136, 198, 230, 286], and CSF-1 is considered a potential target in the treatment of advanced disease. As previously mentioned, using MMTV-PyMT mice crossed with CSF-1 null mutant mice ($Csf1^{op}/Csf1^{op}$), Lin et al. showed that the absence of CSF-1 activity inhibited TAM accumulation in the primary tumour and almost completely abrogated metastasis [199]. Restoration of CSF-1 activity increased TAM numbers in primary tumours and restored metastasis to the wild-type level. Furthermore, elevated expression of CSF-1 in wild-type mice resulted in accelerated tumour progression [199]. However, in a recent study by Denardo et al. using the same MMTV-PyMT model, but in mice with intact CSF-1 activity, treatment with CSF-1R signalling antagonists alone had minimal effects on primary tumour growth or lung metastasis. Only in combination with chemotherapy did anti-CSF-1R treatment reduce metastasis to lung [136]. Therefore the roles of CSF-1 and TAMs in breast cancer metastasis are still unclear.

1.10.4 Neutrophils in metastasis

Neutrophils can increase the invasive and metastatic capacity of tumour cells [424]. Administration of G-CSF in murine models of fibrosarcoma and breast cancer increased neutrophil recruitment and accumulation in primary tumours and blood, which increased metastatic capacity [8, 200]. Neutrophils exposed to tumour conditioned medium increased metastasis of mammary adenocarcinomas, whereas 'normal' neutrophils did not [424]. In two different studies using breast cancer models, it was found that neutrophils were the predominant population in the pre-metastatic lung and depletion of neutrophils reduced metastasis to the lung [3, 8]. Furthermore, it was shown that macrophage numbers decrease in these pre-metastatic lungs when compared to normal lungs [3].

However, as for macrophages, the role of neutrophils at metastatic sites remains controversial. Neutrophils have been implicated in the clearance of experimental metastasis [425]. Depletion of neutrophils via anti-G-CSF antibody treatment in the 4T1 breast cancer model resulted in a marked reduction of lung metastasis [8], although recent work in the same model, by another group, showed that depletion of neutrophils via anti-Ly6G antibody treatment resulted in a significant increase in the number of metastatic events [201]. The authors suggested that during cancer progression, a unique population of cytotoxic neutrophils, called tumour entrained neutrophils (TENs), that slow metastatic growth is generated [201]. However, TENs were not sufficient to abrogate metastatic growth completely and lung nodules eventually grew.

1.10.5 Macrophages and neutrophils may be functionally linked in promoting metastasis

To summarise the published data, macrophage deficiency did not change primary tumour growth but did reduce metastasis in the MMTV-PyMT model [199]. In contrast to this, recent work in the same model showed that anti-CSF-1 therapy alone had minimal effects on tumour progression, and only when used in an adjuvant setting was a significant effect seen on metastasis [136]. Conversely, in the 4T1 mammary tumour model, depletion of neutrophils had a pronounced effect on metastasis [8] but, in another study using this model, inhibition of neutrophils increased metastasis [201].

In a murine model of cervical cancer on a CCR2-deficient background, TAM numbers were reduced. However, a reciprocal increase in TAN numbers was observed and these TANs could promote tumour growth in the absence of TAMs [426]. Thus it appears that targeting macrophages or neutrophils alone may have a minimal effect as a cancer treatment, and for successful treatment of advanced disease it may be necessary to target both of these immune populations. Furthermore, it needs to be determined which cell types are important in different breast cancer subtypes in order to tailor therapies appropriately.

1.11 The 4T1 murine mammary tumour model

The 4T1 mouse mammary tumour model comprises a series of isogenic tumour cell lines of varying metastatic potential. When injected into the mammary gland of BALB/c mice, these tumours mimic the course of human breast cancer, with primary tumour formation from a small inoculum, invasion of cells through the stroma into the circulation and colonisation at distant organs, including lung and bone [427], depending on the subline used. This is a spontaneous metastasis model and thus overcomes the limitations of experimental models of metastasis, where tumour cells are injected directly into the bloodstream and do not encompass all the steps required for physiological metastasis. It is also a syngeneic model and hence does not involve transplantation of human tumour cells into mice. Human cells are not well adapted to grow in a murine environment and hence immunocompromised mice are needed for tumour growth to occur. Given that this project is investigating the role of myeloid cells in breast tumour metastasis, it is important that the immune system is intact, and that the tumours and myeloid cells are of the same species.

The 4T1.2 tumour cell line was isolated by our research group at the Peter MacCallum Cancer Centre from the 4T1 model that was developed by Aslakson and Miller [428]. The 4T1 tumour cell line is a thioguanine-resistant variant selected from the 410.4 cell line [428]. Line 410.4 was isolated from 4.10LM, which was derived from a spontaneous lung nodule growing in a BALB/c/cfC3H mouse carrying the mouse mammary tumour virus, MMTV [429]. Single cell cloning of 4T1 generated the 4T1.2 cell line that is spontaneously metastatic to lung, lymph node, liver and, importantly,

bone [427]. This model is unique amongst spontaneous mammary tumour models as it spontaneously metastasises to bone, and therefore most closely resembles the pattern of metastatic spread seen in human breast cancer.

The 67NR and 66cl4 murine mammary tumour cell lines are isogenic variants of the 4T1 and 4T1.2 cell lines. Similar to the 4.10LM cell line, the 66 and 67 tumour cell lines were derived from the same, spontaneously arising, mammary tumour in a BALB/c/cfC3H mouse carrying the mouse mammary tumour virus (MMTV) [430]. The 66cl4 tumour cell line variant was isolated from a 66 clone after mutagenesis with ethyl methanosulfate [431]. This 66cl4 cell line is weakly metastatic to lung and lymph nodes [427, 428]. The 67NR tumour cell line was obtained by transfecting the 67 cell line with a geneticin-resistance gene [428] and is not able to leave the primary tumour site, making it non-metastatic [428]. Mice bearing the 4T1.2 cell line show metastasis to various organs but most extensively to the lungs, femur and spine, mice bearing the 66cl4 cell line display moderate metastasis to the lung, while mice bearing the 67NR cell line do not display metastasis to any organs (Figure 1.4) [432]. The isogenic tumour cell lines and their metastatic potential are summarised in Figure 1.5.

1.12 Conclusions and objectives of thesis

Metastasis is the primary cause of breast cancer-associated deaths. Currently we have limited resources to treat advanced disease, in part, due to our poor understanding of the cellular and genetic regulators of breast cancer metastasis. At present, tumour size, grade, hormone and HER2 receptor expression, as well as lymph node involvement, are used as markers of disease status. However, these are inadequate in predicting the likelihood of metastasis. Once metastasis has occurred, treatment options can delay disease progression but are not curative. A newly emerging area of research focuses on the link between inflammation and cancer. A more comprehensive understanding of inflammatory pathways, cells and genes involved in breast cancer metastasis would greatly increase our chances of improving treatment of advanced breast cancer.

Both macrophages and neutrophils have been implicated in breast cancer metastasis. Some studies indicate that inhibition of CSF-1, and thus macrophages, inhibits metastasis, whereas other studies have shown that inhibition of CSF-1 may only be

beneficial in an adjuvant setting. Inhibition of neutrophils has also been reported to inhibit breast cancer metastasis, yet other studies suggest that their removal results in increased metastasis.

Thus, more research is required to elucidate how macrophages and neutrophils promote metastasis and whether targeting these processes can prove useful in the treatment of advanced cancer. It also needs to be determined which breast cancer subtypes are likely to respond to anti-neutrophil or anti-macrophage therapy. The functional relationship between macrophages and neutrophils needs to be further explored as it may be necessary to target both populations to successfully treat metastatic disease. Analysis of changes in these myeloid cell populations, both in primary tumours and at metastatic sites, can lead to more tailored therapies for patients with advanced breast cancer.

Hypotheses

1. TAMs and/or TANs are associated with metastasis, and may directly influence the metastatic capacity of tumour cells.
2. Highly metastatic tumours increase myeloid cell mobilisation, and this in turn promotes tumour metastasis.
3. Depletion of monocytes/TAMs will inhibit metastasis.

Project aims

1. To analyse TAMs and TANs from highly metastatic tumours compared to non-metastatic tumours, and to determine if TAMs and/or TANs from highly metastatic tumours can influence the metastatic capacity of weakly metastatic tumours.
2. To analyse monocyte and neutrophil mobilisation by breast tumours of varying metastatic capacity.
3. To determine how depletion of monocytes and macrophages (TAMs) affects metastasis in the 4T1.2 model, and also to determine how blocking the CSF-1R affects monocyte/TAM populations and metastasis.

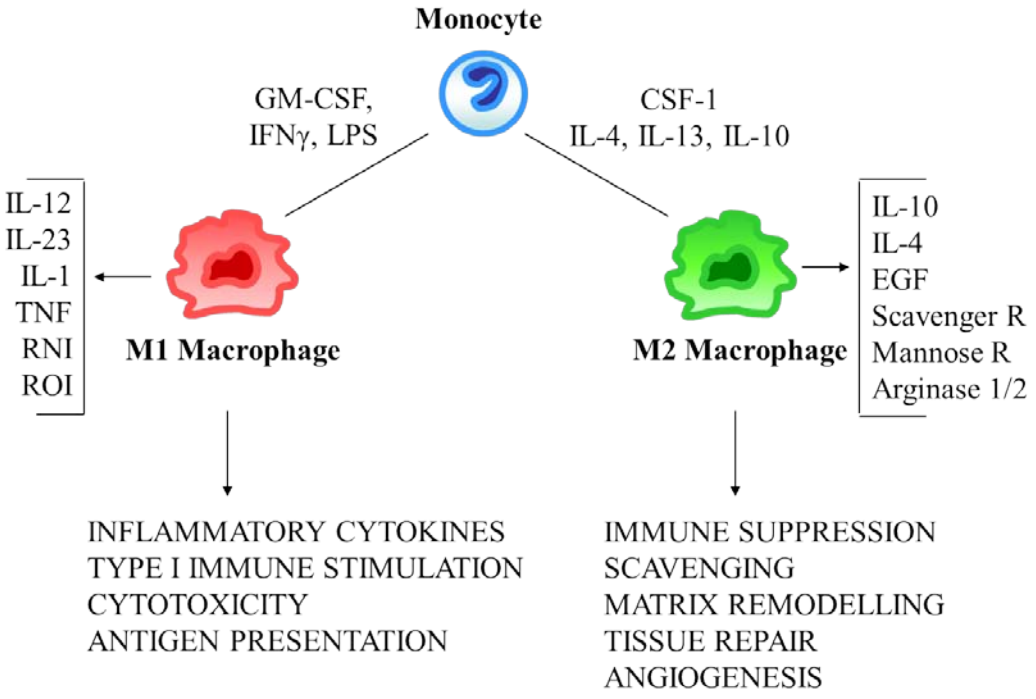


Figure 1.1: M1-like and M2-like macrophage phenotypes.

Macrophages display a continuum of phenotypes of which M1 and M2 are the extremes. M1-like macrophages arise from monocytes encountering pro-inflammatory signals, such as GM-CSF and IFNγ, or bacterial products such as LPS. These signals induce them to mature into macrophages that are pro-inflammatory and cytotoxic, with a robust antigen presenting capacity. M2 macrophages arise from monocytes encountering anti-inflammatory signals, such as CSF-1, IL-4, and IL-10. This stimulates them to mature into M2 macrophages that are trophic, anti-inflammatory, and have reduced cytotoxicity and antigen presenting capacities.

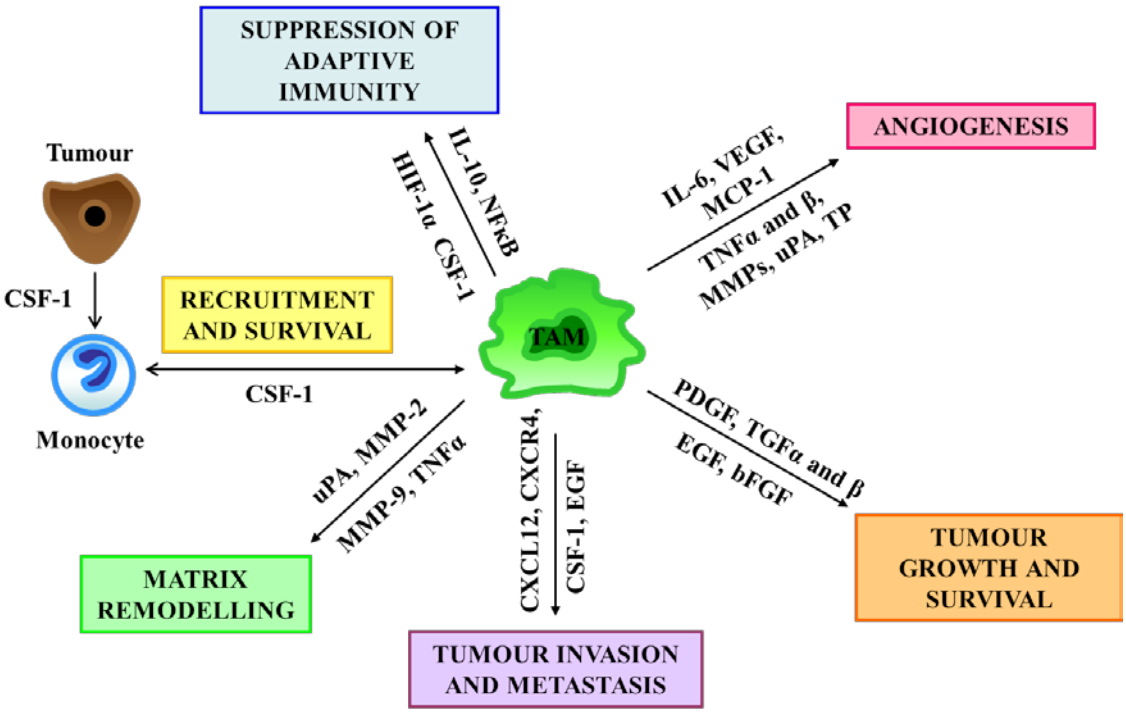


Figure 1.2: TAM functions in tumour progression.

TAMs are involved in every stage of tumour progression. TAMs secrete growth factors, such as EGF, that stimulate tumour survival and growth, and also direct motility of tumour cells. TAMs secrete CSF-1 that promotes TAM survival and proliferation, and further recruits monocytes into the tumour microenvironment. TAMs also suppress the immune system in the tumour microenvironment via secretion of anti-inflammatory cytokines such as IL-10. Finally, by secreting angiogenic factors and proteases, TAMs promote formation of new blood vessels, matrix remodelling, and the invasion of tumour cells through the ECM and into the vasculature.

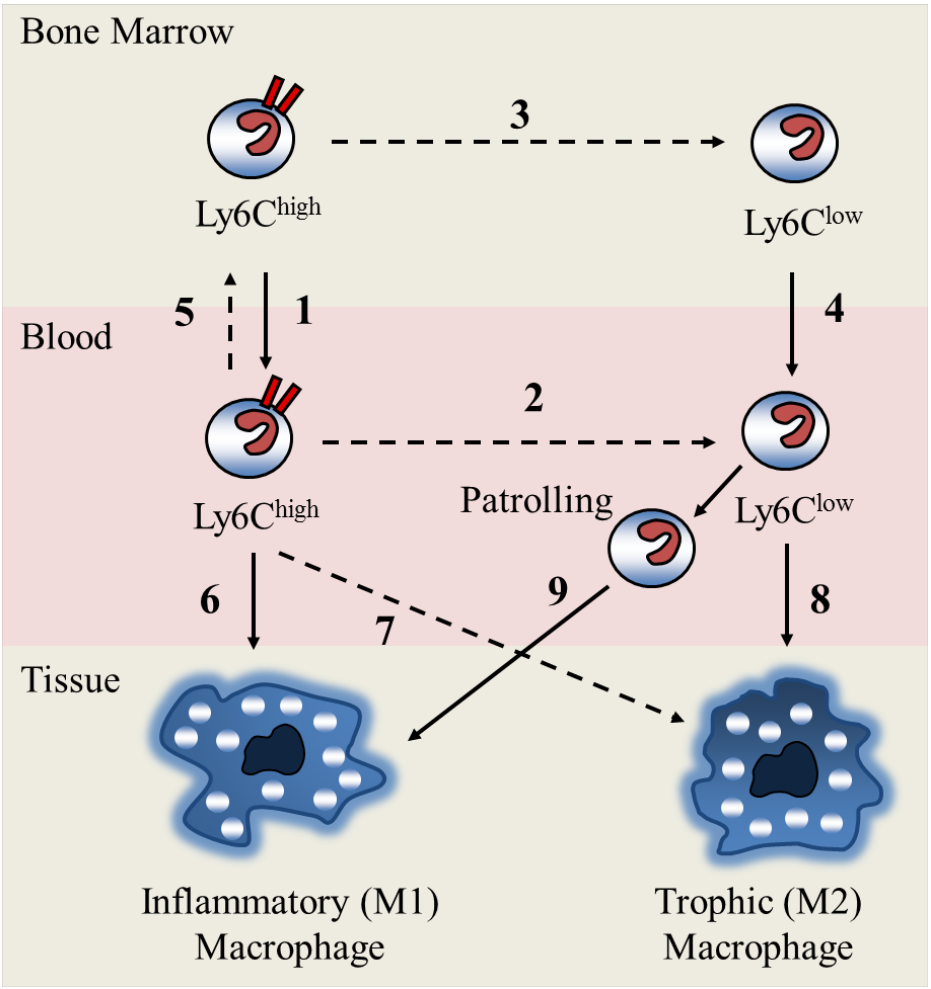


Figure 1.3: A model for murine monocyte differentiation

Monocytes arise in the bone marrow as Ly6C^{high} immature cells. They are released into the bloodstream (1) where they are thought to mature and downregulate their expression of Ly6C, becoming Ly6C^{low} cells (2). However, it is also possible that Ly6C^{high} cells may convert into Ly6C^{low} cells in the bone marrow (3), or that Ly6C^{high} and Ly6C^{low} cells arise independently of each other from precursors in the bone marrow (1 and 4). Ly6C^{high} monocytes may be able to re-enter the bone marrow from the circulation (5). Ly6C^{high} typically give rise to inflammatory macrophages in tissues (6), but may also acquire a trophic phenotype during certain injuries (7). Ly6C^{low} monocytes give rise to trophic or resident macrophages (8), although a fraction of the Ly6C^{low} subpopulation patrols blood vessel walls and can give rise to inflammatory macrophages during infection (9).

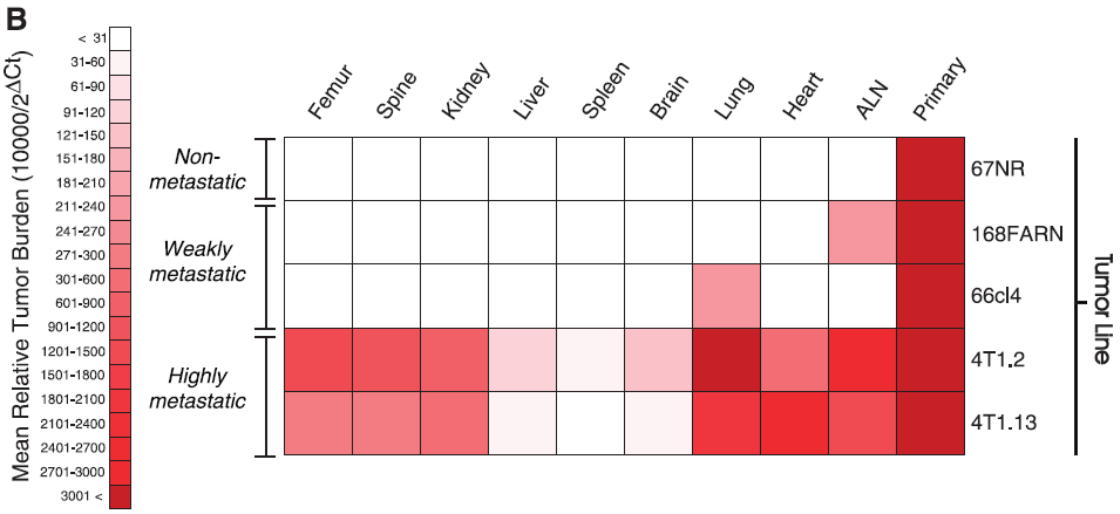
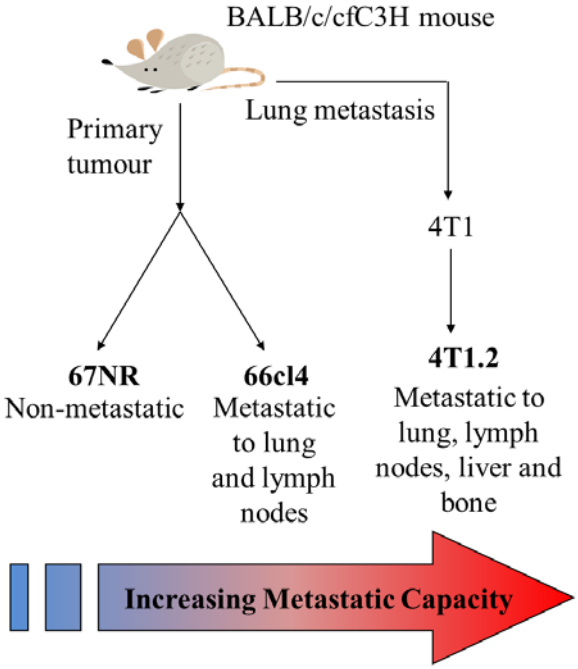


Figure 1.4: Distribution of spontaneous metastases in mice bearing different tumours of varying metastatic capacity.

Tumour cells of varying metastatic capacity were inoculated into the fourth mammary glands of mice. Mice were culled at endpoint and tissues were excised for genomic DNA extraction. A qPCR assay for tumour burden was used to assess the spontaneous metastatic distribution for each tumour cell line. This figure was re-printed from Eckhardt BL, et al. (2005) *Mol Cancer Res. 3*(1):1-13 [432].

A



B Kinetics of 4T1, 66cl4 and 67NR tumour progression

Timepoint	4T1	66cl4	67NR
Day 15*	Tumour cells found in lung	No tumour cells	No tumour cells
Day 19	Tumour cells found in lung	No tumour cells	No tumour cells
Day 22	Tumour cells recovered from vertebrae	No tumour cells	No tumour cells

*First day analysed

Figure 1.5: The 4T1 mammary tumour model.

A) The 4T1 mouse mammary tumour model comprises a series of isogenic tumour cell lines of varying metastatic potential. The 67NR cell line is non-metastatic, the 66cl4 tumour cell line is metastatic to lung and lymph nodes, and the 4T1.2 cell line is metastatic to lung, lymph nodes, liver and bone. B) Analysis of 4T1 (the parent line of 4T1.2) tumour cell dissemination from the mammary fat pad shows 4T1 tumour cells in the lung at day 15 post tumour cell inoculation (first day assessed) and by day 22 4T1 tumour cells could be recovered from the vertebrae. No tumour cells could be recovered from the lung or vertebrae for the 66cl4 or 67NR tumour cell lines. It is unclear at this stage how early 4T1.2 tumour cells can be recovered from lung or bone. This table was adapted from Lelekakis, M., et al. (1999) *Clin Exp Metastasis* 17(2): 163-70 [427]

2 Materials and Methods

2.1 Reagents

The following general laboratory reagents were obtained from commercial sources as follows: acetic acid (HOAc), ammonium acetate (NH_4OAc), ammonium chloride (NH_4Cl), calcium chloride ($CaCl_2$), absolute ethanol (EtOH), glycine, absolute methanol (MetOH), sodium chloride (NaCl), sodium dodecylsulphate (SDS), tris(hyroxymethyl)aminomethane (Tris) from MERCK, NJ, USA. Agarose, dithiothrietol (DTT), non-reducing sample buffer, kaleidoscope pre-stained standards, polyvinylidene fluoride (PVDF) membrane, and Tween-20 from Bio-Rad Laboratories, Richmond, CA, USA. Bovine serum albumin (BSA) and proteinase K from Roche, Basel, Switzerland. Ammonium persulphate (AP), Bovine gelatin B (225 BLOOM), brilliant blue R250, ethylenediaminetetraacetic acid (EDTA), hydrochloric acid (HCl), sodium azide (NaAz), trypan blue, 10% neutral buffered formalin (NBF) and Triton X 100 from Sigma-Aldrich, MO, USA. India ink from Speedball, NC, USA, isoflurane from Lyppards, Worcestershire, UK and phenol/chloroform pH 6.7/8.0 from Astral Scientific, ON, USA. Potassium hydrogen carbonate ($KHCO_3$) and tetramethylethylenediamine (TEMED) from Thermo Fisher Scientific (Ajax Finechem) MA, USA. Alpha-modified minimum essential medium (αMEM), Dulbecco's modified eagle medium (DMEM) and penicillin-streptomycin from GIBCO (Invitrogen), CA, USA. Foetal calf serum (FCS) from CSL Biosciences, Parkville, AUS, heparin sodium (heparin) from DBL® RMH Pharmacy, Parkville, AUS, collagenase I (CLS-1) from Worthington Biochemical Corporation NJ, USA and 70 µm and 100 µm cell strainers from BD Falcon, NJ, USA. Trypsin, random primers, deoxyribonucleotides (dNTPs), first strand buffer and SuperScript III (SSIIIRT) from Invitrogen, CA, USA. TaqMan® Fast Universal PCR Master Mix from Applied Biosystems, CA, USA, 40% Acrylamide 37.5:1 from Amersco, OH, USA and ECL Western blotting detection reagents from Amersham/GE Healthcare, Buckinghamshire, UK. Super RX Fuji medical X-Ray film 100 NIF 18 x 24 was from Fuji, Tokyo, Japan and milk powder from Abbott (Ensure®) IL, USA.

2.2 Antibodies

Monoclonal antibodies (mAbs) against the following antigens used for flow cytometric analysis and cell sorting were obtained from commercial sources as follows: CD11b (Mac-1 α-chain; M1/70-APC), Ly6C (AL-21-FITC), Ly6G (1A8-PE), Ly6G (1A8-PE-Cy7), B220 (CD45R; RA3-6B2-PerCP), CD4 (RM-45-APC), CD8a (53-6.7-PE) and the secondary antibody streptavidin PerCP (Strep-PerCP) from BD Pharmingen, San Diego, CA, USA. CD115 (c-Fms: AFS-98 biotin) from eBioscience, San Diego, CA, USA, and F4/80 (CI; A3-1-biotin) from Abd Serotec, Kidlington, UK. Isotype controls IgG2bκ (A95-1-PC), IgG2aκ (RM4-5-APC), IgMκ (R4-22-FITC), IgG2aκ (R3595-PE), IgG2aκ (RA3-6B2-PerCP) from BD Pharmingen and IgG2b (CI; A3-1-biotin) from Abd Serotec. Polyclonal goat anti-MMP-9 (AF909; R and D Systems, MN, USA) and HRP-conjugated rabbit anti-goat secondary antibody (sc-2922, Santa Cruz Biotechnology, Inc. CA, USA) were used for Western Blotting.

2.3 Reagents that were gifts

The following hybridomas (IgG2a) were gifts: neutralising anti-mouse (CSF-1R) c-Fms (AFS-98) from Dr. S-I Nishikawa, Kobe Japan. Anti-GM-CSF (22E9.11) and anti-β-galactosidase isotype control (GL117.41) from Dr. J Abrams DNAX, Palo Alto, CA, USA. Clodronate liposomes and phosphate buffered saline (PBS) liposomes were a gift from Dr. Jacquie Harper (Malaghan Institute of Medical Research, Wellington, NZ). The CSF-1R kinase inhibitor 5-(3-methoxy-4-((4 methoxybenzyl) oxy) benzyl) pyrimidine-2,4-diamine (GW2580) was a gift from Dr. Ian Holmes (Cancer Therapeutics CRC, CTx, AUS).

2.4 Primers and Probes

The following primers and probes were obtained from Geneworks (SA, AUS).

Table 2.1: Primer and probe sequences used for qPCR analysis of metastatic burden

Primer/Probe	Sequence
mCherry forward primer	5´GCGCCCGGTTCTTTTTG3´
mCherry reverse primer	5´CCTCGTCCTGCAGTTCATTCA3´
vimentin forward primer	5´AGCTGCTAACTACCAGGACACTATTG3´
vimentin reverse primer	5´CGAAGGTGACGAGCCATCTC3´
mCherry probe	6FAMCAGCTGCCCGGCGCCTACATAMRA
vimentin probe	VICCCTTCATGTTTTGGATCTCATCCTGCAGGTAMRA

The following pre-developed TaqMan probe/primer combinations against mouse genes were obtained from Applied Biosystems, CA, USA.

Table 2.2: TaqMan probes/primers used for TAM gene expression analysis

Gene	Catalogue number
HPRT	Mm446968_m1
IL-12A (IL-12 alpha)	Mm00434165_m1
IL-12B (IL-12 beta)	Mm00434174_m1
IL-10	Mm00439615_g1
IL-23A (IL-23 alpha)	Mm_00518984_m1

2.5 Cell lines

The 66cl4 and 67NR murine tumour lines were derived from a spontaneously arising tumour in a BALB/cfC3H mouse carrying the mouse mammary tumour virus, and the 4T1 tumour cell line from a metastatic lung nodule that occurred in the same mouse [428, 430]. The 67NR, 66cl4 and 4T1 cell lines were a kind gift from Dr. F. Miller (Michigan Cancer Foundation, Detroit, MI, USA) and the 4T1.2 cell line was selected from the 4T1 line [427]. 4T1.2, 66cl4 and 67NR cells were maintained in minimal essential medium (αMEM) containing penicillin/streptomycin at 100 U ml^{-1} and 5% foetal calf serum. For experiments where qPCR was used to analyse metastatic burden, the above tumour cell lines expressing mCherry fluorescent protein were used. The E0771 cell line is a C57BL/6 mouse mammary tumour model. This cell line was also developed by our research group at the Peter MacCallum Cancer Centre, and is spontaneously metastatic to lung.

2.6 Mammary tumour model

Female BALB/c mice (6-8 weeks old, Walter and Eliza Hall Institute, Melbourne) were anesthetised and injected into the fourth mammary gland with 2 x 10^5 4T1.2, 66cl4 or 67NR cells in 10 μL PBS. Tumour volume [(length x $width^2$)/2] was measured using electronic callipers. Peripheral blood was harvested via the tail into heparin. Primary tumours, spleens, spines and lungs were excised and primary tumours, spleens and lungs were weighed. Whole femurs were excised and flushed with PBS. All procedures involving mice were conducted in accordance with the National Health and Medical Research Council guidelines and approved by the University of Melbourne Animal Ethics Committee. Tumour growth and organ weight data were plotted using GraphPad Prism® software. For some experiments, metastatic lung nodules were scored as described in Section 2.11 and in others, total tumour burden in lung or spine was measured using a qPCR technique as described in Section 2.12 and 2.12.

2.7 Flow cytometric analysis

Mice were injected with 4T1.2, 66cl4 or 67NR tumour cells or saline alone as a control (see Section 2.5 and 2.6). Peripheral blood and organs were removed as described in Section 2.6. Lungs and primary tumours were digested with 1.5 mg/ml collagenase I in DMEM (Worthington Biochemical Corporation NJ, USA). Disaggregated tumour and lung samples were then filtered through 70 μM followed by 100 μM nylon cell strainers. Spleens were disaggregated using metal mesh cell strainers and collected into PBS. Red blood cells were lysed in primary tumour, peripheral blood, femur, spleen and lung samples by incubation in red blood cell lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA dissolved in MilliQ H_2O) for 5 min at 37ºC. Cells were washed twice in PBS and viable cells were counted by trypan blue exclusion. Cells (5×10^5) were incubated with 25% normal mouse serum at 4ºC for 20 min, primary Abs (Ly6C-FITC 1:200 dilution, Ly6G-PE 1:200 dilution, CD11b/Mac-1-APC 1:1,000 dilution, and c-Fms-biotin 1:50 dilution for blood, lung, bone marrow and spleen samples, or F4/80-biotin (1:50 dilution) for tumour samples, were added. For analysis of B and T cells in primary tumours CD4-APC (1:1,000 dilution), CD8a-PE (1:200 dilution) and B220-PerCP (1:200 dilution) were added; appropriate isotype controls were used for all Abs. Primary Abs were incubated at 4ºC for 20 min. Samples were then washed twice in PBS and samples containing biotin antibody were incubated with secondary Ab (Strep-PerCP, 1:200 dilution) for 20 mins at 4ºC. For flow cytometric analysis, a typical forward and side-scatter gate was set to exclude dead cells and aggregates; a total of 10^4 events in the gate were collected using the Beckman Coulter CyAn ADP Flow Cytometer (Beckman Coulter, CA, USA). Monocytes/macrophages and neutrophils were analysed using Flowjo software (TreeStar, OR, USA).

B cells were defined as $B220^+$ cells and T cells as $CD4^+$ or $CD8^+$ cells. TAMs in primary tumours were characterised as $CD11b^+F4/80^+$ (Figure 2.1A) [8, 223]. For analysis of monocyte/TAM subpopulations in primary tumours, blood, lung and spleen over the course of disease progression, monocytes/macrophages/TAMs were defined as $CD11b^+Ly6G^{neg}Ly6C^{high}$ or $CD11b^+Ly6G^{neg}Ly6C^{low}$ (Figure 2.1B) [303]. Monocytes in the bone marrow were defined as $CD11b^+Ly6G^{neg}Ly6C^{high}$ (Figure 2.1C). All monocyte/macrophage/TAM subpopulations were confirmed to be $CD115^+$ in blood, lung and spleen and $F4/80^+$ in primary tumours. Neutrophils/TANs in primary tumours,

blood, lung, spleen and bone marrow were defined as $CD11b^{+}Ly6G^{high}$ (Figure 2.1D) [8, 223]. Data were plotted using either GraphPad Prism® or Microsoft Excel® software.

2.8 Cell sorting of TAMs and TANs from primary tumours

Mice were injected with 4T1.2 or 67NR mCherry-expressing tumour cells (see Section 2.5 and 2.6). Primary tumours were excised at day 25 post-tumour cell inoculation (one tumour of each variant was used for isolation of TAMs and TANs). Tumours were digested with 1.5 mg/ml collagenase I in DMEM (Worthington Biochemical Corporation NJ, USA). Disaggregated tumour samples were then filtered through 70 μM followed by 100 μM nylon cell strainers. Red blood cells were lysed by incubation in red blood cell lysis buffer (as for Section 2.7) for 5 min at 37ºC. Total cells per tumour were incubated in 1 ml of 25% normal mouse serum at 4ºC for 20 min, 500 μL of primary Abs were then added (CD11b-APC 1:1000 dilution, Ly6G-PE-Cy7 1:200 dilution, and Ly6C-FITC 1:200 dilution) and incubated at 4ºC for 20 min. Samples were washed twice in PBS and re-suspended in 4 ml 1% BSA/PBS. Prior to cell sorting, samples were filtered through a 70 μM nylon cell strainer to remove aggregates. TAMs were characterised as $mCherry^{neg}CD11b^{+}Ly6G^{neg}$ and $Ly6C^{high}$ or $Ly6C^{low}$ [303], and TANs were characterised as $mCherry^{neg}CD11b^{+}Ly6G^{high}$ [8, 223]. TAMs and TANs were collected into DMEM containing 5% FCS.

2.9 RT-qPCR analysis of gene expression

TAMs and TANs were isolated from primary tumours (see Section 2.8). RNA was then extracted using the Ambion *mir*VANA™ miRNA Isolation Kit (Ambion® Life technologies - Invitrogen, CA, USA) as per manufacturer's instructions. RNA concentration was analysed by NanoDrop® (ND-1000 spectrophotometer, BioLab,GA, USA) and two μg of total RNA, in a 20 μL reaction mix containing 100 ng random primer, 10 mM deoxyribonucleotide triphosphates (dNTPs), 5 mM dithiothreitol (DTT) and 1X first strand buffer, was reverse transcribed using 10 units SuperScript III reverse transcriptase (Invitrogen, CA, USA). The reaction mix was incubated for 5 min at 25ºC, 60 min at 50ºC, 15 min at 55ºC, and finally for 15 min at 70ºC. RT-qPCR was carried out using an ABI PRISM 7900HT sequence detection system (Applied Biosystems, CA, USA) and pre-developed TaqMan probe/primer combinations for mouse IL-12A, IL-

12B, IL-23A, and IL-10 (see Section 2.4) and 40 ng of cDNA per reaction. All samples were assayed in triplicate and threshold cycle numbers calculated relative to the housekeeping gene, HPRT. Data were plotted using GraphPad Prism® software.

2.10 Co-injection of TAMs and TANs

TAMs and TANs were isolated from primary tumours (see Section 2.8). TAMs or TANs were then co-injected with 66cl4 tumour cells into the fourth mammary gland at a ratio of 1 TAM/TAN: 10 tumour cells (1.5×10^5 66cl4 tumour cells + 1.5×10^4 TAMs or TANs per mouse). Two further 'top-up' injections of 1.5×10^4 TAMs or TANs were given into the fourth mammary gland at 1 and 2 weeks post 66cl4 tumour cell inoculation. Mice injected with 66cl4 tumour cells alone served as a control and received PBS injections 1 and 2 weeks post-tumour cell inoculation (see Figure 3.8). Tumour growth [volume = (length x width^2)/2] was measured using electronic callipers. Data were plotted using GraphPad Prism® software.

2.11 India ink measurement of lung tumour nodules

4T1.2 and 67NR tumour-bearing mice (see Section 2.5 and 2.6) were killed and the trachea exposed. A cannula was inserted into the trachea and 2 ml of 15% India ink (Speedball, NC, USA) in PBS was injected into the lungs. The trachea was then clamped and held for 2 minutes. India ink-stained lungs were excised, washed in PBS, and fixed overnight in 10% neutral buffered formalin. To count the number of metastatic tumour nodules, lungs were rinsed in H_2O and kept wet for scoring. The five lobes of the lung were separated and visualised under a dissection microscope. Tumour nodules could be seen as raised white areas on a black background of normal tissue (Figure 2.2). Data were plotted using Microsoft® Excel software.

2.12 Phenol/chloroform DNA extraction

Disaggregated or frozen homogenised lungs, and frozen homogenised spines were digested overnight in 500 µL digestion buffer [100 mM sodium chloride NaCl, 10 mM Tris-hydrochloride (TrisHCL; Tris in distilled H_2O, pH adjusted to 8.0 with hydrochloric acid; HCL), 25 mM EDTA and 5% SDS in distilled H_2O]. Proteinase K

(Roche, Basel, Switzerland) was added on the day of tissue digestion at 0.1 mg/ml. After overnight digestion, 500 μL of phenol/chloroform, pH 6.7/8.0 (Astral Scientific, ON, USA), was then added directly to digested lungs and DNA extracted by biofuge centrifugation at maximum speed for 5 min. 400 μL of the aqueous layer was removed into a fresh tube and 200 μL of 5M ammonium acetate (NH_4OAc) was added, followed by 800 μL 100% ethanol (EtOH). Samples were then centrifuged for 10 min at maximum speed and the supernatant removed and discarded. Samples were left to air dry for 10 min before re-suspension in 400 μL 1:4 TE buffer (10 mM Tris-HCL and 1mM EDTA in distilled H_2O). DNA samples were incubated overnight at 50ºC.

2.13 qPCR tumour burden analysis

Lung and spine DNA was extracted using the phenol/chloroform method (see Section 2.12) and DNA concentration analysed by NanoDrop® (ND-1000 spectrophotometer, BioLab, GA, USA). Tumour burden for each individual tissue was measured by qPCR (StepOne™ System – Applied Biosystems, CA, USA) and Taqman chemistry. The cycle threshold (Ct) for vimentin (total mouse tissue) and mCherry (tumour cells only) was detected. Relative tumour burden was calculated by comparing the threshold cycle (Ct) values of vimentin and mCherry (ΔCt), and using the following calculation: relative tumour burden = 10,000 x $1/2^{\Delta ct}$. Using this formula a tissue without any tumour cells scores zero, whereas a tissue composed entirely of tumour cells scores 10,000 [432]. Multiplex PCR reactions consisted of 10 ng of DNA, vimentin probe; 50 nmol/L, vimentin forward and reverse primers; 50 nmol/L, mCherry probe; 50 nmol/L, and mCherry forward and reverse primers; 50 nmol/L. Data were plotted using GraphPad Prism® software.

2.14 RayBio® array analysis of serum factors in tumour-bearing mice

Serum was collected from mice bearing 4T1.2, 66cl4 and 67NR tumours (see Sections 2.5 and 2.6) at day 15 post-tumour cell inoculation, or non-tumour-bearing (saline injected) control mice. A RayBio® Mouse Cytokine Antibody Array G Series 3 and 4 (RayBiotech, Inc., GA, USA) was then carried out according to manufacturer's instructions. Slides were visualised on an Agilent Microarray Scanner with SureScan

technology (G2505B; CA, USA) and spot fluorescence intensity quantified using TotalLab TL120 software (TotalLab, Newcastle, UK). Positive controls (biotinylated protein) were normalised to one membrane. Relative fluorescence intensity was calculated by subtracting the median background (BSA) spot signal from the median sample spot signal and normalising to the total mean intensity of the positive controls. Data was pooled (three mice per group) and fold differences were calculated between tumour-bearing mice compared to saline controls. Any ≥ 1.5 fold-increases or ≤ 0.65-fold decreases in signal intensity were considered significant as per manufacturer's instructions. Data were plotted using GraphPad Prism® software.

2.15 Gelatin zymography

Serum from mice bearing 4T1.2 tumours was collected at day 15 post-tumour cell inoculation (see Sections 2.5 and 2.6). Serum was concentrated and 50, 20 and 8 µL aliquots analysed. Proteins were separated on 8% SDS-PAGE containing 2.5 mg/ml bovine gelatin B (225 BLOOM, Sigma-Aldrich, MO, USA) under non-reducing conditions. Gels were rinsed in MilliQ H_2O and then washed twice for 30 min and once for 1 h in re-naturation buffer (2.5% Triton X 100). Gels were rinsed again in H_2O and incubated in activation buffer (50 mM TrisHCL pH 7.5, 10 mM $CaCl_2$, 150 mM NaCl, 1% Triton X 100 and 0.02% w/v sodium azide) overnight at 37ºC. Gels were rinsed thoroughly in H_2O and stained with Coomassie blue (Brilliant Blue R250, 30 % methanol and 10% acetic acid, filtered) for 1 h. Gels were washed in de-stain solution (30% methanol and 10% acetic acid) until gelatinolytic bands were visible.

2.16 Western blotting

Serum from mice bearing 4T1.2 tumours was collected at day 15 post-tumour cell inoculation (see Sections 2.5 and 2.6). Serum was concentrated and 50, 20 and 8 µL aliquots analysed. Protein was separated on an 8% SDS-PAGE gel, electrotransferred onto PVDF membrane and incubated in blocking buffer (0.05 % Tween and 5-10% milk powder in PBS) for 1 h. Gels were rinsed thoroughly, washed in wash buffer (0.025% Tween-20 and 0.1% BSA in PBS) and incubated overnight at 4 ºC with goat anti-mouse MMP-9 antibody (1:400 dilution). Gels were washed three times for 15 min in wash buffer then incubated with HRP-conjugated rabbit anti-goat secondary

antibody (1:2000 dilution) for 40 min. Gels were washed again three times for 15 min in wash buffer, rinsed in H_2O and visualised using ECL Western blotting detection reagents on a CP1000 AGFA X-ray developer (AGFA GEVAERT, N.V., Germany).

2.17 Extraction of clodronate liposomes

Liposome encapsulated dichloromethylene-bisphosphonate (clodronate) and PBS (control) were prepared as previously described [433, 434] by Dr. Harper. The amount of clodronate liposomes required for injections was removed and centrifuged for 40 min at 3,500 rpm and 10ºC. The clear clodronate solution, under the white band of clodronate liposomes, was removed using a syringe and discarded. The clodronate liposomes were re-suspended in sterile PBS and washed three times by centrifugation for 15 min at 3,500 rpm and 10ºC per wash. In between each wash the upper solution was removed and discarded, and the liposome pellet re-suspended in sterile PBS. The final liposome pellet was re-suspended in sterile PBS and adjusted to the volume required for injections. The flask containing clodronate liposomes in suspension solution was purged with nitrogen gas after each opening to prevent oxidation of phophatidylcholine in the solution. The same protocol as above was used to extract PBS (control) liposomes.

2.18 Clodronate treatment

Mice were injected with 4T1.2 mCherry-expressing tumour cells (see Sections 2.5 and 2.6) and then injected intraperitoneally every 3 days, from day 5 to 21 post-tumour cell inoculation, with 50 μL of clodronate liposomes. 50 μL of PBS liposomes and PBS alone were injected as controls. Tumour volume [(length x width^2)/2] was measured using electronic callipers. Peripheral blood was harvested via the tail vein at day 5 (24 h prior to the first treatment), day 16 (48 h after the third treatment) and end-point (day 28 after tumour cell inoculation). Primary tumours were resected and weighed at end-point. Blood and primary tumour monocytes/macrophages and neutrophils were analysed by flow cytometry (see Section 2.7). Lungs and spines were excised, snap frozen and homogenised for qPCR analysis of metastatic burden (see Sections 2.12 and 2.13). Data were plotted using GraphPad Prism® software.

2.19 Treatment with neutralising antibody

Mice were injected with 4T1.2 mCherry-expressing tumour cells (see Sections 2.5 and 2.6) and then injected intraperitoneally every 4 days, from day 7-21 post-tumour cell inoculation, with antibody, namely, 250 μg of anti-CSF-1R (AFS-98), 150 μg of anti-GM-CSF (22E9) or 250 μg of anti-β-galactosidase isotype control (GL117.41). Tumour volume [(length x width^2)/2] was measured using electronic callipers. Blood was harvested via the tail vein on day 6 (24 h prior to the first treatment), day 19 (24 h after the fourth treatment), and end-point (day 28 after tumour cell inoculation). Primary tumours, lungs and spines were resected at end-point. Primary tumours and lungs were weighed, disaggregated and monocytes/macrophages and neutrophils were analysed by flow cytometry (see Section 2.7). Spines were snap frozen and homogenised. Spine homogenate and the remainder of the disaggregated lungs were used for qPCR analysis of tumour burden (see Sections 2.12 and 2.13). Data were plotted using GraphPad Prism® software.

2.20 Treatment with CSF-1R inhibitor

The CSF-1R kinase inhibitor (GW2580) was used to inhibit CSF-1R signalling. Mice were injected with 4T1.2 mCherry-expressing tumour cells (see Sections 2.5 and 2.6) and then treated by oral gavage every 4 days from day 7-21 post-tumour cell inoculation with 160 mg/kg of GW2580 in 200 μL hydroxypropylmethylcellulose suspension vehicle (HPMC-SV) or 200 μL of vehicle as a control [435, 436]. Tumour growth, primary tumour and lung myeloid cell numbers, as well as metastasis to lung and spine were measured as outlined above in Section 2.19. Data were plotted using GraphPad Prism® software.

2.21 Statistical analysis

A non-paired student's t-test was used to determine statistical significance. Data involving multiple groups and/or time points were analysed using one or two-way ANOVA analysis of variance with Bonferroni correction. $P \leq 0.05$ was considered significant.

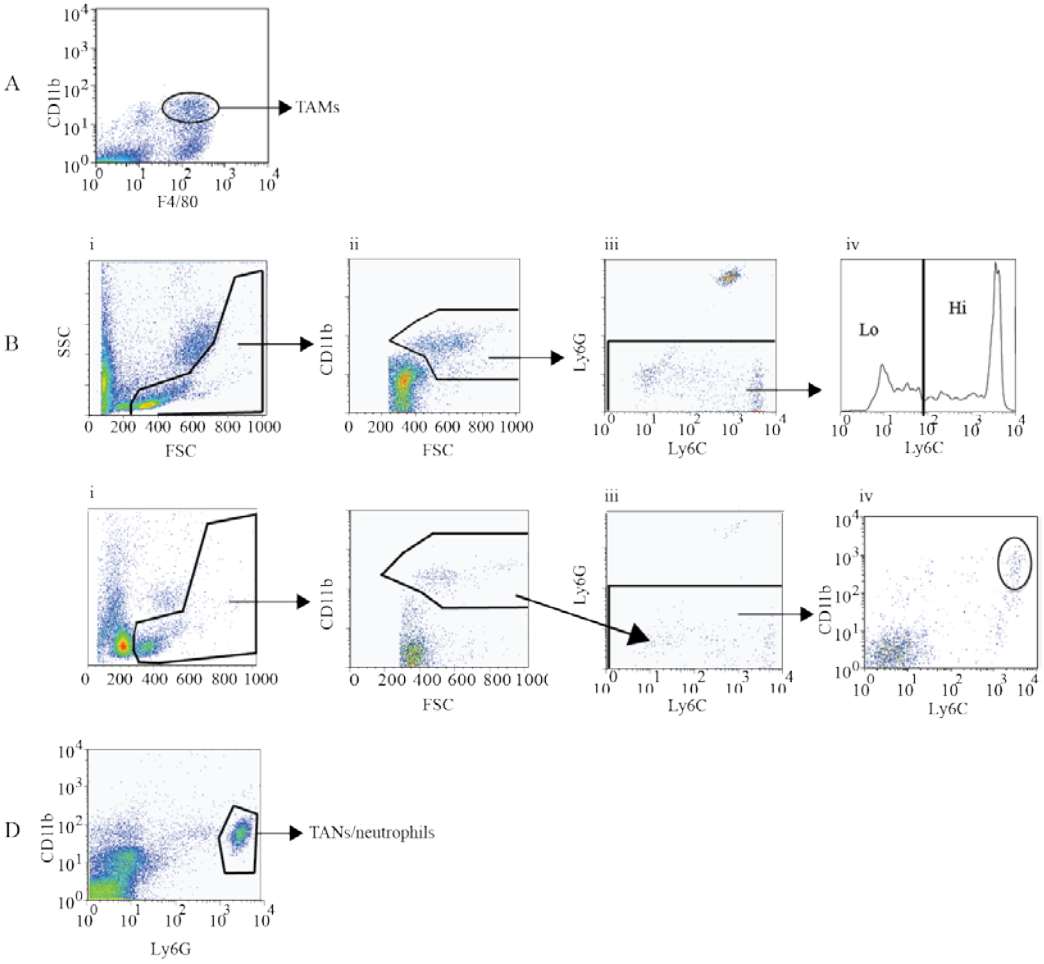


Figure 2.1: Flow cytometric analysis of monocytes/macrophages and neutrophils. Leukocytes were labelled with anti-CD11b, anti-Ly6C, anti-Ly6G and either anti-CD115 or anti-F4/80. A) The total TAM population in primary tumours was characterised as $CD11b^{+}F4/80^{+}$ cells. B) For analysis of monocyte/macrophage/TAM subpopulations (i) a typical side scatter vs. forward scatter gate was set for mononuclear cells from which (ii) $FSC^{high}CD11b^{+}$ myeloid cells were selected and $FSC^{low}CD11b^{+}$ were excluded . (iii) From this $FSC^{high}CD11b^{+}$ population, $Ly6C^{+}Ly6G^{+}$ cells were excluded (thus removing any $Ly6G^{+}$ contaminating neutrophils) and (iv) the remaining monocyte/macrophage/TAMs divided into the $Ly6C^{high}$ and $Ly6C^{low}$ subpopulations. A representative peripheral blood sample is depicted in panel B. C) For analysis of bone marrow samples, (i) mononuclear cells were again selected; (ii) $FSC^{high}CD11b^{+}$ myeloid cells were selected and $FSC^{low}CD11b^{+}$ cells were excluded. (iii) $Ly6C^{+}Ly6G^{+}$ cells (neutrophils) were excluded, and (iii) monocytes characterised as $CD11b^{+}Ly6G^{neg}Ly6C^{high}$ cells. D) Neutrophils/TANs were characterised as $CD11b^{+}Ly6G^{high}$ cells.

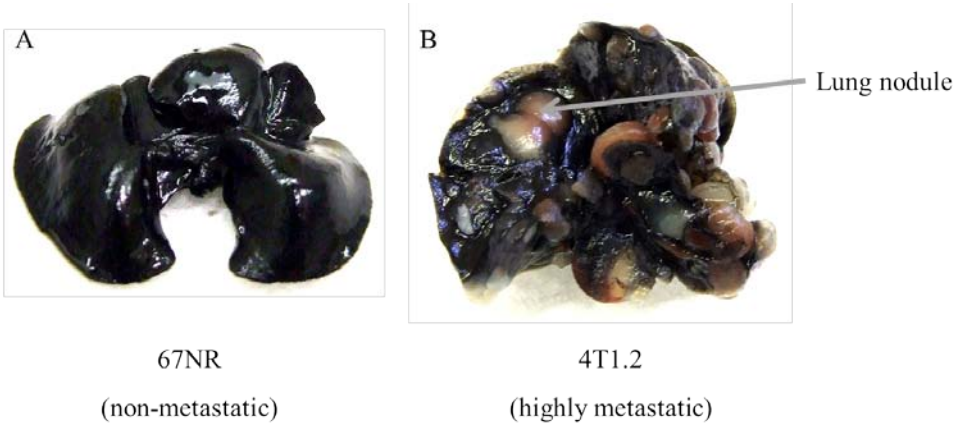


Figure 2.2: Lungs from 67NR and 4T1.2 tumour-bearing mice stained with India ink.

Mice were injected orthotopically into the fourth mammary gland with either 4T1.2 (highly metastatic) or 67NR (non-metastatic) tumour cells. Lungs from tumour-bearing mice were excised every 5 days after tumour cell inoculation, stained with India ink, and lung nodules counted. This figure shows one representative sample of twelve, at day 28 after tumour cell inoculation, from A) 67NR tumour-bearing mice and B) 4T1.2 tumour-bearing mice. Lung tumour nodules can be seen as raised white areas on a black background (B).

3 Chapter 3: Analysis of myeloid cells in metastatic mammary tumours

3.1 Introduction

Investigations into the role of myeloid cells in breast tumour metastasis have been hindered by confusion regarding the heterogeneity of myeloid-derived suppressor cells (MDSCs) [206-208]. Murine MDSCs are characterised as Gr-1$^+$CD11b$^+$ cells. However, Gr-1$^+$ cells are often designated as neutrophils [218, 219] or monocytes [220]. In fact, Gr-1 does not distinguish between neutrophils and monocytes/macrophages and both neutrophil-like and macrophage-like cells have been characterised within tumour-associated MDSCs [218].

TAMs are known to promote breast tumour growth [230-232] and have also been implicated in metastasis [235, 414]. Macrophages are affected by the local environment and display a continuum of phenotypes of which the 'inflammatory' M1-like and 'anti-inflammatory/trophic' M2-like macrophages are the extremes [437]. One way of characterising M1-like and M2-like macrophages is by the different cytokines that they produce [146, 154, 437]. TAMs are considered to have an M2-like phenotype [231, 438], although recent results indicate that TAM subpopulations can display M1- or M2-like phenotypes [225]. However, differences between the Ly6C^{high} (immature, classical) and Ly6C^{low} (mature, non-classical) subpopulations of TAMs have not been investigated in breast tumours or other cancers.

Recent data link TANs to progression of malignancy in a number of cancers [362, 363, 383], but the link between TANs and breast cancer metastasis is still unclear. Co-injection of Gr-1$^+$CD11b$^+$ cells isolated from tumours and spleens of 4T1 tumour-bearing mice with 4T1 tumour cells increases metastasis to lung, whereas there is no increase in lung metastasis following injection of 4T1 tumour cells with Gr-1$^+$CD11b$^+$ cells from spleens of non-tumour-bearing mice [185]. Thus, it is possible that TAMs and/or TANs can influence the metastatic potential of tumours. Therefore the studies discussed in this chapter investigated changes in TAM subpopulations and TANs in mice bearing tumours or varying metastatic capacity and how these changes related to metastasis.

In this chapter it is shown that the Ly6C^{low} TAM subpopulation is predominant in primary tumours and increases in number with increasing metastatic capacity. TANs are the main immune cell type found in highly metastatic 4T1.2 tumours, and are highly increased in number compared to the other tumour variants. These increases in TAMs and TANs occur during the early phases of metastasis. Preliminary data indicate that gene expression may differ in Ly6C^{high} TAMs compared to Ly6C^{low} TAMs. Results from a pilot study indicating that TAMs and TANs isolated from highly metastatic 4T1.2 tumours are able to promote the growth of weakly metastatic 66cl4 primary tumours are also presented.

3.2 Results

3.2.1 TAM and TAN numbers increase in highly metastatic 4T1.2 tumours

The majority of the literature on immune cells in breast tumour metastasis focuses on macrophages [4, 230, 232, 233, 340] and lymphocytes [341, 439-442]. However, emerging evidence implicates neutrophils in tumour development. Given this, and the evidence from a recent study showing that increased TAM numbers and decreased lymphocyte numbers in breast tumours associated with poor prognosis [136], TAM, TAN and lymphocyte numbers were analysed in end-stage primary tumours of varying metastatic capacity. TAMs were characterised as CD11b^{+}Ly6G^{neg}F4/80^{+} or, in order to analyse TAM subpopulations, as CD11b^{+}Ly6G^{neg}Ly6C^{high} or CD11b^{+}Ly6G^{neg}Ly6C^{low} cells [303]. TANs were characterised as CD11b^{+}Ly6G^{high} cells [8, 223]. This approach allowed for a more specific analysis of TAMs and TANs.

TANs were the main immune cell type found in late stage highly metastatic 4T1.2 tumours (Figure 3.1A, $P \leq 0.01$ TANs vs. TAMs and CD4 T cells and $P \leq 0.001$ TANs vs. CD8 T cells and B cells). In weakly metastatic 66cl4 primary tumours, TAMs were increased compared to TANs, CD8 T and B cells, but not CD4 T cells (Figure 3.1B, $P \leq 0.05$ TAMs vs. TANs, CD8 T cells and B cells). In non-metastatic 67NR primary tumours, CD4 T cells were increased compared to TANs, CD8 T and B cells (Figure 3.1C, $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.05$, respectively), but not TAMs.

The same data were used to compare TAM, TAN and lymphocyte numbers between the tumour variants. TAM numbers were increased in 4T1.2 compared to 67NR tumours (Figure 3.2A, $P \leq 0.05$) with a trend towards increased numbers in 66cl4 tumours also observed (Figure 3.2A). TAN numbers were increased markedly in 4T1.2 compared to 66cl4 and 67NR tumours (Figure 3.2B, $P \leq 0.001$). CD4 T cells showed similar numbers between the metastatic tumour variants (Figure 3.2C). CD8 T cell and B cell numbers were also increased in tumours with greater metastatic capacity (Figures 3.2D and 3.2E, $P \leq 0.05$ 4T1.2 vs. 66cl4 and $P \leq 0.01$ 4T1.2 vs. 67NR), although their numbers were relatively low (see Figure 3.1).

3.2.2 Ly6C^{high} and Ly6C^{low} TAM/monocyte numbers are differentially increased in 4T1.2 tumour-bearing mice

Given that little is known about Ly6C^{high} and Ly6C^{low} TAM/monocyte subpopulations in spontaneous metastasis, both subpopulations were measured in end-stage primary tumours, as well as in peripheral blood. In the primary tumour, Ly6C^{low} TAMs were the predominant subpopulation and numbers increased with increasing metastatic capacity (Figure 3.3A, $P \leq 0.05$ 4T1.2 vs. 66cl4 and $P \leq 0.001$ 4T1.2 vs. 67NR), with a trend towards increased numbers in 66cl4 compared to 67NR primary tumours also observed. No statistical difference was seen for Ly6C^{high} TAM numbers between the tumour variants, although, again, a trend towards increasing numbers with increasing metastatic capacity was observed (Figure 3.3A).

In the peripheral blood of mice bearing end-stage tumours, Ly6C^{high} monocytes were the predominant subpopulation, and numbers increased in 4T1.2 compared to 66cl4 and 67NR tumour-bearing mice (Figure 3.3B, $P \leq 0.001$ 4T1.2 vs. 66cl4 and 67NR), with a trend towards increased numbers in 66cl4 compared to 67NR tumour-tumour-bearing mice seen. No statistical difference was seen for blood Ly6C^{low} monocyte numbers between the different tumour variants, although there was a trend towards increasing numbers with increasing metastatic capacity (Figure 3.3B). Blood neutrophil numbers also increased in 4T1.2 tumour-bearing mice compared to the other tumour variants (Figure 3.4, $P \leq 0.0001$ 4T1.2 vs. 66cl4 and 67NR) and, again, a trend towards increased numbers was observed in 66cl4 compared to 67NR tumour-bearing mice.

To see how TAMs and TANs associated with tumour progression, their numbers were analysed during the course of tumour growth and metastasis to lung. 4T1.2 and 67NR primary tumours were collected every 5 days following tumour cell inoculation and TAM and TAN numbers were analysed by flow cytometry. The size of this study limited the number of tumour cell line variants used. The 4T1.2 and 67NR tumour variants were chosen as, given that 4T1.2 tumours are highly aggressive, differences between metastatic and non-metastatic tumours would be more apparent using the 4T1.2 rather than the 66cl4 cell line. Lungs were also collected at these time points and metastasis quantified by India ink staining. No visible lung nodules were detected in mice bearing 67NR tumours hence only data scoring lung nodules in 4T1.2 tumour-bearing mice is shown. Significant increases in both Ly6C^{high} and Ly6C^{low} TAM numbers in highly metastatic 4T1.2 tumours compared to non-metastatic 67NR tumours were first seen at day 20 post-tumour cell inoculation (Figures 3.5A, $P \leq 0.001$ and 3.5B, $P \leq 0.05$). This was also the time that metastatic lung tumour nodules were first seen in mice bearing 4T1.2 tumours (Figure 3.5). The same pattern was seen for TANs, with numbers increasing in 4T1.2 tumours around the time visible lung tumour nodules were first detected (day 20) (Figure 3.5C, $P \leq 0.01$).

Since it is possible that these differences in monocyte/TAM and neutrophil/TAN numbers were due to differences in tumour growth, primary tumour growth was measured and primary tumours were weighed at harvest. No differences were observed between the metastatic variants in regard to tumour growth (Figure 3.6A) or end-stage weight (Figure 3.6B). This indicates that the increases in myeloid cell numbers, in particular TANs and Ly6C^{low} TAMs in the primary tumour and neutrophils and Ly6C^{high} monocytes in the peripheral blood, are related to tumour metastatic capacity and not primary tumour growth and size.

3.2.3 Ly6C^{high} and Ly6C^{low} TAMs display different gene expression profiles

Recent gene expression analysis of TAMs revealed differences in gene expression of TAM subpopulations based on MHC II expression, with MHC II^{high} TAMs displaying a more ‘pro-inflammatory’ M1-like phenotype, and MHC II^{low} TAMs being more ‘anti-

inflammatory/trophic' M2-like in nature [225, 264]. Both of these MHC II^{high} and MHC II^{low} subpopulations were $Ly6C^{low}$ and it has recently been shown that the $Ly6C^{high}$ subpopulation does not express MHC II [226]. The differences in gene expression profiles of $Ly6C^{high}$ and $Ly6C^{low}$ TAMs have not been analysed.

A protocol for the isolation of TAMs from primary tumours using fluorescence-activated cell sorting (FACS) was optimised. Cell sorting was chosen as this method required the least manipulation of cells, thus minimising potential phenotypic changes within TAMs during isolation. TAMs were isolated from tumours at day 25 post-tumour cell inoculation, as by day 28 4T1.2 tumours were often necrotic, making it difficult to obtain sufficient numbers of cells for analysis. Since tumour cells were expressing the mCherry fluorescent protein, TAMs were characterised as $mCherry^{neg}CD11b^{+}Ly6G^{neg}Ly6C^{high}$ or $mCherry^{neg}CD11b^{+}Ly6G^{neg}Ly6C^{low}$. NK cells were excluded, via exclusion from the $CD11b^{+}$ gate. Cells from highly metastatic 4T1.2 tumours (4T1.2 $Ly6C^{high}$ TAMs and 4T1.2 $Ly6C^{low}$ TAMs) were compared to cells from non-metastatic 67NR tumours (67NR $Ly6C^{high}$ TAMs and 67NR $Ly6C^{low}$ TAMs).

Given that TAM subpopulations were reported to show differences in M1, 'inflammatory' and M2, 'trophic' associated gene expression, the classic M1 genes, IL-12 and IL-23 [143] and the characteristic M2 gene, IL-10 [143], were analysed. In a preliminary experiment, 4T1.2 $Ly6C^{high}$ TAMs appeared to express higher levels of both IL-12A and IL-23A mRNA than 4T1.2 $Ly6C^{low}$ TAMs and TAMs from 67NR tumours (Figures 3.7A and 3.7C). Both the IL-12A and IL-23A genes code for the alpha subunits (p35 and p19, respectively), but the IL-12 beta subunit (p40) is required for active heterodimers of these cytokines [443-445]. 67NR $Ly6C^{low}$ TAMs appeared to express the highest levels of IL-12B mRNA, whereas minimal expression was seen in 4T1.2 $Ly6C^{high}$ and $Ly6C^{low}$ TAMs (Figure 3.7B). $Ly6C^{high}$ TAMs appeared to express higher levels of IL-10 mRNA than $Ly6C^{low}$ TAMs in both 4T1.2 and 67NR tumours (Figure 3.7D). As this experiment was repeated only once, albeit each time with samples in triplicate, no rigorous statistical analysis was applied.

3.2.4 Co-injection of TAMs and TANs isolated from highly metastatic (4T1.2) or non-metastatic (67NR) primary tumours with weakly metastatic (66cl4) tumour cells

Primary tumour Gr-1^{+}CD11b^{+} cells (neutrophils and macrophages) can increase the metastatic capacity of tumours [92, 185]. It is therefore possible that TAMs and/or TANs isolated from highly metastatic tumours may be able to enhance the metastatic potential of less metastatic tumours. TAMs and TANs were isolated from day 25 4T1.2 (highly metastatic) and 67NR (non-metastatic) mCherry-expressing primary tumours. TAMs were characterised as before (see Section 3.2.3) and TANs as mCherry^{neg}CD11b^{+}Ly6G^{high}. 4T1.2 TAMs or TANs or 67NR TAMs or TANs were then co-injected with 66cl4 tumour cells (at a ratio of 1 TAM or TAN: 10 tumour cells) into the mammary fat pad. Two further intra-tumoural injections of TAMs or TANs (the same numbers as used for the first co-injection) were given one and two weeks after the initial tumour cell inoculation (Figure 3.8).

Co-injection of 66cl4 tumour cells with 4T1.2 TAMs or 4T1.2 TANs increased 66cl4 tumour growth (Figure 3.9, $P \leq 0.01$-0.001 4T1.2 TAMs or 4T1.2 TANs vs. 67NR TAMs, 67NR TANs and 66cl4 alone). Unfortunately in this experiment tumours regressed in the control groups (Figure 3.9: 67NR TAM or TAN co-injected with 66cl4 tumour cells and 66cl4 tumour cells injected alone). Furthermore, tumours that did grow became palpable much later (day 25) than the expected day 7-10 post-tumour cell inoculation (see Figures 3.9 and 3.6A). Metastasis was not analysed due to the lack of control samples for comparison to experimental samples.

3.3 Discussion

Increased TAM numbers have been associated with metastasis in breast cancer [234, 235], and TAMs are known to carry out a number of functions associated with tumour progression and metastasis [446]. Emerging data associate increased TAN numbers and malignancy in a number of other cancers [361-363], although a link between TANs and breast tumour metastasis has not been conclusively shown. In this chapter it is reported that Ly6C^{low} TAMs were the predominant subpopulation in primary tumours, and numbers increased with increasing metastatic capacity (Figure 3.3A). Ly6C^{low} TAMs

have been reported to be the predominant subpopulation in other mammary tumour models [202, 225], but an increase in Ly6C^{low} TAM numbers with increasing tumour metastatic capacity has not been previously described. TANs were increased only in the 4T1.2 primary tumours (Figures 3.1A and 3.2B). TANs have been linked to metastasis in some rodent mammary tumour models [5, 8] but an association between increased TAN numbers and metastasis has not been reported before in breast cancer models. Analysis of TANs in human breast tumours is required to determine if increased TAN numbers correlate with metastasis in the human disease.

Presumably the differences in TAM and TAN numbers described above reflect the different microenvironments generated by primary tumours of different metastatic capacity. 67NR tumours may be non-metastatic in part due to their impaired ability to recruit TAMs and/or TANs. On the other hand, 66cl4 tumours can metastasise to lung to some degree, potentially due to their ability to attract higher numbers of TAMs, whereas 4T1.2 tumours are extremely aggressive and attract high numbers of both TAMs and TANs.

CD8 T cells and B cells also increased with increasing metastatic capacity of the primary tumour (Figures 3.2D and 3.2E). Increased CD8 T cell counts have been associated with increased survival in human breast cancer [447]; hence CD8 T cell numbers would possibly be expected to decrease in the highly metastatic 4T1.2 tumours. The increases in B and CD8 T cell numbers reported in this thesis may be indicative of failed anti-tumoural immune responses, as TAMs and TANs have both been shown to suppress anti-tumour T cell activity [193, 215, 256], and were found in high numbers in 4T1.2 tumours (Figures 3.2A and 3.2B).

Increases in Ly6C^{high} and Ly6C^{low} TAMs, as well as TANs, were seen early in tumour progression, around the time that visible lung tumour nodules were first detected (Figure 3.5), even though there were no differences in growth rates or weights of the different tumour variants (Figure 3.6). Thus, TAM and TAN numbers appear to be increasing during the early phases of metastasis, possibly at the micrometastatic or macrometastatic stages, given that their numbers increased around the time that visible lung nodules were first detected. These data indicate that the increases in Ly6C^{high} and Ly6C^{low} TAMs, as well as TANs, are associated with metastatic capacity of the primary

tumour rather than tumour size or growth. Thus, it appears that not only Ly6C^{low} TAMs, but also TANs are associated with metastasis in the 4T1.2 mammary tumour model, and are possibly involved early in tumour progression to metastasis. Depletion of these cell populations and individual subpopulations, either after metastasis is established or during early metastatic events, could indicate the importance of these cells in different stages of metastatic progression.

Recent gene expression profiling data have shown that, although TAMs express many genes associated with the M2 macrophage phenotype [225, 257, 258], they can also express M1 phenotype-associated genes [225, 259]. Based on MHC II expression, two distinct TAM subpopulations were reported in murine mammary TS/A tumours and hepatocellular carcinomas [225, 264]. MHC II^{high} TAMs upregulated expression of genes such as iNOS, IL-1β and IL-12, and were more M1-like in phenotype, whereas MHC II^{low} TAMs upregulated genes such as ARG1 and STAB1 and were more M2-like in phenotype [225, 264]. MHC II^{low} TAMs were found to reside preferentially in hypoxic regions of tumours and were pro-angiogenic [225]. These studies indicate that TAM subpopulations may have specialised functions depending on the microenvironmental influences of the tumour region that they occupy.

IL-12 and IL-23 are associated with the M1 'pro-inflammatory' macrophage phenotype [143] and preliminary data in this thesis indicated that Ly6C^{high} TAMs express higher levels IL-12A and IL-23A mRNA than Ly6C^{low} TAMs (Figures 3.7A and 3.7C) and expression was upregulated in 4T1.2 compared to 67NR tumours (Figures 3.7A and 3.7C). Despite the fact that IL-23 is associated with a pro-inflammatory macrophage phenotype, increased IL-23 serum levels have recently been linked to poor prognosis in human breast cancer [178]. Hence the role of IL-23 in breast tumour metastasis is unclear. On the other hand, IL-12 increases T and NK cell cytotoxicity against tumours [173, 174, 448]. Treatment with IL-12 reduces murine mammary tumour metastasis [175], and IL-12 is considered a potential adjuvant in the treatment of cancer [449]. However, IL-12B, the binding partner that is required for heterodimer activity of IL-12 and IL-23 [443-445] was not expressed in 4T1.2 Ly6C^{high} or Ly6C^{low} TAMs (Figure 3.7B); it is therefore possible that Ly6C^{high} TAMs are not making active IL-12 and IL-23. This observation needs to be confirmed at the protein level.

On the other hand, IL-10 is typically associated with the M2/trophic phenotype in macrophages [143, 154, 437] and 4T1.2 Ly6C^{high} TAMs also appeared to express higher IL-10 mRNA levels than 4T1.2 Ly6C^{low} TAMs (Figure 3.7D). Increased IL-10 serum levels have been associated with poor prognosis in human breast cancer [177], and with reduced T cell activation [176, 450], suggesting that IL-10 may suppress anti-tumour immune responses. Surprisingly, IL-10 appeared to be most highly expressed in 67NR Ly6C^{high} TAMs (Figure 3.7D). Macrophages display a phenotypic spectrum, of which M1 and M2 are the extremes *in vitro*, thus Ly6C^{high} TAMs may express a combination of 'pro-inflammatory' M1 and 'trophic' M2 associated genes. Analysis of additional M1 and M2-associated genes is required to develop a comprehensive gene expression profile for these cells. This is discussed further in Chapter 6, Section 6.3.1.

Gr-1^{+}CD11b^{+} cells isolated from metastatic tumours can increase metastasis to lung upon co-injection with tumour cells [185]. Given that Gr-1 labels both macrophages and neutrophils, it is possible that either TAMs and/or TANs can influence the metastatic capacity of primary tumours. In line with this, results from the pilot experiment in this chapter indicated that co-injection of both TAMs and TANs from highly metastatic 4T1.2 tumours increased 66cl4 tumour growth compared to TAMs and TANs isolated from non-metastatic 67NR tumours (Figure 3.9). However, this result was confounded by the fact that 66cl4 tumours injected with saline alone regressed, as did 66cl4 tumours co-injected with TAMs or TANs from 67NR tumours (Figure 3.9). Given the failure of the controls, metastasis was not assessed, as no comprehensive comparison could be made for the experimental groups.

For co-injection of 66cl4 tumour cells with TAMs or TANs, a ratio of 10 tumour cells to 1 TAM/TAN was used, as this most closely represented what was seen *in vivo*. To achieve this ratio, fewer numbers (1.5×10^5 cells per mouse) of 66cl4 tumour cells than normally used (2×10^5 cells/mouse) were injected, and this may not have been sufficient for 66cl4 tumour growth to occur. In the tumours that did grow, tumour onset occurred much later than expected, around day 24 post-tumour cell inoculation, as opposed to the expected day 7-10 (see Figures 3.9 and 3.6A).

Another possible complication was contamination of 4T1.2 cells during cell sorting, as this cell line can grow tumours successfully from very few cells, just with a longer lag phase. To address this, 4T1.2 TAMs or TANs were injected alone into the fourth mammary fat pad. No tumour growth occurred in mice that received 4T1.2 TANs alone. However, one mouse out of six that received 4T1.2 TAMs grew a tumour. This indicated that, in the 4T1.2 TAM group at least, there were contaminating 4T1.2 tumour cells. This contamination may involve only a few cells, as 4T1.2 tumours can grow from as little as 50 cells (Restall and Anderson, unpublished data). However, because no tumour growth was seen upon injection of 4T1.2 TANs alone, the preliminary results showing that TAMs and TANs from 4T1.2 tumours can enhance 66cl4 tumour growth are promising. This experiment needs to be repeated after further optimisation of the cell sorting protocol. Gradient separation of tumour samples or CD11b microbead selection may help to minimise tumour cell contamination. Furthermore, the use of mCherry-expressing tumour cells for isolating TAMs and TANs from primary tumours, and a different tag, such as neomycin, for the 66cl4 cells would allow for verification of which cell line gave rise to metastases, depending on the reporter gene expressed.

3.4 Conclusions

Ly6C^{low} TAMs were the predominant subpopulation in tumours and their number increased with increasing metastatic capacity. Both Ly6C^{low} TAM and TAN numbers increased during the early stages of metastasis, although it is unclear at this stage if this was during the micrometastatic or macrometastatic phases. Preliminary data indicate that Ly6C^{high} and Ly6C^{low} TAM gene expression profiles may differ, but this needs further validation. TAN numbers were also increased in highly metastatic primary tumours. TAMs and TANs isolated from highly metastatic 4T1.2 tumours might be able to increase the growth of weakly metastatic 66cl4 tumours but again, this observation needs to be confirmed. This implies that, not only TAMs, but importantly, TANs are associated with metastasis of 4T1.2 mammary tumours. Depletion of TANs will clarify if this cell population is also important for metastasis.

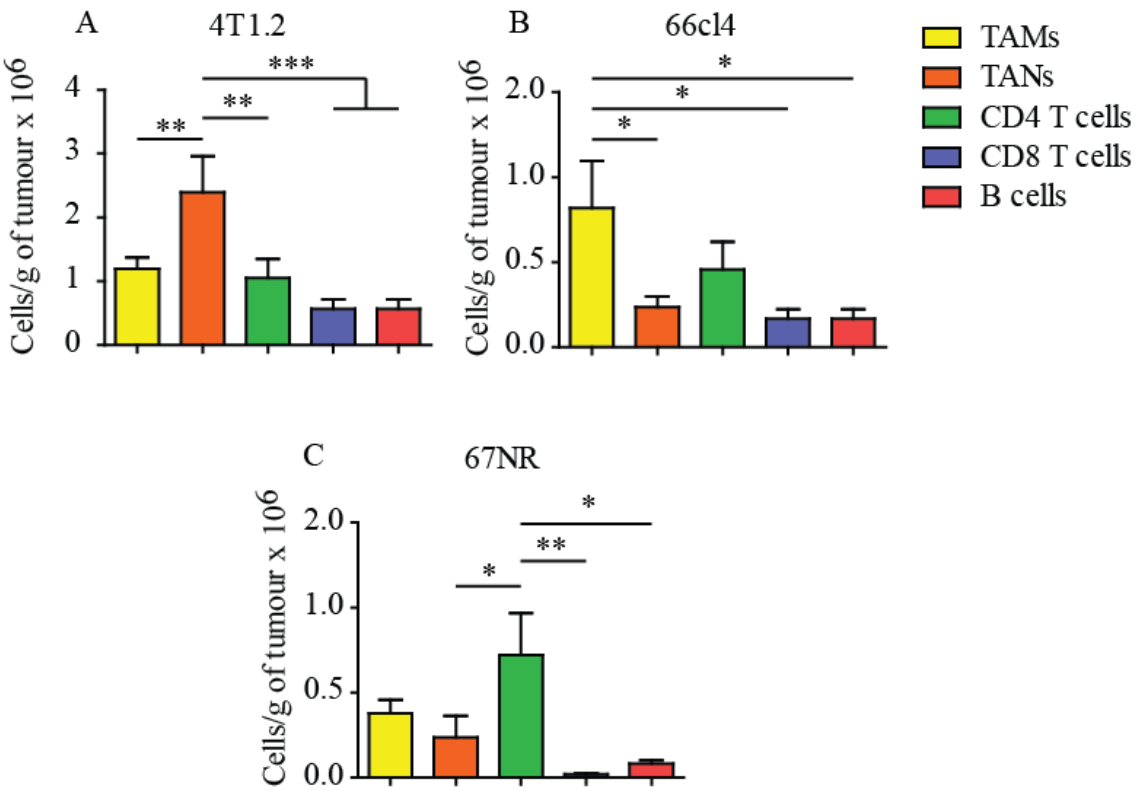


Figure 3.1: Comparison of immune cell populations in 4T1.2 (highly metastatic), 66cl4 (weakly metastatic) and 67NR (non-metastatic) end-stage primary tumours. Mice were injected orthotopically into the fourth mammary gland with either 4T1.2 (highly metastatic), 66cl4 (weakly metastatic) or 67NR (non-metastatic) tumour cells. Primary tumours were excised at end-point (day 28), disaggregated, and myeloid cell populations were analysed by flow cytometry. TAMs were characterised as $CD11b^{+}Ly6G^{neg}F4/80^{+}$ cells, TANs as $CD11b^{+}Ly6G^{high}$ cells, T lymphocytes as $CD4^{+}$ cells or $CD8^{+}$ cells, and B lymphocytes as $B220^{+}$ cells. A) 4T1.2, B) 66cl4 and C) 67NR end-point primary tumour myeloid and lymphoid cell population numbers. Data are expressed as cells per gram of tumour. Mean±SEM; n=6 mice/group. A) $^{**}P \leq 0.01$ TANs vs. TAMs and CD4 T cells, $^{***}P \leq 0.001$ TANs vs. CD8 T cells and B cells. B) $^{*}P \leq 0.05$ TAMs vs. TANs, CD8 T cells and B cells. C) $^{*}P \leq 0.05$ CD4 T cells vs. TANs and B cells, $^{**}P \leq 0.01$ CD4 T cells vs. CD8 T cells, one-way repeated measures ANOVA.

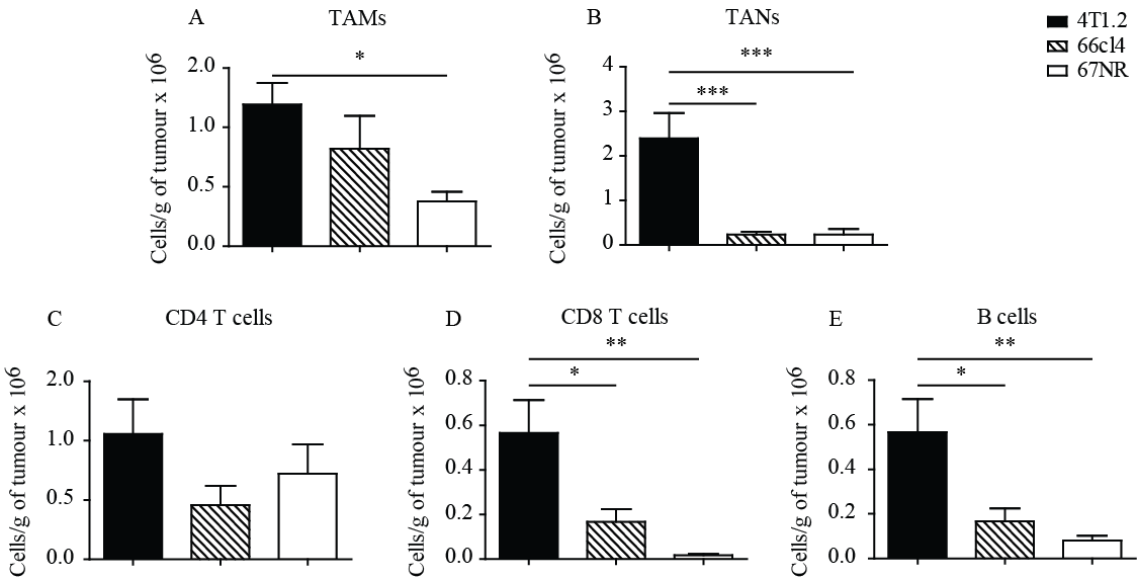


Figure 3.2: TAMs, TANs, CD8 T cells and B cells increase with increasing metastatic capacity of primary tumours.

Primary tumour immune cell populations were analysed by flow cytometry as described in Figure 3.1. A) TAM, B) TAN, C) $CD4^+$ T cell, D) $CD8^+$ T cell and E) B cell numbers. Data are expressed as cells per gram of tumour. Mean±SEM; *n*=6 mice/group. A) *$P \leq 0.05$ 4T1.2 vs. 67NR, B) ***$P \leq 0.001$ 4T1.2 vs. 66cl4 and 67NR, D and E) *$P \leq 0.05$ 4T1.2 vs. 66cl4, **$P \leq 0.01$ 4T1.2 vs. 67NR, one-way repeated measures ANOVA.

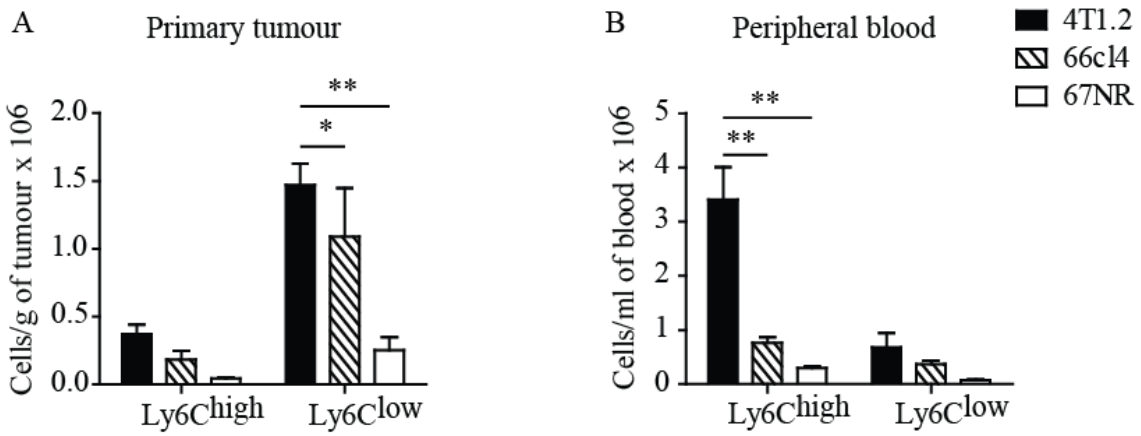


Figure 3.3: Ly6C^{low} TAM and Ly6C^{high} blood monocyte numbers increase with increasing tumour metastatic capacity.

Mice were injected orthotopically into the fourth mammary gland with 4T1.2 (highly metastatic), 66cl4 (weakly metastatic) or 67NR (non-metastatic) tumour cells. Primary tumours and peripheral blood were harvested at end-point (day 28). Primary tumours were disaggregated. Primary tumour TAM and blood monocyte subpopulations were characterised by flow cytometry as described in Figure 3.1. A) Primary tumour Ly6C^{high} and Ly6C^{low} TAM numbers. B) Blood Ly6C^{high} and Ly6C^{low} monocyte numbers. Data are expressed as A) cells per gram of tumour and B) cells per ml of blood. Mean±SEM; *n*=8 mice/group. A) *$P \leq 0.05$ 4T1.2 vs. 66cl4. **$P \leq 0.001$ 4T1.2 vs. 67NR and B) **$P \leq 0.001$ 4T1.2 vs. 66cl4 and 67NR, two-way repeated measures ANOVA.

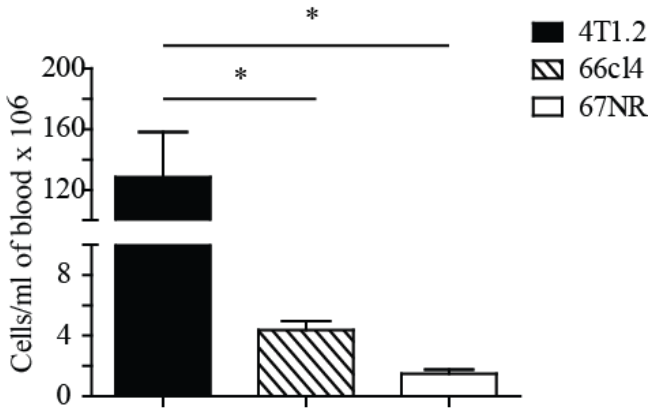


Figure 3.4: Blood neutrophil numbers increase with increasing tumour metastatic capacity.

Mice were injected orthotopically into the fourth mammary gland with 4T1.2 (highly metastatic), 66cl4 (weakly metastatic) or 67NR (non-metastatic) tumour cells. Peripheral blood was harvested at end-point (day 28) and neutrophil numbers analysed by flow cytometry as described in Figure 3.1. Data are expressed as cells per ml of blood. Mean±SEM; *n*=8 mice/group. A) *$P \leq 0.0001$ 4T1.2 vs. 66cl4 and 67NR, one-way repeated measures ANOVA.

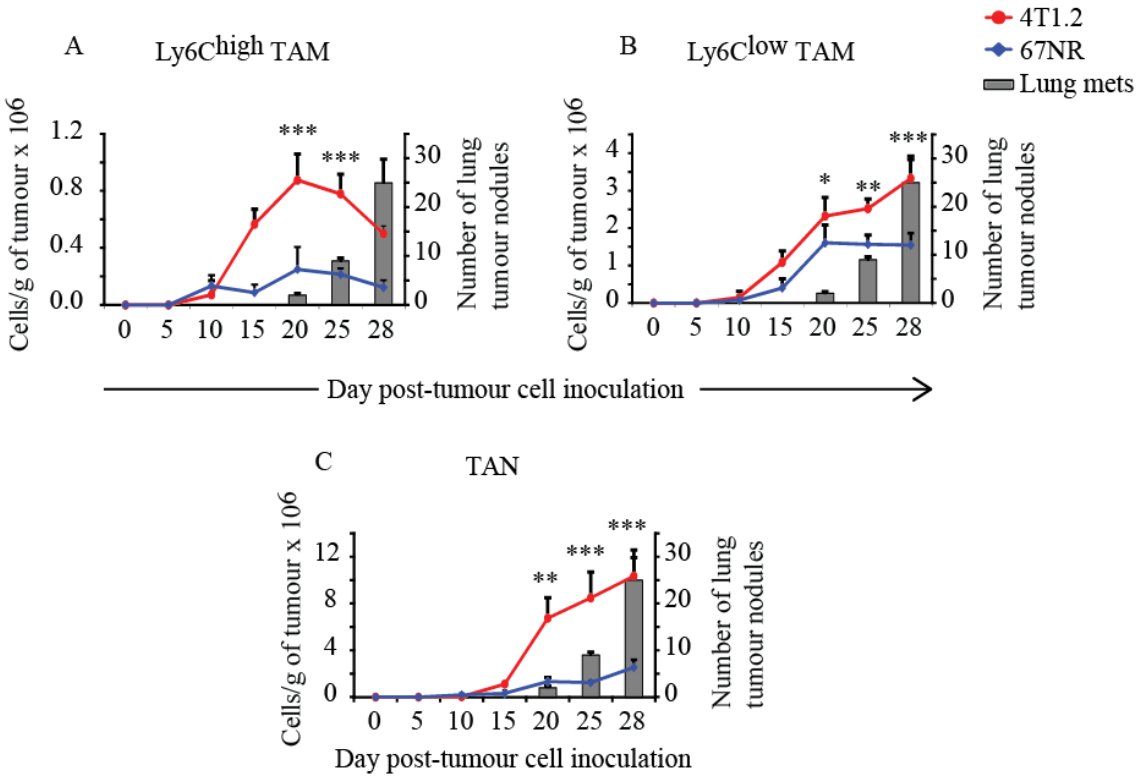


Figure 3.5: Primary tumour Ly6C^{high} and Ly6C^{low} TAM, as well as TAN numbers, increase around the time visible lung tumour nodules are first observed.
Mice were orthotopically injected into the fourth mammary gland with either 4T1.2 (highly metastatic) or 67NR (non-metastatic) tumour cells. A cohort of mice (*n*=12/group) was culled at each of the time points shown. Primary tumours were excised every 5 days after tumour cell inoculation, disaggregated, and TAM and TAN numbers analysed by flow cytometry as described in Figure 3.1 (left axis). Lungs from tumour-bearing mice were excised every 5 days after tumour cell inoculation, stained with India ink, and lung nodules counted (right axis). No lung nodules were detected in the lungs of 67NR tumour-bearing mice hence only the lung nodule scores from 4T1.2 tumour-bearing mice are shown. Primary tumour A) Ly6C^{high} TAMs, B) Ly6C^{low} TAMs and C) TANs. Data are expressed as cells per gram of tumour (left axis) and number of total surface lung tumour nodules (right axis). Mean±SEM. **P*≤0.05, ***P*≤0.01 and ****P*≤0.001 4T1.2 vs. 67NR, two-way repeated measures ANOVA.

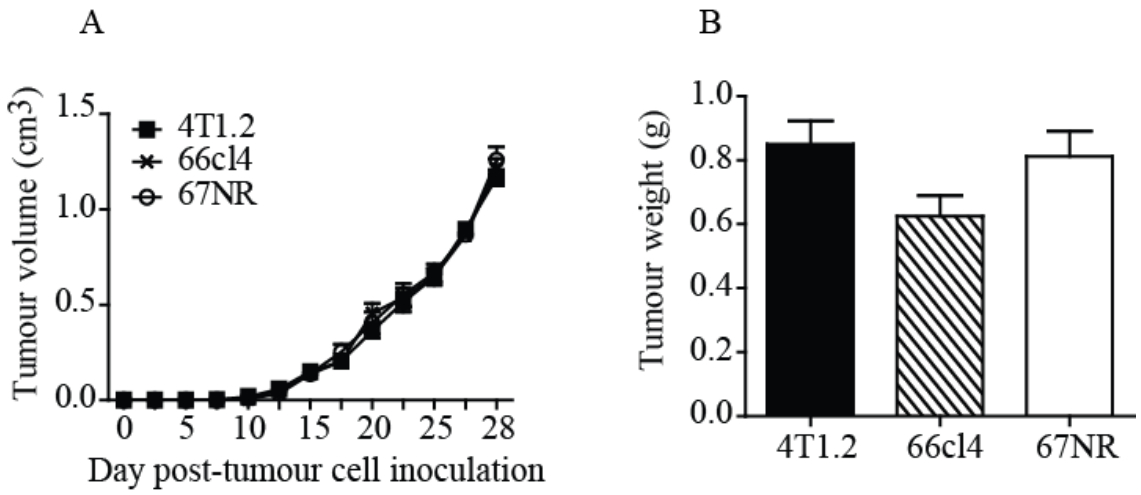


Figure 3.6: 4T1.2, 66cl4 and 67NR primary tumours have the same growth rates and end-stage weights.

Mice were injected orthotopically into the fourth mammary gland with either 4T1.2 (highly metastatic), 66cl4 (weakly metastatic) or 67NR (non-metastatic) tumour cells. Tumour growth was measured using electronic callipers and tumours were excised and weighed at day 28. Primary tumour A) growth (volume, cm^3) and B) end-stage weights (grams). Mean±SEM; *n*=8 mice/group.

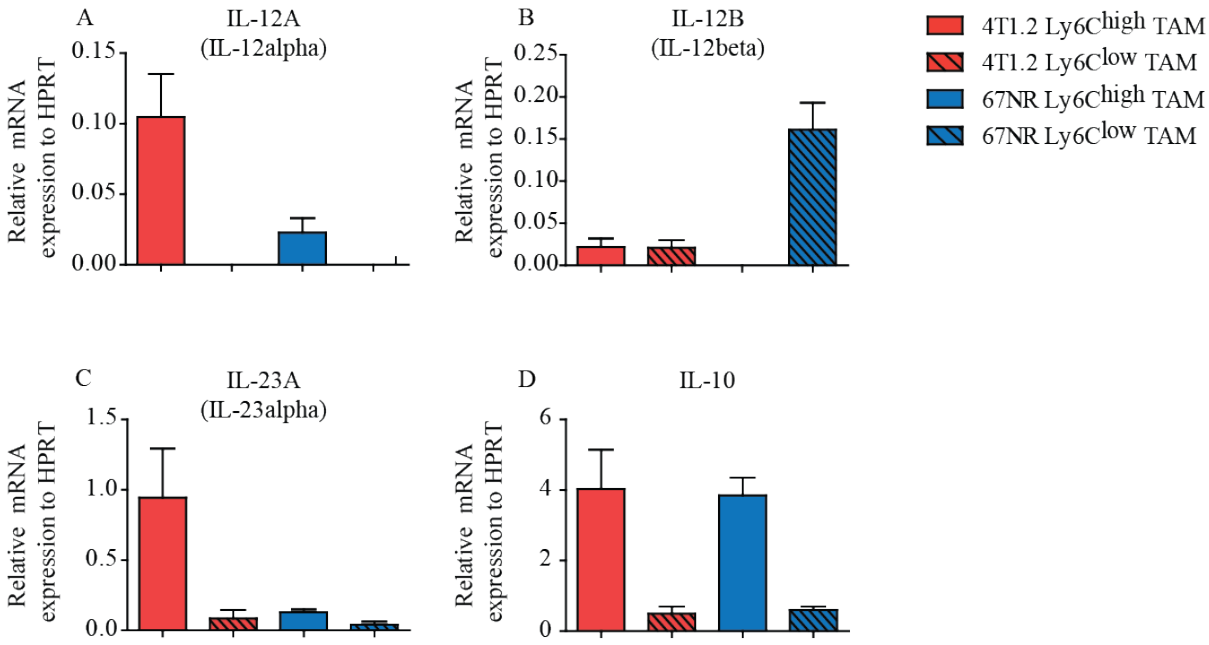


Figure 3.7: IL-12A, IL-12B, IL-23A and IL-10 mRNA expression in Ly6C^{high} and Ly6C^{low} TAMs from 4T1.2 and 67NR primary tumours.

Mice were injected orthotopically into the fourth mammary gland with either 4T1.2 (highly metastatic) or 67NR (non-metastatic) mCherry-expressing tumour cells. Primary tumours were excised at day 25 post-tumour cell inoculation, disaggregated and Ly6C^{high} and Ly6C^{low} TAMs were isolated by cell sorting. TAMs were characterised as mCherry^{neg}CD11b^{+}Ly6G^{neg}Ly6C^{high} or mCherry^{neg}CD11b^{+}Ly6G^{neg}Ly6C^{low}. RNA was extracted from sorted cells and gene expression analysed by qRT-PCR. A) IL-12A (IL-12 alpha), B) IL-12B (IL-12 beta), C) IL-23A (IL-23 alpha) and D) IL-10 mRNA expression. Data are expressed as relative expression of mRNA compared to that of the housekeeping gene, HPRT. Mean±SEM; *n*=2 biological samples, each in triplicate.

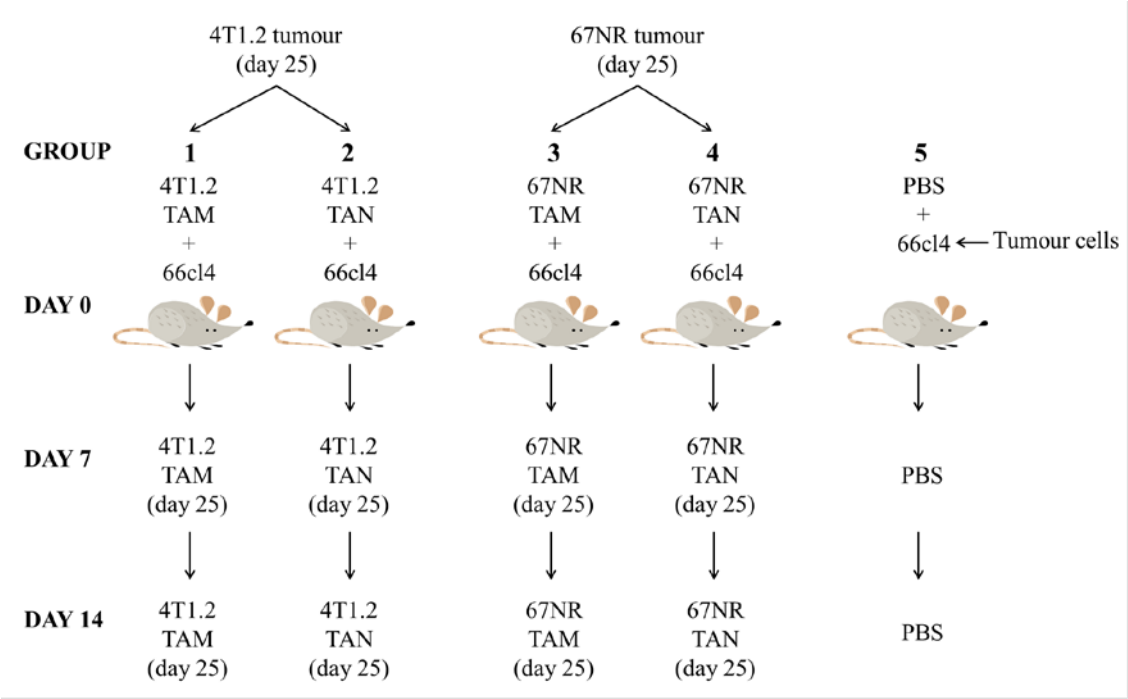


Figure 3.8: Schematic of co-injection of TAMs and TANs isolated from either highly metastatic (4T1.2) or non-metastatic (67NR) primary tumours with weakly metastatic (66cl4) tumour cells.

Mice were injected orthotopically into the fourth mammary gland with either 4T1.2 (highly metastatic) or 67NR (non-metastatic) mCherry-expressing tumour cells. Primary tumours were excised at day 25 post-tumour cell inoculation, disaggregated, and TAMs and TANs were isolated by cell sorting. TAMs were characterised as $mCherry^{neg}CD11b^{+}Ly6G^{neg}F4/80^{+}$ cells, and TANs as $mCherry^{neg}CD11b^{+}Ly6G^{high}$ cells. TAMs and TANs isolated from 4T1.2 or 67NR primary tumours were then co-injected into the fourth mammary gland with 66cl4 tumour cells at a ratio of 1 TAM or TAN:10 tumour cells (1.5×10^5 66cl4 tumour cells + 1.5×10^4 TAMs or TANs per mouse). 66cl4 tumour cells injected in PBS alone served as a control. Two further 'top-up' injections of 1.5×10^4 TAMs or TANs, isolated from 4T1.2 or 67NR tumours, were then given into the fourth mammary gland at 1 and 2 weeks post initial co-injections. Mice that received 66cl4 tumour cells alone were injected with PBS 1 and 2 weeks post 66cl4 tumour cell inoculation.

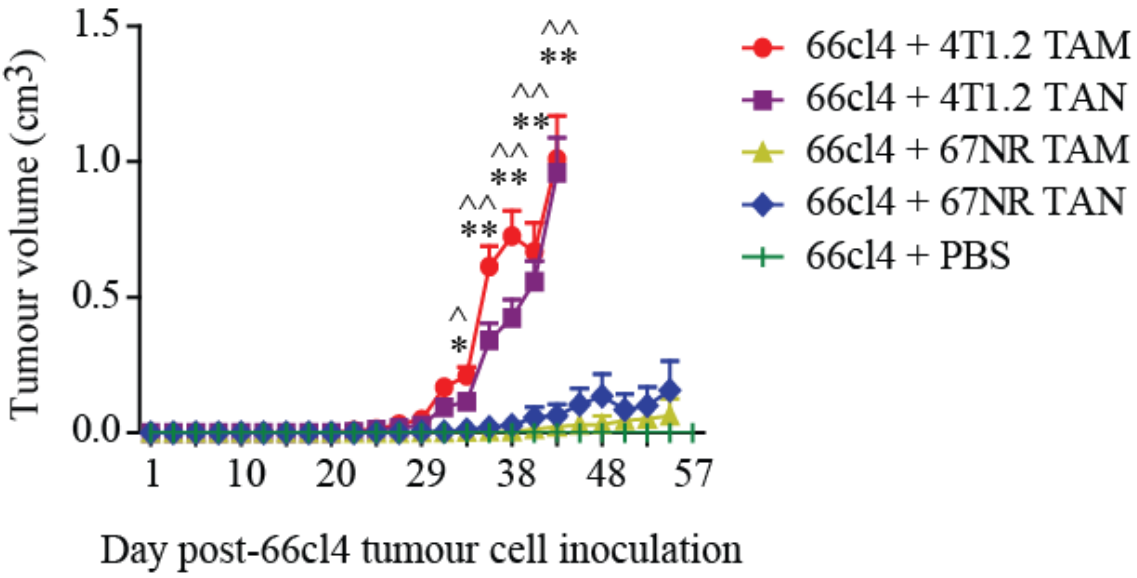


Figure 3.9: Co-injection of TAMs and TANs from 4T1.2 or 67NR primary tumours increases 66cl4 tumour growth.

TAMs and TANs isolated from 4T1.2 or 67NR primary tumours were co-injected into the fourth mammary gland with 66cl4 tumour cells at a ratio of 1 TAM or TAN:10 tumour cells (1.5 x 10^5 66cl4 tumour cells + 1.5 x 10^4 TAMs or TANs per mouse). Two further 'top-up' injections of 1.5 x 10^4 TAMs or TANs were then given at 1 and 2 weeks post 66cl4 tumour cell inoculation. 66cl4 tumour cells injected in PBS alone served as a control (see Figure 3.8). Primary tumour growth (volume, cm^3) was measured by callipers. Mean±SEM; *n*=15 mice/group. *$P \leq 0.01$ and **$P \leq 0.001$ 4T1.2 TAMs vs. 67NR TAMs, 67NR TANs and 66cl4 alone. $^{\wedge}P \leq 0.01$ and $^{\wedge\wedge}P \leq 0.001$ 4T1.2 TANs vs. 67NR TAMs, 67NR TANs and 66cl4 alone, two-way repeated measures ANOVA.

4 Chapter 4: Monocyte and neutrophil mobilisation by highly metastatic 4T1.2 tumours

4.1 Introduction

Systemic monocytes and neutrophils have not been studied extensively in metastasis. Increases in Gr-1$^+$CD11b$^+$ cells have been reported in the spleen [215], bone marrow [216] and circulation [217] of mice bearing lymphoma, Lewis lung carcinoma and mammary and colon tumours, and at all three sites in the c-erbB-2 (HER-2/neu) mouse model of breast cancer [451]. Gr-1$^+$ cells have also been shown to form clusters in the lung prior to the arrival of 4T1 murine mammary tumour cells [3]. However, Gr-1 cells can be either monocytes or neutrophils.

Murine monocytes can be loosely grouped into two functionally specialised subpopulations, the Ly6C^{high}, 'immature, classical' and the Ly6C^{low}, 'mature, non-classical' [298, 309]. Blood monocytes are increased in breast cancer patients [326] and high tumour expression of monocyte/macrophage chemoattractants and growth factors, such as CCL2 and CSF-1, has been correlated with advanced disease [330]. In the MMTV-PyMT murine breast tumour model, Ly6C^{high} monocyte recruitment increased in lungs with metastases compared to normal lungs [202]. However, this was demonstrated in the context of experimental metastasis, which does not encompass all the steps of physiological metastasis. Analysis of systemic Ly6C^{high} and Ly6C^{low} monocyte subpopulations in spontaneous metastasis may afford a better understanding of the contribution of these cell populations to this process.

Neutrophils have not been comprehensively studied in breast cancer. Aeed et al. found that circulating neutrophil numbers increased with increasing metastatic capacity in a mammary adenocarcinoma model [5]. Neutrophils exposed to tumour-conditioned medium increased metastasis of mammary adenocarcinomas, whereas 'normal' neutrophils did not [424]. However, neutrophils have also been implicated in the clearance of experimental metastasis [425] and a recently described neutrophil population termed 'tumour entrained neutrophils (TENs)' inhibited metastatic seeding of lungs in a mouse mammary tumour model [201]. Therefore, further investigation of systemic neutrophils is required to clarify their role in breast tumour metastasis.

It was shown in Chapter 3 that Ly6C^{low} TAMs and TANs increased in 4T1.2 highly metastatic tumours, and that these increases occurred early in tumour progression. In this chapter, systemic monocytes/macrophages and neutrophils were analysed in the context of primary tumour progression to metastasis. It was found that highly metastatic 4T1.2 tumours increased monocyte and neutrophil mobilisation and preliminary data implicated MMP-9 in this process.

4.2 Results

4.2.1 Monocytes and neutrophils are mobilised early during highly metastatic 4T1.2 tumour progression

Monocytes and neutrophils were analysed by flow cytometry during tumour progression. Monocytes were characterised as CD11b^{+}Ly6G^{neg}Ly6C^{high} or CD11b^{+}Ly6G^{neg}Ly6C^{low} [303], and neutrophils as CD11b^{+}Ly6G^{high} [8, 223]. Significant increases in blood Ly6C^{high} monocyte and neutrophil numbers in highly metastatic 4T1.2 compared to weakly metastatic 66cl4 or non-metastatic 67NR tumour-bearing mice and naive controls first occurred at day 20 post-tumour cell inoculation (Figures 4.1A, $P \leq 0.0001$ and 4.1C, $P \leq 0.05$). Ly6C^{low} monocyte numbers increased later in highly metastatic 4T1.2 tumour-bearing mice, at day 25 post-tumour cell inoculation (Figure 4.1B, $P \leq 0.0001$). As reported in Chapter 3, Ly6C^{high} monocytes were the predominant blood monocyte subpopulation.

Ly6C^{high} and Ly6C^{low} monocytes, as well as neutrophils, therefore increased early during highly metastatic 4T1.2 tumour progression. However, it was unclear how this mobilisation related to metastasis. The experiment was repeated, this time comparing the numbers of mobilised Ly6C^{high} and Ly6C^{low} monocytes, as well as neutrophils to the extent of 4T1.2 metastasis to lung. Weakly metastatic 66cl4 tumour-bearing mice were not analysed again due to their similarity to non-metastatic 67NR tumour-bearing mice in regard to blood monocyte and neutrophil numbers.

Increases in blood Ly6C^{high} monocytes and neutrophils in 4T1.2 tumour-bearing mice coincided with detection of the first visible lung tumour nodules (Figures 4.2A, $P \leq 0.001$ and 4.2C, $P \leq 0.05$). As before, blood Ly6C^{low} monocytes increased later (day 25) in

4T1.2 tumour-bearing mice, after lung nodules were first observed (Figure 4.2B, $P \leq 0.001$). In the same experiment, neutrophil and Ly6C^{high} monocyte numbers were analysed in the bone marrow (Figure 4.3), as this is where monocytes and neutrophils originate. Ly6C^{low} cells in the bone marrow did not express CD11b (a myeloid cell marker) and hence could not be confirmed as monocytes. As a result, these cells were not included in the analysis. Neutrophils and Ly6C^{high} monocytes were significantly increased in the bone marrow of highly metastatic 4T1.2 compared to non-metastatic 67NR tumour-bearing mice at day 15, before the appearance of visible lung tumour nodules (Figure 4.3, $P \leq 0.0001$ and $P \leq 0.001$, respectively), but declined after day 20 (Figure 4.3). This is most likely due to their mobilisation into the circulation.

Also in the same experiment, splenic Ly6C^{high} and Ly6C^{low} monocytes, as well as neutrophils were analysed in relation to 4T1.2 metastasis to lung (Figure 4.4). The spleen is known to act as a reservoir of immune cells, and is itself an important participant in the generation of inflammatory reactions [452-454]. Splenic neutrophil numbers increased significantly in 4T1.2 tumour-bearing mice around the time visible lung tumour nodules were first seen (Figure 4.4C, $P \leq 0.05$). A trend towards increased Ly6C^{high} monocytes was also observed at this time point and these cells were significantly increased by day 25 (Figure 4.4A, $P \leq 0.05$). Ly6C^{low} monocytes accumulated in the spleens of 4T1.2 tumour-bearing mice only at later stages of tumour growth (day 28) (Figure 4.4B, $P \leq 0.01$).

In summary, Ly6C^{high} monocyte and neutrophil numbers in highly metastatic 4T1.2 tumour-bearing mice increased in the bone marrow before the appearance of visible lung tumour nodules (Figure 4.3). Ly6C^{high} monocytes and neutrophils increased in the blood and spleen around the time visible lung tumour nodules first occurred, albeit splenic Ly6C^{high} monocytes increased later (Figures 4.2 and 4.4). Circulating and splenic Ly6C^{low} monocyte numbers increased later in 4T1.2 tumour progression (Figures 4.2B and 4.4B). Since tumour growth and weights at harvest were not seen to differ between the highly metastatic 4T1.2 and non-metastatic 67NR variants (Figure 4.5), the monocyte and neutrophil expansion described above was associated with metastasis, and not tumour growth and size.

Monocyte and neutrophil numbers from day 20 of this time course experiment (when visible lung tumour nodules were first detected) are summarised in Figure 4.6. This figure shows that there was a greater increase in the numbers of neutrophils compared to monocytes in the blood, bone marrow and spleen of 4T1.2 tumour-bearing mice.

4.2.2 Neutrophils increase in 4T1.2 metastasis-associated lungs around the time tumour cell DNA is first detected

Given that Ly6C^{high} monocytes were shown to be recruited to pre-metastatic lungs in the MMTV-PyMT model [202], a new experiment was set up to analyse Ly6C^{high} and Ly6C^{low} macrophages, as well as neutrophils, in the lungs of highly metastatic 4T1.2 compared to non-metastatic 67NR tumour-bearing mice. Tumour variants expressing the mCherry fluorescent protein were used to assess metastatic lung burden. Since visible nodules were first seen at day 20 post-tumour cell inoculation (see Figures 4.2-4.4), tumour cells must lodge in the lungs at earlier time points. Hence, lungs were analysed at day 5, 8, 11 and 13 post-tumour cell inoculation. Tumour-derived mCherry DNA was first detected in the lungs at day 11 after 4T1.2 tumour cell inoculation (Figure 4.7A), whereas mCherry DNA was not found in lungs of 67NR tumour-bearing mice. As before, no differences were seen in primary tumours weights between the 4T1.2 and 67NR variants (Figure 4.7B).

Neutrophil numbers increased significantly in the lungs of 4T1.2 compared to 67NR tumour-bearing mice at day 11 post-tumour cell inoculation (Figure 4.8A, $P \leq 0.05$). Lung Ly6C^{high} and Ly6C^{low} macrophage numbers did not differ between mice bearing the different tumour variants at these time points and appeared to decrease in number after day 5 (Figures 4.8B and 4.8C). Peripheral blood was also harvested from these mice. Blood neutrophils were increased significantly at day 13 post-tumour cell inoculation in 4T1.2 tumour-bearing mice (Figure 4.8D, $P \leq 0.05$). As for lung macrophages, blood Ly6C^{high} and Ly6C^{low} monocyte numbers were similar between 4T1.2 and 67NR tumour-bearing mice at these time points (Figures 4.8E and 4.8F). This experiment has only been completed once and needs to be confirmed. However, these preliminary data indicate that increases in lung neutrophils rather than macrophages were associated with the arrival of tumour cells in the lung.

4.2.3 MMP-9 and CXCL12 are increased in the serum of 4T1.2 tumour-bearing mice at day 15 post-tumour cell inoculation

Little is known about the factors involved in mobilisation of monocytes and neutrophils from the bone marrow during tumour progression. To address this, serum from mice bearing 4T1.2, 66cl4 and 67NR tumours was taken at day 15 post-tumour cell inoculation, at the time of myeloid cell mobilisation from the bone marrow in this tumour model. Mice injected with saline were used as non-tumour-bearing controls. A RayBio® cytokine array (RayBiotech, Inc., GA, USA) that measures ninety-six inflammation-associated factors including cytokines, chemokines, growth factors and proteases was utilised. A number of factors were found to be changed in tumour-bearing mice compared to saline controls, as summarised in Tables 4.1 and 4.2.

Serum from mice bearing 4T1.2 tumours contained elevated levels of pro-angiogenic factors, including members of the VEGF family, growth factors such as bFGF, members of the IGF family, and the adhesion factors E-selectin and ICAM-1 (Table 4.1). Amongst others, CCL2, CCL5 and IL-10 were downregulated in serum of 4T1.2 tumour-bearing mice (Table 4.2). Pro-inflammatory cytokines IL-1α, IL-12 and IFNγ, and a number of proteins associated with APC and lymphocyte activation, including IL-2, CD30 and CD40, were also downregulated (Table 4.2).

In serum from weakly metastatic 66cl4 tumour-bearing mice, upregulation of L-selectin, members of the IGF growth factor family and the pro-inflammatory cytokines IL-12 and IL-2 was seen (Table 4.1). No factors were found to be downregulated in serum from these mice (Table 4.2). In serum from non-metastatic 67NR tumour-bearing mice, the pro-inflammatory cytokines IL-12 and IL-2 were upregulated (Table 4.1) and a number of growth factors and adhesion molecules, including P-selectin and ICAM-1 were downregulated (Table 4.2).

MMP-2 and MMP-3 were decreased in the serum of mice bearing highly metastatic 4T1.2 and non-metastatic 67NR tumours. However, this decrease was minor (Table 4.2). Conversely, Pro-MMP-9 was highly increased in the serum of mice bearing all three tumour variants with the highest increase seen in mice bearing highly metastatic 4T1.2 tumours (12.1-fold, Table 4.1). The data are shown in more detail in Figure 4.9,

where analysis of the relative fluorescence intensity values revealed that pro-MMP-9 was increased significantly in serum from 4T1.2 tumour-bearing mice compared to mice bearing 66cl4 and 67NR tumours and naive controls (Figure 4.9A, $P \leq 0.001$). MMP-9-mediated degradation of CXCL12 (SDF-1α) has been implicated in mobilisation of cells from the bone marrow after G-CSF stimulation [455]. Serum CXCL12 levels were also found to be increased in 4T1.2 tumour-bearing mice compared to 66cl4 and 67NR tumour-bearing mice and naive controls (Figure 4.9B, $P \leq 0.01$). However, serum G-CSF levels were similar between the metastatic variants and naive controls (Figure 4.9C).

MMP-9 levels were then measured in serum of 4T1.2 tumour-bearing mice at day 15 post-tumour cell inoculation using Western blot and zymography. Culture supernatant from a 4T1.2 BM2/LM-511 tumour cell line variant provided by Dr. Normand Pouliot [456], known to contain MMP-9, was used as a positive control. MMP-9 protein was present in the culture supernatant of BM2/L-511 tumour cells by Western blot (Figure 4.10A lanes 7-9) but could not be detected in the serum from 4T1.2 tumour-bearing mice (Figure 4.10A lanes 3-5). Zymography was also used to assess MMP-9 levels. Again, MMP-9 gelatinase activity was detected in the positive controls (Figure 4.10B lanes 7-9) but not in the serum from 4T1.2 tumour-bearing mice (Figure 4.10B lanes 3-5).

4.3 Discussion

The use of Gr-1 as a marker of myeloid cells in breast tumours has resulted in some confusion regarding the role of monocytes/TAMs and neutrophils/TANs in metastasis. Analysis of myeloid cells in cancer models using antibodies against Ly6C (found on both monocytes and neutrophils) and Ly6G (found on neutrophils) allows for a clearer delineation of monocytes/macrophages from neutrophils [8, 201, 223-225]. It is reported in this chapter that highly metastatic 4T1.2 tumours increased mobilisation of $Ly6C^{high}$ monocytes, as well as neutrophils, early in progression to metastasis. Furthermore, MMP-9 is implicated in this enhancement of monocyte and neutrophil mobilisation.

Blood monocytes increase in patients with metastatic tumours compared to normal individuals [326], and elevated serum neutrophil numbers, neutrophil factors or neutrophil to lymphocyte ratios generally predict poor prognosis in a number of human cancers, including gastric [350, 351], lung [354], cervical [357] and ovarian [358, 359]. In breast cancer, increased levels of the neutrophil specific protease, NE, correlate with poor prognosis [360]. Nevertheless, the mobilisation of monocytes and neutrophils in metastatic tumours compared to non-metastatic tumours has not been well investigated.

In this chapter it was found that Ly6C^{high} monocyte and neutrophil numbers increased in the bone marrow of 4T1.2 compared to 67NR tumour-bearing mice before visible lung nodules were first observed (Figure 4.3), whereas blood Ly6C^{high} monocytes and neutrophils increased around the time the first visible lung nodules were detected (Figure 4.2). Thus it appears that systemic monocytes and neutrophils, similar to TAMs and TANs, increase during the early stages of metastasis, probably at the pre-metastatic or micrometastatic phases given that bone marrow monocytes and neutrophils increased prior to the appearance of visible lung nodules and circulating monocytes increased around the time of lung nodule detection.

It is unclear at this stage if monocytes and neutrophils mature in the bone marrow or circulation. Given that Ly6C^{low} monocytes were not detected in the bone marrow, but were seen in the circulation, it is likely that maturation occurs in the blood. However, the maturation of monocytes from Ly6C^{high} to Ly6C^{low} has recently been questioned [297]. For example, Sunderkotter et al showed that, after depletion of all blood monocytes in the steady state, the returning circulating monocytes were predominantly Gr-1^{high} and after 5 days converted to Gr-1^{low} [304], but reduction of Gr-1^{+}/Ly6C^{high} monocytes does not always result in reduction of Gr-1$^{neg/low}$/Ly6C^{low} monocytes [307, 308] suggesting that Ly6C^{high} do not always convert into Ly6C^{low} (for further discussion see Chapter 1, Section 1.8.2). Therefore, it also remains unclear if Ly6C^{high} monocytes convert into Ly6C^{low} TAMs as they migrate into tumours or if Ly6C^{low} monocytes are directly recruited to tumours from the blood and give rise to Ly6C^{low} TAMs. Blood Ly6C^{high} monocytes may be recruited chiefly to metastatic sites such as the lung as it has been shown that immature bone marrow derived cells and immature (Gr-1^{high}/Ly6C^{high}) monocytes are recruited to pre-metastatic and metastatic lungs [92, 202].

In the spleen, neutrophil, and possibly Ly6C^{high} monocyte, numbers increased around the time that visible lung tumour nodules occurred (Figure 4.4). The spleen has been suggested to act as a reservoir for TAMs and TANs in a murine model of lung adenocarcinoma [452]. In the 4T1.2 model this seems less likely, at least for TAMs, as splenic monocyte numbers increased later in tumour progression. However, it is possible that the spleen contributes TAMs in late stage mammary tumours. On the other hand, the spleen may be acting as a reservoir of TANs early in 4T1.2 tumour progression.

Analysis of monocyte and neutrophil mobilisation in other tumour models and mouse strains is required to confirm that this process is associated with aggressive tumours and whether or not this process is specific to the 4T1.2 mammary tumour model or BALB/c mice.

The data discussed above indicate that mobilisation of monocytes and neutrophils from the bone marrow occurs either before metastasis, or at least at the time of early metastatic events. In support of this, preliminary experiments showed that neutrophil numbers increased in lungs of 4T1.2 tumour-bearing mice at the time that tumour cell DNA was first detected there (Figures 4.7A and 4.8A).

In contrast, Ly6C^{high} and Ly6C^{low} macrophage numbers did not differ in the lungs of 4T1.2 and 67NR tumour-bearing mice, and in fact appeared to decrease in number (Figures 4.8B and 4.8C). It is unclear why this decrease occurred. CD11c^{+} resident lung macrophages were not analysed and it is possible that these cells increase in number during metastasis. However, a recent study in the MMTV-PyMT model has suggested that a unique CD11b^{+} macrophage population that promotes metastatic tumour growth was recruited to lungs from the circulation and that the resident CD11c^{+} lung macrophage population did not change in number and was not important for metastasis to this site [335]. Furthermore, increases in bone marrow progenitor cells, such as CD113^{+}CD34^{+}CD117^{+}VEGFR-1^{+} cells (which have been shown to increase in the pre-metastatic lung [92]), may have been missed during the analysis described in this thesis as these cells are not likely to express Ly6C and Ly6G. Lung macrophages may also 'spike' at day 5 post-tumour cell inoculation and then decrease, but a comparison with macrophage numbers in naive lungs is required to confirm this. Such a comparison was

not done here, as the focus of these experiments was on the differences between metastatic and non-metastatic tumours to distinguish between tumour- and metastasis-driven effects. It would be informative to analyse macrophage and neutrophil numbers in the lungs of tumour-bearing mice at day zero to establish a baseline value for comparison of later timepoints.

It would also be beneficial to determine if clustering of macrophages or neutrophils is occurring in the lung (possibly by immunohistochemistry) as this has been shown to be an important step in pre-metastatic niche formation [3, 92]. However, there is currently a lack of Ly6C and Ly6G antibodies suitable for immunohistochemistry. Furthermore, it would be difficult to assess macrophage sub-populations by immunohistochemistry using the Ly6C marker as it would be difficult to distinguish between differences in fluorescence intensity in the $Ly6C^{high}$ and $Ly6C^{low}$ cells. It has recently been shown that $Ly6C^{high}$ monocytes do not express MHC II, whereas $Ly6C^{low}$ monocytes do [226] and this may provide a better method of discriminating between these sub-populations.

Neutrophils were more numerous in the circulation, spleen and bone marrow of 4T1.2 tumour-bearing mice compared to $Ly6C^{high}$ and $Ly6C^{low}$ monocytes/macrophages around the time visible lung tumour nodules were first observed (Figure 4.6). This could be explained by the fact that monocytes make up 5-10% of circulating white blood cells, whereas neutrophils make up 45-70%. Nevertheless, given that TANs were the predominant immune cell type in 4T1.2 primary tumours (see Chapter 3, Figure 3.1); these systemic increases in neutrophils may indicate that neutrophils/TANs are also important for metastasis in the 4T1.2 model. This is in line with a recent study showing that neutrophils were important for metastasis to lung in the 4T1 murine model of breast tumour metastasis [8].

However, as previously mentioned, Granot et al. showed that neutrophils (TENs) inhibited metastatic seeding in the 4T1 model [201]. In this study neutrophils were characterised as $Ly6G^{+}$ cells and were depleted using an anti-Ly6G antibody [201]. During spontaneous 4T1 tumour metastasis neutrophils were shown to accumulate in the lungs of tumour-bearing mice early during metastatic progression and depletion of these cells increased the number of metastatic lung nodules. It is unclear why conflicting results were seen in this thesis, although neutrophils were not depleted.

Depletion of neutrophils in the 4T1.2 tumour-bearing mice would confirm if these cells are also relevant for metastasis in this model (this is discussed further in Chapter 6).

As discussed before, it has been suggested that a unique macrophage population that promotes metastatic tumour growth is recruited to metastasis-associated lungs [335] and in MMTV-PyMT mice, a monocyte subpopulation recruited via CCL2 was shown to be important for metastasis [202]. Hence the contribution of monocytes and macrophages in metastatic tumour growth cannot be discounted. Furthermore, it is possible that macrophages may increase in metastasis associated lungs later than neutrophils, given that neutrophils are typically first to appear at sites of inflammation. Analysis of later time points will clarify if/when macrophage numbers increase during tumour progression.

It is likely that tumour cells arrive in the lungs earlier than day 11 post-tumour cell inoculation. It is difficult to detect single tumour cells at metastatic sites by qPCR as the signal from a single cell is weak. Detection of tumour cells by flow cytometry or immunofluorescence in early metastatic lungs has been used successfully in a previous study with DsRed-expressing tumour cells (mCherry is a DsRed mutant) [92]. It would be worthwhile confirming the timing of tumour cell arrival in the lungs during 4T1.2 mammary tumour progression using these methods.

It is unknown if circulating monocytes or neutrophils can migrate back into the bone marrow, and hence if these cells are also recruited from the blood to bone metastases. It has been suggested that Ly6C^{high} monocytes can move bi-directionally between the circulation and bone marrow [296, 305], thus they may be recruited back into the bone marrow during metastasis. However, given that both monocytes/macrophages and neutrophils are already present in the bone marrow it seems more likely that bone metastases may induce proliferation of these cells here, rather than recruitment from the blood.

The factors involved in mobilisation of monocytes and neutrophils in metastatic breast cancer are not resolved, nor have the changes in circulating factors early in tumour progression been fully investigated. Factors in the serum of tumour-bearing mice were analysed, primarily to identify those involved in mobilisation of cells from the bone

marrow. However, given that preliminary experiments showed tumour cells present in lungs at day 11 post 4T1.2 tumour cell inoculation (Figure 4.7A), some of the factors analysed may be involved in progression to metastasis. Analysis of serum at earlier times would provide additional information on changes in these factors during early metastatic events.

IL-12 and IL-2 were upregulated in serum from weakly metastatic 66cl4 and non-metastatic 67NR tumour-bearing mice, but were downregulated in highly metastatic 4T1.2 tumour-bearing mice (Tables 4.1 and 4.2). IL-12 is a key factor in activating anti-tumour responses in T and NK cells [173, 174, 448] and treatment with IL-12 reduced mammary tumour metastasis in both the C3L5 and 4T1 murine models [175]. IL-2 also induces anti-breast tumour responses in NK cells [457, 458]. Thus it appears that 66cl4 and 67NR tumours have an impaired ability to suppress anti-tumour responses and this may limit their metastatic potential, whereas 4T1.2 tumours are highly aggressive in part due to their ability to suppress these host responses.

Several angiogenic factors including VEGFR1, 2 and 3 were upregulated predominantly in serum from 4T1.2 tumour-bearing mice (Table 4.1). This implies greater angiogenic and lymphangiogenic activity. A previous study from our research group at the Peter MacCallum Cancer Centre has shown that the 4T1.2, 66cl4 and 67NR tumour variants do not differ in their ability to induce angiogenesis [432]. However, lymphangiogenesis was not investigated. It would therefore be worthwhile to confirm this finding for angiogenesis in the day 15 tumour variants, for example by CD31 immunostaining, and to determine if the tumour variants differ in their ability to induce lymphangiogenesis, for instance by podoplanin or LYVE-1 immunostaining [459-461].

These preliminary results give some insight into the factors that may be involved in early metastatic events. Naturally, further investigation of the relevance of these factors is required. Additionally, it would be of interest to determine which cell types are secreting these factors.

Surprisingly, monocyte chemoattractants CCL2 and CCL5 were downregulated in the serum of 4T1.2 tumour-bearing mice, as well as the neutrophil chemoattractants LIX and KC (Table 4.2). Other key neutrophil chemoattractants, such as CXCL1 and

CXCL2 were not present on the array and were therefore not analysed. Given that only one time point was analysed, it is possible that these chemoattractants increase later in disease progression. However, pro-MMP-9 was markedly increased in the serum of mice bearing highly metastatic 4T1.2 tumours compared to weakly metastatic 66cl4 and non-metastatic 67NR tumour-bearing mice and non-tumour-bearing controls (Figure 4.9A).

MMPs have been shown to promote tumour progression to malignancy [462] and increased expression of MMP-9 in human breast cancer tissues has been proposed to be a prognostic factor for metastasis [463, 464]. MMP-9 plays a key role in breast tumour invasion [203]. However, host-derived MMP-9 has also been shown to be essential for tumour angiogenesis in murine models of pancreatic cancer [465] and ovarian cancer [466]. MMP-9-mediated ECM degradation is important for tumour angiogenesis [389] as degradation of the ECM releases sequestered angiogenic factors such as VEGF [242, 467].

Aside from its pro-tumourigenic role, MMP-9 is important in the reconstitution of bone marrow progenitor cells after bone marrow suppression [468] and MMP-9-mediated degradation of CXCL12 has been implicated in mobilisation of cells from the bone marrow into the circulation [455]. Jin et al. suggested that G-CSF stimulation activated MMP-9 in the bone marrow, but to a lesser degree in the blood, with the subsequent decrease in bone marrow CXCL12 levels creating a gradient causing cells to egress into the circulation [455]. Analysis of CXCL12 and G-CSF expression in the serum of mice bearing highly metastatic 4T1.2 tumours revealed that CXCL12 was also upregulated, but G-CSF was not (Figures 4.9B and 4.9C). However, MMP-9 may be promoting mobilisation of monocytes and neutrophils from the bone marrow in a mechanism that is independent of G-CSF. Furthermore, CXCL12 has been implicated in the directional metastasis of tumour cells [469]. Thus it is possible that the increased levels of CXCL12 seen in the serum of 4T1.2 tumour-bearing mice may be promoting metastasis in this way.

No MMP-9 protein or gelatinase activity could be detected in serum from mice bearing highly metastatic 4T1.2 tumours at day 15 post-tumour cell inoculation (Figure 4.10). However, the RayBio® cytokine array is a more sensitive assay than Western blot or

zymography, and may have detected small changes that were not apparent in the Western blot and zymography assays. Analysis of monocyte and neutrophil numbers in MMP-9 deficient mice bearing 4T1.2 tumours would determine if this protease is involved in tumour-driven mobilisation of these cells. This will be discussed further in Chapter 6.

4.4 Conclusion

It is shown here that highly metastatic 4T1.2 tumours enhanced mobilisation of monocytes and neutrophils from the bone marrow early in tumour progression to metastasis. Preliminary experiments indicate that neutrophils also increased in metastasis-associated lungs around the time of tumour cell arrival. In contrast, macrophages did not increase at this time. These increases in systemic monocyte and neutrophil numbers were associated with primary tumour metastatic capacity and not with tumour size. It appears that systemic monocytes and neutrophils increase during the early stages of metastasis, possibly at the pre-metastatic or micrometastatic phases. MMP-9 was implicated in this 4T1.2 tumour-driven enhancement of monocyte and neutrophil mobilisation. These data indicate that increases in systemic neutrophil numbers associate with 4T1.2 tumour metastasis and further investigation of these cells in breast cancer may provide valuable information for the treatment of breast cancer. Given that the increases in monocyte and neutrophil numbers occurred early in tumour progression to metastasis, analysis of changes in these cells in human disease may also aid in the assessment of disease status and likelihood of metastasis.

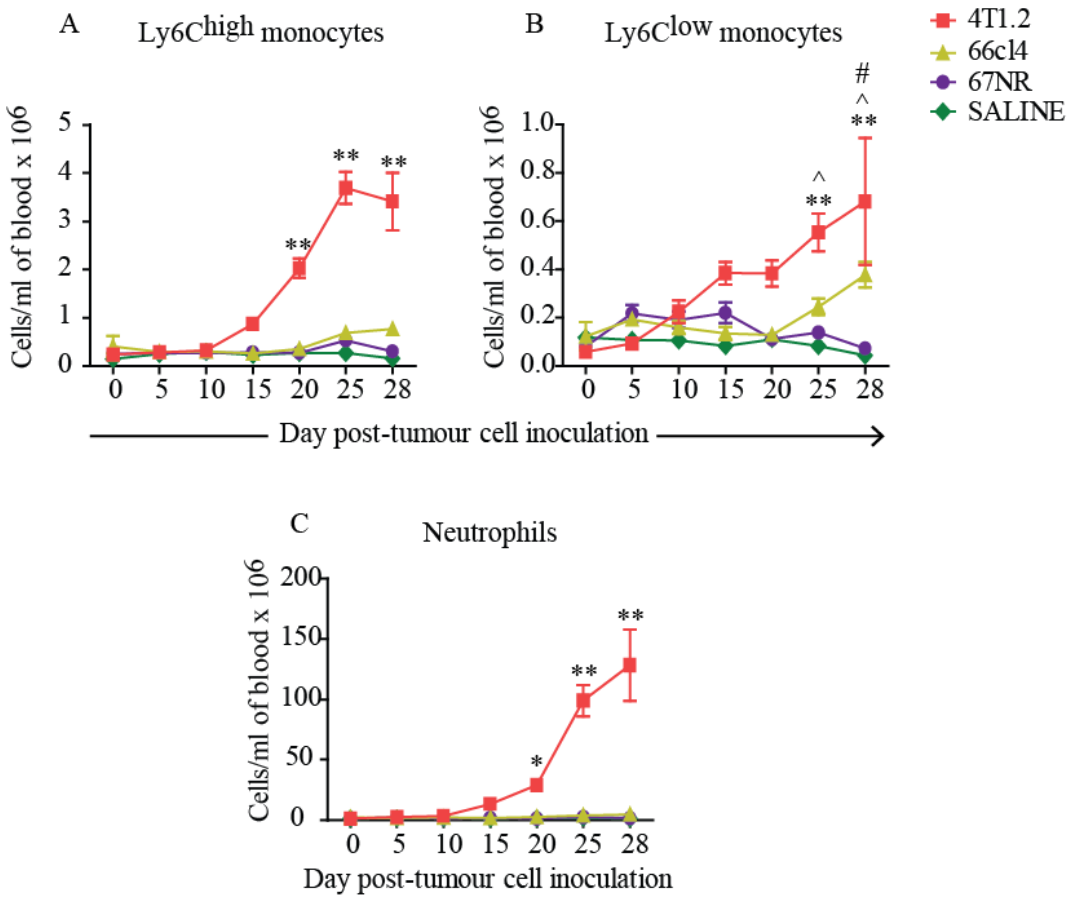


Figure 4.1: Blood monocyte and neutrophil numbers increase early in highly metastatic 4T1.2 compared to weakly metastatic 66cl4 and non-metastatic 67NR tumour progression.

Mice were injected orthotopically into the fourth mammary gland with 4T1.2 (highly metastatic), 66cl4 (weakly metastatic) or 67NR (non-metastatic) tumour cells. Saline was injected into a control group of mice. Peripheral blood was harvested by tail vein every 5 days after tumour cell inoculation and monocytes were characterised as $CD11b^{+}Ly6G^{neg}Ly6C^{high}$ or $CD11b^{+}Ly6G^{neg}Ly6C^{low}$ and neutrophils as $CD11b^{+}Ly6G^{high}$ by flow cytometry. Peripheral blood A) $Ly6C^{high}$ monocyte, B) $Ly6C^{low}$ monocyte, and C) neutrophil numbers. Data are expressed as cells per ml of blood. Mean±SEM, *n*=8 mice/group. **P*≤0.05 4T1.2 vs. 66cl4, 67NR and saline, ***P*≤0.0001 4T1.2 vs. 66cl4, 67NR and saline, B) ^*P*≤0.05 4T1.2 vs. 66cl4, $^{\#}P$≤0.01 66cl4 vs. 67NR and saline, two-way repeated measures ANOVA.

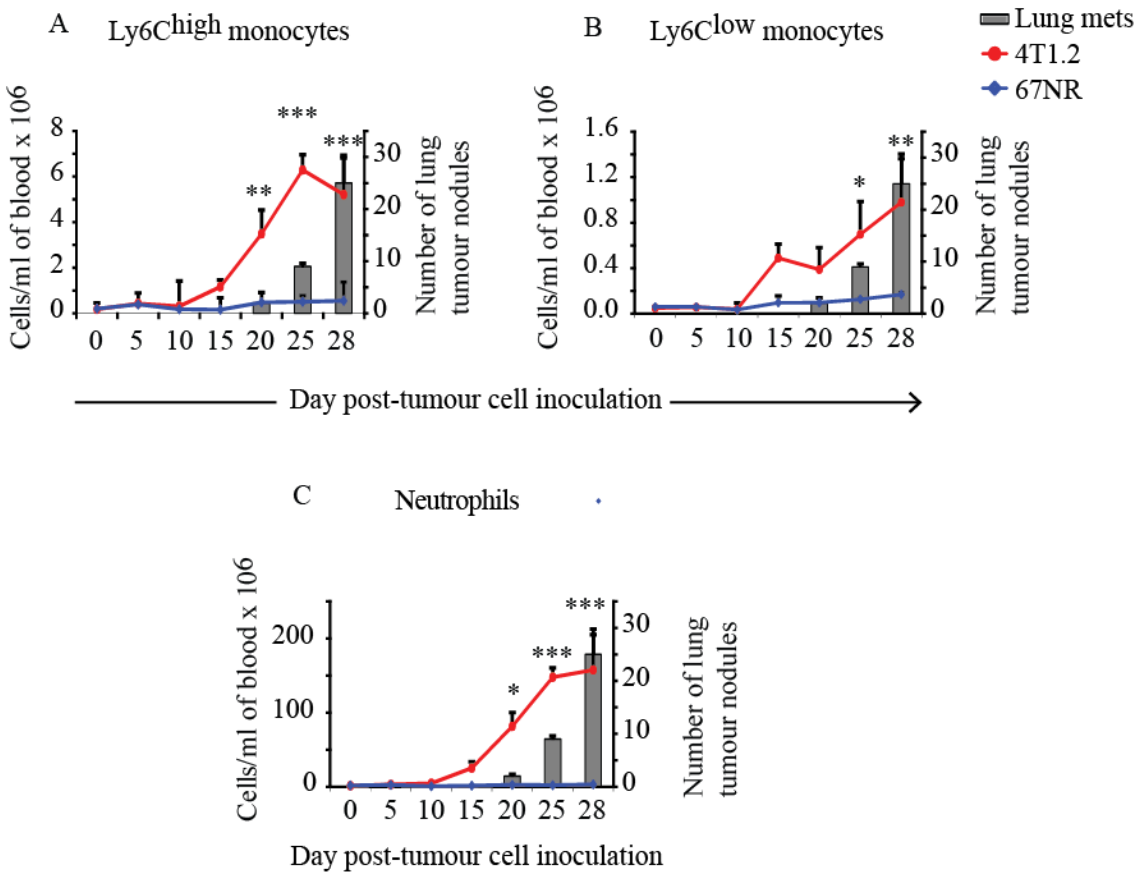


Figure 4.2: Peripheral blood Ly6C^{high} and monocyte and neutrophil numbers increase around the time visible lung metastases are first detected.

Mice were injected orthotopically into the fourth mammary gland with either 4T1.2 (highly metastatic) or 67NR (non-metastatic) tumour cells. A cohort of mice (*n*=12/group) was culled at each of the time points shown. Peripheral blood was harvested by tail vein and monocytes and neutrophils were characterised by flow cytometry as described in Figure 4.1 (left axis). Lungs from tumour-bearing mice were also excised at these time points, stained with India ink, and lung nodules counted (right axis). No lung nodules were detected in the lungs of 67NR tumour-bearing mice hence only the lung nodule scores from 4T1.2 tumour-bearing mice are shown. Blood A) Ly6C^{high} monocytes, B) Ly6C^{low} monocytes and C) neutrophils. Data are expressed as cells per ml of blood (left axis) and number of total surface lung tumour nodules (right axis). Mean±SEM. *P≤0.05, **P≤0.001 and ***P≤0.001 4T1.2 vs. 67NR, two-way repeated measures ANOVA.

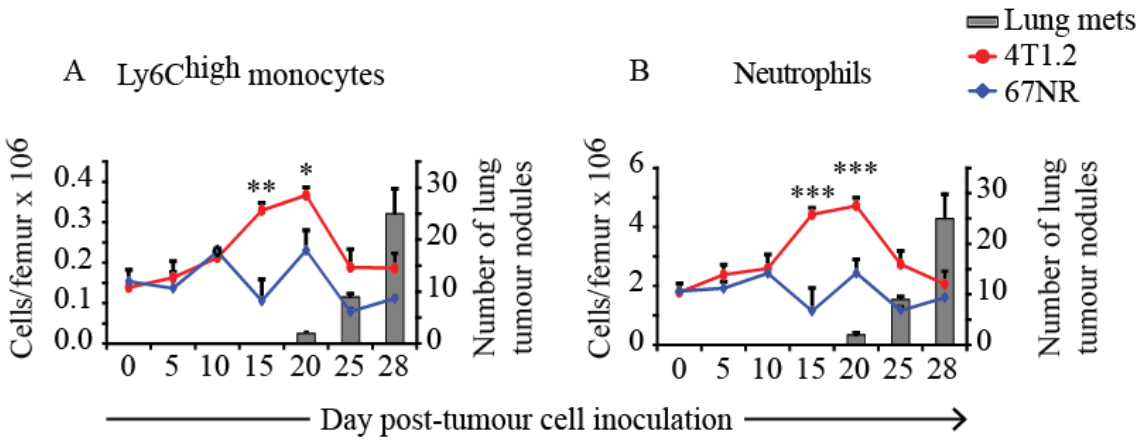


Figure 4.3: Bone marrow Ly6C^{high} monocyte and neutrophil numbers increase before visible lung metastases are first detected.

Whole femurs were excised and monocyte and neutrophil numbers were analysed by flow cytometry and surface lung nodules counted as described in Figure 4.2. A cohort of mice (n=12/group) was culled at each of the time points shown. Bone marrow A) Ly6C^{high} monocytes and B) neutrophils. Data are expressed as cells per femur (left axis) and number of total surface lung tumour nodules (right axis). Mean±SEM. *P≤0.05, **P≤0.001 and ***P≤0.001 4T1.2 vs. 67NR, two-way repeated measures ANOVA.

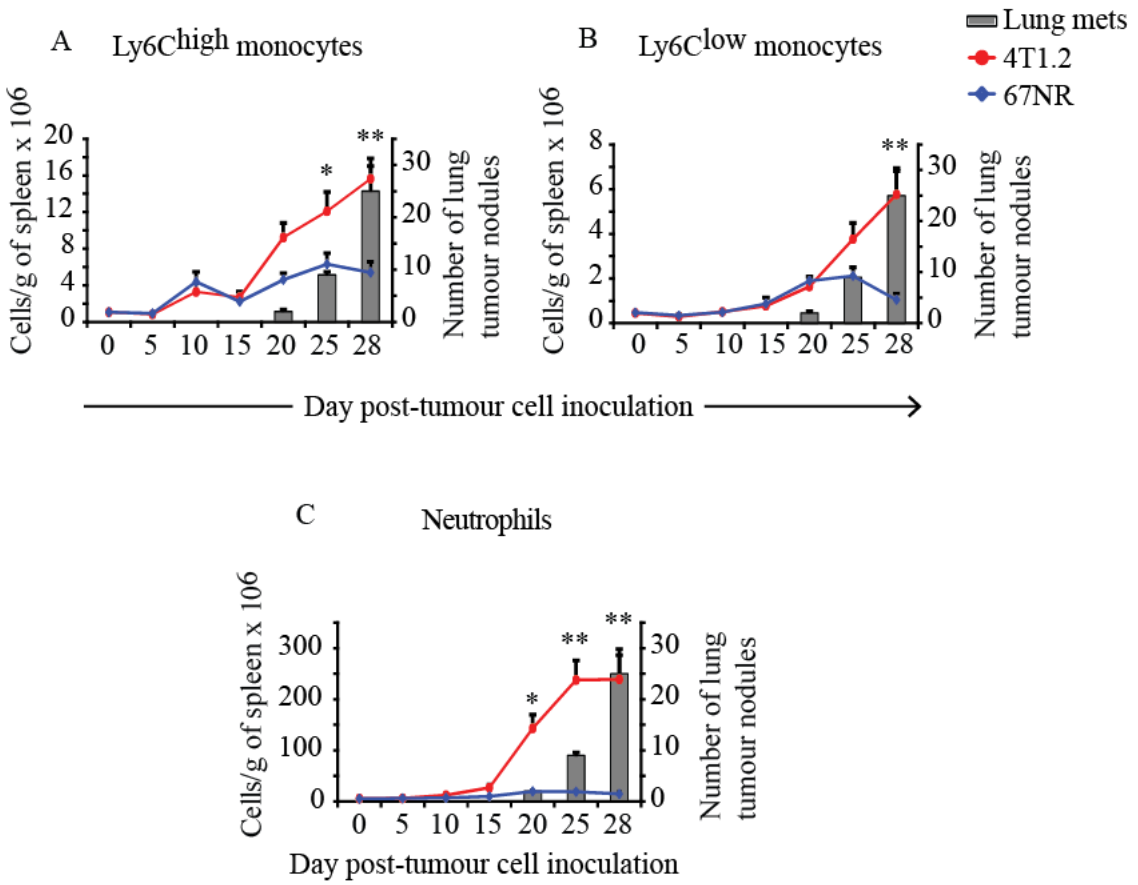


Figure 4.4: Splenic Ly6C^{high} and neutrophil numbers increase around the time visible lung metastases are first detected.

Spleens were excised, disaggregated and monocyte and neutrophil numbers were analysed by flow cytometry and surface lung nodules counted as described in Figure 4.2. A cohort of mice (*n*=12/group) was culled at each of the time points shown. Splenic A) Ly6C^{high} monocytes, B) Ly6C^{low} monocytes and C) neutrophils. Data are expressed as cells per gram of spleen (left axis) and number of total surface lung tumour nodules (right axis). Mean±SEM. *P≤0.01 and **P≤0.001 4T1.2 vs. 67NR, two-way repeated measures ANOVA.

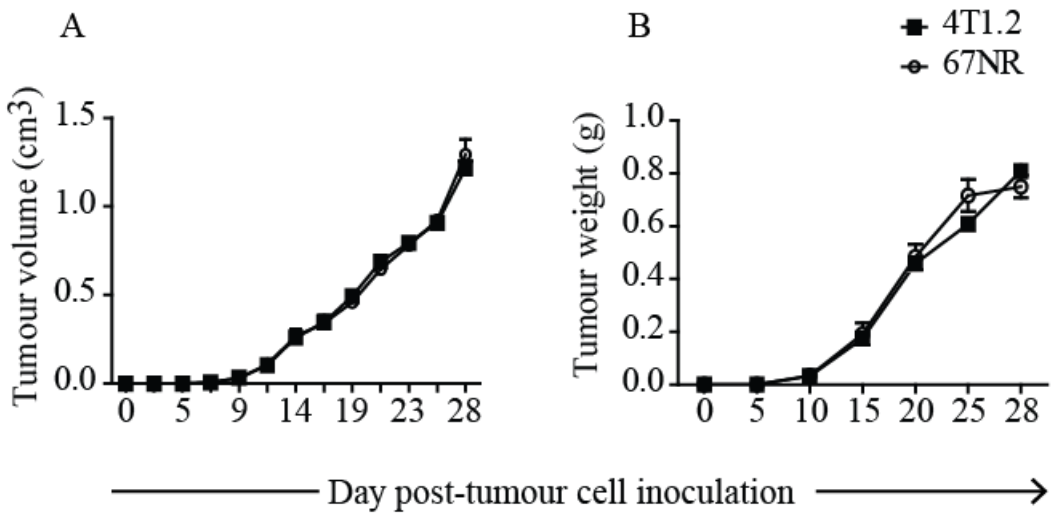


Figure 4.5: Primary tumour growth and weight are similar between highly metastatic 4T1.2 and non-metastatic 67NR tumours.

In the same experiments described in Figures 4.2-4.4 primary tumour growth was measured using electronic callipers and primary tumours were excised and weighed every 5 days after tumour cell inoculation. A cohort of mice (*n*=12/group) was culled at each of the time points shown. Primary tumour A) growth (volume, cm^3) and B) weights (grams). Mean±SEM.

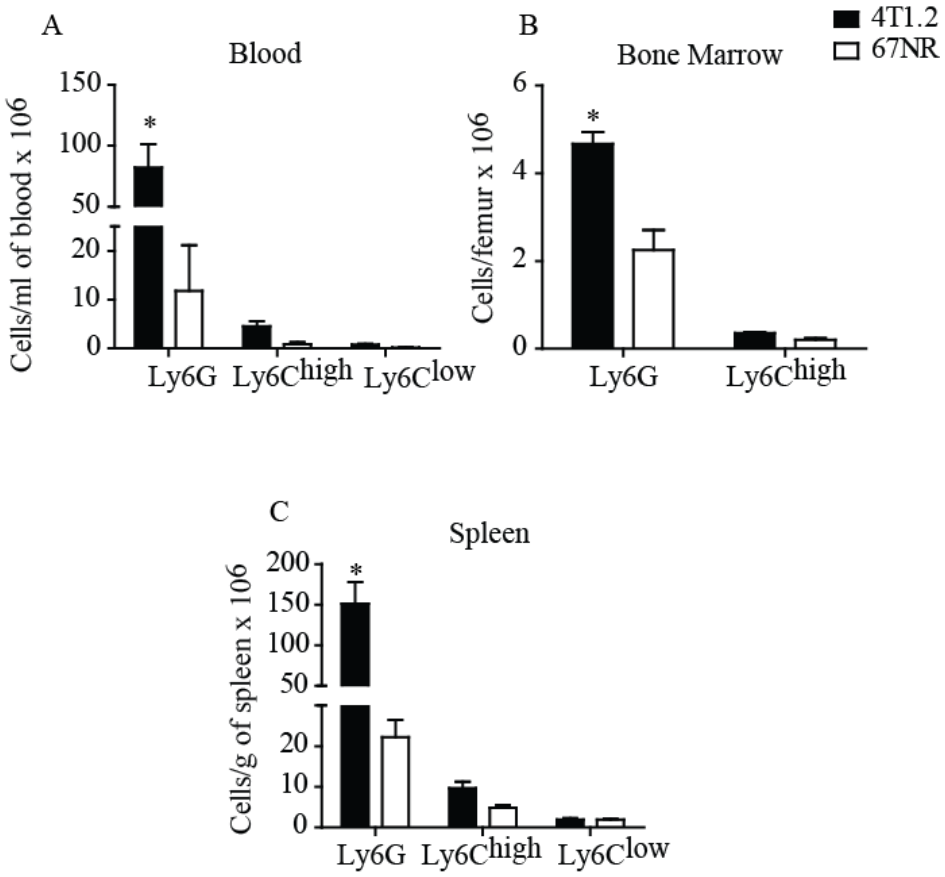


Figure 4.6: Neutrophils are highly increased in peripheral blood, bone marrow and spleen of 4T1.2 tumour-bearing mice.

As described in Figures 4.2-4.4 primary tumours, femurs, and spleens were excised, and peripheral blood was harvested by tail vein at day 20 post-tumour cell inoculation and monocytes/macrophages and neutrophils were characterised by flow cytometry. A) Blood, B) bone marrow and C) splenic neutrophil (Ly6G), and Ly6C^{high} and Ly6C^{low} monocyte numbers. Data are expressed as A) cells per ml of blood, B) cells per femur and C) cells per gram of spleen. Mean±SEM; *n*=12 mice/group. *$P \leq 0.001$ neutrophils vs. Ly6C^{high} and Ly6C^{low} monocytes (4T1.2 and 67NR), two-way repeated measures ANOVA.

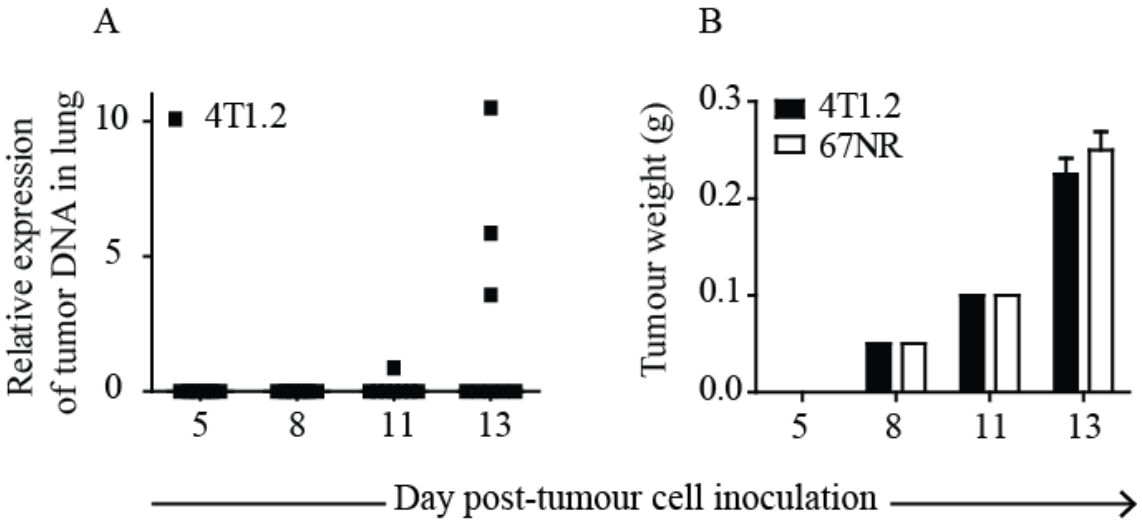


Figure 4.7: Tumour cells are first detected in lungs at day eleven post-tumour cell inoculation.

Mice were injected orthotopically into the fourth mammary gland with either 4T1.2 (highly metastatic) or 67NR (non-metastatic) mCherry-expressing tumour cells. Lungs were excised at day 5, 8, 11 and 13 post-tumour cell inoculation, disaggregated and genomic DNA was isolated. Primary tumours were also excised and weighed at these time points. Metastatic tumour DNA was measured by qPCR. However, as no tumour DNA was detected in lungs from mice bearing 67NR tumours, only data from 4T1.2 tumour-bearing mice is shown. A cohort of mice (n=8/group) was culled at each of the time points shown. A) Tumour DNA in lungs. B) Primary tumour weights. Data are expressed as A) relative expression of mCherry DNA (tumour cells only) to that of the housekeeping gene, vimentin (all cells) and B) grams. Mean±SEM. For day 8 and 11 time points in (B) the error bars are too small to be visible.

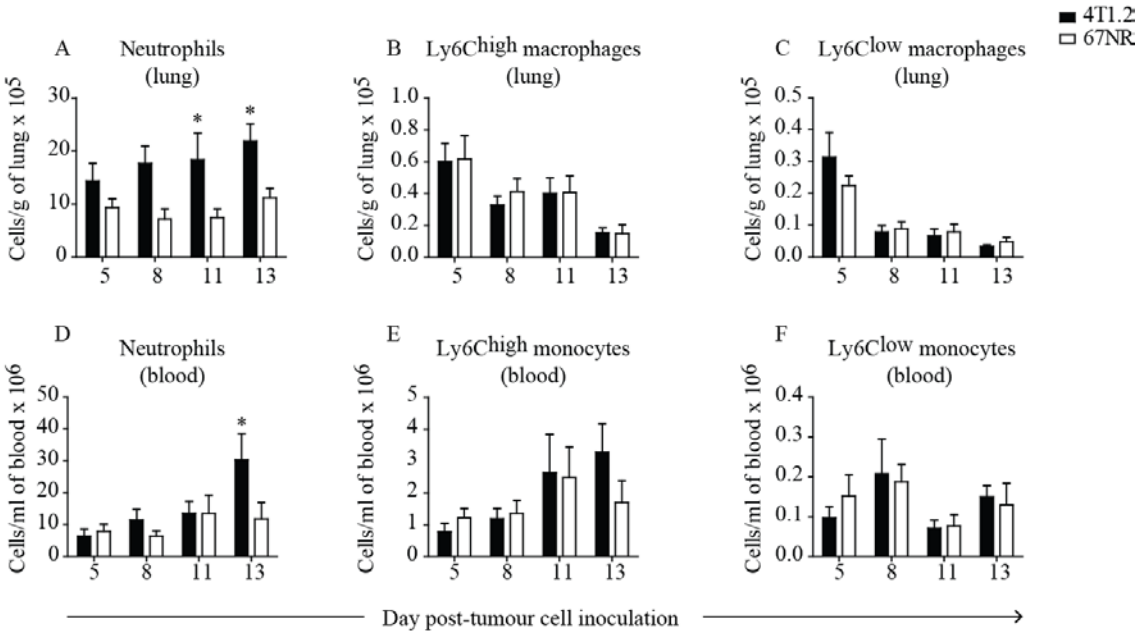


Figure 4.8: Neutrophil numbers increase in 4T1.2 metastasis-associated lungs at day eleven post-tumour cell inoculation.

Mice were injected orthotopically into the fourth mammary gland with either 4T1.2 (highly metastatic) or 67NR (non-metastatic) mCherry-expressing tumour cells. Lungs were excised and disaggregated, and peripheral blood was harvested by tail vein at day 5, 8, 11 and 13 post-tumour cell inoculation. Monocytes/macrophages and neutrophils were characterised by flow cytometry as described in Figure 4.1. A cohort of mice (*n*=8/group) was culled at each of the time points shown. Lung A) neutrophil, B) Ly6C^{high} macrophage, and C) Ly6C^{low} macrophage numbers. Blood D) neutrophil E) Ly6C^{high} monocyte and F) Ly6C^{low} monocyte numbers. Data are expressed as A-C) cells per gram of lung, and D-F) cells per ml of blood. Mean±SEM. *$P \leq 0.05$ 4T1.2 vs. 67NR, two-way repeated measures ANOVA.

Table 4.1: Factors upregulated in serum of 4T1.2, 66cl4 and 67NR tumour-bearing mice compared to naive controls.

4T1.2		66cl4		67NR	
Factor	Fold change	Factor	Fold change	Factor	Fold change
Pro-MMP-9	12.083	Pro-MMP-9	2.261	MIG	1.816
DPPIV/CD26	3.386	IL-3	2.214	IL-12 p40/p70	1.569
E-selectin	3.006	IGFBP-3	2.126	Pro-MMP-9	1.566
TRANCE	2.62	IL-5	2.009	IL-2	1.542
Lungkine	2.453	IL-2	1.976		
VEGF R3	2.403	L-Selectin	1.874		
Flt-3 Ligand	2.366	IL-12 p70	1.832		
I-TAC	2.308	TNFα	1.824		
TIMP-2	2.273	IL-12 p40/p70	1.778		
bFGF	2.166	IL-6	1.755		
IGFBP-2	2.135	Lymphotactin	1.726		
Dtk	2.04	GM-CSF	1.716		
VEGF R1	2.009	KC	1.693		
VEGF-D	1.954	PF-4	1.577		
Thymus CK-1	1.912	IGFBP-6	1.573		
SDF-1α	1.857	IL-17	1.566		
Osteoprotegerin	1.814	sTNF RII	1.521		
IL-15	1.808	IL-4	1.501		
ICAM-1	1.75				
IL17B R	1.746				
BLC	1.702				
IGF-II	1.701				
IL-7	1.686				
TSLP	1.668				
VEGF R2	1.643				
TIMP-1	1.62				
Lymphotactin	1.565				

Table 4.1: Mice were injected orthotopically into the fourth mammary gland with 4T1.2 (highly metastatic), 66cl4 (weakly metastatic) or 67NR (non-metastatic) tumour cells. Saline was injected into another cohort as a control. Cardiac blood was recovered on day 15 and serum was prepared. A RayBio® mouse cytokine antibody array (G series 3 and 4) was used to analyse changes in serum factors. Any fold change ≥1.5 was considered to be a significant increase in expression, as per manufacturer's instructions. Factors that were significantly upregulated in tumour-bearing mice compared to naive mice are shown. Data are expressed as fold change in fluorescent signal intensity compared to saline controls. *n*=3 mice/group.

Table 4.2: Factors downregulated in serum of 4T1.2, 66cl4 and 67NR tumour-bearing mice compared to naive controls.

4T1.2		66cl4		67NR	
Factor	Fold change	Factor	Fold change	Factor	Fold change
IL-17	0.119			Resistin	0.363
IL-3	0.166			IGF-I	0.371
CCL2	0.293			Thymus CK-1	0.383
IL-2	0.31			IGF-II	0.446
IFNγ	0.312			Fc gammaRIIB	0.465
IL-12 p70	0.332			Axl	0.53
GM-CSF	0.336			MMP-3	0.543
IL-6	0.394			GITR	0.572
MMP-2	0.404			IGFBP-2	0.588
IL-3 Rβ	0.432			DPPIV/CD26	0.593
CTACK	0.449			LIX	0.604
IL-9	0.474			Shh-N	0.607
CRG-2	0.48			IL17B R	0.611
IL-4	0.482			TROY	0.625
IL-10	0.485			Dtk	0.626
TECK	0.521			MDC	0.629
VEGF	0.526			TIMP-2	0.633
LIX	0.538			P-Selectin	0.638
TROY	0.54			HGF R	0.64
IL-1α	0.55			ICAM-1	0.645
Leptin R	0.562				
MMP-3	0.568				
CD30 L	0.572				
Axl	0.576				
IL-1 beta	0.58				
IGFBP-3	0.582				
PF-4	0.594				
Fractalkine	0.621				
KC	0.623				
CCL5	0.64				
TCA-3	0.641				
CD40	0.643				
CD30 T	0.643				

Table 4.2: Samples were prepared and analysed as described in Table 4.1. Any fold change ≤0.65 was considered to be a decrease in expression, as per manufacturer's instructions. Factors that were significantly downregulated in tumour-bearing mice compared to naive mice are shown. Data are expressed as fold change in fluorescent signal intensity compared to saline controls. *n*=3 mice/group.

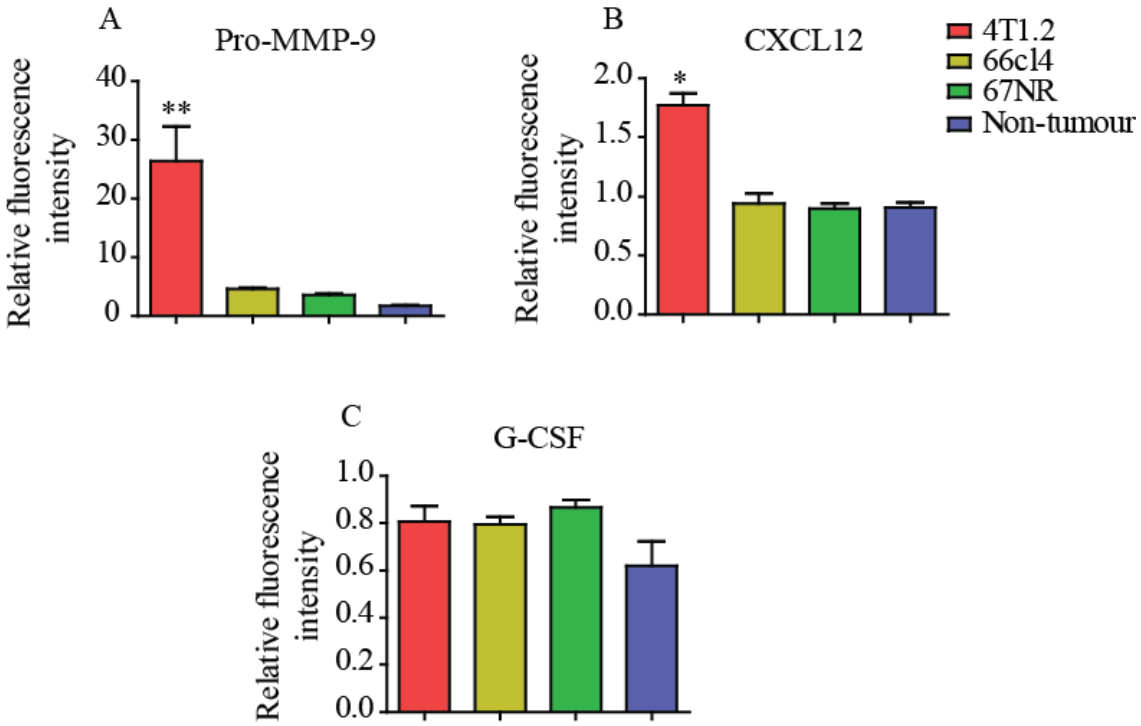


Figure 4.9: Pro-MMP-9 and CXCL12 are increased in serum of mice bearing 4T1.2 (highly metastatic) compared to 66cl4 (weakly metastatic), 67NR (non-metastatic) tumours and naive controls.

Samples were prepared and analysed as described in Table 4.1. Serum A) pro-MMP-9, B) CXCL12 (SDF-1α) and C) G-CSF levels in tumour-bearing mice and non-tumour-bearing (naive) controls. Data are expressed as fluorescent signal intensity normalised to in-built positive and negative controls. Mean±SEM; *n*=3 mice/group. **P*≤0.01 and ***P*≤0.001 4T1.2 vs. 66cl4, 67NR and non-tumour-bearing mice, one-way ANOVA.

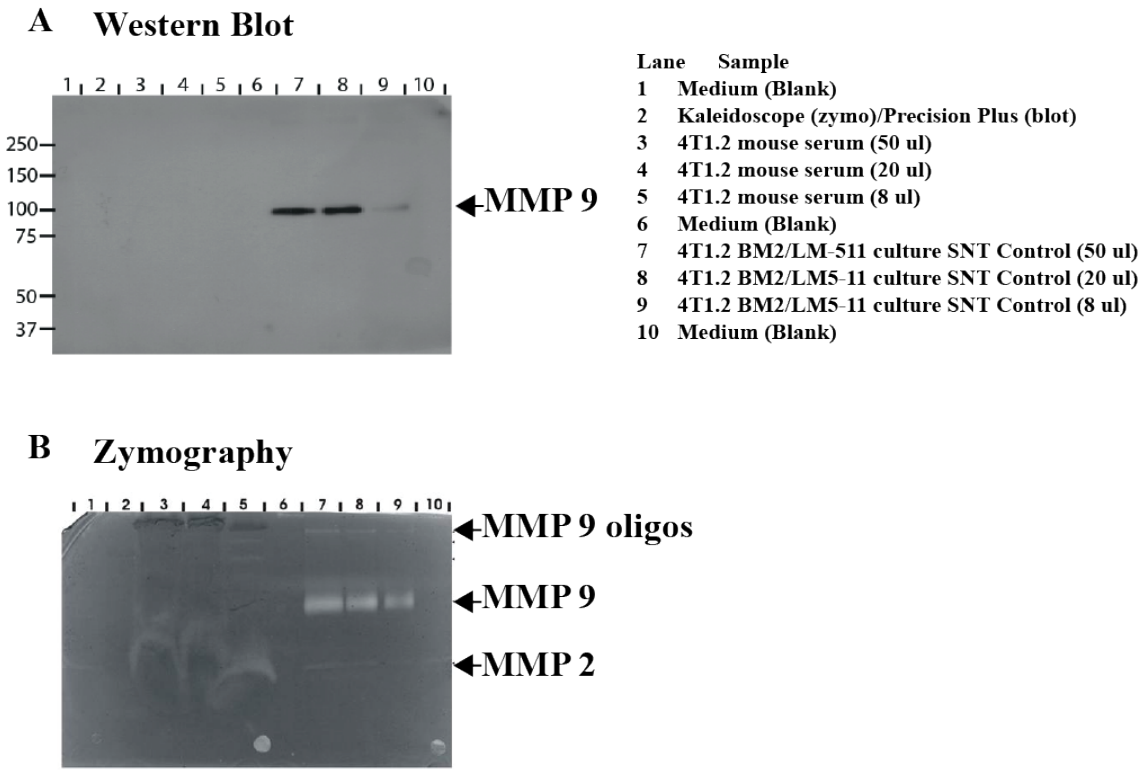


Figure 4.10: Analysis of serum MMP-9 levels in 4T1.2 tumour-bearing mice by Western blot and zymography.

Samples were prepared and analysed as described in Table 4.1. A) Western blot analysis of MMP-9 protein levels in serum from 4T1.2 tumour-bearing mice (lanes 3-5) and culture supernatant (SNT) from 4T1.2 BM2/LM5-11 positive controls (lanes 7-9). B) Zymographic analysis of MMP-9 in serum from 4T1.2 tumour-bearing mice (lanes 3-5) and culture supernatant from 4T1.2 BM2/LM5-11 positive controls (lanes 7-9). Mean±SEM; *n*=3 mice/group.

5 Targeting Monocytes in Breast Cancer Metastasis

5.1 Introduction

As discussed in Chapter 1 (Section 1.7.6), colony stimulating factor-1 (CSF-1/M-CSF) is a key macrophage growth factor [266, 267], the effects of which are mediated through the receptor tyrosine kinase, CSF-1R (c-Fms/CD115) [267]. Treatment of human mammary xenografts (in immunocompromised mice) with CSF-1 antisense oligonucleotides [292], anti-CSF-1 antibodies [293], or small interfering RNAs (siRNAs) against CSF-1 [294] suppressed tumour growth. In mice deficient in an active *Csf1* gene, MMTV-PyMT metastasis to lung was reduced [199]. However, in more recent studies using the same MMTV-PyMT model, but in mice with intact CSF-1 activity, treatment with CSF-1R signalling antagonists alone had minimal effects on lung metastasis [136]. Only when used in combination with chemotherapy did anti-CSF-1R treatment reduce metastasis to lung [136]. CSF-1 signalling is therefore considered a potential target in breast cancer treatment and clinical trials are underway [135].

GM-CSF is involved in both macrophage and neutrophil function [470, 471] and is proposed to regulate activation of these cells during inflammation [472, 473]. Treatment with GM-CSF has been shown to reduce murine Met-1 mammary tumour growth and metastasis [474]. However, GM-CSF has also been suggested to promote metastatic breast tumour growth in bone [475]. Knockdown of GM-CSF by short hairpin RNAs in MDA-MB-231 human breast tumour cells or antibody-mediated depletion of GM-CSF reduced osteolytic bone metastases in a murine model of experimental metastasis [475].

In immunodeficient mice bearing human breast carcinoma cells, inhibition of the monocyte chemoattractant, CCL2, reduced metastasis to lung [476]. However, these mice did not have an intact immune system, which alone could have affected metastasis. In experimental models of metastasis, using human breast tumour cell lines, enhanced expression of CCL2 increased metastatic growth, and was associated with increased monocyte migration [477]. In another experimental metastasis study in the MMTV-PyMT model, blocking Ly6C^{high} monocytes either by anti-CCL2 antibodies, or using CCR2 deficient mice, delayed tumour cell extravasation into the lung [202]. However,

these experimental metastasis models do not encompass all the steps of metastasis. In this chapter, the effect of monocyte depletion on metastasis was studied in the context of spontaneous metastasis.

It is reported in this chapter that treatment with neutralising GM-CSF antibody does not alter tumour growth or metastasis, nor are blood monocyte and neutrophil numbers reduced with this treatment. Treatment with clodronate depletes Ly6C^{low}, non-classical blood monocytes and reduces metastasis to lung. Surprisingly, increased metastasis to the lung and spine is seen upon neutralisation of CSF-1R signalling. Neutrophils, and possibly Ly6C^{high} monocytes, are implicated in this enhanced metastasis.

5.2 Results

In Chapter 4 it was shown that increased mobilisation of monocytes was associated with metastasis. Given this, and data from other studies showing that inhibition of monocyte recruitment reduces metastasis to lung [202, 476], it was hypothesised that depletion of monocytes would reduce TAM numbers and consequently metastasis, and possibly primary tumour growth as well.

5.2.1 Treatment with clodronate liposomes depletes peripheral blood Ly6C^{low} monocytes and reduces metastasis to lung

Clodronate is toxic to cells but does not cross phospholipid bilayers easily; hence when encapsulated in liposomes, it is rendered harmless. Phagocytic cells such as monocytes can internalise clodronate liposomes [434]. Once inside the cell, the liposomes are degraded, releasing the clodronate, which then accumulates and destroys the cell [434]. Clodronate liposomes have been shown to deplete monocyte and macrophage populations without affecting neutrophil and lymphocyte numbers [478].

The route of injection dictates which monocyte or macrophage subpopulations are depleted; intravenous (i.v.) injection of clodronate liposomes depletes macrophages in the liver (Kupffer cells) and spleen [479], as well as circulating monocytes [304]. Subcutaneous (s.c.) injection of clodronate liposomes depletes macrophages in the draining lymph nodes [480]. Intraperitoneal (i.p.) injection of clodronate liposomes

depletes macrophages in the peritoneal cavity, lymph nodes, liver and spleen, as well as circulating monocytes [481].

To assess the effect of monocyte depletion on 4T1.2 metastasis, clodronate treatment via the i.p. route was chosen. This route minimises any tissue damage due to multiple injections, as injections can be administered alternatively into the left and right sides of the peritoneum, whilst still depleting a number of different resident macrophage cell types, as well as circulating monocytes [481, 482]. Mice bearing 4T1.2 mCherry-expressing tumours were treated i.p. with clodronate liposomes every three days, from day 5 to 21 post-tumour cell inoculation. PBS liposomes and PBS alone were used for the control groups.

To determine how clodronate liposome treatment alters circulating monocyte and neutrophil populations, blood was collected 24 hours prior to the first treatment and 48 hours after the third treatment (day 5 and 16 post-tumour cell inoculation, respectively). Monocytes were defined as $CD11b^{+}Ly6G^{neg}Ly6C^{high}$ or $CD11b^{+}Ly6G^{neg}Ly6C^{low}$ [303], and neutrophils as $CD11b^{+}Ly6G^{high}$ [8, 223]. Treatment with clodronate liposomes depleted $Ly6C^{low}$ monocytes in the peripheral blood compared to treatment with PBS liposomes and PBS (Figure 5.1A, $P \leq 0.05$). $Ly6C^{high}$ monocytes and neutrophils were not affected (Figures 5.1B and 5.1C).

Treatment with clodronate liposomes did not alter primary tumour growth or endpoint weights (Figure 5.2), but significantly reduced metastasis to lung (Figure 5.3A, $P \leq 0.05$). Metastasis to spine was significantly reduced upon clodronate treatment compared to PBS-treated mice (Figure 5.3B, $P \leq 0.05$) but only a trend towards reduced metastasis to spine was seen when clodronate liposome-treated mice were compared to PBS liposome-treated mice (Figure 5.3B).

5.2.2 Treatment with clodronate liposomes does not alter end-stage peripheral blood and primary tumour monocyte/TAM and neutrophil/TAN numbers

End-stage TAM and TAN numbers were analysed to determine if treatment with clodronate liposomes altered the number of these cells in the primary tumour. Peripheral blood and primary tumours were harvested at day 28 post-tumour cell inoculation (seven days after the last clodronate liposome treatment). TAMs and TANs were characterised as described above for circulating monocytes and neutrophils. No differences were seen in end-stage $Ly6C^{high}$ or $Ly6C^{low}$ TAM or TAN cell numbers in primary tumours after clodronate treatment (Figure 5.4A-C), nor were there any differences in end-stage $Ly6C^{high}$ and $Ly6C^{low}$ monocytes or neutrophils in the peripheral blood (Figure 5.4D-F).

5.2.3 Treatment with neutralising anti-CSF-1R antibody increases metastasis to lung and spine

The anti-CSF-1R antibody, AFS-98, has been shown to deplete macrophages in atherosclerotic lesions [483], in renal allografts [484], during skeletal muscle damage [485], and in diabetic nephropathy [486]. Previous work in our research group at the Arthritis and Inflammation Research Centre reported that AFS-98 reduces macrophages in the steady state and during inflammation [224]. Kubota et al. have reported that AFS-98 treatment reduces TAM numbers in osteosarcoma [487].

Given the literature linking CSF-1 and TAM numbers with breast cancer metastasis [4, 136, 198, 230, 286], it was expected that treatment with AFS-98 would reduce metastasis, and potentially primary tumour growth, due to depletion of TAMs. However, treatment of mice bearing 4T1.2 mCherry-expressing tumours with AFS-98, although not affecting primary tumour growth (Figure 5.5), significantly increased metastasis to lung (Figure 5.6A, $P \leq 0.01$) and spine (Figure 5.6B, $P \leq 0.05$).

5.2.4 Treatment with neutralising anti-GM-CSF antibody does not alter metastasis to lung and spine

GM-CSF has been implicated in progression to advanced disease in cancer [475, 488]. It was therefore hypothesised that neutralising GM-CSF may also reduce metastasis in 4T1.2 tumour-bearing mice. However, treatment of mice bearing 4T1.2 mCherry-expressing tumours with anti-GM-CSF antibody (22E9) had no effect on primary tumour growth (Figure 5.5), although a non-significant trend towards increased metastasis was observed to both lung and spine (Figure 5.6).

5.2.5 Treatment with neutralising anti-CSF-1R antibody increases neutrophil numbers in the primary tumour and lung

Previous research from our group at the Arthritis and Inflammation Research Centre has demonstrated that AFS-98 treatment reduced tissue macrophages, but only the Ly6C^{low} monocyte subpopulation in the blood, both in the steady state and during inflammation [224]. Treatment with 22E9 was shown to limit increases in circulating Ly6C^{high} monocytes and neutrophils in a collagen-induced arthritis model [489]. Given the unexpected result with AFS-98 treatment for metastasis, and the trend towards increased metastasis upon treatment with 22E9, macrophage and neutrophil numbers were measured in primary tumours and lungs, following anti-CSF-1R (AFS-98) and anti-GM-CSF (22E9) treatment. Macrophages and neutrophils were characterised as described in Section 5.2.1.

Treatment with anti-CSF-1R antibody did not reduce TAM numbers (Figure 5.7A) but, surprisingly, increased TAN numbers in primary tumours (Figure 5.7B, P=0.02). A trend towards higher numbers of Ly6C^{high} lung macrophages was observed (Figure 5.7C) but neutrophils were markedly increased in lungs after anti-CSF-1R antibody administration (Figure 5.7D, P=0.001). Treatment with 22E9 did not alter TAM or TAN (Figures 5.8A and 5.8B), or lung macrophage and neutrophil (Figures 5.8C and 5.8D) numbers.

5.2.6 Treatment with neutralising anti-CSF-1R antibody further enhances tumour-driven increases of circulating neutrophils and Ly6C^{high} monocytes

The unexpected increase in primary tumour and lung neutrophils (and perhaps lung Ly6C^{high} macrophages) following anti-CSF-1R administration may have been due to changes in cell trafficking. In Chapter 4 it was shown that peripheral blood neutrophils and monocyte subpopulations increased during 4T1.2 tumour progression in the absence of antibody treatment (see Chapter 4, Figure 4.1).

To assess the effect of anti-CSF-1R and anti-GM-CSF antibody treatment on these cell populations, blood was collected 24 hours prior to the first treatment and 24 hours after the fourth treatment (day 6 and 19 post-tumour cell inoculation, respectively). Monocytes and neutrophils were characterised as described in see Section 5.2.1. As reported in Chapter 4, the expansion of neutrophils, as well as Ly6C^{high} and Ly6C^{low} monocytes was again observed in the isotype control-treated tumour-bearing mice (Figure 5.9; $P \leq 0.01$, isotype control: pre- vs. post-treatment). Administration of anti-CSF-1R antibody further increased this tumour-mediated expansion of blood neutrophils and Ly6C^{high} monocytes (Figures 5.9A and 5.9B; $P \leq 0.05$, anti-CSF-1R vs. isotype control post-treatment) but not of Ly6C^{low} monocytes (Figure 5.9C). GM-CSF blockade did not further enhance tumour-mediated expansion of monocytes and neutrophils in the blood (Figure 5.10).

5.2.7 Treatment with the CSF-1R inhibitor, GW2580, shows a trend towards increased metastasis

GW2580 is an orally bioavailable kinase inhibitor of CSF-1R that binds competitively to the ATP binding site in the CSF-1R and prevents CSF-1R-mediated survival and proliferation of macrophages *in vitro* and *in vivo* [436]. The intracellular domain of CSF-1R is similar in structure to its other family members, but GW2580 has a high specificity for the CSF-1R compared to related kinases such as c-kit and Flt3 [436, 490]. Mice bearing 4T1.2 mCherry-expressing tumours were treated with 160 mg/kg of GW2580 daily, as this treatment regime was shown by Priceman et al. to have maximum bioavailability and minimal toxicity [435].

Treatment with GW2580 has been reported to reduce macrophage numbers in mouse models of autoimmune encephalomyelitis [491] and rheumatoid arthritis [492]. Priceman et al. reported that treatment with GW2580 depleted TAMs in mouse melanoma, as well as in lung and prostate carcinomas [435]. In this thesis, no changes were seen in 4T1.2 primary tumour growth over 28 days following treatment with the CSF-1R inhibitor compared to vehicle (Figure 5.11A), nor were there any differences seen in end-stage primary tumour weights (Figure 5.11B). However, a trend towards increased metastasis to lung and spine was observed following treatment with the CSF-1R inhibitor, but this did not attain significance (Figures 5.11C and 5.11D).

5.2.8 Treatment with the GW2580 CSF-1R inhibitor depletes Ly6C^{low} TAMs in primary tumours

Given the trend towards increased metastasis with the CSF-1R inhibitor, and the unexpected observation that AFS-98 treatment increased metastasis, macrophage and neutrophil numbers were measured in primary tumours and lungs following treatment with GW2580. TAMs/macrophages and TANs/neutrophils were analysed as described in Section 5.2.1. Treatment with GW2580 depleted Ly6C^{low} TAMs in the primary tumour (Figure 5.12A, $P \leq 0.05$) and yielded a trend towards increased Ly6C^{high} TAM numbers (5.12A). Therefore treatment with GW2580 may be inhibiting maturation of Ly6C^{high} TAMs into Ly6C^{low} TAMs. TAN numbers were not altered by treatment with GW2580 (Figure 5.12B), nor were lung Ly6C^{high} macrophage, Ly6C^{low} macrophage and neutrophil numbers (Figures 5.12C and 5.12D).

5.2.9 Treatment with the GW2580 CSF-1R inhibitor does not enhance tumour-driven increases of circulating monocytes and neutrophils

As mentioned previously, peripheral blood neutrophils and monocyte subpopulations increase during 4T1.2 tumour progression in the absence of antibody treatment (see Chapter 4, Figure 4.1). To assess the effect of CSF-1R inhibitor treatment on these cell populations, blood was collected 24 hours prior to the first treatment and 24 hours after the fourth treatment (day 6 and 19 post-tumour cell inoculation, respectively) and

monocytes and neutrophils were characterised as described in Section 5.2.1. The expansion of neutrophils and Ly6C^{high} monocytes was observed in the vehicle-treated group (Figure 5.13A and 5.13B; $P \leq 0.05$, vehicle: pre- vs. post-treatment). However, in this case, only a trend towards increased Ly6C^{low} monocyte numbers was seen in 4T1.2 tumour-bearing mice without treatment (Figure 5.13C; vehicle: pre- vs. post-treatment).

This latter result is in contrast to the previous findings from Chapter 4 and what was observed upon treatment with the isotype control for AFS-98 and 22E9 (Figure 5.9C and 5.10C; $P \leq 0.01$, isotype control: pre- vs. post-treatment). However, this discrepancy could simply be due to the smaller sample size of the groups treated with GW2580 and vehicle, as a trend towards increased numbers of Ly6C^{low} monocytes was observed (Figure 5.13C; inhibitor vs. vehicle post-treatment). These group sizes were limited by the availability of GW2580. Treatment with GW2580 had no impact on the tumour-driven increases in circulating monocyte and neutrophil numbers (Figure 5.13). However, a trend towards reduced Ly6C^{low} blood monocytes was observed upon treatment with GW2580 (Figure 5.13C; inhibitor vs. vehicle post-treatment).

5.2.10 Enhanced expression of G-CSF in tumours increases metastasis

Given the increases in systemic and tumour neutrophils reported following blockade of CSF-1R signalling in this thesis, it was hypothesised that enhanced recruitment of neutrophils in the 4T1.2 mammary tumour model would further promote metastasis. In a collaborative experiment with Dr. Yuan Cao, Peter MacCallum Cancer Centre, the weakly metastatic 66cl4 cell line was engineered to express increased levels of the neutrophil chemoattractant, G-CSF, and the effects on tumour growth, metastasis, as well as blood myeloid cell populations were analysed. The 66cl4 tumour cell line was chosen as it is weakly metastatic and any increase in metastasis would be more apparent than in the highly aggressive 4T1.2 cell line. The weakly metastatic C57BL/6-derived E0771 tumour cell line was also engineered to express G-CSF to confirm that any changes in metastasis or myeloid cell numbers were not specific to the BALB/c 66cl4 model.

Dr. Cao engineered the G-CSF over-expressing cells and completed the animal work. I completed the analysis of myeloid cells at our research laboratory at the Arthritis and Inflammation Research Centre. Both 66cl4 and E0771 tumours engineered to express G-CSF showed a marked increase in G-CSF levels compared to empty vector controls (Figures 5.14A and 5.14B, P=0.0001). Enhanced expression of G-CSF in 66cl4 or E0771 tumour cells did not alter primary tumour growth or end-stage weights (Figure 5.14C-F). However, lung and spleen weights were increased in mice bearing 66cl4 G-CSF over-expressing tumours, (Figures 5.14G, P=0.02 and 5.14H, P=0.0001, respectively), which is indicative of increased metastasis [493]. A trend towards increased lung and spleen weights was observed in mice bearing E0771 G-CSF over-expressing tumours (Figures 5.14I and 5.14J)

To determine the effect of elevated G-CSF expression by tumours on circulating myeloid cells, blood was collected at end-point and monocytes and neutrophils were analysed by flow cytometry. Monocytes and neutrophils were characterised as described in Section 5.2.1. Circulating neutrophil and Ly6C^{high} monocyte numbers were increased in 66cl4-G-CSF-expressing tumours (Figures 5.15A, P=0.02 and 5.15B, P=0.01, respectively), whereas Ly6C^{low} blood monocyte numbers were not altered (Figure 5.15C). Elevated expression of G-CSF in E0771 tumours markedly increased circulating neutrophil (Figure 5.15D, P=0.001), as well as Ly6C^{high} (Figure 5.15E, P=0.0001) and, to a lesser degree, Ly6C^{low} blood monocyte numbers (Figure 5.15F, P= 0.02).

5.3 Discussion

In Chapters 3 and 4, it was shown that highly metastatic 4T1.2 tumours promoted mobilisation of monocytes and neutrophils from the bone marrow, resulting in increased circulating Ly6C^{high} monocytes and neutrophils, as well as increased TAMs and TANs in the primary tumour. However, it is unclear if these changes in tumour and systemic cell numbers are important for metastasis. In this chapter, it was reported that treatment with clodronate liposomes depleted peripheral blood Ly6C^{low} monocytes (Figure 5.1A) and that this was associated with reduced metastasis to lung (Figure 5.3A) and possibly spine (Figure 5.3B). It has been reported previously that treatment with clodronate liposomes reduced lung cancer metastasis to bone [494]. However, this was an

experimental metastasis model and, thus, did not include all the steps of metastasis. The 4T1.2 model is a spontaneous model of metastasis and this may explain why a significant reduction in spine metastasis was not seen following clodronate treatment (Figure 5.3B).

Blood Ly6C^{high} monocytes were not depleted upon treatment with clodronate (Figure 5.1B). This may be due to the relatively low dose used (50 µl), as the resident peritoneal macrophages and the more phagocytic Ly6C^{low} monocytes [495] could have engulfed all of the clodronate liposomes first. This dose was chosen as, given the number of injections, higher doses proved toxic. It is possible that with a higher dose of clodronate, Ly6C^{high} monocytes would also have been depleted, as total blood monocyte depletion was achieved in another study where 200 µl of clodronate liposomes were injected i.v. [304]. A higher dose was possible for the above study as fewer injections of clodronate were given than for the study in this thesis. However, highly aggressive 4T1.2 tumours markedly increase circulating monocyte and neutrophil numbers (see Chapter 4, Figures 4.1 and 4.2); thus the toxic effects seen upon clodronate administration may be due to destruction of large numbers of cells. In this regard, a better result may have been achieved with a less aggressive metastatic tumour cell line, such as 4T1 or 66cl4.

It is possible that depletion of both Ly6C^{high} and Ly6C^{low} blood monocytes may have a more profound effect on 4T1.2 tumour metastasis, as Ly6C^{high} monocytes increased in MMTV-PyMT metastasis-associated lungs, and inhibition of Ly6C^{high} monocytes via blockade of CCL2 reduced metastatic seeding at this site [202]. Treatment with clodronate liposomes can also reduce CD4^{+} T cell proliferation [496], and inhibit APCs [478, 497]. Hence the reduction in 4T1.2 tumour metastasis reported in this chapter after clodronate treatment may not necessarily be due to depletion of Ly6C^{low} blood monocytes.

Both end-stage Ly6C^{high} and Ly6C^{low} TAM numbers were the same in clodronate and control treatment groups (Figures 5.4A and 5.4B). However, this was seven days after the last clodronate treatment. Treatment with clodronate liposomes has been shown to deplete TAMs in models of rhabdomyosarcoma [498], lung cancer [494] and melanoma [499]. However, in all these studies TAM numbers were analysed within four days of the last clodronate treatment and splenic macrophages have been shown to begin to re-

populate by one week after clodronate treatment [500]. Thus, it is possible that TAM numbers in 4T1.2 tumours were reduced during clodronate treatment, and re-populated after the last dose was given. Hence a possible effect on metastasis due to TAM reduction cannot be completely ruled out. Clodronate liposomes are viable for 2 weeks, after which they begin to degrade [482]. Since the aim of the experiment for this thesis was to investigate monocytes in metastasis, mice were treated from day 5 to 21 post-tumour cell inoculation to target key stages of the metastatic process.

Previous studies by our research group at the Arthritis and Inflammation Research Centre using the anti-GM-CSF antibody (22E9) found that neutralising GM-CSF activity limited increases in circulating Ly6C^{high} monocytes and neutrophils in a collagen-induced arthritis model [489] and reduced neutrophil numbers in a model of lung inflammation [501]. In this chapter it was found that treatment with neutralising GM-CSF antibody did not affect blood or lung monocyte/macrophage and neutrophil numbers (Figures 5.8 and 5.10), nor were primary tumour TAM and TAN numbers altered (Figure 5.8). Furthermore, inhibition of GM-CSF activity did not affect metastasis (Figure 5.6). This in line with a previous study using the 4T1 mammary tumour model, showing that treatment with GM-CSF neutralising antibody did not alter blood or lung neutrophil numbers and did not appear to be important for metastasis [8].

GM-CSF plays a key role in a number of inflammatory and autoimmune diseases [472]. However, in these settings a more ‘pro-inflammatory’ reaction is induced, contrary to the ‘trophic, anti-inflammatory’ reaction generated by metastatic breast tumours [265]. Thus, GM-CSF inhibition may not have any impact on the ‘trophic-like’ environment of breast cancer.

Surprisingly, anti-CSF-1R antibody administration, although not affecting primary tumour growth, enhanced metastasis of 4T1.2 tumours to both lung and spine (Figures 5.5 and 5.6). Furthermore, treatment of mice bearing 4T1.2 tumours with the CSF-1R inhibitor, GW2580, again while not affecting primary tumour growth (Figures 5.11A and 5.11B), showed a trend towards increased metastasis to lung and spine (Figures 5.11C and 5.11D). Prior to this report, various approaches disrupting CSF-1R/CSF-1 signalling in a number of solid tumour models resulted in suppression of growth and/or metastasis but never an increase in either parameter [136, 199, 293, 294]. In this

chapter, TAMs were not depleted by anti-CSF-1R treatment but (Figure 5.7A), unexpectedly, TANs were increased 3-fold (Figure 5.7B). The increase in neutrophil numbers was also manifested in metastasis-involved lungs and in peripheral blood (Figures 5.7D and 5.9A).

Tumour-driven increases in blood neutrophils and Ly6C^{high} monocytes seen without anti-CSF-1R treatment (see Chapter 4, Figure 4.1) could be actively contributing to metastasis. Neutralising anti-CSF-1R treatment further enhanced these increases in neutrophils in the blood (Figure 5.9A) and lung (Figure 5.7D), and possibly, as a result of this, further promoted metastasis. Additionally, increases in Ly6C^{high} monocytes/macrophages in the blood (Figure 5.9B), and possibly in the lung (Figure 5.7C), were further enhanced by anti-CSF-1R administration. An increase in Ly6C^{high} blood monocytes after neutralisation with AFS-98 was not observed in our previous study [224], but has been reported by another group after administration of a different anti-CSF-1R antibody (M279) in the steady state [502]. Ly6C^{high} monocytes have been implicated in experimental MMTV-PyMT tumour metastasis to lung [202], and hence may be involved in the enhanced metastasis reported after CSF-1R neutralisation in this thesis.

It is possible that depletion of monocytes or TAMs may have been achieved with higher doses of anti-CSF-1R or anti-GM-CSF antibodies. The doses used for the above experiments were guided by the analysis of the half-life of the anti-GM-CSF (22E9) antibody carried out by MorphoSys (Martinsried/Planegg, Germany), which revealed that a protocol of 150 ug of antibody administered twice weekly was sufficient to guarantee ample levels of antibody. Similar studies were not completed for the AFS-98 antibody hence a higher dose of 250 ug was chosen to ensure sufficient levels of antibody. These doses have been shown to deplete monocytes and macrophages with minimal cytotoxicity in our laboratory at the Arthritis and Inflammation Centre (Cook et al., unpublished data).

Elevated expression of the key neutrophil chemoattractant, G-CSF [503], in weakly metastatic 66cl4 tumours increased lung and spleen weights (Figures 5.14G and 5.14H), and, in E0771 tumours, yielded a trend towards increased lung and spleen weights (Figures 5.14I and 5.14J) and this was associated with increased circulating neutrophils

and Ly6C^{high} monocytes (Figure 5.15). Increased lung and spleen weights are indicative of increased metastasis [493]. These data support the concept that the increased neutrophil, and possibly Ly6C^{high} monocyte mobilisation seen after AFS-98 administration enhances 4T1.2 tumour metastasis. It would also be of value to assess G-CSF, GM-CSF and CSF-1 serum levels in mice bearing tumours of varying metastatic capacity at different stages of metastatic progression. In particular, assessment of serum CSF-1 levels in mice treated with the anti-CSF-1R neutralising antibody will give an indication of the effectiveness of the AFS-98 antibody.

Unlike treatment with neutralising CSF-1R antibody, treatment with the GW2580 inhibitor did not enhance tumour-driven mobilisation of monocytes or neutrophils (Figure 5.13) which could explain why a significant increase in metastasis was not seen following this treatment. This result with GW2580 treatment may also be due to the smaller group sizes in this study. Enhanced mobilisation of Ly6C^{high} monocytes and neutrophils, as well as a significant increase in metastasis with GW2580 treatment may become evident with larger sample sizes.

Treatment with GW2580 did, however, deplete Ly6C^{low} TAMs (Figure 5.12A), with a trend towards reduced Ly6C^{low} blood monocytes also observed (Figure 5.13C). It has been shown previously by another group that, after 14 days of treatment with GW2580, a significant reduction in TAMs, as well as monocytes in the bone marrow and spleen, and a trend towards reduced monocytes in the blood, occurred in a mouse model of lung carcinoma [435]. In this study, it was claimed that GW2580 affected only Ly6C^{high} monocytes and not neutrophils. However, Ly6C^{high} monocytes were classified as CD11b^{+}Gr-1^{low}Ly6C^{high} cells, whereas CD11b^{+}Gr-1^{high}Ly6C^{low} cells were considered neutrophils. Since Gr-1 labels both Ly6C and Ly6G, cells that are Gr-1^{high} by all rights should be Ly6C^{high} as well. Therefore it remains unclear whether GW2580 affected both monocyte subpopulations or only Ly6C^{high} monocytes in the above study [435].

A second ligand for the CSF-1R that shows functional overlap with CSF-1, IL-34, has been described recently [277, 278, 504]. Thus the possibility that the effects on metastasis seen upon treatment with CSF-1R antibody or inhibitor may result from inhibition of IL-34 signalling cannot be ruled out completely. However, the two ligands appear to bind different parts of the CSF-1R [504], and show differences in bioactivity

and signal activation; for example IL-34 and CSF-1 showed differences in their ability to induce production of CCL2 in macrophages, and IL-34 induced a stronger, albeit brief, phosphorylation of the CSF-1R [505].

5.4 Conclusion

Depletion of blood Ly6C^{low} monocytes by clodronate liposomes associated with reduced metastasis to lung. However, secondary effects due to inhibition of APCs and impairment of T cell function cannot be ruled out. Blockade of CSF-1R signalling increased metastasis to lung and spine and was associated with enhanced tumour-driven mobilisation of neutrophils, and to a lesser degree, Ly6C^{high} monocytes. Enhanced expression of G-CSF in weakly metastatic tumours increased metastasis to lung and this was associated with enhanced mobilisation of neutrophils and Ly6C^{high} monocytes. These findings provide evidence that neutrophils are also likely to be important in 4T1.2 metastasis, and it may be necessary to deplete both macrophages and neutrophils in order to successfully treat advanced breast cancer.

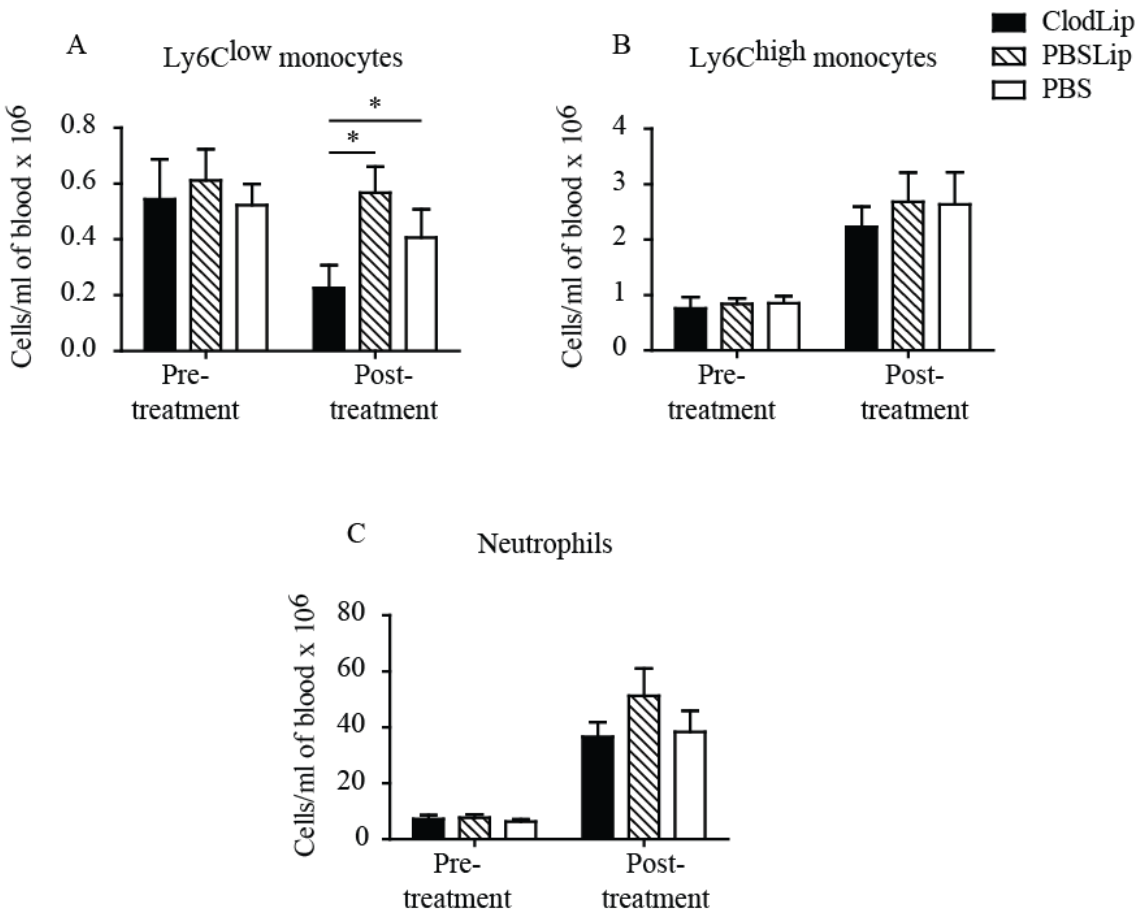


Figure 5.1: Clodronate liposome treatment depletes blood Ly6C^{low} monocytes. Mice were injected orthotopically into the fourth mammary gland with 4T1.2 mCherry-expressing tumour cells and treated every 3 days, from day 5 to 21 post-tumour cell inoculation, with 50 µl of clodronate liposomes (ClodLip), PBS liposomes (PBSLip) or PBS alone as controls. Peripheral blood was harvested via the tail vein at day 5 and 16 post-tumour cell inoculation (24 h prior to the first, and 48 h after the third treatment, respectively). Monocytes were characterised as CD11b^{+}Ly6G^{neg}Ly6C^{high} or CD11b^{+}Ly6G^{neg}Ly6C^{low} and neutrophils as CD11b^{+}Ly6G^{high} by flow cytometry. Blood A) Ly6C^{low} monocyte, B) Ly6C^{high} monocyte and C) neutrophil numbers. Data are expressed as cells per ml of blood. Mean±SEM; n=20 mice/group. *P≤0.05 ClodLip vs. PBSLip and PBS, two-way repeated measures ANOVA.

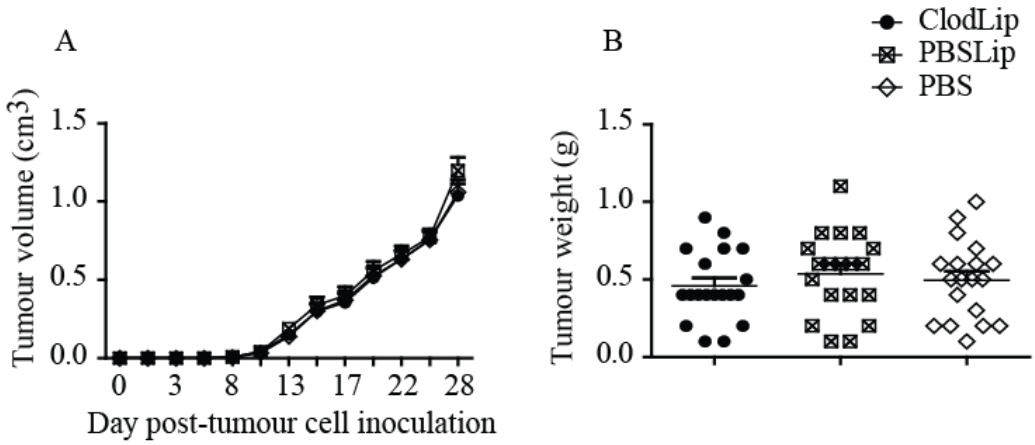


Figure 5.2: Clodronate liposome treatment does not alter primary tumour growth. 4T1.2 (mCherry) tumour-bearing mice were treated as described in Figure 5.1. Tumour growth was measured using electronic callipers and tumours were excised and weighed at end-point (day 28). Primary tumour A) growth (volume, cm^3) and B) end-point weights (grams). Mean±SEM; *n*=20 mice/group.

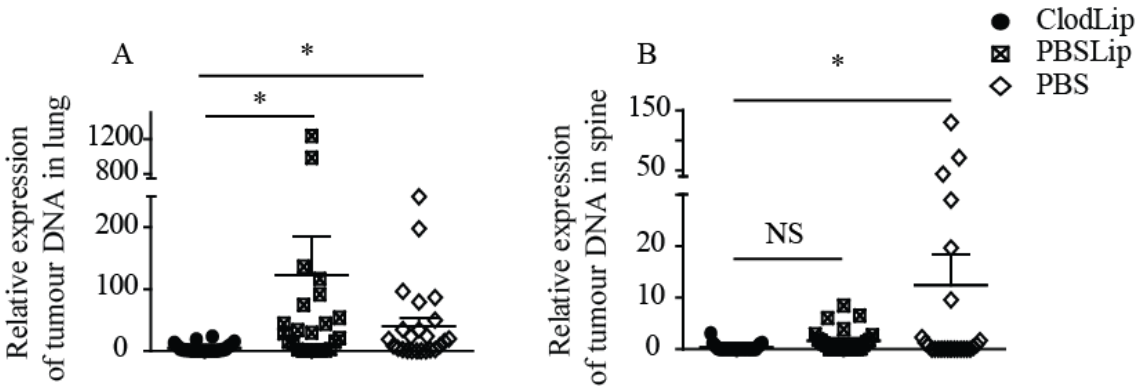


Figure 5.3: Clodronate liposome treatment reduces metastasis to lung.

4T1.2 (mCherry) tumour-bearing mice were treated as described in Figure 5.1. Lungs and spines were excised at end-point (day 28), snap frozen and homogenised. Genomic DNA was isolated and tumour mCherry DNA measured by qPCR. A) Lung metastasis and B) spine metastasis. Data are expressed as relative expression of mCherry DNA (tumour cells only) to that of vimentin DNA (all cells). Mean±SEM; *n*=20 mice/group. *$P \leq 0.05$ ClodLip vs. PBSLip and/or PBS, NS = not significant, two-way repeated measures ANOVA.

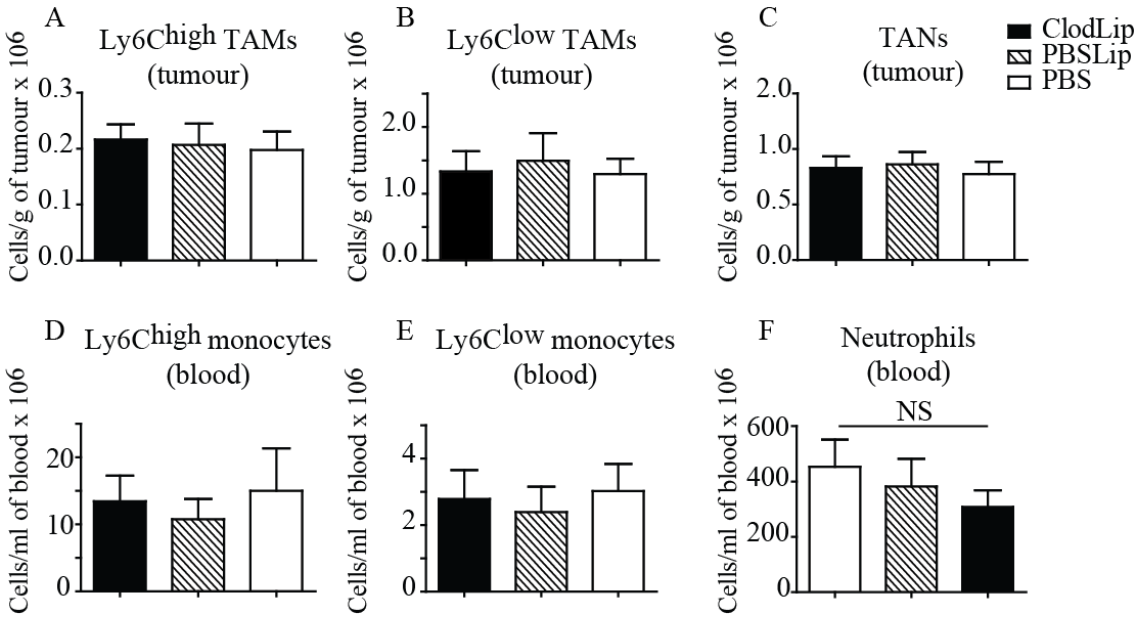


Figure 5.4: Clodronate liposome treatment does not alter end-stage TAM or TAN numbers or blood monocyte and neutrophil numbers.

4T1.2 (mCherry) tumour-bearing mice were treated as described in Figure 5.1. Peripheral blood was harvested via the tail vein and primary tumours were excised and disaggregated at end-point (day 28). Primary tumour TAMs and TANs, and peripheral blood monocytes and neutrophils, were analysed by flow cytometry, again as described in Figure 5.1. Primary tumour A) Ly6C^{high} TAM, B) Ly6C^{low} TAM and C) TAN numbers. Blood D) Ly6C^{high} monocyte E) Ly6C^{low} monocyte and F) neutrophil numbers. Data are expressed cells per g of tumour (A-C) or cells per ml of blood (D-F). Mean±SEM; *n*=20 mice/group. NS = not significant, two-way repeated measures ANOVA

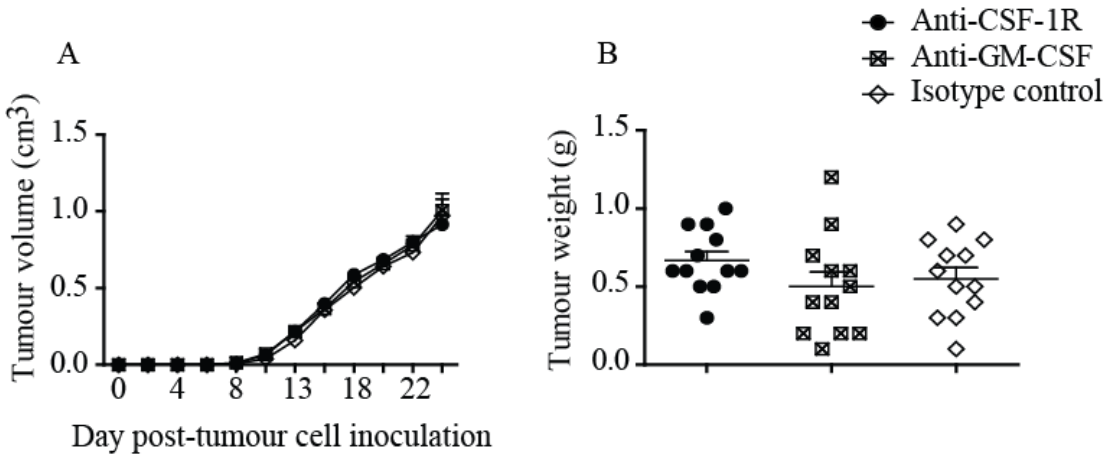


Figure 5.5: Anti-CSF-1R and anti-GM-CSF antibody treatment does not alter primary tumour growth.

Mice were injected orthotopically into the fourth mammary gland with 4T1.2 mCherry-expressing tumour cells and treated intraperitoneally every 4 days from day 7-21 post-tumour cell inoculation with either 250 μg of anti-CSF-1R antibody (AFS-98), 150 μg of anti-GM-CSF antibody (22E9) or 250 μg of anti-β-galactosidase isotype control [224]. Tumour growth was measured using electronic callipers and tumours were excised and weighed at end-point (day 28). Primary tumour A) growth (volume, cm^3) and B) end-stage weights (grams). Mean±SEM; *n*=15 mice/group.

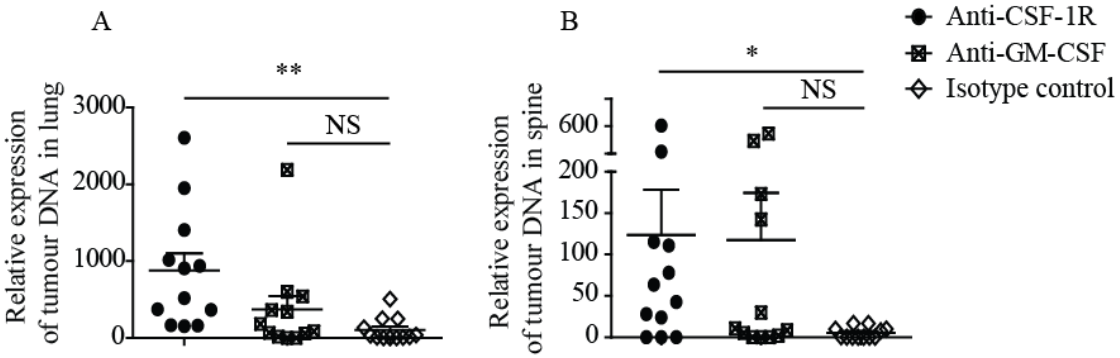


Figure 5.6: Anti-CSF-1R antibody treatment increases metastasis to lung and spine.

4T1.2 (mCherry) tumour-bearing mice were treated as described in Figure 5.5. Lungs were excised at end-point and disaggregated. Spines were excised at end-point, snap frozen and homogenised. Genomic DNA was isolated from lung and spine samples and tumour mCherry DNA measured by qPCR. A) Lung metastasis and B) spine metastasis. Data are expressed as relative expression of mCherry DNA (tumour cells only) to that of vimentin DNA (all cells). Mean±SEM; *n*=15 mice/group. **P*≤0.05. ***P*≤0.01 anti-CSF-1R vs. isotype control, NS = not significant, non-paired student's T test.

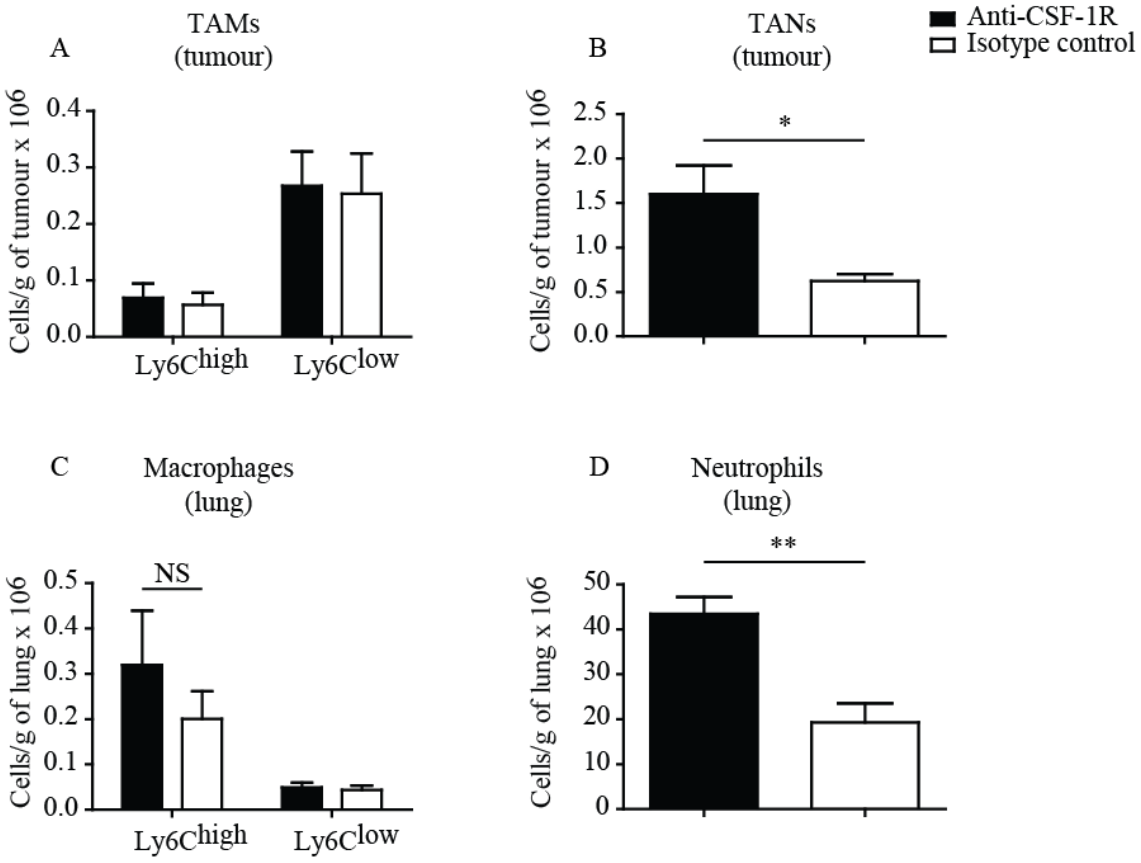


Figure 5.7: Anti-CSF-1R antibody treatment increases primary tumour TAN and metastasis-associated lung neutrophil numbers.

4T1.2 (mCherry) tumour-bearing mice were treated with 250 μg of AFS-98 or isotype control as described in Figure 5.5. Primary tumours and lungs were excised and disaggregated at end-point (day 28). TAMs/macrophages and TANs/neutrophils were analysed by flow cytometry as described in Figure 5.1. Primary tumour A) $Ly6C^{high}$ and $Ly6C^{low}$ TAM and B) TAN numbers. Lung C) $Ly6C^{high}$ and $Ly6C^{low}$ macrophage and D) neutrophil numbers. Data are expressed as cells per gram of tumour (A and B) or cells per gram of lung (C and D). Mean±SEM; *n*=15 mice/group. **P*=0.02. ***P*=0.001 anti-CSF-1R vs. isotype control, NS = not significant, non-paired student's T test.

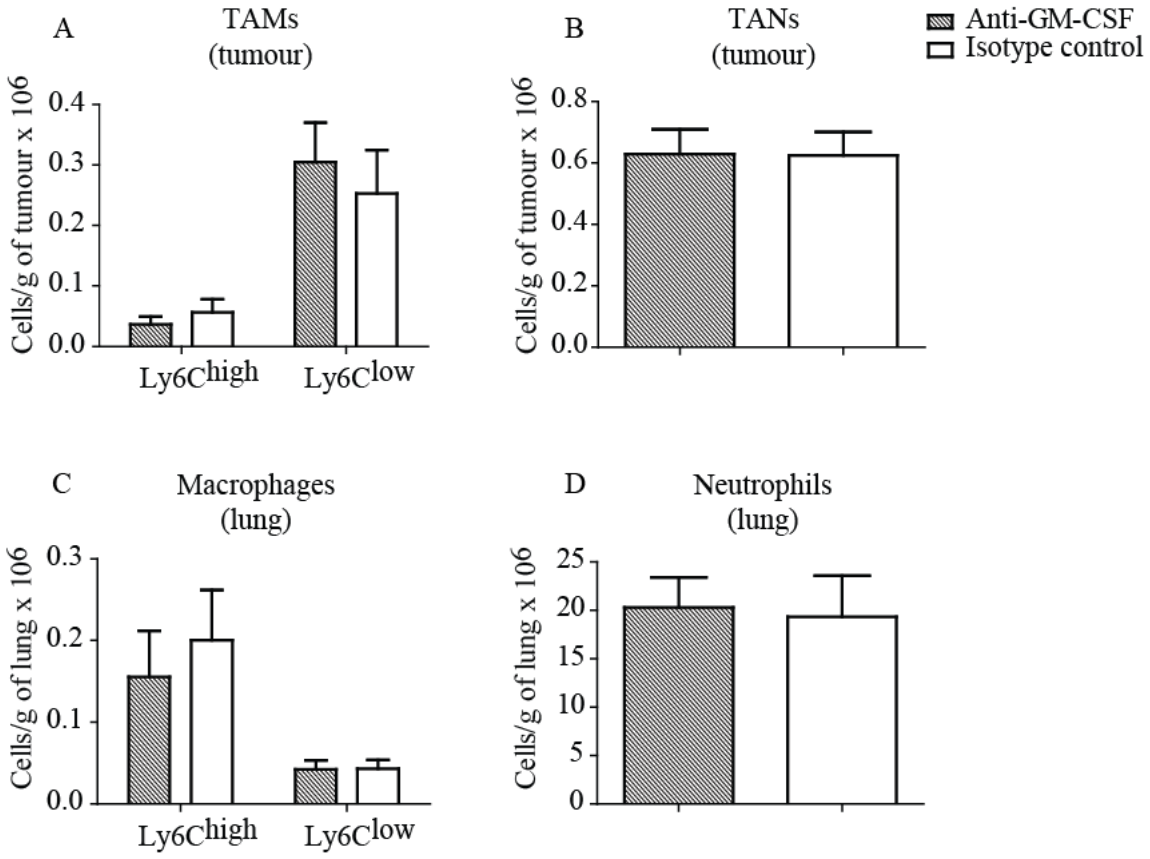


Figure 5.8: Anti-GM-CSF antibody treatment does not alter primary tumour TAM and TAN, or metastasis-associated lung neutrophil and macrophage numbers.

4T1.2 (mCherry) tumour-bearing mice were treated with 150 μg of 22E9 or 250 μg of isotype control as described in Figure 5.5. Primary tumours and lungs were excised and TAMs/macrophages and TANs/neutrophils were analysed by flow cytometry as described in Figure 5.1. Primary tumour A) $Ly6C^{high}$ and $Ly6C^{low}$ TAM and B) TAN numbers. Lung C) $Ly6C^{high}$ and $Ly6C^{low}$ macrophage and D) neutrophil numbers. Data are expressed as cells per gram of tumour (A and B) or cells per gram of lung (C and D). Mean±SEM; *n*=15 mice/group.

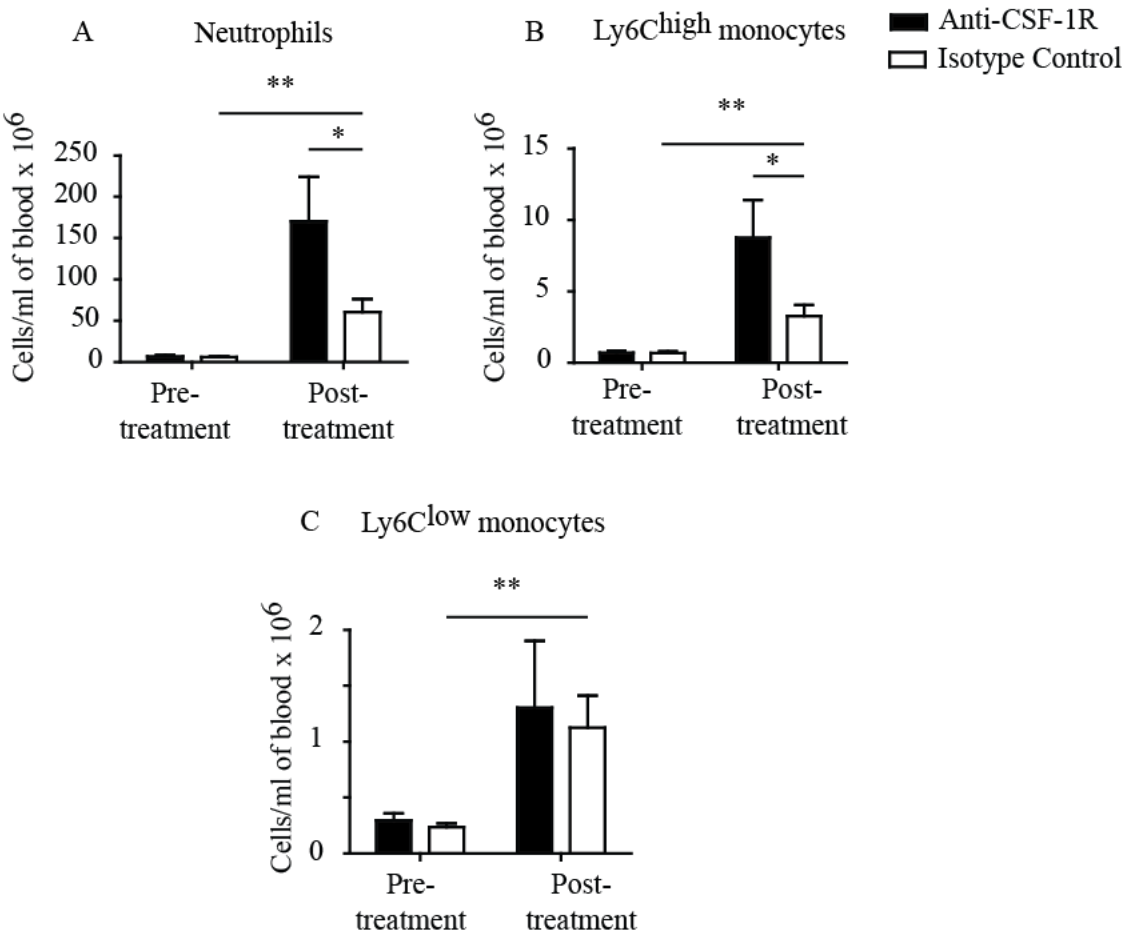


Figure 5.9: Anti-CSF-1R antibody treatment enhances tumour-driven increases of blood neutrophils and Ly6C^{high} monocytes.

4T1.2 (mCherry) tumour-bearing mice were treated with 250 µg of AFS-98 or isotype control as described in Figure 5.5. Peripheral blood was collected 24 h prior to the first and 24 h after the fourth antibody treatment (day 6 and 19 post-tumour cell inoculation, respectively). Monocyte and neutrophil numbers were analysed by flow cytometry as described in Figure 5.1. Blood A) neutrophil, B) Ly6C^{high} monocyte and C) Ly6C^{low} monocyte numbers. Data are expressed as cells per ml of blood. Mean±SEM; *n*=15 mice/group. A-C) ***P*≤0.01 isotype control pre-treatment vs. post-treatment. A and B) **P*≤0.05 anti-CSF-1R vs. isotype control, two-way repeated measures ANOVA.

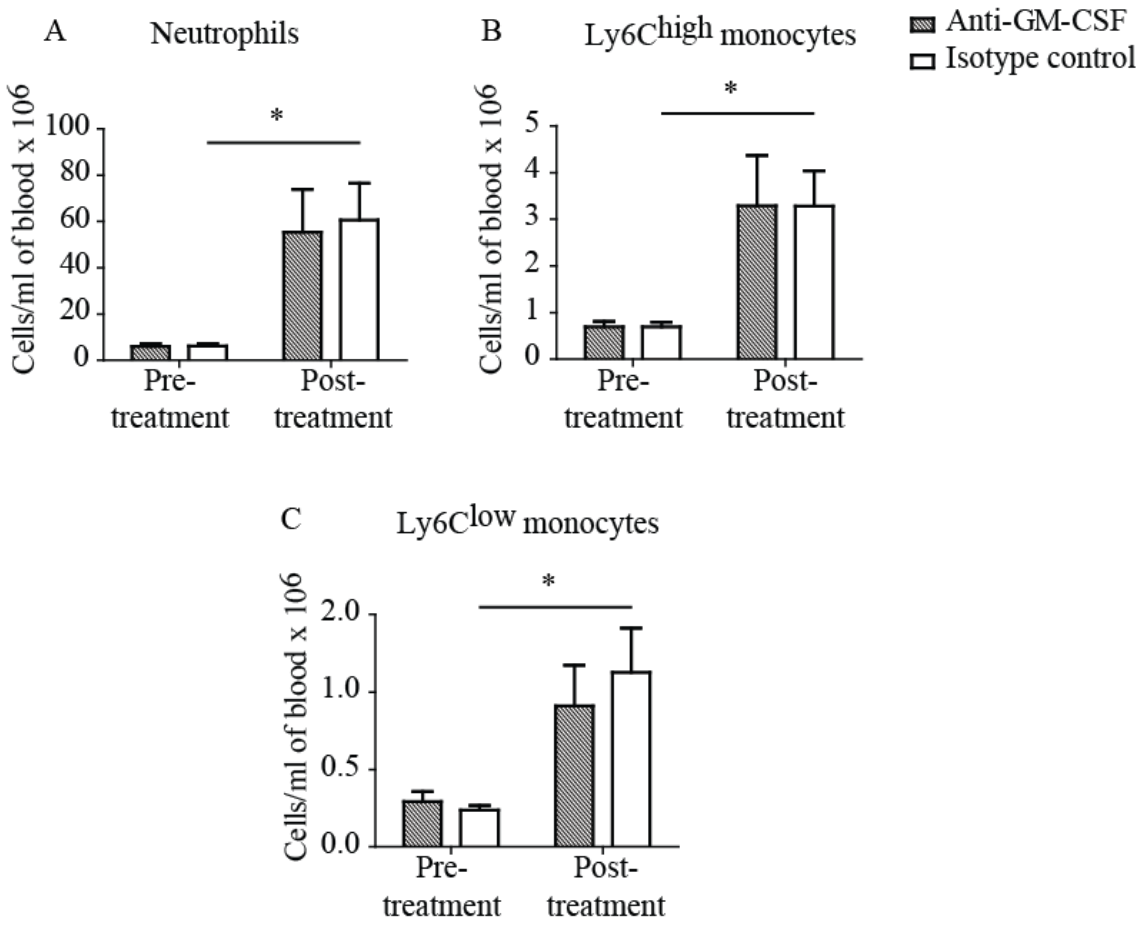


Figure 5.10: Anti-GM-CSF antibody treatment does not enhance tumour-driven increases of blood neutrophils and Ly6C^{high} monocytes.

4T1.2 (mCherry) tumour-bearing mice were treated with 150 μg of 22E9 or 250 μg of isotype control as described in Figure 5.5. Peripheral blood was collected 24 h prior to the first and 24 h after the fourth antibody treatment (day 6 and 19 post-tumour cell inoculation, respectively). Monocyte and neutrophil numbers were analysed by flow cytometry as described in Figure 5.1. Blood A) neutrophil, B) Ly6C^{high} monocyte and C) Ly6C^{low} monocyte numbers. Data are expressed as cells per ml of blood. Mean±SEM; n=15 mice/group. *$P \leq 0.01$ isotype control pre-treatment vs. post-treatment two-way repeated measures ANOVA.

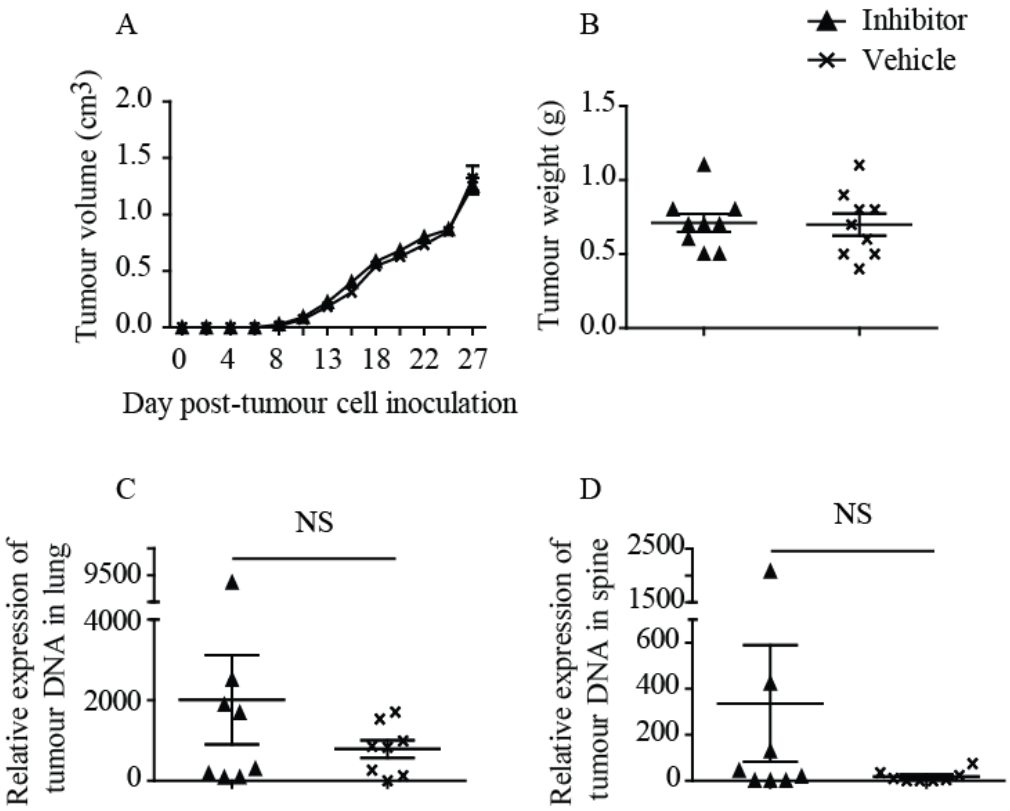


Figure 5.11: CSF-1R inhibitor treatment does not alter primary tumour growth but shows a trend towards increased metastasis.

Mice were injected orthotopically into the fourth mammary gland with 4T1.2 mCherry-expressing tumour cells and treated by oral gavage daily from day 7-21 post-tumour cell inoculation with 160 mg/kg of GW2580 or 200 µl of vehicle. Tumour growth was measured using electronic callipers and tumours were excised and weighed at end-point (day 28). Primary tumour A) growth (volume, cm^3) and B) end-point weights (grams). Lungs and spines were excised at end-point, spines were snap frozen and homogenised and lungs were disaggregated. Genomic DNA was isolated from lung and spine samples and tumour mCherry DNA measured by qPCR. C) Lung metastasis and D) spine metastasis. Data are expressed as relative expression of mCherry DNA (tumour cells only) to that of vimentin DNA (all cells). Mean±SEM; *n*=9 mice/group. NS = not significant, non-paired student's T test.

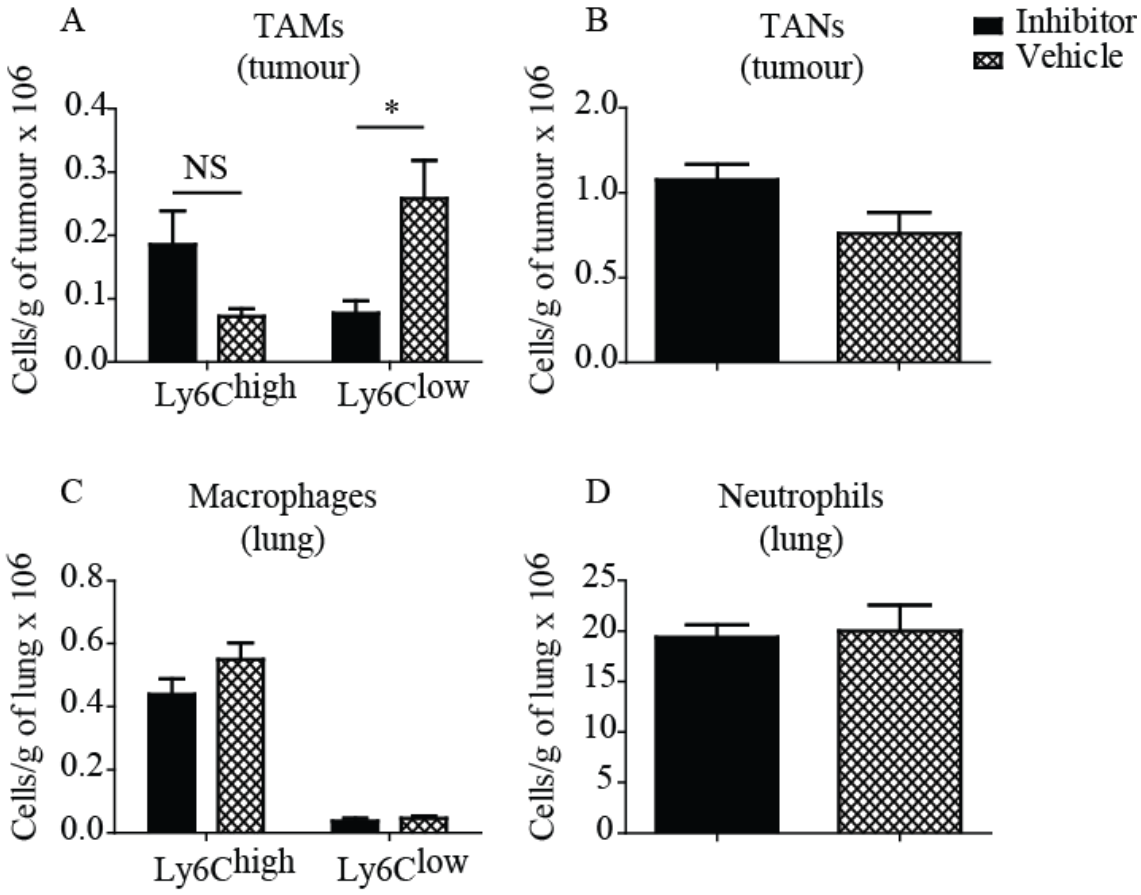


Figure 5.12: CSF-1R inhibitor treatment depletes Ly6C^{low} TAMs.

4T1.2 (mCherry) tumour-bearing mice were treated as described in Figure 5.11. Primary tumours and lungs were excised and disaggregated at end-point (day 28). TAMs/macrophages and TANs/neutrophils were analysed by flow cytometry as described in Figure 5.1. Primary tumour A) Ly6C^{high} and Ly6C^{low} TAM and B) TAN numbers. Lung C) Ly6C^{high} and Ly6C^{low} macrophage and D) neutrophil numbers. Data are expressed as cells per gram of tumour (A and B) or cells per gram of lung (C and D). Mean±SEM; *n*=9 mice/group. *$P \leq 0.05$ CSF-1R inhibitor vs. vehicle, NS = not significant, two-way repeated measures ANOVA.

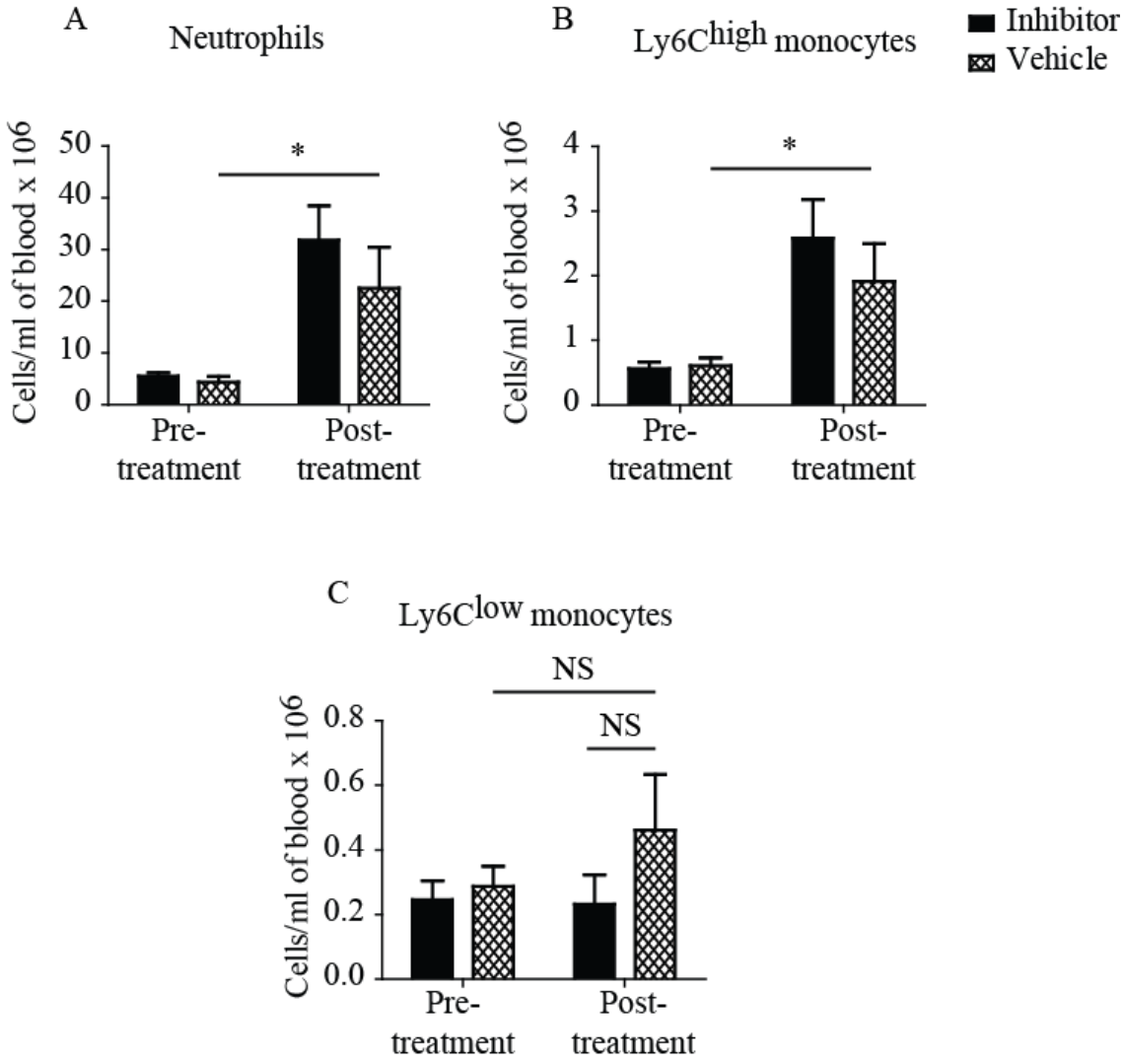


Figure 5.13: CSF-1R inhibitor treatment does not enhance tumour-driven increases of blood neutrophils and Ly6C^{high} monocytes.

4T1.2 (mCherry) tumour-bearing mice were treated as described in Figure 5.11. Peripheral blood was collected 24 h prior to the first and 24 h after the fourth inhibitor treatment (day 6 and 19 post-tumour cell inoculation, respectively). Monocyte and neutrophil numbers were analysed by flow cytometry as described in Figure 5.1. Blood A) neutrophil, B) Ly6C^{high} monocyte and C) Ly6C^{low} monocyte numbers. Data are expressed as cells per ml of blood. Mean±SEM; *n*=9 mice/group. *$P \leq 0.05$ pre-treatment vs. post-treatment, NS = not significant, two-way repeated measures ANOVA.

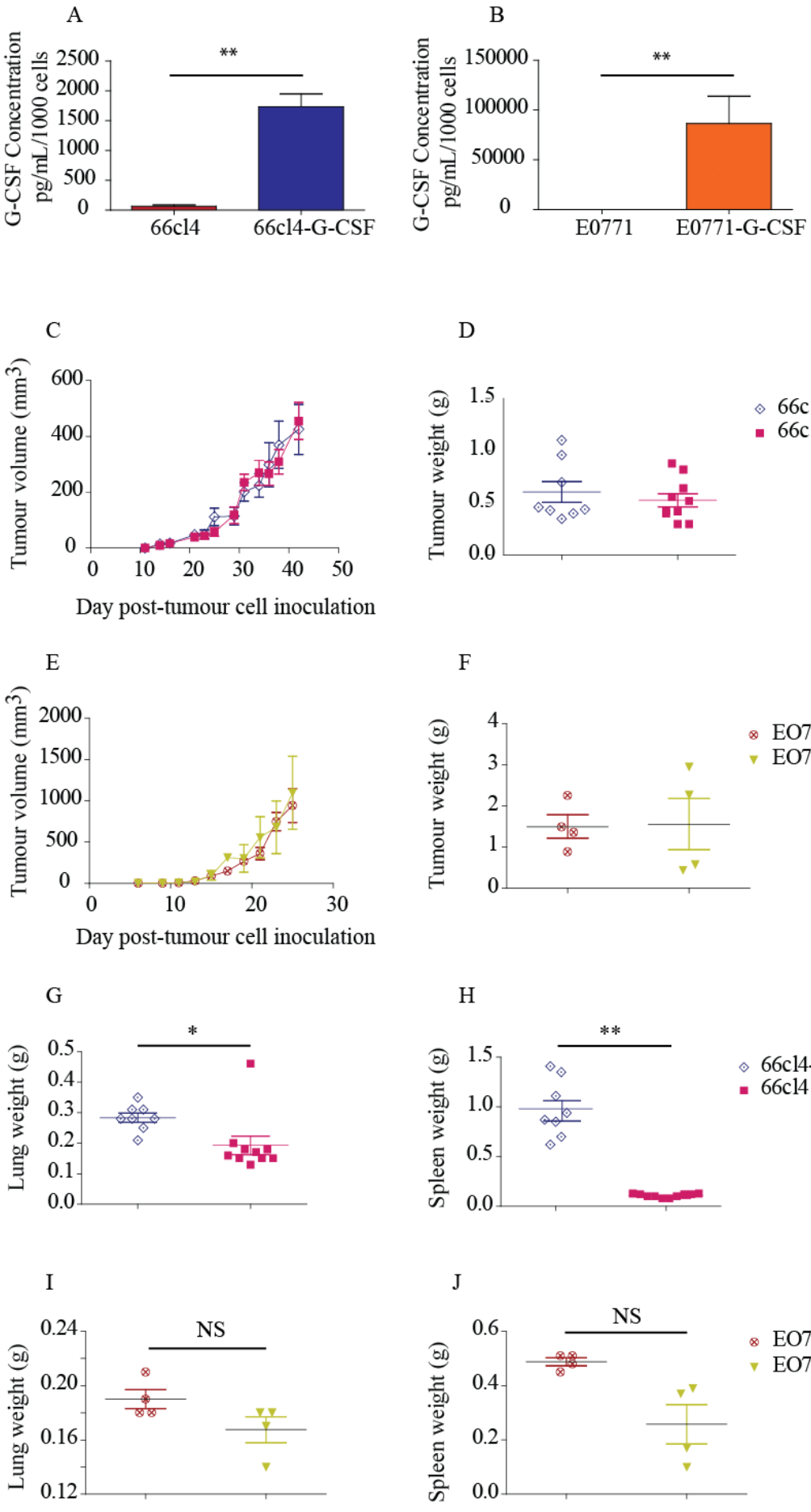

A
G-CSF Concentration pg/mL/1000 cells
2500
2000
1500
1000
500
0
**
66cl4
66cl4-G-CSF
B
G-CSF Concentration pg/mL/1000 cells
150000
100000
50000
0
**
E0771
E0771-G-CSF
C
Tumour volume (mm3)
600
400
200
0
0 10 20 30 40 50
Day post-tumour cell inoculation
D
Tumour weight (g)
1.5
1.0
0.5
0.0
66cl4-G-CSF
66cl4
E
Tumour volume (mm3)
2000
1500
1000
500
0
0 10 20 30
Day post-tumour cell inoculation
F
Tumour weight (g)
4
3
2
1
0
EO771-G-CSF
EO771
G
Lung weight (g)
0.5
0.4
0.3
0.2
0.1
0.0
*
H
Spleen weight (g)
1.5
1.0
0.5
0.0
**
66cl4-G-CSF
66cl4
I
Lung weight (g)
0.24
0.20
0.16
0.12
NS
J
Spleen weight (g)
0.6
0.4
0.2
0.0
NS
EO771-G-CSF
EO771

Figure 5.14: Enhanced expression of G-CSF in 66cl4 and E0771 tumours does not alter primary tumour growth but increases lung and spleen weights.

This experiment was completed by Dr. Yuan Cao at the Peter MacCallum Cancer Centre. 66cl4 or E0771 tumour cells engineered to express G-CSF were injected orthotopically into the fourth mammary gland of BALB/c or C57BL/6 mice, respectively. 66cl4 or E0771 tumours expressing vector controls were used for comparison. Tumour growth was measured using electronic callipers and tumours were excised and weighed at end-point. A) G-CSF levels expressed by 66cl4 ± G-CSF tumours and B) G-CSF levels expressed by E0771 ± G-CSF tumours. Primary tumour C) growth (volume, mm^3) and D) end-stage weights (grams) for 66cl4 ± G-CSF tumours. Primary tumour E) growth (volume, mm^3) and F) end-stage weights (grams) for E0771 ± G-CSF tumours. G) End-stage lung weights (grams) and H) end-stage spleen weights (grams) for 66cl4 ± G-CSF tumour-bearing mice. I) End-stage lung weights (grams) and J) end-stage spleen weights (grams) for E0771 ± G-CSF tumour-bearing mice. Mean±SEM; *n*=5 mice/group. **P*=0.02, ***P*=0.0001, NS = not significant, non-paired student's T test.

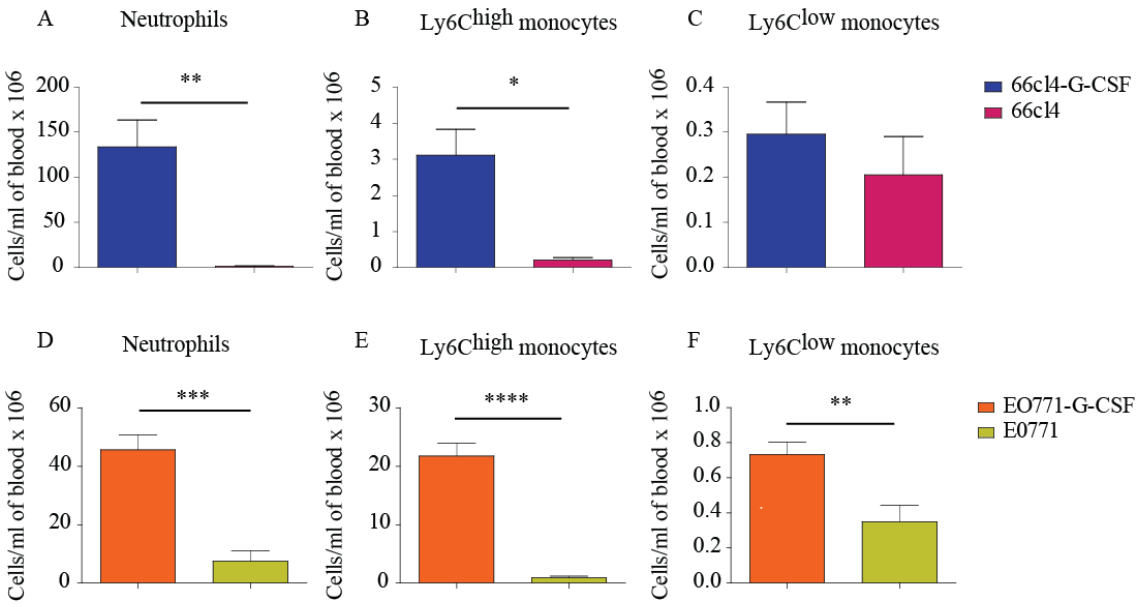


Figure 5.15: Elevated expression of G-CSF in 66cl4 and E0771 tumours increases blood neutrophils and Ly6C^{high} monocytes.

This experiment was completed in collaboration with Dr. Yuan Cao at the Peter MacCallum Cancer Centre. Dr. Cao recovered the peripheral blood at end-point from mice bearing 66cl4 or E0771±G-CSF tumours (see Figure 5.14). I analysed monocyte and neutrophil numbers by flow cytometry as described in Figure 5.1. Blood A) neutrophil, B) Ly6C^{high} and C) Ly6C^{low} monocyte numbers in 66cl4 ± G-CSF tumour-bearing mice. Blood D) neutrophil, E) Ly6C^{high} and F) Ly6C^{low} monocyte numbers in E0771 ± G-CSF tumour-bearing mice. Data are expressed as cells/ml of blood. Mean±SEM; n=5 mice/group. A) ***P*=0.02, B) **P*=0.01, D) ****P*=0.001, E) *****P*=0.0001 and F) ***P*=0.02, non-paired student's T test.

6 Discussion

My thesis has investigated how macrophages/TAMs and their precursors, monocytes, influence mammary tumour metastasis, and if targeting these cells reduces systemic spread of tumours. Furthermore, the association between neutrophils/TANs and metastasis of mammary tumours was examined. This general discussion reviews the broad findings of this project and the impact that they could have on the classification and treatment of advanced breast cancer. The challenges remaining and strategies for further investigation of the contribution of these cells to metastasis are also considered.

6.1 Highly metastatic 4T1.2 tumours enhance mobilisation of monocytes and neutrophils

The results from this thesis showed that 4T1.2 tumours increased mobilisation of monocytes and neutrophils from the bone marrow into the circulation, and this was associated with metastasis but not tumour growth and size. As discussed in Chapter 1, Section 1.6, confusion regarding the heterogeneity of MDSCs has impeded a thorough analysis of individual myeloid cell populations and subpopulations in metastatic breast tumours [206-208]. This thesis is the first body of work to include a comprehensive analysis of changes in monocyte/macrophage subpopulations and neutrophils during tumour progression.

6.1.1 Increases in TAM and TAN numbers are associated with tumour metastatic capacity

Ly6C^{low} TAMs were the predominant TAM subpopulation in tumours and their numbers increased with increasing metastatic capacity. A similar trend was seen for the less prevalent Ly6C^{high} TAM subpopulation (Figure 3.3A). Other studies have also reported Ly6C^{low} TAMs to be the predominant subpopulation in mammary tumour models [202, 225]. However, increased Ly6C^{low}, and possibly Ly6C^{high}, TAM numbers with increasing metastatic capacity has not been described previously.

The Ly6C^{low} subpopulation is often considered to be the more mature monocyte/macrophage population and it is thought that Ly6C^{high} monocytes convert into Ly6C^{low} macrophages as they infiltrate tissues and mature [304]. Ly6C^{high} monocytes have been reported to be the precursors for TAMs in murine mammary TS/A tumours [225]. Conversely, in studies of MMTV-PyMT tumours, Ly6C^{low} monocytes were preferentially recruited [202]. Hence, it is unclear whether Ly6C^{high} monocytes convert into Ly6C^{low} cells within tumours, or if they do so in the blood, and then migrate into 4T1.2 tumours. Since, in this thesis Ly6C^{high} TAMs were seen in primary tumours (Figure 3.3A), it is possible that the former mechanism takes place. To clarify this, tagged Ly6C^{high} monocytes could be adoptively transferred into mice with established tumours. The presence of tagged Ly6C^{high} TAMs in primary tumours would indicate that conversion from Ly6C^{high} to Ly6C^{low} occurs there. However, Ly6C^{low} monocytes were also observed in the blood of tumour-bearing mice (Figure 3.3B) and it is possible that Ly6C^{high} and Ly6C^{low} monocytes are recruited independently of each other. Intravital confocal microscopy imaging in real time could be used to determine the mechanism of monocyte recruitment to primary tumours. This technique was used successfully to track monocytes in the circulation [313], and also to track TAM and tumour cell co-migration within primary tumours [261, 506].

Both Ly6C^{low} and Ly6C^{high} TAM numbers were increased in primary tumours around the time that visible lung tumour nodules were first detected in 4T1.2 tumour-bearing mice (Figures 3.5A and 3.5B). It is therefore possible that Ly6C^{low} TAMs, as well as Ly6C^{high} TAMs (even though they are less numerous), may be involved early in tumour progression to metastasis.

TANs were the predominant myeloid cell population found in highly metastatic 4T1.2 tumours, and were also increased in number around the time of visible lung metastatic nodule detection (Figures 3.1A and 3.5C). Thus TANs were also associated with metastatic capacity in the 4T1.2 mammary tumour model, and may be involved early in metastasis. Increased TAN numbers have been associated with poor prognosis in renal cell carcinoma [361], bronchioloalveolar and gastric carcinomas [6, 349], and melanoma [363], but this has not yet been shown for breast cancer. To the best of my knowledge this thesis is the first report of increased TAN numbers associating with metastasis in an animal model of breast cancer.

Increased neutrophil numbers have recently been proposed to be associated with an anti-metastatic response in the 4T1 model (the parent cell line of 4T1.2) [201]. However, this accumulation of neutrophils occurred in the lungs, rather than primary tumour [201], and hence increased numbers of TANs in the primary tumour may still indicate poor prognosis in mammary tumour models. Analysis of TAN numbers in other mammary tumour models will confirm this.

A pilot study in this thesis indicated that TAMs and TANs isolated from highly metastatic 4T1.2 tumours can increase growth of weakly metastatic 66cl4 tumours, whereas TAMs and TANs from non-metastatic 67NR tumours cannot (Figure 3.9). It has been reported previously by another group that Gr-1^{+}CD11b^{+} cells isolated from tumours and spleens of 4T1 tumour-bearing mice increased metastasis to lung when co-injected with 4T1 tumour cells, whereas 4T1 tumour cells co-injected with Gr-1^{+}CD11b^{+} cells from normal spleens did not [185]. However, Gr-1^{+} cells can be macrophages/TAMs or neutrophils/TANs. Given the data from this study, and the pilot study from this thesis showing that TAMs and TANs may affect tumour growth, it is possible that either TAMs and/or TANs can also influence metastasis. Analysis of the effect of co-injecting TAMs and TANs from highly metastatic 4T1.2 tumours with weakly metastatic 66cl4 tumour cells on 66cl4 metastasis will determine whether or not TAMs and TANs can regulate metastasis. If TAMs are found to influence tumour metastatic capacity, it would also be of interest to investigate if Ly6C^{high} and/or Ly6C^{low} TAMs differ in their capacity to do so.

Furthermore, it appears that TAMs and TANs 'educated' by highly metastatic primary tumours retain this phenotype upon transfer into another microenvironment. It would be of interest to ascertain if there are phenotypic differences between TAMs and TANs from 4T1.2 tumours co-injected with less metastatic 66cl4 tumour cells (once the tumour is established) compared to TAMs and TANs isolated from un-manipulated 66cl4 tumours.

6.1.2 Increases in systemic monocyte/macrophage and neutrophil numbers are associated with metastasis

In contrast to primary tumours, Ly6C^{high} monocytes were found to be the predominant monocyte subpopulation in the circulation. Highly metastatic 4T1.2 tumours markedly enhanced circulating Ly6C^{high} monocytes and neutrophils and, to a lesser extent, Ly6C^{low} monocytes, whereas non-metastatic 67NR tumours did not (Figure 4.1). Weakly metastatic 66cl4 tumours somewhat enhanced Ly6C^{high} monocyte mobilisation at end-stages of tumour progression (Figure 4.1) As seen for TAM and TAN numbers, increases in blood monocytes and neutrophils in 4T1.2 tumour-bearing mice occurred around the time that visible lung nodules were first detected (Figures 4.2A and 4.2C). Splenic neutrophils were also found to increase at this time (Figure 4.4C). Additionally, bone marrow Ly6C^{high} monocyte and neutrophil numbers increased prior to the appearance of visible lung tumour nodules (Figure 4.3).

Ly6C^{high} monocytes have been reported to be recruited to pre-metastatic lungs in the MMTV-PyMT model [202]. However, preliminary experiments in this thesis have shown that neutrophils, rather than macrophages, increase in 4T1.2 metastasis-associated lungs (Figures 4.7 and 4.8). This is in line with a recent study using the 4T1 model showing that neutrophils, rather than macrophages, were important in metastasis to lung [8]. However, as neutrophils are typically the first cells to arrive at sites of inflammation and only early time points were analysed in this thesis, it is possible that macrophages may increase later in the lung and still contribute to metastatic tumour growth.

Both blood monocytes and lung macrophages have been found adjacent to tumour cells in the lung, and facilitated tumour cell extravasation and growth in models of Lewis lung carcinoma and melanoma [92]. Neutrophils increased melanoma cell arrest and extravasation through a fibroblast monolayer in an *in vitro* flow extravasation assay [422]. It would be of interest to determine if either Ly6C^{high} or Ly6C^{low} monocytes, as well as neutrophils, are found in close proximity to 4T1.2 tumour cells in the circulation or in metastasis-associated lungs, as they may also facilitate tumour cell extravasation and growth in this model.

Little is currently known about the factors that are involved in the mobilisation of myeloid cells by tumours. Studies from this thesis revealed that pro-MMP-9 was increased in serum from mice bearing metastatic 4T1.2 tumours around the time of monocyte and neutrophil mobilisation (Table 4.1 and Figure 4.9A). MMP-9 is important in the reconstitution of bone marrow progenitor cells after bone marrow suppression [468] and it has been proposed that, after G-CSF stimulation, MMP-9 is activated and degrades CXCL12 in the bone marrow resulting in a gradient that causes egress of cells into the circulation [455]. It is possible that there is low level MMP-9 activity in the blood, or that MMP-9 is present primarily in the inactive zymogen form, pro-MMP-9, and upon G-CSF stimulation, MMP-9 activity is increased predominantly in the bone marrow. Elevated levels of pro-MMP-9 have been reported in serum of BALB/c-neuT mice compared to serum from normal BALB/c mice and in this same study it was suggested that tumour-derived MMP-9 may be one of the factors involved in mobilisation of Gr-1^+ cells from the bone marrow in tumour-bearing mice [451].

Based on the studies discussed above, and the data in Chapters 3 and 4, a model is proposed in which metastatic breast tumours generate an inflammatory reaction that mobilises neutrophils and Ly6C^{high} monocytes from the bone marrow. This occurs, at least in part, via degradation of CXCL12 by MMP-9 activity triggered by the presence of an aggressive tumour. Ly6C^{high} monocytes then either convert to Ly6C^{low} cells in the blood or in the primary tumour, ultimately amplifying TAN and Ly6C^{low} TAM numbers in primary that then promote tumour cell homing to distant tissues such as the lung. Increases in circulating neutrophils and Ly6C^{high} monocytes may also facilitate tumour cell migration through the circulation, and arrest, extravasation and growth at secondary sites (Figure 6.1).

In the c-erbB-2 (HER-2/neu) mammary tumour model, diminished MMP-9 expression was shown to reduce Gr-1^+ cell expansion in the bone marrow and peripheral blood [507]. In the 4T1.2 model, MMP-9 is secreted primarily from stromal cells in tumours, and no compensatory increase in MMP-9 expression is seen primary tumour cells grown in MMP-9 deficient mice, despite 4T1.2 cells in culture having robust MMP-9 expression (Pouliot and Anderson, unpublished data). Analysis of monocyte and neutrophil numbers in bone marrow and blood of MMP-9 deficient mice bearing 4T1.2 tumours could be carried out to determine if MMP-9 is involved in mobilisation of cells

from the bone marrow during 4T1.2 tumour progression. If MMP-9 is important for mobilisation of monocytes and neutrophils from the bone marrow in the 4T1.2 model, the absence of MMP-9 should result in a reduction in monocyte and neutrophil numbers compared to wild type controls.

6.2 Monocytes/macrophages as therapeutic targets

6.2.1 Clodronate treatment depletes blood Ly6C^{low} monocytes and reduces metastasis to lung

Treatment with clodronate liposomes depleted Ly6C^{low} monocytes in peripheral blood and this was associated with reduced metastasis to lung and possibly spine as well (Figures 5.1A and 5.3). End-stage TAM numbers were not reduced after clodronate treatment (Figure 5.4). This may be due to cell re-population, as the last clodronate treatment was given seven days prior to this measurement [500]. However, given that Ly6C^{high} blood monocytes were not affected (Figure 5.1B), it is also possible that they were contributing to TAM numbers in the primary tumour [304, 508]. Toxic depletion of both Ly6C^{high} and Ly6C^{low} blood monocytes may have a more profound effect on metastasis.

6.2.2 CSF-1R neutralisation enhances metastasis to lung and bone and increases neutrophil/TAN numbers

Previous studies have reported that CSF-1R/CSF-1 blockade reduces primary tumour growth and metastasis either alone [199, 292-294], or in combination with chemotherapy [136]. CSF-1 signalling is therefore considered a potential target for adjuvant therapy of breast cancer and clinical trials are underway [135]. In contrast, it is reported in this thesis that treatment with a neutralising CSF-1R antibody (AFS-98), while not affecting primary tumour growth, actually enhanced metastasis of breast carcinoma to both lung and spine (Figure 5.6). This was associated with an increase in TANs and metastasis-associated lung neutrophils, but not with a reduction in TAMs or lung macrophages (Figures 5.7). Treatment with a small molecule inhibitor of CSF-1R also showed a trend towards increased metastasis to lung and spine (Figures 5.11C and 5.11D).

The data in Chapter 4 indicate that tumour-driven increases in systemic neutrophils and Ly6C^{high} monocytes seen without treatment may be actively promoting metastasis. Treatment with the anti-CSF-1R antibody further enhanced these tumour-driven increases in circulating neutrophils and Ly6C^{high} monocytes (Figures 5.9A and 5.9B), and this may be contributing to the enhanced metastasis seen upon CSF-1R blockade. Along these lines, it has been suggested that neutrophils can acquire a 'metastatic phenotype' under certain conditions [411]. G-CSF mobilised Ly6G^{+}Ly6C^{+} granulocytes enhanced 4T1 metastasis to lung [8] and Gr-1^{+} myeloid cells (which contain granulocytic lineage cells) can tip the balance to tumour promotion in the pre-metastatic lung [3]. However, the role of neutrophils in metastasis in murine breast cancer models is controversial with an anti-metastatic activity of lung neutrophils recently being proposed [201].

It has been shown that a unique population of cytotoxic neutrophils, termed 'tumour entrained neutrophils' or TENs, were induced by the primary tumour to accumulate in the lungs early in 4T1 tumour metastasis [201]. Depletion of neutrophils by treatment with anti-Ly6G antibody increased the number of metastatic events in the lung indicating that TENs were limiting metastatic seeding [201]. However, neutrophils were only successfully depleted until day 14 of treatment, thus a possible rebound effect resulting in the generation of cytotoxic neutrophils cannot be completely ruled out. Furthermore, metastatic growth eventually occurred indicating that TENs were insufficient to completely inhibit metastasis [201]. The primary tumour appeared to be protected from TEN-mediated cytotoxicity, possibly through the activity of TGFβ, which can suppress anti-tumour neutrophil responses [201, 412]. It was suggested by the authors that TENs were only effective in suppressing micrometastases, where TGFβ levels were low, whereas in large metastases and primary tumours, increased TGFβ levels overcame the anti-metastatic properties of these cells [201]. Hence, neutrophils may play a dual role in metastasis, and, like macrophages, their contribution to metastatic growth is dependent on the complex milieu generated by primary tumours and metastatic nodules.

Further investigation of the contribution of neutrophils to 4T1.2 metastasis, and how primary tumours and metastatic nodules can affect the phenotype of these cells is required. Since Ly6C^{high} blood monocytes facilitate pulmonary metastasis in the

MMTV-PyMT transgenic mouse [202], it is possible that this major monocyte subpopulation may also contribute to the increased metastasis seen in the 4T1.2 model, given that a trend towards increased numbers with increasing metastatic capacity was seen and their numbers increased early in tumour progression (Figures 3.3B and 4.2A). Further investigation of these cells in metastasis is also required.

Treatment with the small molecule inhibitor of CSF-1R did not further enhance tumour-driven neutrophil and monocyte mobilisation (Figures 5.13A and 5.13B), and this may explain why only a trend towards increased metastasis was seen after such treatment. The differences seen between CSF-1R neutralising antibody treatment and the small molecule CSF-1R inhibitor may be due to differences in the mechanisms by which they inhibit CSF-1R signalling [509], or due to the extent to which they block signalling through CSF-1R.

Previous research completed in the Arthritis and Inflammation Research Centre, along with other studies, has reported that neutralisation with AFS-98 preferentially reduced the Ly6C^{low} subpopulation in blood and tissue in the steady state [224, 502] and during acute inflammation [224]. These findings are consistent with the notion that CSF-1 (and/or IL-34) acts later in macrophage lineage development [224, 502]. In these studies, an increase in Ly6C^{high} blood monocytes after neutralisation with AFS-98 was not observed [224]; although it has been reported by another group that after administration of a different anti-CSF-1R antibody (M279) in the steady state, an increase in Ly6C^{high} blood monocytes was seen [502].

The reason for the increased neutrophil numbers following treatment with the anti-CSF-1R antibody is unknown, although compensatory increases in neutrophils during infection in *Csf1*^{op}*/Csf1*^{op} mice have been noted [510], and increased granulocyte numbers have also been reported with M279 treatment during inflammation [502]. Even though a reduction in TAMs was not seen, it is possible that their function is altered such that their influence on neutrophil trafficking in or out of the tumour has changed, or perhaps their ability to clear the neutrophils by phagocytosis is compromised [511].

Disposal of apoptotic neutrophils is an important step in resolution of inflammation [512] and macrophages are thought to influence neutrophil accumulation during

inflammation through phagocytosis of apoptotic neutrophils [511] and may even induce neutrophil apoptosis directly [513]. Blockade of monocytes/macrophages delays clearance of apoptotic neutrophils during inflammation [140]. Tumours have been described as 'wounds that do not heal'[514, 515] and TAMs (or at least some TAM subpopulations) are 'trophic'-like in nature [257]. Thus it is possible that TAMs are attempting to resolve tumour-mediated inflammation and are inhibiting TANs. Thus, depletion of TAMs may abrogate inhibition of neutrophil recruitment to tumours, resulting in increased numbers of TANs that are influenced by the tumour microenvironment to promote tumour growth and progression. In support of this, compensatory increases in TANs capable of promoting tumour development have been reported after TAM reduction in a model of ovarian cancer [426]. However, it is also possible that there is enhanced mobilisation of the neutrophils from the bone marrow. It was shown in Chapter 5 that increased expression of the key neutrophil chemoattractant, G-CSF, in tumours enhanced blood neutrophil (and Ly6C^{high} monocyte numbers) and promoted metastasis (Figures 5.15 and 5.14G-J, respectively), providing further evidence that mobilisation of these cell populations can enhance metastasis.

Previous studies inhibiting CSF-1/CSF-1R signalling in breast tumour models found a reduction in TAMs [136, 293, 294, 435]. It is not clear why significant reductions in TAMs or blood Ly6C^{low} monocytes were not found in the 4T1.2 model with anti-CSF-1R antibody treatment. This was unexpected as a similar protocol was used to that in the previous study from our research group where a significant reduction in Ly6C^{low} monocytes was observed within 4 days of a single injection of AFS-98 [224]. It is possible that a treatment regime commencing earlier in disease progression may result in reduced TAMs, metastasis and primary tumour growth. However, this study aimed to target metastasis using a protocol that reflects the limited number of options available for treatment of advanced disease. Beginning treatments earlier than day 7 would not have allowed delineation between possible secondary effects on metastasis due to disrupted primary tumour development.

Treatment with the small molecule inhibitor of CSF-1R, on the other hand, did deplete Ly6C^{low} TAMs, with a trend towards reduced circulating Ly6C^{low} monocytes also observed (Figures 5.12A and 5.13C). This is similar to what was seen in a lung carcinoma model where treatment with the same inhibitor reduced TAMs, and possibly

blood monocytes [435]. Given that clodronate-mediated depletion of Ly6C^{low} blood monocytes was associated with reduced metastasis to lung, it is unclear why CSF-1R inhibitor treatment did not reduce metastasis, especially given this reduction in Ly6C^{low} TAMs and blood monocytes. Decreased CD4^{+} T cell proliferation [496] and reduced APC numbers have also been associated with clodronate treatment [478, 497]. Thus it may be that the depletion of Ly6C^{low} blood monocytes by clodronate did not affect metastasis, but rather, reduced metastasis occurred due to changes in T cells and APCs. This may also explain why reduced metastasis was not seen after CSF-1R inhibitor-mediated reduction of Ly6C^{low} monocytes/TAMs.

In a previous study using the isogenic, weakly metastatic 66cl4 tumour cell line variant, stress-induced metastasis was suppressed by CSF-1R inhibitor treatment and was associated with a decrease in TAM numbers [516]. It is possible that the 4T1.2 model is more dependent on neutrophils for tumour progression to metastasis, and hence depletion of TAMs may not inhibit this process. However, it was reported in Chapter 5 that elevated expression of the neutrophil factor, G-CSF, in 66cl4 tumours enhanced blood neutrophil numbers and metastasis to lung (Figures 5.15A, 5.14G and 5.14H, respectively), indicating that neutrophils can also enhance metastasis in this isogenic tumour variant.

These data raise a cautionary note for the use of anti-CSF-1/CSF-1R therapy, even in an adjuvant setting, for patients at high risk of extensive metastasis. It may be prudent to attempt to stratify patients based on the properties and features of the primary lesions [136], such as myeloid cell content or aggressiveness, before undertaking CSF-1/CSF-1R therapy. Importantly these studies provide further evidence that neutrophils may be active participants in breast tumour metastasis.

6.3 Future research questions

6.3.1 Analysis of TAMs and TANs and systemic monocytes/macrophages and neutrophils in breast cancer metastasis may help to tailor treatments for advanced breast tumours

Inflammatory cells have been implicated in metastasis of breast tumours [152], and it has been suggested that analysis of inflammatory components in the tumour microenvironment may aid in better classification of aggressive breast tumours [46-48]. The studies from this thesis have shown that increased numbers of circulating 'classical' Ly6C^{high} monocytes and neutrophils associated with 4T1.2 tumour metastasis. Human monocytes have subpopulations corresponding to those seen in mice [298] and increases in circulating monocytes, in particular the 'non-classical' CD16^{+} subset , have been reported in breast cancer patients [326, 327]. At present, limited analyses are available for predicting the likelihood of metastasis; therefore it would be of value to determine if increases in circulating monocyte subpopulation numbers, as well as neutrophil numbers, associate with metastasis in breast cancer. This approach, in conjunction with current methods that include tumour size, grade, lymph node biopsies, hormone and HER2 receptor expression, may aid in diagnosing the tumours that are likely to and/or have metastasised.

Macrophages are influenced by the local environment and display a continuum of phenotypes of which the 'inflammatory' M1-like and 'trophic' M2-like macrophages are the extremes [437]. Recent studies have shown that TAM subpopulations differ in their gene expression profiles. For example, immature TAMs, most likely representing Ly6C^{high} TAMs, express pro-inflammatory genes, whereas mature TAMs, most likely representing Ly6C^{low} TAMs, express trophic genes [225, 257, 264]. Preliminary data in this thesis support this finding in that Ly6C^{high} TAMs appeared to express a more pro-inflammatory gene profile (Figure 3.7). However, these data need to be confirmed with analysis of further biological replicates. Furthermore, the studies described above reported that TAM subpopulation phenotypes appear to associate with the region of the tumour in which these subpopulations reside [225, 261, 262]. MHC II^{high} TAMs expressed genes associated with inflammation in TS/A tumours. Conversely, MHC II^{low}

TAMs expressed genes associated with wound healing (M2), displayed a higher pro-angiogenic activity and were found to reside preferentially in hypoxic regions of the tumour [225]. However, these MHC II^{high} and MHC II^{low} subpopulations were also Ly6C^{low}. Moreover, it has recently been shown that Ly6C^{high} cells do not express MHC II [226]. It is therefore possible that the Ly6C^{high} and Ly6C^{low} subpopulations themselves are not associated with a specific phenotype but are influenced by the region of the tumour in which they are found, regardless of their Ly6C status. It would important to determine whether the Ly6C^{high} and Ly6C^{low} subpopulations preferentially reside in different tumour regions and if this relates to any functional differences, or whether they are found in the same tumour regions and display a similar phenotype based on the microenvironmental influences of the particular region they reside in.

Classification of M1-like and M2-like phenotypes via gene expression is not an ideal approach as macrophages display a phenotypic spectrum, and some of the 'classical' M1 and M2 genes do not cluster with M1-like or M2-like macrophages [517]. Rather than attempt to characterise Ly6C^{high} and Ly6C^{low} TAMs as M1- or M2-like, it would be better to identify genetic differences that may infer functional differences using these phenotypic extremes as a reference point.

Recently, a 'metastatic gene set' was identified in TAMs isolated from late stage MMTV-PyMT mammary tumours comprising of ARG1, VEGFA, CXCL4, CCL2, APOE, ADAM8, SCL16A3, CATHB, APOC, STAB1, ECM1, WNT7B and MMP-12 [257, 262]. It was somewhat surprising that MMP-9 was not identified in this 'metastatic gene set' as it has been implicated in breast/mammary tumour metastasis in a number of studies [92, 203, 204, 518, 519]. Analysis of gene and protein expression of the 'metastatic set' and of 'pro-inflammatory' mediators such as iNOS, TNFα, and IL-1, as well as 'trophic' factors, including IL-4, EGF and MMP-9, would provide a good foundation for further phenotypic analysis.

Given that both Ly6C^{low} and Ly6C^{high} TAM numbers increase early in disease progression, it would also be of interest to assess gene expression profiles of Ly6C^{high} and Ly6C^{low} TAMs earlier in disease progression. Such an analysis would allow us to assess if TAM phenotypes change over the course of disease progression, and if gene expression may be differentially regulated during metastatic progression.

TAN numbers were also found to associate with metastasis in the 4T1.2 mammary tumour model. Given that few studies have assessed the role of neutrophils in breast tumour metastasis, inhibition of TANs by anti-G-CSF, anti-G-CSF-1R and/or anti-Ly6G antibody treatment, not only in the 4T1.2 model but also in other mammary tumour models, is required to confirm that these cells are necessary for mammary tumour metastasis. Furthermore, analysis of human breast tumours is required to determine if TAN numbers associate with prognosis in human disease. It has been proposed that neutrophils, like macrophages, display a phenotypic spectrum [411, 520]. Similar studies investigating TAN gene expression in tumours of varying metastatic capacity, as described above for TAMs, may prove to be informative.

Primary tumours have recently been shown to influence macrophages at distant sites [521, 522] and tumour cell gene expression profiles have been shown to differ between primary tumours and metastases [65, 66]. Therefore, macrophages and neutrophils at metastatic sites may express different genetic profiles to TAMs and TANs in primary tumours. Thus, it would be beneficial to investigate gene expression in macrophages and neutrophils at metastatic sites, since treatment targets arising from gene profiling of TAMs and TANs in primary tumours may be different to those found in metastases and may not necessarily inhibit metastatic tumour growth.

6.3.2 Targeting monocytes and neutrophils in advanced breast cancer

Depletion of Ly6C^{low} monocytes by clodronate was associated with reduced metastasis (Figures 5.1A and 5.3), although Ly6C^{high} monocytes were unaffected by this particular treatment regime (Figure 5.1B). It would therefore be of interest to determine if specific depletion of Ly6C^{high} monocytes affects metastasis. In experimental models of breast tumour metastasis, enhanced expression of CCL2 in tumour cells increased metastatic growth in both lungs and bone [477] and inhibition of CCL2 reduced Ly6C^{high} monocyte recruitment to metastasis-associated lungs [202]. Depletion of CCR2 by RNA interference in mice bearing lymphomas resulted in reduced primary tumour growth and reduction of Ly6C^{high}, but not Ly6C^{low} TAMs. The effects of this therapy on metastasis were not assessed, nor were changes in blood or lung monocyte numbers investigated [523]. It would therefore be valuable to determine if anti-CCL2 and/or anti-CCR2

depletion of Ly6C^{high} monocytes and/or TAMs in the 4T1.2 mammary tumour model can impact on metastasis. However, given that CCL2 is also a chemoattractant for memory T lymphocytes and NK cells [524], and depletion of these cells may also affect metastasis, any anti-CCL2/CCR2 effects on T cells and NK cells would need to be ascertained. Furthermore, determining how depletion of both Ly6C^{high} and Ly6C^{low} subpopulations affects metastasis compared to depletion of either of these populations alone may yield more insight into their contributions to metastasis.

In this thesis, blockade of CSF-1R signalling was shown to enhance metastasis, and this was associated with increases not only in TAN (Figure 5.7B), but also in lung (Figure 5.7D) and blood neutrophil (Figure 5.9A) numbers. This is contrary to what has been reported in other studies where CSF-1/CSF-1R signalling has been inhibited [292-294]. However, in a recent publication by Denardo et al., treatment of mice bearing MMTV-PyMT mammary tumours with a small molecule inhibitor of CSF-1R signalling, or with an antibody targeting CSF-1, did not alter either primary tumour growth or metastasis to lung. Only when CSF-1/CSF-1R blockade was employed in conjunction with chemotherapy was a reduction in metastasis seen [136]. 4T1.2 tumours are more aggressive than the MMTV-PyMT tumours, showing extensive metastasis to several sites [432] and thus may respond differently to such treatment. Nonetheless it is necessary to determine if CSF-1R neutralisation can lead to increased metastasis and increased primary tumour TAN, and systemic neutrophil and Ly6C^{high} monocyte/macrophage numbers in other tumour models. It would be of interest to investigate if depletion of neutrophils can reverse the increase in metastasis seen upon CSF-1R blockade. Furthermore, it would be of value to determine if an inverse relationship exists between CSF-1/CSF-1R expression and TAN numbers in aggressive human breast tumours as it may be necessary to target both macrophages and neutrophils in order to successfully treat advanced breast cancer.

The acute loss of monocytes and tissue macrophages seen upon treatment with AFS-98 may reflect either direct antibody toxicity or clearance of monocytes/macrophages [509]. AFS-98 is a rat IgG2a which binds the MHC class I-related receptor, FcRn with high affinity [525] and this may result in destruction of cells via antibody mediated cytotoxicity [509]. The M279 antibody, on the other hand, is a rat IgG1 which binds poorly to FcRn and hence does not stimulate this kind of cytotoxicity [525]. To confirm

that the increase in metastasis seen upon treatment with AFS-98 is due to CSF-1R blockade, rather than any toxic effects on monocytes/macrophages, the same experiments would need to be repeated with M279 (or a similar monoclonal antibody), or with an anti-CSF-1 monoclonal antibody.

6.3.3 Targeting the cross talk between TAMs, TANs and other cancer-associated immune cells

The tumour microenvironment comprises a number of tumour-associated immune cells that create a signalling network capable of modifying immune responses towards the tumour. For example, the cross talk between NK or NKT cells and TAMs is important in mediating NK and NKT cell anti-tumour responses [526, 527]. In a model of neuroblastoma, TAMs in hypoxic tumour regions could suppress NKT cell activity [526]. Expression of IL-15 in NKT cells abrogated this TAM-mediated suppression, enhanced NKT cell destruction of TAMs and, as a result, reduced metastasis [526]. In another study, macrophages activated by Poly:IC, which is likely to induce an M1-like phenotype, enhanced NK cell anti-tumour cytotoxicity but were themselves protected from NK cell-mediated destruction [527]. This anti-tumour cytotoxicity of NK cells was dependent, in part, on macrophage secretion of IL-15, IL-18 and IL-12 [527]. A number of factors involved in NK or NKT cell activation were downregulated in the serum of 4T1.2 tumour-bearing mice (Table 4.2). Hence it is possible that TAMs and NK or NKT cells are also functionally linked in this model, although IL-15 was upregulated in serum from 4T1.2 tumour-bearing mice (Table 4.1).

Given that TAM subpopulations appear to differ in phenotype, it would be interesting to investigate the cross-talk between NK and/or NKT cells and the different subpopulations. The enhancement of the M1-like phenotype seen in some TAM subpopulations [225, 264] may increase NK or NKT cell activation and lead to suppression of metastasis. Furthermore, such a treatment/therapy may result in destruction of M2-like pro-tumour TAMs, while the M1-like anti-tumour TAMs may be protected. The presence of a similar cross-talk between TANs and NK/NKT cells could also be explored. To treat metastatic disease effectively, it may be necessary to inhibit and/or enhance pathways within the signalling network generated by these tumour-

associated immune cells. Therefore, a thorough analysis of the relationships between different classes of immune cells associated with breast tumours and metastasis is required.

6.4 Conclusions

In this thesis it is reported that increases in both primary tumour Ly6C^{low} TAMs and TANs, as well as systemic Ly6C^{high} macrophages/monocytes and neutrophils were associated with metastasis in the 4T1.2 mammary tumour model of breast cancer. Furthermore, increases in these cell populations occurred early in tumour progression to metastasis. Thus it appears that more aggressive tumours escalate monocyte and neutrophil mobilisation, possibly via MMP-9, which in turn promotes metastasis. Further investigation of neutrophils, as well as Ly6C^{high} and Ly6C^{low} monocyte/macrophage subpopulations, not only in the primary tumour, but also systemically and at metastatic sites may yield novel targets to help classify and treat advanced breast tumours.

In contrast to previous studies, inhibition of CSF-1R signalling increased metastasis to both lung and spine, and this was associated with increased TAN and lung neutrophil numbers, as well as an enhancement of tumour-driven neutrophil and Ly6C^{high} monocyte mobilisation. These data indicate that caution is warranted for the use of anti-CSF-1/CSF-1R therapy for patients at high risk of extensive metastasis. Importantly, the above data also highlight the need to further investigate the contributions of neutrophils to breast tumour metastasis, as well as the functional relationships between macrophages/TAMs and neutrophils/TANs. Different tumour subtypes may recruit and utilise different inflammatory cell populations, and stratification of patients, possibly based on primary tumour TAM or TAN numbers and/or immune cell content, may better predict not only the likelihood of metastasis, but also response to therapy.

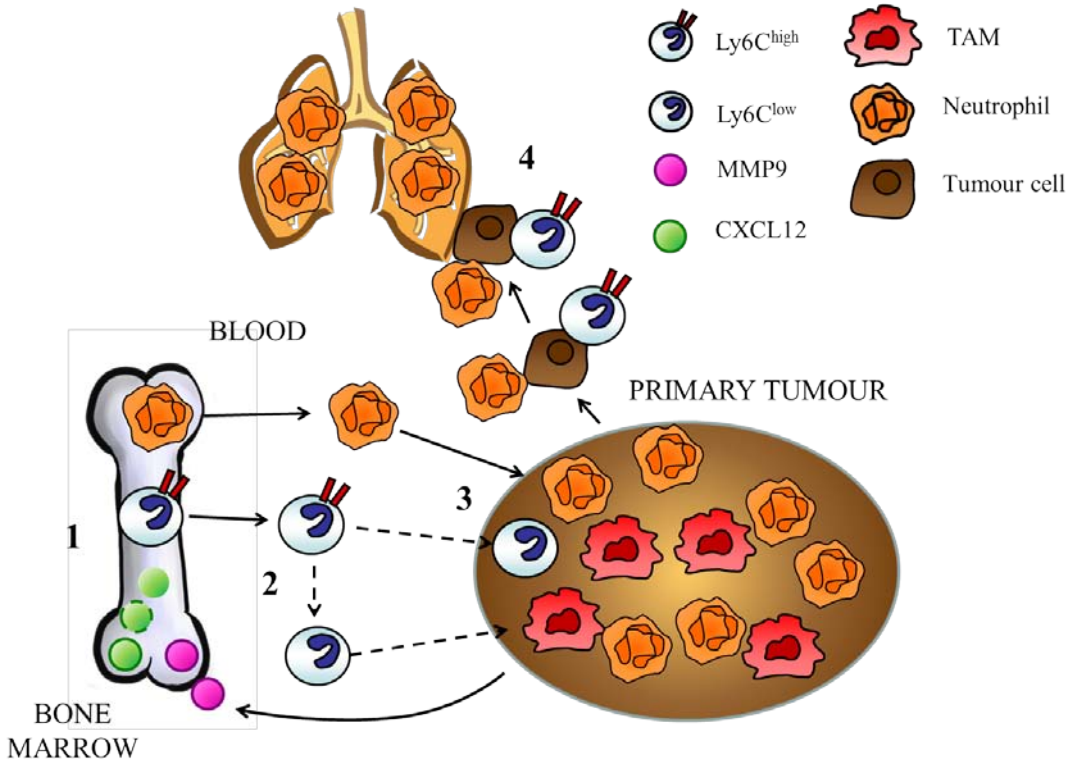


Figure 6.1: Proposed model for mobilisation of monocytes and neutrophils by metastatic tumours.

Metastatic breast tumours generate an inflammatory reaction whereby 1) CXCL12 degradation by tumour-derived MMP-9 mobilises neutrophils and Ly6C^{high} monocytes from the bone marrow. 2) Monocytes either convert to Ly6C^{low} cells in the primary tumour or in the blood. 3) The amplified monocyte and neutrophil numbers increase the number of TAMs and TANs which then 4) promote tumour cell homing to distant tissues such as the lung. 4) Neutrophils and Ly6C^{high} monocytes may also aid tumour cell migration through the circulation, arrest, extravasation and growth at secondary sites.

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