THE IMPACT OF HISTAMINE RECEPTOR 2 ANTAGONISTS ON BREAST CANCER DEVELOPMENT AND MONOCYTIC POPULATIONS

by

Ava Vila

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ABSTRACT

Breast cancer is the most commonly diagnosed cancer in women and the second leading cause of cancer-related death. There is an urgent need for immunomodulatory drugs which specifically target immune cells to activate an antitumour response, or inhibit the immunosuppressive microenvironment induced by the tumour. Monocytes have been shown to be involved in many aspects of tumour development, including tumour growth, invasion, metastasis, and immunosuppression. One population of monocytes involved in immunosuppression are the monocytic myeloid-derived suppressor cells (M-MDSCs), an immature population of monocytic cells capable of suppressing an antitumour immune response and directly enhancing tumour development.

Histamine is an immune mediator that has previously been studied in the context of cancer. Histamine can signal through four histamine receptors. Signaling through histamine receptor 2 (H2) is often immunosuppressive. We evaluated whether blockade of H2 signaling would alleviate this immunosuppression and therefore inhibit breast tumour development. We utilized two injectable orthotopic breast cancer models and one spontaneous breast cancer model.

Our results showed that continuous oral treatment with the commonly used H2 antagonist ranitidine decreased tumour progression. The nature of this response differed between models, ranging from increased tumour latency to decreased metastasis. These effects were dependent on the presence of monocytes and/or M-MDSCs. Ranitidine did not impact tumour development when monocytes were depleted or their recruitment was inhibited. In one model, this process was found to be independent of $CD8^+$ T cells. Ranitidine treatment led to decreased immune suppression and decreased monocyte numbers consistent with reduced monocyte development in the context of H2 blockade. Overall, our results indicate that ranitidine decreased MDSCs, thereby enhancing immunosurveillance, antitumour immunity, and subsequently decreasing tumour development. These findings indicate an urgent need for clinical studies to investigate the use of commonly used H2 antagonists in the prevention and treatment of breast cancer.

LIST OF ABBREVIATIONS USED

-APC	Allophycocyanin
ADCC	Antibody-dependent cell-mediated cytoxicity
AML	Acute myeloid leukemia
APC	Antigen presenting cell
Arg1	Arginase 1
CD	Cluster of differentiation
CMoP	Common monocyte progenitor
CMP	Common myeloid progenitor
CRC	Colorectal cancer
CSF	Colony stimulating factor
CSF1R	Colony stimulating factor 1 receptor
Ct	Critical threshold
CTC	Circulating tumour cell
CTL	Cytotoxic T lymphocyte
Cy7	Cyanine7
DAO	Diamine oxidase
DC	Dendritic cell
ER	Estrogen receptor
FITC	Fluorescein isothiocyanate
GFP	Green fluorescent protein
GMP	Granulocyte-macrophage progenitor
H1	Histamine receptor 1
H2	Histamine receptor 2
H_2O_2	Hydrogen peroxide
H3	Histamine receptor 3
H4	Histamine receptor 4
HDC	Histidine decarboxylase
HER2	Epidermal growth factor receptor
HGF	Hepatocyte growth factor
HNMT	Histamine-*N*-methyltransferase
EMT	Epithelial-mesenchymal transition
IDO	Indoleamine-pyrrole 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
Lin	Lineage
LPS	Lipopolysaccharde
MDSC	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MMTV	Mouse Mammary Tumour Virus
mTOR	Mammalian target of rapamycin
NIC	Neu/Her2-MMTV-Cre
NK	Natural killer

NO	Nitrogen oxide
NOS2	Inducible nitric oxide synthase
NOX	NADPH oxidase
PARP-1	Poly-ADP ribose-polymerase-1
PE	Phycoerythrin
PGE_2	Prostaglandin E_2
PGF	Placental growth factor
PR	Progesterone receptor
ROS	Reactive oxygen species
TAM	Tumour-associated macrophage
TAN	Tumour-associated neutrophil
TCR	T cell receptor
TGFβ	Transforming growth factor β
T_H	T helper cell
TIMP	Tissue inhibitors of metalloproteinases
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
Treg	T regulatory cell
VEGF	Vascular endothelial growth factor

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1 CHAPTER 1 INTRODUCTION

1.1 Cancer

In Hanahan and Weinberg's seminal paper, they initially described the hallmarks of cancer as sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis, and resisting cell death [1]. Although all these hallmarks can be induced by the cancer cells themselves, cancerous cells and the tumour microenvironment can also induce immune cells to aid in key processes, such as enhancement of angiogenesis, induction of metastasis, and enhancing tumour growth. An emerging hallmark of cancer is avoiding immune destruction [1]. This occurs by cancer cells altering antigen presentation, releasing mediators that will suppress immunosurveillance, and mediators that will cause immune cells to become immunosuppressive. Combinations of such processes subsequently suppress an antitumour response.

In Canada, cancer is the leading cause of death [2], while in the United States, it is the second leading cause of death after heart disease [3]. The greatest cause of death in breast cancer patients is due to metastasis. Many new therapies are aimed at activating the immune system to clear the tumour, but many of these treatments are not successful, due to the immune microenvironment that the tumour has created [4-6]. Therefore there are now more attempts at using combination therapies that not only enhance an immune response against the tumour, but also target immunosuppressive cells and cause their inhibition or cell death [7-10]. Some of these treatments include using recombinant

cytokines to enhance an immune response [11-13] and blocking certain immune mediators that hinder an immune response [14-16].

Cancer development involves a variety of different factors that can involve aberrant signaling, genetic mutations leading to uncontrolled proliferation and immortality, and recruitment of normal cells to allow these cancer cells to expand. However many of these pathways are beyond the scope of this thesis, which will primarily be the impact of immune cells, particularly myeloid cells on breast cancer progression.

1.1.1 Breast cancer

1.1.1.1 Immune cell populations associated with breast cancer

Local resident immune cells have mainly been examined in breast tumour samples, analyzing what cellular infiltrates are found (reviewed in [17]). Few studies have analyzed the resident cells in normal breast tissue. Mast cells and macrophages are been shown to be important for mammary gland development during puberty [18-20]. In a study performed by Degnim *et al* [21], they showed that in healthy human breast tissue, $CD8^+$ T cells, monocytes, and dendritic cells (DCs) are part of the resident immune cells found in the lobules. The exact functions of these cells are unknown, but it can be speculated that these cells are involved in tumour surveillance.

In breast tumours, there are substantial infiltrates of immune cells, including $CD4^+$ T helper cells, T regulatory cells (Tregs), $CD8^+$ T cells, DCs, and natural killer (NK) cells [22]. Mast cells have also been found to be present peritumorally [23] and shown to enhance tumour development [24-26], but can be activated to enhance antitumour immunity [27]. Neutrophils and macrophages are also present within the tumour, in

populations known as tumour associated neutrophils (TANs) and tumour associated macrophages (TAMs) [28], respectively. Subsets of granulocytes and monocytes found within the tumour microenvironment are collectively known as myeloid derived suppressor cells (MDSCs) [29, 30]. Under ideal conditions, there would be clearance of tumour cells via cytolytic activity by $CD8^+$ cytolytic T lymphocytes (CTLs) [31], NK cells [32], or macrophages [33]. However, this clearance does not always occur, due to the presence of immunosuppressive cells, including MDSCs (reviewed in [34]).

1.1.1.2 Signaling

Genetic instability is found in most tumour types and can be due to failures in DNA maintenance or DNA repair during replication, which can lead to point mutations within genes. If the genes that are mutated are involved in regulating key steps of proliferation or cell death, known as tumour suppressor genes, then aberrant growth may follow. Accumulation of defects in these genes can then lead to cancer progression. In breast cancer, common mutations are found in the BRCA1 and BRCA2 tumour suppressor genes (Figure 1.1). If DNA is damaged, other tumour suppressors known as ATM and ATR become activated (depending on the type of DNA damage that occurs) [35, 36], which go on to, directly or indirectly, activate BRCA1. BRCA1 and BRCA2 can also colocalize to the damaged DNA with the protein RAD51 [37, 38], which is involved in double strand DNA repair. BRCA1 activation can then interact with other proteins, such as Chk1 [39], and p21 and Rb [40, 41], which are important regulators of cell cycle progression, leading to cell cycle arrest. BRCA1 and BRCA2 are also involved in DNA repair, and mutations or absence of these proteins increases the risk of developing breast

cancer. ATM and ATR can also lead to phosphorylation of MDM2, leading to subsequent activation of p53 [42, 43].

P53 is a major checkpoint protein in normal cell growth and proliferation. Under normal situations, p53 is kept at low concentrations in the cell, via ubiquitination and proteolysis [44]. In response to some types of intracellular stress, including DNA damage or hypoxia [45, 46], p53 can become activated, leading to cell cycle arrest and potentially apoptosis [47, 48]. In more than 50% of human tumours, p53 is mutated [49], so that even in the presence of DNA damage and hypoxia that is typically associated with cancer, there is no inhibition of growth via this pathway. P53 can also be involved in the mTOR pathway; alterations in nutrient levels in the cell lead to p53 activation [49]. Activation of p53 leads to activation of AMPK, and enhanced transcription of PTEN and TSC2 [49], leading to inhibition of the mTOR pathway.

mTOR is a serine-threonine protein kinase composed of two protein complexes, mTORC1 and mTORC2 (Figure 1.2) [50]. Under normal conditions, growth factors stimulate the PI3K/AKT pathway [51], LKB1/AMPK/TSC pathway [52] and/or mTOR directly [53], all culminating in mTOR activation of downstream regulators of translation, S6 kinase and eIF4E binding protein 1 [54, 55]. mTOR activation can inhibit autophagy and promote cell growth and proliferation [51], and inhibition of mTOR inhibits cell growth and enhances autophagy. In cancer, mTOR is upregulated, usually due to alterations in negative regulators, including PTEN [56], TSC [57], and LKB1 [58], or upregulation of positive regulators, such as AKT [59] and PI3K [60].

In many cancer types, there is also the shift from typical oxidative phosphorylation to aerobic glycolysis, also known as the Warburg effect. Even in the presence of oxygen, many cancer cells will use glycolysis for energy supply, where glucose is broken down into lactate, generating 2 ATPs per glucose molecule, as opposed to 36 ATPs per glucose molecules during oxidative phosphorylation. Through incomplete breakdown of glucose, there is accumulation of macromolecular precursors, such as acetyl-CoA for fatty acids and glycolytic intermediates for amino acid synthesis. Furthermore, incomplete breakdown of glucose and catabolism of glutamine allows for more NADPH to be synthesized [61]. This excess lactate can be recycled by the liver, or used as fuel for surrounding tumour cells in oxidative phosphorylation [62]. Mutations involved in the Warburg effect include mutations in the PI3K/AKT/mTOR pathway [58, 63]. Aberrant signaling in this pathway has been shown to lead to resistance to treatment in epidermal growth factor receptor $(HER2)^+$ and estrogen receptor $(ER)^+$ breast cancers [64-66] .

Some mutations can lead to cancer cells producing their own growth factors that act in an autocrine manner [67, 68], or enhance growth factor production by normal surrounding stromal cells [67]. Some mutations can also lead to creating constitutively active receptors. HER2 can become constitutively active [69], leading to activation of MAPK, JAK/Stat, and mTOR pathways that are all involved in growth, metabolism, cell survival, and proliferation. Although all these pathways individually can impact cancer cell development, they are also all intertwined, therefore one mutation/alteration in one pathway, can lead to alterations in other pathways.

1.1.1.3 Metastasis

For cancer cells to invade and spread to peripheral sites of the body, cancer cells go through alterations in adhesion molecules, decreases in proliferation and morphologically alter into a mesenchymal phenotype, where they become more elongated, mobile, and invasive [70]. This is known as epithelial-mesenchymal transition (EMT). EMT can be induced by a variety of mediators, including transforming growth factor β (TGFβ), hepatocyte growth factor (HGF), fibroblast growth factors, and insulin growth factor [71, 72]. Some of the main transcriptional mediators of EMT include ZEB 1 and ZEB 2, Snail, Slug, and Twist, which not only regulate each other, but also cause downregulation of epithelial markers and upregulation of mesenchymal markers [73]. During EMT, there is downregulation of adhesion molecules such as E-cadherin, occludins, epithelial cellular adhesion molecule and epithelial cytokeratins, followed by upregulation of mesenchymal proteins such as vimentin, fibronectin, and N-cadherin, and reorganization of the cytoskeleton [71, 72, 74]. TGFβ can also support survival of these cells with increased expression of anti-apoptotic protein Bcl-x_L, when typically loss of adhesion can lead to apoptosis [72]. While undergoing EMT, tumour cells can secrete TGFβ and thrombospondin-1, which leads to impairment of DC function and induction of Tregs, therefore creating a suppressive microenvironment during the start of metastasis [70]. Furthermore, during EMT the cancer cells are resistant to immunotherapy, via induction of immunosuppression, and also decreased sensitivity to CTL-mediated cytolysis [70]. This phenotype is seen in circulating tumour cells (CTCs), although there are some CTCs that express both epithelial and mesenchymal markers, and even CTCs that are strictly epithelial [74]. Once the cells seed at a distant metastatic site, these cells go back to

having an epithelial phenotype through a process known as mesenchymal to epithelial transition.

For a cell to be able to seed at a distant site there needs to be priming of the environment to support tumour cell seeding growth, known as setting up a “pre-metastatic niche”. This niche can be induced by the tumour itself, causing a recruitment of immune cells to a distant site, such as the lung or liver, before metastasis occurs [75]. This niche is initiated by tumour-derived secreted factors that can then influence the site of metastasis to create mediators to facilitate stromal and immune cell recruitment. Some of these mediators are secreted under hypoxic conditions, and hypoxia-associated factors such as hypoxia-inducible factor 1α, have been shown to be involved in secretion of mediators for formation of metastasis, such as CCL2, colony stimulating factor 3 (CSF3), tumour necrosis factor (TNF), vascular endothelial growth factor (VEGF), and matrix metalloproteinase 9 (MMP9) [75, 76]. There is potential for variability of secretion factors from tumour to tumour, which can then influence to which site metastasis occurs. Secretion of VEGF and placental growth factor (PGF) has been shown to promote recruitment of hematopoietic cells in secondary organs [75]. Other mediators such as TGFβ and lysyl oxidase can also drive formation of the pre-metastatic niche [77-79]. A blockade in CSF3 has been shown to suppress metastasis in several breast cancer models [80]. At the pre-metastatic site, there is usually an increase in mediators to induce recruitment of cells such as monocytes and granulocytes, and also tumour cells, such as CCL2 [81-83], interleukin (IL)-6 [84], S100A8 and S100A9, TNF, TGFβ, CXCL2 [78, 80], CCL2 and CXCL12 [85]. There is also an increase of growth factors and cytokines to alter the immune microenvironment, such as fibroblast growth factor and insulin

growth factor, IL-4, IL-5, IL-9, and IL-10, as in the case of the 4T1 pre-metastatic niche [85].

In the pre-metastatic lung, there is accumulation of hematopoietic cells, with the population primarily being made up of MDSCs, as well as immature myeloid cells, alveolar macrophages, DCs, $CD4^+$ T cells and $CD8^+$ T cells [86], NK cells [76], granulocytic cells [80], and hematopoietic progenitor cells [75]. Further examination of these cell types show that DCs have decreased major histocompatibility complex (MHC) class II and CD86, signifying that they have decreased ability for antigen presentation. The NK cells recruited to the metastatic lung are immature with decreased cytotoxic activity [76]. The $CD4^+$ T cell population is also predominantly polarized to a T helper cell (T_H) 2 subset [86]. This signifies that at the metastatic site similarly to the primary tumour, there is reduction in the effective antitumour immune response.

In the pre-metastatic niche there are alterations in the basement membrane; VEGF and PGF can induce fibroblast proliferation and production of fibronectin [87], and increased fibronectin is important for recruitment of myeloid cells via VLA-4 binding [75]. Lysyl oxidase binds to the fibronectin and can cause cross-linking of collagen and elastin, which allows for enhanced adherence of myeloid cells in the lung. These myeloid cells can then secrete MMPs to further remodel the extracellular matrix and cause leaky vasculature, cause further recruitment via byproducts of collagen cleavage [79, 85], and even enhance metastasis [75]. The recruitment of these cells is also important for decreasing immunosurveillance, therefore allowing the tumour to grow in the absence of an effective antitumour response. Depletion of these myeloid cells leads to ablated metastasis [75], indicating the importance of these cells for supporting metastasis.

It is important to note that metastasis does not occur randomly in any organ; there is organotropism, where certain cancers will primarily metastasize to specific organs. In breast cancer, the most common sites of metastasis are lung, bone, liver, brain and local lymph nodes. Primary breast cancer cells and breast cancer cells from a metastatic site have been shown to have increased levels of CXCR4 compared to normal breast cells [88]. The ligand for CXCR4, CXCL12, is upregulated in lymph nodes, lung, liver and bone marrow, replicating the common sites of breast metastasis [88]. Furthermore, CXCL12 can also induce an invasive phenotype in breast cancer, in this case showing a mechanism by which a pre-metastatic site can impact tumour metastasis. Melanoma has been shown to express CCR10, whose ligand CCL27 is highly expressed in the skin [88], implying that differences in chemokine receptor expression, by cancer cells, leads to the specific organotropism seen in different cancer types. Furthermore, with release of mediators from the tumour, recruitment of immune cells is only seen in sites of metastasis, not to sites where metastasis does not occur [75, 80]. Notably, when a mouse is co-injected with tumour-conditioned medium and a second different tumour cell, the tumour will metastasize to the sites associated with the first tumour [75]. The mechanism behind this was not further investigated.

1.1.1.4 Therapies

Breast cancer has traditionally been subdivided into five groups, depending on the expression of ER, progesterone receptor (PR), and HER2 [89]: Basal-like ($ER^-PR^-HER2^-$), HER2-enriched ($ER^-PR^-HER2^+$), normal breast-like ($ER^{-/+}HER2^-$), luminal A ($ER^+PR^+HER2^-$), luminal B ($ER^{+/-}PR^{+/-}HER2^{+/-}$). However, since the classification of these groups there have been other subgroups created whereby other

markers, such as molecular markers associated with specific genetic mutations, are required to completely characterize the breast tumour type.

Some tumour types, such as luminal B and basal-like, are still treated with chemotherapy while other tumour types are treated with antibody therapies that target highly upregulated markers. Trastuzumab is an antibody that targets HER2, and has been used for the treatment of HER2 positive breast cancer. Binding of HER2 by trastuzumab has a variety of effects; downregulation of HER2 expression [90], inhibition of HER2-HER3 dimerization thereby inhibiting constitutive activity of HER2 signaling [91], and inhibition of HER2 cleavage into active p95-HER2 which can dimerize and signal with HER2 [92]. Trastuzumab can also target the tumour cells for antibody-dependent cell-mediated cytoxicity (ADCC) by NK cells [93].

For ER^+ tumours, hormonal aromatase inhibitors and selective estrogen receptor modulators such as tamoxifen would often be utilized for treatment. Tamoxifen binds to estrogen receptor, and instead of the standard recruitment of coactivator, there is recruitment of corepressors, that leads to inhibition of estrogen-dependent genes [94], leading to inhibition of tumour growth and sometimes tumour cell death [95]. Aromatase is the enzyme responsible for the synthesis of estrogen, therefore aromatase inhibitors cause decreases in circulating estrogen, leading to lack of signaling on ER^+ cancer cells and causing growth arrest. However, even with treatments against specific targets, there are still differences in response from subject to subject. Other treatments that are now being tested are PI3K/AKT/mTOR pathway inhibitors, including PI3K and mTOR inhibitors, and AMPK activators [60, 96, 97]. For the basal-like group, which does not express any of the three key receptors but does appear to have BRCA1 mutation [98, 99],

poly-ADP ribose-polymerase-1 (PARP-1) inhibitors have been used. PARP-1 is involved with DNA repair, and inhibition of PARP-1 in combination with the BRCA1 and/or BRCA2 mutation that is found in the basal-like group, can lead to cell death [100-102].

1.1.1.5 Statistics of breast cancer

Even with the decrease in breast cancer-related mortality due to early screening and better treatments, breast cancer is the most common cancer in females, being the most commonly diagnosed cancer, and causing the second highest number of cancer-related mortalities in North America, after lung cancer [2]. Metastasis is the main cause of mortality associated with breast cancer. Approximately 20-30% of patients develop metastatic disease, with a median survival of 2-4 years [100, 103], and there is no standard method of treatment, although trastuzumab in combination with other chemotherapeutics has been shown to be beneficial for metastatic $HER2^+$ breast cancer [104, 105]. Immunotherapeutics have been shown in laboratories to hinder breast tumour development, and immunotherapeutics have been shown to be beneficial in some cancer types such as lymphoma and melanoma, but whether it would be beneficial for significant numbers of patients with breast cancer is still being tested in clinical trials.

1.1.1.6 Murine breast cancer models

Mouse models are widely used for investigating tumour immunology. It is important to note that different mouse strains have a propensity towards a specific immunological phenotype; in BALB/c mice, there is an increased M2 macrophage phenotype and T_H2 T cell phenotype, while in C57BL/6 mice there is an increased M1and T_H1 phenotype [106, 107]. BALB/c mice also have higher levels of Tregs and their T effector cells are more susceptible to suppression compared to C57BL/6 cells [107]. Therefore the effect drugs

have on an immune response may be different from one model to another due to this skewing of phenotype, leading to an altered impact on cancer development from one model to another.

1.1.1.6.1 Tumour cell injection

4T1 is a p53 null [108], poorly immunogenic mouse mammary carcinoma cell line isolated from a BALB/c mouse. As this model is derived from an immunocompetent mouse, it can be used for orthotopic models of breast cancer to determine the impact of immunomodulation on the tumour. When orthotopically injected into a mammary fat pad, it is capable of metastasizing in a similar fashion as seen in humans; it can metastasize to the lung (first site starting at approximately 7 days post-injection), liver, brain, and bone [109]. For detection of micrometastases, the organs of interest are digested and resuspended in media containing 6-thioguanine, a chemotherapeutic that 4T1 cells are naturally resistant to, therefore all normal cells (eg. lung cells, infiltrates) will die, while 4T1 cells will survive. After plating this suspension in petri dishes and incubation, the individual tumour cells that were present will grow into individual colonies, and by counting the colonies it can be determined the number of tumour cells present in the organ.

The 4T1 tumour microenvironment and effect on the immune system has been extensively studied. Of the infiltrating cells in the 4T1 tumour, the majority are myeloid cells, with a high proportion being immature myeloid cells [110]. 4T1 cells can express MHC class I, therefore making it a target for CTL-dependent cytolysis [111]. However, 4T1 cells can express PD-L1 when IFNγ is present *in vivo* [16], therefore they are capable of causing death of CTLs. 4T1 cells can also express CCL2, CXCL1, and CCL5

[110], enhancing recruitment of monocytes, neutrophils, and T cells. 4T1 can secrete CSF2 and CSF3 [112], therefore in combination with the enhanced recruitment of monocytes, can cause induction of MDSCs. With high levels of CSF3 production, there is enhanced granulocytosis in these mice; in the metastatic lung, the immune infiltrate is primarily granulocytic cells [110]. In the blood there is high levels of granulocytosis, and in the bone marrow there is increased myelopoiesis and increased levels of immature myeloid cells [112]. 4T1-bearing mice have splenomegaly due to increased levels of immature cells [112], specifically immature granulocytic cells [113].

E0771 is a mouse ER^{+} [114] mammary adenocarcinoma cell line isolated from a C57BL/6 mouse, similarly allowing for studies on an antitumour response. However, it does not metastasize in the same manner as 4T1 cells, as metastasis of E0771 cells to the lung or peritoneal organs are only found in 50% of cases [114]. The primary tumour is highly vascularized. In my studies I found that E0771 cells alone will not grow well or consistently in mice; therefore I injected E0771 cells in Matrigel®, a solubilized basement membrane mixture composed of laminin, collagen IV, heparan sulfate proteoclycans and entactin, to allow for better support of the tumour cells, and found there is better tumour take in the mice and more consistent tumour growth. As there was no unique marker that could be used for detection of metastasis, we transduced a plasmid coding green fluorescent protein (GFP) into E0771 cells and selected for a cell line that expressed detectable amounts of GFP, but not high expressing cells, in case these cells would be cleared by the immune system due to high levels of GFP expression. There are also fewer studies into how E0771 tumours alter an immune response, although E0771 cells can cause immunosuppression *in vivo* [114]. In part this may be due to induction of

MDSCs in the spleen, but the induction of MDSCs by E0771 cells is not as high as in the 4T1 model (40 vs 20%) [115].

1.1.1.6.2 Spontaneous tumour model

Lkb1$^{-/-}$/Neu/HER2-MMTV-Cre (Lkb1$^{-/-}$/NIC) mice are a spontaneous breast cancer model in which mammary epithelial cells have a hyperactive mTOR activity due to specific knockout of LKB1 [58]. 100% of mice will develop breast cancer, with 50% incidence of tumour at 147 days after birth [58].

These mice are created by crossing mouse mammary tumour virus (MMTV) -NIC male mice with female mice with floxed LKB1 (Figure 1.3). The MMTV promotor is found throughout the mice but is only activated via glucocorticoid stimulation, in mouse mammary epithelial cells and mouse mammary stem cells [116]. Only in female mice will it be activated. In the MMTV-NIC mice, MMTV activation leads to transcription of mRNA containing both HER2 and Cre enzyme, where between the HER2 and Cre cistrons there is an internal ribosomal entry sequence, allowing both HER2 and Cre to be translated separately [117]; therefore these mice will have high HER2 expression on their mammary epithelial cells and Cre expression. When crossed with floxed LKB1 mice, the Cre enzyme will recognize the lox sequence on either side of LKB1 gene, excise the gene, and ligate the DNA in the absence of LKB1, therefore only in mammary cells will LKB1 be missing in the mice. This deficiency in LKB1 leads to aberrant glucose metabolism and hyperactive mTOR activation, leading to mammary tumour development [58]. There are no studies in regards to the immune response in LKB1$^{-/-}$/NIC mice, but for MMTV neu mice, MDSCs and Tregs are increased compared to the wild type FVB background strain of mice [118, 119].

1.1.1.7 Tumour antigens

Tumour antigens can be categorized into overexpressed cellular antigens, mutated proteins, viral antigens if the cancer is induced by viral infection of the cells, or antigens that are typically expressed in germ cells, but are not present in typical somatic cells. The mutated proteins or germ cell proteins are expressed due to mutations in the genome that are associated with cancer, leading to point mutations that cause alterations in the level of proteins, or alterations that lead to inappropriate expression of proteins. In optimal conditions, these proteins are presented in the context of MHC class I, leading to subsequent clearance of the tumour via CTL-mediated cytolysis; however, due to the immunosuppression associated with tumour development, this typically does not occur.

Peptide vaccines for breast cancer have been studied, with the use of commonly overexpressed tumour antigens. It is difficult to predict what sort of mutated proteins can be present in tumours to create a vaccine. However, there are currently clinical trials for peptides that are overexpressed in some breast cancers, such as HER2 [120, 121] and mucin 1 [122], an overexpressed glycoprotein. These clinical trials are for breast cancer patients, therefore are more of an adjuvant vaccine to boost a weak T cell response rather than a prophylactic vaccine. For the peptide vaccine to work, it must be able to be presented on DCs in the context of MHC class II, and induce a potent T cell response that will activate a T_H1 response, leading to increased interferon (IFN) γ production to induce a CTL and NK cell response. An antibody response by B cells and memory induction would also be optimal. There are currently algorithms to predict the optimal peptide sequence that will induce this response, which then leads to testing the predicted peptide against T cells *in vitro* and *in vivo* to see which will give the most optimal response.

Typically with the vaccine there is the use of an adjuvant to increase DC maturation and to steer the DCs towards production of T_H1-specific cytokines. These adjuvants can include toll-like receptor (TLR) agonists [123] and CSF2 [124].

1.1.1.8 Tumour surveillance

Under optimal conditions, mutated cancerous cells would be cleared by immune cells. Cells undergoing stress can also release mediators, such as damage-associated molecular pattern molecules and alarmins, which can then activate DCs [125, 126]. Released mutated peptides can be taken up by DCs and presented in the lymph nodes to activate $CD4^+$ T cells and induce a T_H1 phenotype that can induce cytolytic activity via production by IFNγ. Whole tumour cells can also migrate to the lymph nodes and induce an antitumour response [127]. DCs can also be induced to cross-present the peptide to activate $CD8^+$ T cells. Cancerous cells would express mutated or overexpressed proteins in the context of MHC class I, which could then be detected by CTLs, leading to cytolytic activity to cause apoptosis of the tumour cells. If the cancer cells downregulate MHC class I, NK cells can target these cells for cytolysis.

Cancer cells are capable of evading this immunosurveillance and develop into large tumours that can spread to other parts of the body. There are several mechanisms that tumours utilize to evade immunosurveillance, including antigen modulation [128] and expression of inhibitory molecules that can cause apoptosis of T cells [129]. The focus of this thesis will be tumour-induced immunosuppression, specifically altering myeloid cells into immunosuppressive phenotypes, including development of TAMs and MDSCs.

1.1.1.9 Cancer and Immunology

1.1.1.9.1 Protumourigenic

There are myeloid cells that are involved in a protumourigenic response, which will be covered in later sections.

1.1.1.9.1.1 T regulatory cells

Tregs are $CD4^{+}CD25^{+}FoxP3^{+}$ that suppress effector T cell activity. FoxP3 is a transcription factor that drives genes that are involved in Treg development, and inhibits genes involved in T effector cell development [130]. There are different categories of Tregs, including natural Tregs, and inducible Tregs. For both populations TGFβ is important for development and maintenance (with IL-2 playing an important role in maintenance) [131]. Natural Tregs develop in the thymus while inducible Tregs are induced in the periphery, where naïve T cells under the presence of mediators such as TGFβ become Th3 Tregs [132]. Another category of inducible Tregs is the Tr1 population, which is induced by IL-10 [133].

Tregs can suppress a wide variety of cells, including $CD4^{+}$ and $CD8^{+}$ T cells, B cells, NK cells and DCs [134, 135]. The main mechanism of suppression by Tregs is IL-10, TGFβ, and IL-35 production [136, 137]. Other mechanisms of suppression include having a higher affinity for IL-2, leading to deprivation of IL-2 for effector T cells [138], engagement of B7 on T effector cells with CTLA-4 on Tregs [139], and induction of indoleamine-pyrrole 2,3-dioxygenase (IDO) on DCs by B7-CTLA-4 interaction, which depletes the non-essential amino acid tryptophan from the environment, which can lead to inhibition of T cell proliferation [140, 141]. Tregs are known to predominantly

suppress an antitumour response, but through the production of TGFβ, can also have an impact on angiogenesis and metastasis [142, 143]. Breast cancer cells have been shown to induce recruitment of Tregs via secretion of chemokines, such as CCL22, that mediates chemotaxis through CCR4 on Tregs [144]. Furthermore, within the tumour, Tregs have been shown to proliferate. In breast cancer, elevated levels of Tregs are present in the peripheral blood [145] and within the tumour itself [143], and can be an indicator of relapse [144, 146].

1.1.1.9.2 Antitumourigenic

1.1.1.9.2.1 NK cells

NK cells are part of the innate immune response, capable of detecting stressed or transformed cells and directly targeting them for cytolysis. NK cells are also major producers of IFNγ [147, 148], which can induce T_H1 phenotype development [148] and DC activation, leading to $CD8^+$ T cell activation and memory induction [147]. NK cells are capable of killing tumour cells through a variety of mechanisms. MHC class I expression is an inhibitory signal to NK cells which signals through Ly49 on mice or KIR receptor family members in humans, and if tumours downregulate expression of MHC class I to evade CTL detection, there is no inhibitory signal, therefore leading to activation of NK cells. Stressed cells can also upregulate receptors that are ligands for activating receptors on NK cells, such as MICA and MICB on stressed cells, interacting with NKG2D. NK cells also express Fc receptors, and are therefore capable of targeting tumour cells that are bound by antibodies [149].

The main mechanism of NK cell-mediated cytolysis is via perforin and granzyme B. When activated, NK cells release vesicles that contain perforin and granzyme B. Perforin

inserts itself into the target cells membrane and forms a pore that allows granzyme B to enter into the cell. It is worth noting that new studies reveal that perforin and granzyme B get endocytosed first, and perforin creates a pore to allow granzyme B to enter the cytosol [150]. Once granzyme enters the cell, it can cleave several caspases, leading to apoptosis. NK cells are also capable of causing apoptosis by Fas ligand-Fas interaction on tumour cells, and release of soluble TNF-related apoptosis-inducing ligand (TRAIL) leading to interaction with TRAIL receptors on the tumour cell [151].

In the tumour microenvironment, NK cells can be inhibited by a variety of mechanism, some of which will be addressed in later sections. In breast cancer patients, an increase in activation receptors on NK cells correlates with a decrease in tumour recurrence [152]. However, NK cells in the tumour-bearing patient's blood have decreased activating receptors and increased inhibitory receptors, while tumour-infiltrating NK cells had similar alterations in receptors but also decreased cytotoxic ability and decreased ADCC function [153].

1.1.1.9.2.2 $CD4^+$ and $CD8^+$ T cells

$CD4^+$ T effector cells are important regulators of the immune response. Once presented with a tumour peptide by an antigen-presenting cell (APC), such as a DC, B cell, or macrophage, these T cells become activated and release cytokines that can lead to activation of other immune effector cells. The nature of the cytokines that are released by T cells are dependent on the presence of cytokines during activation. If DCs are producing IL-12 during activation, the T cell displays a T_H1 phenotype, which includes secretion of IFNγ and TNF. If during activation IL-4 is present, the T cell displays a T_H2 phenotype, including the secretion of IL-4, IL-5, and IL-13, which has been shown to be

suppressive to T_H1 development and IFNγ production [154]. The T_H1 phenotype is considered antitumourigenic, as IFNγ can induce activation of $CD8^+$ T cells and NK cells. IFNγ can also upregulate MHC class I expression on tumour cells [155], making them more susceptible to CTL-mediated cytolysis. $CD4^+$ T cells are important for the maintenance of $CD8^+$ T cells, including recruitment to the tumour [156]; IL-2 production by $CD4^+$ T cells can also help enhance $CD8^+$ proliferation and IFNγ expression, leading to an enhanced antitumour response [157]. Furthermore, IFNγ and TNF can synergize to cause tumour cell death [158, 159]. One study showed that $CD4^+$ T cells can interact with peptide-MHC class II on tumour cells and become activated to release granzyme and perforin to induce cytolysis [160]. T_H1 cells can also activate DCs via CD40L-CD40 interaction to cross-present peptide on MHC class I to activate $CD8^+$ T cells.

$CD8^+$ T cells are involved in directly targeting tumour cells for apoptosis. Once activated by DCs in the context of peptide-MHC class I, $CD8^+$ T cells become activated into CTLs. If an activated CTL comes into contact with a target cell expressing the specific peptide on MHC class I, the CTL is capable of killing the cell, via the same perforin and granzyme method that NKs utilize. CTLs can also induce apoptosis via Fas-Fas ligand interaction [161]. There are also some CTLs that express CD16, and therefore can mediate ADCC [162].

In the tumours there are T_H2 cells, which by the production of IL-13 can sometimes enhance tumour development [163]. $CD8^+$ T cells can be found in the tumour microenvironment, but many tumours downregulate MHC class I, therefore CTLs cannot readily become activated. Furthermore, the tumour microenvironment can suppress CTLs via a variety of mechanisms, including interaction with MDSCs. In the peripheral blood

of breast cancer patients, there are decreased $CD8^+$ T cells, and the $CD8^+$ T cells present have decreased levels of costimulatory molecules CD28 and CD80, and increased Fas expression [164]. In some cases, the presence of $CD8^+$ T cells in breast tumours led to a decreased mortality rate [165]. In another study, there was a correlation between increased CD4 and CD8 expression and decreased survival [166], although these authors only looked at CD4 expression without differentiating between Tregs and T effector cells. In metastasis, CD4 lymphopenia in the blood correlates with poor survival [167].

1.1.1.9.2.3 Dendritic cells

DCs are important innate immune cells which activate many aspects of the immune response. DCs can phagocytose peptides, migrate to the lymph nodes, and then present these peptides in the context of MHC class II. DCs can then activate naïve $CD4^+$ T cells. Dendritic cells can also become activated by $CD4^+$ T cells to present the peptide on MHC class I, leading to activation of $CD8^+$ T cells. In cancer patients, there can be defects in DCs; tumour cells have been shown to induce DC apoptosis [168]. DCs can be induced by breast tumours to induce a T_H2 response as opposed to the antitumourigenic T_H1 response [163]. DCs, as previously stated, can also be induced to express IDO, leading to depletion of tryptophan in the environment, inhibiting T cell activation [169]. Immature DCs, which are known to induce anergic T cells, can get recruited by the tumour, and induce Treg proliferation [170]. Some antigens, including mucin 1 and HER2, can get taken up by DCs, but are not able to be processed to be presented on MHC class II [171]. There can also be decreases in DCs due to improper differentiation of monocytes into DCs. In breast cancer patients, there is an increase in apoptotic DCs in the blood [172]. If

there is an increase in mature DCs infiltrating the tumour, there is decreased metastasis to the lymph node, and increased relapse-free survival and overall survival [173].

1.1.1.9.2.4 B cells and antibodies

Although there is a now categorized group of B cells known as B regulatory cells that have immunosuppressive activities and can affect cancer development [174, 175], this section will focus on the antitumourigenic activities of B cells. A large number of studies suggest that B cells enhance tumour development, but these results may be attributed to the newly categorized B regulatory cell population. Antibodies against cancer cells can be produced by B cells. In the lymph node, B cells can take in peptide from DCs and present them on MHC class II. Then, via activation with $CD4^+$ T cells specific for the peptide, B cells can begin to produce antibodies against the tumour cells. In the presence of cytokines, there can be class switching, leading to production of immunoglobulin G (IgG) or IgE antibodies against the tumour. In mice, a T_H1 environment induces IgG2a production, while a T_H2 environment induces IgG1 production. B cells are also capable of altering an immune response; interaction between B cells and CTLs or $CD4^+$ T cells can enhance T cell survival and proliferation, and IFNγ production [176, 177].

Antibodies bound to tumour cells can lead to a variety of effects. As previously stated, some antibodies can cause inhibition of signaling if bound to some receptors on the tumour cells. NK cells and macrophages can detect and become activated by cells bound with antibodies, leading to ADCC. Bound antibodies can lead to complement activation [178], leading to the classical complement pathway activation causing the membrane attack complex to form pores on the cell membrane, inducing lysis. There are mechanisms by which tumour cells can avoid antibody detection, including antigen

shedding, and antigen mutations. In breast cancer, high tumour-infiltrating B cell activity is associated with decreased metastasis [179, 180]. Increased levels of mucin 1-specific IgG and IgM in early breast cancer patients correlates with increased survival and decreased metastasis [181]. As a result of tumour cell apoptosis, there is an increase in antibodies against tumour proteins produced by tumour-infiltrating B cells [182].

1.2 Monocytes/Macrophages and Neutrophils

1.2.1 Monocytes and macrophages

Mononuclear phagocytes represent 10-15% of the total cells in many organs [183]. Monocytes are found in the peripheral blood, where they comprise of 4 and 10% of leukocytes in mice and humans, respectively [184]. Monocytes in the peripheral blood come from the bone marrow and have a half-life of 22 hours [185]. In humans there are two main monocyte populations: the $CD14^{+}$ monocyte (which can be divided into $CD16^{+}$ and $CD16^{-}$ groups) which is inflammatory, and $CD14^{low}CD16^{+}$ monocytes which are resident monocytes [186, 187]. In mice, the primary marker for monocytes is Ly6C, and monocytes can also be subdivided based on the levels of Ly6C expression: $Ly6C^{hi}CCR2^{+}CD62L^{+}CX_3CR1^{mid}$ monocytes, and $Ly6C^{low}CX_3CR1^{hi}$ monocytes [188].

Monocytes are capable of differentiating into DCs and macrophages. The primary role of macrophages was once thought to involve phagocytizing dying cells and bacterial pathogens, as well as tissue remodeling. However, they are also important for modulating an immune response, and have been found to be an important aspect of tumour development. Macrophages can differentiate further into specialized cells depending on the inflammatory environment and the location; in some locations there are specific tissue

resident macrophages (eg. Kupffer cells in the liver, alveolar macrophages in the lungs, microglia in the brain). In humans, macrophages are $CD14^{+}CD68^{+}$ [189-191]. In mice, macrophages are detected primarily as $CD11b^{+}Ly6C^{low}F4/80^{+}$ [191, 192].

1.2.1.1 Life cycle and development

During fetal development, hematopoiesis starts in the yolk sac with the synthesis of fetal erythrocytes [193]. In the yolk sac a group of macrophages termed “primitive macrophages” are seen, and from these cells another subset of cells known as “fetal macrophages” are formed, that are phagocytic [194, 195]. It is thought that these macrophages develop through an accelerated pathway, in which there is no intermediate monocyte stage [194, 195]. Whether these fetal macrophages persist into adulthood is unknown. Later in development, hematopoietic stem cells derived from the yolk sac [195] and from aorta-gonads-mesonephros [196] colonize the fetal liver [193]. In the fetal liver primitive and fetal macrophages are still present, but there are also greater numbers of traditional monocytes and monocyte precursors [195]. At a later time point the stem cells then colonize the bone marrow [193].

Monocyte development in mice is dependent on CSF1 [197, 198]. CSF1 is shown to be important for proliferation, differentiation, adaptation, and survival of mononuclear cells. However, certain populations of macrophages and monocytes are not altered in the absence of CSF1 [183], including lymph node macrophages and bone marrow monocytes. Depletion of CSF1 receptor (CSF1R) positive cells does not deplete all populations of resident macrophages, and there is only partial depletion of monocytes in the blood, which does not include depletion of inflammatory monocytes [183]. Selective

gene deletions in mice that include CSF1 or CSF1R knockout have deficiencies in the central nervous system, mammary glands, bones, and reproductive organs [199-201].

The general development of monocytes begins with hematopoietic stem cells, as stained as lineage (Lin)$^{-}$cKit^{+}Sca-1^{+}. Lineage negative indicates the cells do not express markers typically seen in fully mature immune cells, such as B220 for B cells, Thy1.1 for T cells, and Ter119 for erythroid cells [202]. Hematopoietic stem cells can then develop into a strictly myeloid cell progenitor called common myeloid progenitor (CMPs; Lin^{-}Thy1^{-} IL-7Rα^{-}Sca1^{-}cKit^{+}FcγR1^{lo} CD34^{+}) which can develop into granulocyte-macrophage progenitors (GMPs; Lin^{-}Thy1^{-}IL-7Rα^{-}Sca1^{-}cKit^{+}FcγR1^{hi}CD34^{+}) [203]. These go on to develop macrophage and dendritic cell precursor (Lin^{-}cKit^{+}CSF1R^{+}CD135^{+}Ly6C^{-} CD11b^{-}) [204, 205], and then common monocyte progenitor (cMoP; Lin^{-} cKit^{+}CSF1R^{+}CD135^{-}Ly6C^{+}CD11b^{-}) [205]. These cMoPs can then develop into monocytes [205] which are Ly6C^{hi}, but can also differentiate into Ly6C^{low} in the bone marrow [204, 206]. These Ly6C^{hi} monocytes can then leave the bone marrow to be part of the peripheral blood monocyte population [204].

There are several theories as to the source of tissue resident macrophages. Some tissue-resident macrophages are thought to develop from embryonic progenitors that begin seeding the tissue during fetal development [206], as opposed to recruitment of monocytes from circulation [207]. From the yolk sac these myeloid cells can be recruited to become the precursor of skin resident cells and microglia [208, 209]. Some monocytes leave the blood to fill the pool of residential macrophages in tissue during inflammation in some locations [185]. However, recent studies show that during steady state, it is the local macrophages that replicate and replenish the local population [207], although in

some sites, such as in the skin [210] and gut [211], resident tissue macrophages can also be replenished by circulating monocytes. With lethal irradiation and subsequent bone marrow transplantation, macrophage recovery in the tissue is thought to be dependent on recruitment of circulating progenitors [207]. Under certain situations, such as in the presence of IL-4 [212] or CSF1 and CSF2 [207], tissue macrophages can proliferate [213], which allows for maintenance of the local population of macrophages. In humans, CSF1, CSF2, and IL-3 are shown to induce proliferation of monocytes [214].

There are monocyte reserves found in the spleen [215], which leave during inflammation and get recruited to the site of inflammation [216]. There is also the discovery of extramedullary hematopoiesis that occurs in the spleen to create more monocytes. Increased mobilization of hematopoietic stem cells can be induced by CSF3 [217]; the mechanism is by decreasing CXCL12 levels in the bone marrow, which is important for retainment of stem cells [218]. Under certain conditions such as chronic inflammation or cancer development, these rare hematopoietic cells in the blood can get recruited to the spleen and create a monocyte and neutrophil reservoir in the spleen that can then go into circulation [219, 220], in a process known as emergency myelopoiesis, or abnormal myelopoiesis in cancer. Extramedullary progenitor cells have also been shown in human cancer patients [28]. In the peripheral blood of cancer patients, hematopoietic cells constitute around 1% of the population [221]. This process is driven by IL-3, CSF2, CSF3 and type 1 IFNs, and is enhanced by TLR signaling [222-224], while cytokines enhanced in the tumour microenvironment, such as VEGF, CSF1-3, IL-3, and stem cell factor, can also induce abnormal myelopoiesis [191, 225]. What is different with myelopoiesis in cancer as opposed to typical emergency myelopoiesis, is that abnormal

myelopoiesis leads to an increase in immature myeloid cells in circulation [191]. These immature myeloid cells can become MDSCs.

The Ly6C^{hi} population in the bone marrow is recruited to blood circulation, which can then differentiate into Ly6C^{low} monocyte population in circulation [204, 206]. The Ly6C^{hi} population is the predominant population that is recruited to sites of inflammation and tissue remodeling [188]. These inflammatory monocytes usually express CCR2^{hi}CX$_3$CR1^{low}. The Ly6C^{hi} monocytes can also under steady state migrate back into the bone marrow and contribute to both the circulating monocyte pool or the local Ly6C^{low} monocyte population [204]. In humans, there are differences found in the inflammatory CD14$^+$CD16$^+$ and CD14$^+$CD16$^-$ monocytes. They are both capable of phagocytosis, but CD14$^+$CD16$^-$ monocytes produce high levels of reactive oxygen species (ROS), IL-6, CXCL8, CCL2 and CCL3 in the presence of lipopolysaccharide (LPS), while CD14$^+$CD16$^+$ do not produce ROS and produce IL-1β, TNF, IL-6, and CCL3 [187].

The Ly6C^{low} population “patrols” the endothelium via a mechanism dependent on LFA-1, Mac-1 and CX$_3$CR1 [226, 227]. These cells are also required for the extravasation and tissue invasion of inflammatory monocytes during infection [226]. During inflammation Ly6C^{low} monocytes can also be recruited to the site of inflammation and are the primary source of TNF [226]. Ly6C^{low} monocytes are primarily involved in repair of the endothelium, and can also be involved neutrophil recruitment to help with endothelial repair [227]. These monocytes have a steady-state half-life of 2 days [206]. What supports the survival of Ly6C^{low} is CSF1R and NUR77 signaling [183, 206, 228], although NUR77 signaling is also important for controlling macrophage polarization, as

NUR77 knockouts have increased M1 proinflammatory macrophages [229]. These monocytes typically express CX_3CR1^{hi} [226, 227] and are thought to be the precursor of some tissue resident macrophages [230]. In humans, the equivalent population is $CD14^{dim}CD16^+$ [187]. $CD14^{dim}CD16^+$ do not respond to bacterial antigens, but are capable of producing proinflammatory cytokines in response to viruses [187].

Typically, upon migration into tissue, monocytes differentiate into macrophages or DCs, but a study by Jazubzick *et al* [231] showed that under steady state conditions, monocytes can migrate into tissue without differentiating into macrophages and DCs, and can also leave the tissue as monocytes. Furthermore, these monocytes can take up antigens [232]; specifically, $Ly6C^+$ monocytes in the blood can increase MHCII expression by a mechanism that is dependent on transendothelial migration, to become antigen-presenting monocytes [231]. These monocytes then enter the lymph nodes, both via the blood or lymphatic vessels [231], and can then go on to differentiate into DCs [233].

The CCR2-CCL2 axis is important for the recruitment of monocytes from bone marrow to circulation, and lack of CCR2 leads to accumulation of monocytes in the bone marrow [234]. CCL2 is also important for recruitment of $Ly6C^{hi}CCR2^+$ inflammatory monocytes into peripheral lymph nodes during inflammation [235], although in some infection models, it is shown that once in circulation, monocyte recruitment to inflamed sites is CCR2-independent [234]. In an infection model IL-10 expression led to a decrease in CCL2 expression and therefore decreased monocyte recruitment [236]. CCR2 signaling is also important for recruitment of inflammatory monocytes from the splenic reservoir [237]. Once monocytes differentiate into macrophages, there is downregulation of CCR2 and CX_3CR1 [238, 239]. With this differentiation there is an increase in CCR1 and CCR5

[230, 240]; CCL3 signals through CCR1 and CCR5, and CCL3 is upregulated at the site of inflammation for recruitment of macrophages [241].

1.2.1.2 Function

At steady state, tissue macrophages have anti-inflammatory functions. In the intestines, if there is breakdown of the IL-10 production by macrophages, severe intestinal inflammation can occur [242]. Also in the marginal zone of the spleen, macrophages are suppressive to prevent self-reactivity to apoptotic cells found in the blood, and the loss of these macrophages can lead to increased inflammation associated with autoimmunity [243]. Macrophages in the tissue are important for immune surveillance and are part of the first line of immune cells to be activated when a pathogen is introduced into the system [244]. Macrophages are professional phagocytes that can ingest and destroy the foreign pathogen. Furthermore macrophages are important for maintenance of healthy tissue, by ingesting dead cells and any foreign materials that are in the system (eg. alveolar macrophages remove allergens in the lungs, Kupffer cells clear pathogens and toxins in the liver) [245-247].

Macrophages can become polarized into two different groups; the classically activated macrophages, also known as M1 macrophages, and alternatively activated macrophages, also known as M2 macrophages. Bacterial LPS (via induction of IFNβ), IFNγ, and CSF2 can promote M1 generation [106, 248, 249]. In general, M1 macrophages are associated with a T_H1 response; M1 is characterized by increased microbicidal activity (as indicated by elevated expression of inducible nitric oxide synthase [NOS2] [106]), increased antigen presentation and T_H1 stimulation associated with MHC class II expression [249], and increased IL-12 production [250]. M1 can also secrete TNF, IL-1, IL-6, IL-18, and

IL-23 [249] to promote inflammation. As IL-12 and IL-23 can promote T_H1 and T_H17 environment, M1 is involved in autoimmune disease [251]. M1 macrophages can switch gene expression to become anti-inflammatory M2 macrophage; in a study by Arnold *et al*, inflammatory M1 macrophages upregulated secretory leukocyte protease inhibitor, which is upregulated by proinflammatory signaling but causes downregulation of proinflammatory signaling [252]. Phagocytosis of apoptotic cells by M1 macrophages were also shown to induce the M2 phenotype [253]. This switch in phenotype is a mechanism of inhibiting hyperinflammation once the immunogen has been cleared.

M2 macrophages are associated with T_H2 responses, including allergy, parasite clearance, tissue remodeling and tumour promotion. CSF1 induces monocyte to macrophage differentiation and causes M2 development [238], suggesting that under homeostatic conditions, M2 is the "default setting" for macrophages. M2 macrophages can be induced by a variety of mediators, including IL-4 and IL-13 [212], antibody-antigen complexes and TLR and IL-1R ligands [254, 255], and IL-10, which give slightly altered M2 phenotypes [230]. Interestingly, in the presence of T_H1 $CD4^+$ T cells that express CD40 ligand and IFNγ, M2 macrophages differentiate into M1 macrophages [250], a mechanism which allows for induction of M1 macrophages during an inflammatory response. Although M2 cells are capable of phagocytosis, they do not stimulate T cell proliferation [249] due to decreased antigen presentation, but are capable of being APCs that induce T_H2 and Tregs. M2 macrophages also engulf dead cells and extracellular matrix components, which would otherwise promote an M1 phenotype. M2 macrophages express the enzyme Arg1, which hydrolyzes arginine into urea and ornithine which can stimulate wound healing [106]. Arg1 activity is also capable of inhibiting immune cells

by depleting the environment of arginine, which is needed for both T_H1 and T_H2 cell proliferation [256]. M2 macrophages can secrete IL-10 [249], TGFβ, platelet-derived growth factor, MMPs and tissue inhibitors of metalloproteinases (TIMPs), which are important for immune suppression and tissue repair. TGFβ produced by M2 is capable of inhibiting NOS2, therefore inhibiting an M1 response [106]. Arg1 and NOS2 compete with each other for the same substrate, which would therefore be another mechanism of regulating M1 function.

1.2.1.3 Tumour associated monocytes and TAMs

There is debate on where TAMs are derived, whether from blood monocytes or tissue-resident macrophages. A study by Cortez-Retamozo *et al* showed that splenectomized mice have decreased TAMs, suggesting that extramedullary hematopoiesis may be important for TAM formation [28], with low contribution from the bone marrow. A study by Qian *et al* showed that at the primary breast tumour, the macrophages were primarily resident tissue macrophages, while at the metastatic niche, they are predominantly recruited inflammatory monocytes [82]. In a study by Leuschner *et al*, silencing of CCR2 *in vivo* in mice with palpable tumours decreased tumour growth and TAM numbers, which would suggest that recruitment is important for the TAM population [237]. In another study, MDSCs were shown to differentiate into macrophages and DCs in the spleen, while in the tumour they would differentiate into immune suppressive TAMs with high levels of NOS2 and Arg1 [257], which were thought to be induced by the hypoxic environment in the tumour [258]. Therefore, the origin of TAMs may depend on the tumour type and whether it is the primary or secondary site of tumour development.

TAMs make up the largest population of leukocytes in most solid tumours [259]. TAMs can be found localized throughout the tumour; there are fewer macrophages deep within the tumour unless associated with tumour vasculature, and higher levels of TAMs in the tumour periphery, where TAMs enhance the ability of tumour cells to invade surrounding tissue [260, 261] for tumour expansion and intravasation. TAMs are not a stable consistent population; they are continuously being replaced during tumour progression [28]. TAMs are known to promote tumour growth [28, 262], progression [28, 263], invasion [260], metastasis [260, 264], vascularity [262, 265], and chemotherapeutic resistance [262, 266, 267]. Increased levels of TAMs or monocytes are associated with decreased survival in some cancers [10, 81, 268-270]. A variety of chemokines and cytokines are capable of recruiting monocytes to the tumour, which differ depending on the tumour type. These mediators include CSF1 [263], CCL2 [28], CCL3, CCL4, CCL5, CCL8, CXCL12, and VEGF [259, 271]. Some cancers cause an increase in inflammatory monocyte circulation, but upon entering the tumour microenvironment, their phenotype changes to a more immunosuppressive type; tumours are shown to secrete CSF1 to shift the monocyte population to an M2/TAM macrophage phenotype [81]. TAM induction may also be dependent on IL-4 production by T_H2 $CD4^+$ T cells [260]. In humans, M2 macrophages found in tumours are thought to be induced by the tumour via production of IL-6 and CCL2 [272]. However, there are also some studies that suggest that TAMs have a distinct transcriptome from M1 or M2 [273]. One study showed that they express many properties of M2 macrophages but also express IFN-inducible chemokines [271]. Although there are differences in opinion regarding TAM phenotype, it is widely

accepted that M2 macrophages are protumourigenic, while induction of M1 macrophages would lead to decreased tumour burden.

TAMs are capable of having suppressive functions on immune cells. A study by Biswas *et al* showed that IL-10 and TGFβ levels are higher in TAMs compared to MDSCs [271]. Another study also showed that TAMs were more immunosuppressive than MDSCs due to enhanced ROS synthesis [274]. TAMs can express MHC class II but express low levels of costimulatory molecules CD80 and CD86 [273], which would create anergic T cells. TAMs can suppress $CD8^+$ T cell proliferation [10, 264], effector function [275], and induce apoptosis [273], and depletion of TAMs leads to increased CTL infiltration [10]. TAMs are also capable of recruiting Tregs (via CCL17 and CCL22 [275, 276]) and also induce differentiation of naïve T cells into Tregs via IL-10 and CCL18 [275]. Although typically not thought of as one of the main mechanisms of tumour suppression in the tumour microenvironment, TAMs can still have immunosuppressive functions that can alter tumour outcome.

Tumour invasion is facilitated by TAMs by their secretion of proteolytic enzymes and MMPs to help breakdown extracellular matrix [275]. TAMs facilitate tumour cell extravasation with the secretion of VEGF and epidermal growth factor [82, 260, 261]. TAMs also secrete CCL5 [275], which can directly promote tumour cell invasion, EMT, and metastasis [277-279]. In some tumour models at a pre-metastatic site, there is an increase in CCL2 production, followed by increased inflammatory monocyte recruitment [81-83], suggesting the importance of these monocytes in setting a metastatic niche. This effect has been shown to be important in lung and bone metastasis in breast cancer, where CCL2 increased activation of osteoclasts, myeloid cells involved in bone remodeling

[83]. Interestingly, in weakly metastatic tumour cell lines, CCL2 is capable of inducing lung metastasis but not bone metastasis, indicating some organotropic specificity [83].

1.2.1.4 Antitumourigenic monocytes and macrophages

M1 macrophages are thought of as antitumourigenic, since they can directly affect tumour development. IFN can induce cytolytic activity by macrophages [33], which includes secretion of superoxides and nitrogen radicals [33, 280, 281]. STAT6-deficient mice have decreased M2 macrophages and increased M1 macrophages, and these macrophages are cytotoxic towards tumour cells in a NOS2-dependent manner [282, 283]. M1 macrophages can also alter metastasis. In the absence of IL-4 signaling, TAMs are shown to have an increased M1 phenotype, which can prevent tumour cell invasion and metastasis [260].

M1 macrophages can present antigens and produce IL-12 [250], allowing for stimulation of the T_H1 phenotype [106, 249]. In the presence of IFNγ, TAMs were induced to produce IL-12 and decrease IL-10 production, and allowed for increased costimulatory activity with T cells and enhanced CTL activity [275]. Macrophages stimulated under certain conditions can cause increases in NK cell cytotoxicity against tumour cells due to macrophage-dependent induction of NKG2D expression and IFNγ synthesis [284]. Macrophages also had increased NKG2D ligand expression that could induce NK cell activation, and produced and secreted IL-15, IL-18, IL-12, and IFNβ, which are all capable of inducing NK cell activation.

1.2.2 Neutrophils

Neutrophils are the most populous leukocyte group in circulation with a half-life of 6-8 hours [285]. They are the first immune infiltrate at the site of inflammation, and are

involved in clearance of pathogens and also release cytokines and chemokines to increase recruitment of immune cells to the site and cause activation. Human neutrophils are $CD11b^{+}CD16^{+}CD15^{+}CD49d^{-}$ [286, 287]. Mouse neutrophils are $CD11b^{+}Ly6G^{+}Ly6C^{low}$ [288, 289].

1.2.2.1 Development and Life cycle

During fetal development neutrophils start appearing at the stage where the fetal liver is the primary site of hematopoiesis [195]. After that neutrophil development occurs in the bone marrow in a process that takes 6-8 days [285], where it is regulated primarily by CSF3[290], but also by IL-6, CSF2, and IL-3 [285]. Similar to monocytes, GMPs are a precursor to neutrophils; to become neutrophils, GMPs differentiate into myeloblasts ($Lin^{-}cKit^{hi}Ly6G^{-}$) [291], then promyelocytes ($Lin^{-}cKit^{int}Ly6G^{-}$) [291]. Promyelocytes become myelocytes ($Lin^{-}cKit^{int}Ly6G^{low}$), then metamyelocytes ($Lin^{-}cKit^{low}Ly6G^{int}$), then band cells, segmented cells ($Lin^{-}cKit^{low}Ly6G^{hi}$) [291], and finally neutrophils [292]. At this point, neutrophils can either be part of a reserve in the bone marrow to be released at a later time [285], or are released into circulation, which is controlled by CXCL8 and CSF3, by causing a decrease in CXCL12 in the bone marrow [290] and by decreasing CXCR4 expression on neutrophils [285]. Endotoxin can also lead to increased neutrophil release from bone marrow [285].

Upon getting close to the end of their life cycle, aged neutrophils upregulate CXCR4, CD11b, and CD49d, and decrease CD62L [293]. This CXCR4 upregulation allows for localization of neutrophils to bone marrow and spleen [285, 294]. Aged neutrophils also mobilize to the liver but it is not dependent on CXCR4 [294]. Apoptosed neutrophils are phagocytosed by bone marrow macrophages [293], which leads to release of CSF3 by

macrophages to induce granulopoiesis and neutrophil release to replenish the neutrophil population [294].

1.2.2.2 TANs

Similarly to TAMs, TAN progenitors are found in the spleen and TANs are continuously being replaced during tumour progression [28]. TANs have been associated with poor clinical outcome in several tumour types, including melanoma, colorectal cancer (CRC), hepatocellular carcinoma, ovarian cancer, and lung cancer [295]. In this study there were increased neutrophils in circulation, which correlated to a decreased response to cytokines in immunotherapy, chemotherapy, and/or targeted therapy. Depletion of neutrophils leads to decreased tumour growth [296]. Neutrophils can have a direct impact on T cells via production of hydrogen peroxide (H_2O_2), which can decrease T cell receptor (TCR)ζ, IFNγ, TNF, and IL-2 expression in T cells [297]. Fridlender *et al* [298] showed that lack of TGFβ led to an increase in neutrophil recruitment to tumours, and depletion of these neutrophils led to increased tumour growth. These neutrophils have increased tumour cytolytic activity dependent on high levels of H_2O_2 and superoxides. A comparison of normal TANs and TANs in the absence of TGFβ showed that when normal TANs are depleted, there is enhanced $CD8^+$ T cell activation, while depletion of TANs that were not stimulated with TGFβ lead to a decrease in $CD8^+$ T cell. These authors suggest that there are two TAN phenotypes similar to macrophages, called N1 (the TAN population without TGFβ) and N2 (normal TANs).

Similarly to M1 and M2 phenotypes, N1 is antitumourigenic, while N2 are protumourigenic. N1 has increased proinflammatory cytokines, decreased arginase activity and increased tumour cytolytic activity. N2 produce mediators involved in

angiogenesis, support EMT, and suppress an antitumourigenic response [297, 298]. Similar to MDSCs, N2 cells express NOS2 and Arg1, and also express IL-10 and IL-4, while N1 cells have decreased Arg1, increased IL-12 and myeloperoxidase, and express CD49d [298, 299]. In Tsuda *et al* [299], N1 can induce M1 phenotype via IL-12 and CCL3, and N2 can induce M2 phenotype via IL-10 and CCL2 [299].

Neutrophils have been shown to directly impact cancer development by causing genetic damage and inducing proliferation. Increases in CXCL8 and prostaglandin E_2 (PGE_2) lead to increased neutrophil infiltrates, resulting in enhanced tumour necrosis and higher genetic mutations [300, 301]. During a chronic inflammatory response, there is an increase in neutrophil degranulation which can impact normal cells. Myeloperoxidase production of hypochlorous acid can induce DNA damage and mutagenicity in lung cells [301, 302]. HGF, which is stored in vesicles and can be released upon degranulation [303], can also induce epithelial cell proliferation, leading to hyperplasia [304]. The absence of neutrophil elastase leads to decreased tumour burden [305], while low concentrations of elastase can induce proliferation. The mechanism was due to elastase being taken up by the cell and degrading insulin receptor substrate 1, a regulator of PI3K, resulting in unregulated signaling by PI3K [305].

Metastasis can also be induced by TGFβ and elastase produced by neutrophils [306]. Neutrophil elastase can cleave E-cadherin, which induces EMT in cancer cells [307]. Inhibition of neutrophil recruitment to the tumour has led to a decrease in metastasis [308]. In bronchoalveolar cancer patients, neutrophils produce HGF which induces tumour cell migration [304]. Neutrophils can induce, via elastase, aggregation of breast cancer cells into a spheroid formation. This is important as these spheroids can form an

embolism in lymphatic and blood vessels and metastasize to another organ [309]. Neutrophils can also enhance metastasis in circulation; coinjection of tumour cells with normal neutrophils in the tail vein in experimental metastasis does not alter metastasis, but coinjection with TANs enhanced invasion and metastasis [310]. In melanoma, neutrophils can impact extravasation of melanoma cells from circulation to the metastatic site. Neutrophils can bind endothelium, then bind melanoma cells in circulation, and "assist" with extravasation [311]. Integrin-β2 on neutrophils interacts with ICAM-1 on endothelial cells, and LFA-1 and Mac-1 on neutrophils is important for tethering the melanoma cells [312]. CXCL8 is also important for Mac-1 upregulation and melanoma extravasation. Melanoma cells can also produce CXCL8 and induce neutrophils to produce CXCL8 [313] which can recruit neutrophils to the lung to help with extravasation of melanoma cells [312].

1.2.2.3 Antitumourigenic neutrophils

Regression in some instances have been shown to be dependent on the presence of neutrophils in the tumour [314-316]. When TAMs decrease, even in presence of TANs, there was a decrease in tumour growth [28], indicating TANs did not hinder tumour clearance. Neutrophils are capable of producing cytokines and chemokines that can cause recruitment of NKs and CTLs [317]. Neutrophil recruitment leads to enhanced $CD8^+$ T cell infiltration and tumour regression, and depletion of neutrophils or $CXCR2^{-/-}$ mice leads to a decrease in $CD8^+$ T cell infiltration into the tumour, therefore decreasing tumour regression [315, 318].

Neutrophils have been shown to be important for tumour cell clearance [314], where IL-2 and TNF can directly induce neutrophil-mediated cytolysis by tumours [317]. Neutrophil

production of hypochlorous acid can also cause tumour cell lysis [319]. In human neutrophils, defensins can cause tumour cell lysis, and it can act synergistically with H_2O_2 to increase lysis [320]. Neutrophils and monocytes treated with IFNα upregulate and secrete TRAIL, and patients treated with IFNα have increased soluble TRAIL which can potentially kill TRAIL-susceptible leukemia cells [321]. Interestingly neutrophil elastase can be taken up by breast cancer cells; intracellular elastase can then break down cyclin E, which can then be processed and presented on the surface, making the breast cancer more susceptible to CTL cytolysis [322]. Neutrophils can also clear tumour cells via ADCC. FcRII, FcRIII and Mac-1 are required for ADCC by neutrophils, and CSF2 can enhance this activity [323].

In a 4T1 breast cancer study, neutrophils were present in high number in pre-metastatic niche in the lung, and depletion of TANs led to enhanced metastasis in the lung [324]. Neutrophils from tumour-naïve mice did not have the same impact. These antitumourigenic TANs were induced by CCL2 and CCL5, and they were highly cytotoxic and were capable of discriminating between tumour cells and normal cells. This induction of apoptosis was cell contact dependent via NADPH oxidase (NOX2) and H_2O_2. This effect is also seen in breast cancer patients, where TANs accumulate in lungs [324]. However, as previously stated, TGFβ significantly inhibits neutrophil cytotoxicity, and 4T1 cells can secrete TGFβ [324], showing another mechanism by which 4T1 cells can subvert an antitumourigenic response.

1.2.3 MDSCs

MDSCs are an immature population of myeloid cells with suppressive activity. In humans, there is variability in markers for MDSCs. Many studies use $CD14^{+}HLA\text{-}DR^{low/-}$

[325-329]. Other studies have used $Lin^{lo}CD11b^{+}CD33^{+}CD34^{+}$ $CD14^{-}HLA\text{-}DR^{-}$ [329-331]; specifically granulocytic MDSCs (G-MDSCs) are $Lin^{-}CD33^{+}CD15^{+}$, and monocytic MDSCs (M-MDSCs) are $CD14^{+}HLA\text{-}DR^{low/-}CD86^{low/-}$ $CD80^{low/-}$ [332]. In mice, the markers for identification are $CD11b^{+}Gr1^{+}$. These MDSCs can be divided into $Gr1^{hi}$ (G-MDSCs) and $Gr1^{int}$ (M-MDSCs) [333]. The Gr1 epitope is composed of Ly6C and Ly6G, which can further divide MDSCs into $CD11b^{+}Ly6C^{hi}$ M-MDSCs and $CD11b^{+}Ly6G^{+}Ly6C^{low}$ G-MDSCs. These markers in mice are shared with monocytes and granulocytes, respectively. Recent literature has shown that a marker for MDSCs can include CD49d (also known as VLA-4). M-MDSCs are $CD49d^{+}$, and G-MDSCs are $CD49d^{-}$ [334, 335]. However, the popular method of identifying whether these cells are suppressive is with a T cell proliferation suppression assay.

Typically, there are higher MDSC numbers in tumour-bearing mice. Immature myeloid cells (IMCs) from the spleen and bone marrow naïve mice are unable to inhibit T cell activation, but IMCs from the spleen and bone marrow of tumour-bearing mice inhibit IFNγ production [336-338]. MDSCs are increased in a variety of cancers, including colorectal, pancreatic, gliomas, and breast, and are strongly correlated with the immunosuppression that is present in cancer patients [328, 331, 332, 339-343]. Furthermore, increases in MDSCs correlate with a worse prognosis [330, 331], including increases in tumour size [119], decreases in $CD4^{+}$ T cells in the tumour [119] and increased metastasis [330]. In humans, $CD14^{+}HLA\text{-}DR^{low}$ MDSCs, compared to mature $CD14^{+}HLA\text{-}DR^{+}$ monocytes from the same patients, have much higher levels of Arg1, which causes suppression of T cell proliferation and IFNγ production [328].

MDSCs are the primary source of splenomegaly in cancer development [344]. Numbers of MDSCs in tumour-bearing mice can range from 20-40% of splenocytes depending on the tumour model, while normal mice have approximately 2-4% [34]. MDSCs can also be found in the tumour tissue, sites of metastasis, and the lymph nodes [345]. In the tumour-draining lymph node, with MDSC depletion, there is enhanced T cell activation, even in the presence of Tregs [344]. Analysis of the tumours in one study suggested that M-MDSCs were distributed throughout the tumour while G-MDSCs were predominantly in the center [346]. In this study MDSCs were recruited from the bone marrow to the tumour [346, 347], although this is controversial. M-MDSCs can proliferate more rapidly than the G-MDSCs, but the survival of both populations is supported by the tumour microenvironment [346, 347].

It should be noted that not all tumours increase MDSCs; some tumour cell lines induce >15% MDSC expansion, including 4T1 and EL4, while others have <5% expansion of MDSCs. There were also tumour cell lines that have intermediate expansion (10-15% increase), such as LLC1 and B16-F10 [113]. Furthermore, not all tumour models have the same ratio of G-MDSCs to M-MDSCs [333]. This is in part due to a direct effect by the tumour cells [113]. MDSCs can also accumulate during pregnancy [348], experimental autoimmune encephalomyelitis [349], inflammatory bowel disease [350], Crohn's disease, autoimmune disease, transplantation tolerance [351, 352], parasite infection [353], and sepsis [354]. Interestingly, in naïve mice, there is an increased percentage of MDSCs in older mice compared to young mice [355]. MDSCs from older mice but not younger mice can inhibit cytotoxic T cell activity and proliferation in a contact-dependent

manner, due to increased arginase activity. Furthermore adoptive transfer of MDSCs from older mice to younger mice delayed tumour regression [355].

Continuous stimulation of myelopoiesis and tumour-derived soluble factors leads to development of MDSCs [34]. In the peripheral blood of cancer patients, one third of circulating cells are immature monocytes/macrophages and DCs with immunosuppressive activity [221]. MMP9 expression by MDSCs can also release soluble Kit ligand in bone marrow, which can induce hematopoietic stem cell proliferation, and therefore expand the MDSC population [356]. Tumour-produced mediators can also induce MDSC accumulation, including stem cell factor, urokinase, CSF2, CSF1, IL-1β, IL-6, VEGF, and IDO [30, 357-361]. MDSCs are also induced by tumours to increase fatty acid oxidation, and when this fatty acid oxidation is inhibited, suppressive activity against T cells is decreased, due to decreased Arg1 and NOS2 activity [362]. Lactate, which is elevated in the tumour microenvironment, can also induce MDSC development and increase their ability to suppress NK cell activity [363]. MDSCs can also produce mediators that induce MDSC differentiation including CSF2, CSF3, IL-6, and IL-10 [362]. In the 4T1 model if IL-1R is knocked out, MDSCs accumulated at a later time, which was dependent on IL-1 signaling inducing IL-6 which can signal on MDSCs [364]. Conversely, increases in IL-1β can lead to increases in MDSCs at the primary tumour, metastatic sites, blood, and spleen [220, 365]. MDSCs express receptors EP1-4, which allows them to respond to PGE_2 [366]. PGE_2 causes an increase in MDSC levels in bone marrow cells [366]. EP2-deficient mice show retarded tumour growth and reduced MDSC accumulation, and the MDSCs that were present were less suppressive [366], which may be due to PGE_2 enhancing Arg1 expression in MDSCs [367].

As MDSCs are immature myeloid cells, there is the potential for them to differentiate into mature myeloid cells, particularly M-MDSCs to differentiate into macrophages and DCs. Several different methods have been shown to enhance differentiation of MDSCs into mature cells. 25-hydroxyvitamin D_3, a derivative of vitamin D_3, can lead to decreased immature myeloid cells in circulation due to differentiation into DCs, which included increases in IL-12 and HLA-DR, and increased T cell proliferation and IFNγ production in cancer patients [368]. Blockade of cyclooxygenase 2, therefore leading to decreases in prostaglandin E_2, induces a decrease in MDSCs and an increase in myeloid DCs, which is followed by a decrease in NOS2 and Arg1, and decreased tumour infiltration by myeloid cells [366, 369]. CSF2 and IL-4 have been shown to induce differentiation of M-MDSCs into DCs *in vitro* [221], although this is not observed in all cases [328]. All-trans retinoic acid has also been shown to induce MDSC differentiation into mature myeloid cells in cancer patients. In the presence of CSF2, IL-4, and all-trans retinoic acid, MDSCs differentiate and lose their T cell suppressive capabilities [370].

MDSCs express CXCR2, CXCR7 and CXCR4, and therefore chemokines such as CXCL5 and CXCL12 can recruit MDSCs [371]. There are also differences in chemokine receptor expression between MDSC subtypes; G-MDSCs express CXCR2 and CXCR4, and M-MDSCs express CCR2, CCR5, CXCR4, and CX_3CR1 [346, 372]. In CCR2 knockout mice, there are increased levels of CSF1, CXCL1 and CXCL2, which interact with CXCR2, leading to increased neutrophil and G-MDSC recruitment to the tumour, compared to wild type [346]. When there is CSF2 production by the tumour, there is greater recruitment of $CCR2^+$ suppressive M-MDSCs to the tumour [372]. CCR2 knockout mice had no MDSCs in the tumour or spleen [372]. G-MDSC in some models

can be preferentially recruited to primary tumours, which was dependent on tumours expressing CCL19, CXCL1, CXCL2, and CXCL5, and G-MDSCs express CXCR2 which is the receptor for CXCL1, CXCL2, and CXCL5 [373].

STAT3 hyperphosphorylation and DNA binding can occur in DCs and MDSCs in the presence of mediators released by tumours, including VEGF, CSF3, and CSF1 [374]. In the presence of tumour supernatant there is accumulation and proliferation of IMC, and removal of tumour supernatant allowed for the IMCs to differentiate [374]. Tumour derived factors can induce STAT3 phosphorylation in DCs, and cause them to be unable to induce T cell proliferation, due to decreased MHC II and costimulatory molecules [374]. Inhibition of STAT3 signaling *in vitro* causes a decrease in immature myeloid cells and an increase in DC development, and leads to increased macrophages and DCs in tumour models *in vivo*, but not in non-tumour-bearing mice [375]. These DCs are capable of inducing $CD4^+$ T cell production of IL-2, IFNγ, and IL-10 [375]. Inhibition also leads to increased apoptosis in IMCs [375]. MDSCs can produce IL-6, which can also activate STAT3, which leads to inhibition of DC maturation [376], therefore showing another mechanism by which MDSCs can enhance MDSC accumulation. STAT6 is also thought to be important for MDSC function; STAT6 deficient mice have decreased metastasis that is dependent on the presence of IFNγ, which may correlate with alterations in MDSCs [282]. Furthermore, IL-4 and IL-13 signal through STAT6, which then leads to upregulation of Arg1 [377].

1.2.3.1 MDSC mediators and functions

MDSCs mediate immunosuppression through multiple mechanisms; two of the main mechanisms are through the actions of NOS2 and Arg1. Arginine is a non-essential

amino acid, but under certain conditions that include tumour microenvironments there is increased arginine metabolism and it is a conditional essential amino acid required for T cell activity. Both enzymes deplete arginine from the system, albeit using different mechanisms; NOS2 oxidizes arginine to generate nitric oxide (NO) and citrulline, while arginase breaks down arginine into urea and ornithine [378]. NOS2 has a significant K_m advantage over Arg1 and will out-compete Arg1 for arginine, therefore Arg1 must be present before NOS2 is upregulated to deplete arginine for NOS2 to not be able to synthesize NO [377]. Absence of arginine causes a decrease in CD3ζ protein expression and mRNA expression [379]. Decreased arginine can also lead to decreased proliferation of T cells, with T cells arrested in the G_0-G_1 phase, due to decreases in cyclins (specifically cyclin D3) and decreased phosphorylated Rb [380]. Most studies show that the mechanism is contact-dependent between MDSCs and T cells [220]; even if MDSCs were separated from the T cells by a transwell membrane, but the MDSCs are stimulated with T cells, the separated T cells are not suppressed [381]. The mechanism is predominantly due to the mediators produced by NOS2 and Arg1 being short lived, therefore the T cell needs to be in close proximity to be affected by the mediator.

Whether it is NOS2 or Arg1 that is the main mechanism of suppression, or whether both need to be present for suppressive activity appears to differ between studies and models. Some studies show that the predominant mechanism of T cell proliferation is through NOS2 activity [381]. In a study by Mazzoni *et al*, MDSCs through NOS2 potently inhibit T cell proliferation by preventing T cells from entering the cell cycle, and NOS2 knockout MDSCs lacked suppressive activity [382]. This mechanism was found to be effective in both antigen-dependent and independent manner, and was reversible; the

mechanism was found to be dependent on IL-2 and IL-2 receptor levels, with inhibition of phosphorylation of downstream mediators including STAT5b, Erk1/2, and Akt [382]. In one study, not only did NO suppress $CD4^+$ and $CD8^+$ T cell proliferation, but also had a direct effect on inducing tumour cell proliferation [339].

NOS2, aside from depletion of arginine and nitric oxide production, can also cause inhibition with the production of peroxynitrite. Peroxynitrite ($ONOO^-$) is a chemical reaction between NO and superoxide. It can cause nitration and nitrosylation of amino acids. Nitrosylation of the TCR affects its conformational flexibility, which then hinders its ability to bind to MHC. Peroxynitrite can also cause nitrosylation of MHC class I on tumour cells, affecting MHC binding to peptide, therefore protecting the tumour cells from CTL-mediated cytolysis [383]. Depletion of arginine by Arg1 triggers generation of superoxide from the reductase domain of NOS2, which can contribute to peroxynitrite generation [384]. Kusmartsaev *et al* showed that NOS2 inhibition and addition of a superoxide dismutase mimetic alleviated MDSC suppressive activity, suggesting that the mechanism of action of NOS2 was through production of NO and peroxynitrite [338, 385]. Peroxynitrite can inhibit T cell activity through tyrosine nitration of TCRs, leading to inhibition of CTL IFNγ production and cytotoxic activity [385, 386]. Clearance of peroxynitrite with uric acid *in vivo* did not impact tumour development, but when combined with a vaccine, causes a significant decrease in tumour development [385]. Interestingly, peroxynitrite can also cause nitrosylation of chemokines, including CCL2 and CXCL12, making these chemokines have a weaker affinity for their chemokine receptor, and therefore abrogating T cell recruitment to the tumour. However, myeloid

cells have higher expression of CCR2, therefore the myeloid cells can still migrate in the presence of nitrosylated CCL2 [387].

In other studies Arg1 activity was important for suppression [388]. Bunt *et al* showed that suppression of antigen-specific $CD4^+$ T cells was Arg1-dependent [365], but in another study Arg1 was suppressive against $CD4^+$ and $CD8^+$ T cells [364]. Arg1 activity can cause a decrease in CD3ζ expression on T cells, and lead to a decrease in the production of IL-2, IFNγ, IL-4, and IL-10, and depletion of MDSCs causes the reversal of CD3ζ downregulation [389]. Activated neutrophils and G-MDSCs can release Arg1, but after this activation, neutrophils go through apoptosis, while G-MDSCs survive. This release of Arg1 by G-MDSCs into circulation leads to extracellular depletion of arginine [342]. In melanoma patients, MDSCs had increased Arg1, and Arg1 and ROS were shown to be the main mechanism of suppression, with decreased $CD4^+$ and $CD8^+$ T cells proliferation and IFNγ production [343].

Another mechanism of immunosuppression is the production of ROS, such as H_2O_2. ROS are produced by MDSCs and are regulated by NOX2 [360, 390]. As ROS are short lived, it is typically effective in a cell contact-dependent manner. IFNγ can upregulate expression of NOX2 [335]. In mice with NOX2 knockouts, MDSCs are more readily able to mature into macrophages and DCs, revealing ROS may be important for maintaining the MDSC phenotype [390]. ROS can also decrease IFNγ production and CTL activity, due to decreased TCR affinity to MHC class I [385, 390]. TCR ζ chain expression levels decrease in advanced cancer patients, and there is decreased IFNγ and IL-4 release, which was dependent on H_2O_2 production by G-MDSCs [297].

MDSCs can also produce TGFβ and IL-10 which can be enhanced by IFNγ [391]. In one study MDSCs were the main source of TGFβ, which decreased CTL activity during the induction phase [392, 393]. In melanoma patients, a peptide vaccine in combination with CSF2 injection increases $CD14^{+}CD11b^{+}$ MDSCs, which suppressed IFNγ and perforin production in $CD8^{+}$ T cells in patients in a TGFβ-dependent manner [327]. In another study, G-MDSCs expressed TGFβ which induced EMT [373]. MDSCs in cancers patients can express high levels of IL-10 [325, 332]. MDSCs in direct contact with M2 macrophages lead to a synergistic increase in IL-10 production by MDSCs [394].

MDSCs are also capable of affecting NK cell activity. MDSCs are capable of suppressing NK cells, leading to increases in metastasis [348]. Increased MDSC presence is correlated with decreased NK cell activity [395]. MDSCs can inhibit NK cell cytotoxicity and IFNγ secretion by NK cells in a contact-dependent manner via interaction with NKp30 on NK cells [396]. MDSCs are capable of decreasing IFNγ secretion and NKG2D expression [395]; in this study the mechanism was found to be membrane-bound TGFβ on MDSCs. MDSCs in this study were found to be the most potent suppressor of NK cells, and the absence of MDSCs caused NK cell-dependent tumour clearance [395]. MDSCs stimulated with IL-1β are capable of inhibiting NK cell maturation, and the NK cells that are present have decreased NKG2D expression and decreased cytolytic activity [397].

It is important to note that some studies have shown suppressive differences between M-MDSCs and G-MDSCs. M-MDSCs are more immunosuppressive on a per cell basis when compared to G-MDSCs (in terms of their ability to suppress IFNγ production and proliferation) [113, 333, 334]. Another study showed that NOS2 and Arg1 activity is

higher in $CD49d^+$ M-MDSCs, while synthesis of ROS is higher in $CD49d^-$ G-MDSCs [334]. In a study by Movahedi *et al*, NOS2 via the synthesis of NO, and to an extent cyclooxygenase 2, is a mechanism by which M-MDSCs suppress T cell proliferation, and Arg1 is a mechanism by which G-MDSCs have suppressive effects, but does not appear to be the most prominent mechanism [335]. G-MDSCs but not M-MDSCs were capable of suppressing NK cell IFNγ and granzyme B production via ROS production [398]. In a study by Youn *et al*, both MDSC subsets inhibited T cell proliferation, while $CD8^+$ T cell IFNγ production was inhibited only by G-MDSCs [113]. M-MDSCs are also capable of producing peroxynitrite, and are capable of inducing $CD8^+$ T cells to synthesize peroxynitrite [113]. In another study, G-MDSC specific depletion led to decreased primary tumour size, even in the presence of M-MDSC [373]. One study by Dolcetti *et al* showed that M-MDSCs from a variety of tumour models were immunosuppressive, while G-MDSCs were only suppressive from some tumour models and in only some suppression assays [333].

Interestingly it has been suggested that MDSCs need to work in concert with macrophages to cause immunosuppression. In an IL-4 receptor knockout model, where there is increased M1 macrophages, there is no enhancement of tumour clearance due to the presence of MDSCs [388]; depletion of MDSCs leads to resistance to metastasis [394]. Conversely, a reduction of MDSCs in the absence of M1 macrophages was not sufficient enough to decrease metastasis [282]. In this study, the presence of M1 macrophages and a decrease in MDSCs was needed to have a significant effect on tumour metastasis. MDSCs are also capable of skewing macrophages towards an M2 phenotype,

with decreased IL-12 secretion by macrophages due to IL-10 production by MDSCs [394].

MDSCs can also interact with mast cells and be altered by the presence of mast cells. MDSCs can enhance IgE-mediated IL-6, TNFα, and IL-13 production from mast cells [399]. As IL-13, IL-6, and TNFα are involved in MDSC suppressive activity, differentiation, and recruitment [400], this interaction can enhance MDSC function and numbers. This interaction between MDSCs and mast cells are shown to be important for parasite clearance and exacerbation of allergies [399]. Interestingly, if MDSCs were adoptively transferred into $Kit^{Wsh/Wsh}$ mast cell deficient mice, there was no enhancement of tumour metastasis compared to wild type mice [353]. Mast cell activation in a colorectal cancer model increased mast cell protease 1 release, which directly caused recruitment of MDSCs [401]. Injection of mast cells into mice causes enhancement of MDSC numbers, which was dependent on stem cell factor secretion by the mast cells [402]. These mast cells caused increased CCL2 levels in tumour, therefore enhancing MDSC recruitment. Mast cells also caused enhanced IL-10 and IL-13 levels that helped increase Arg1 levels in recruited MDSCs [402]. The mast cells also enhanced IL-17 secretion by MDSCs, which has been shown to be important for MDSC-dependent Treg recruitment [402].

MDSCs can also have an impact on metastasis in an immunosuppressive-independent manner. G-MDSC depletion causes decreased cancer cell proliferation, which was dependent on mediator secretion by the MDSCs, and the presence of G-MDSCs increased tumour dissemination, EMT, and metastasis [373]. MDSCs can be found in the invasive front of tumours in breast cancer patients [371], and M-MDSCs levels are

increased in metastatic breast cancer [332]. In a cancer model where TGFβ receptor 2 was knocked out in mammary epithelial cells, there was increased metastasis, and on the invasive front of tumour tissue there was increased MDSC recruitment [371]. In an injectable tumour model where MDSCs were coinjected with 4T1 tumour cells there was enhanced tumour cell invasion [371]. These MDSCs express MMP14, MMP13, and MMP2 which aided in the enhanced invasion by causing breakdown of extracellular matrix [371]. Blocking recruitment of MDSCs to the tumour led to decreased metastasis [371]. IL-6 has been shown to induce recruitment of MDSCs to the tumour and to an extent to the metastatic site; tumour cells are capable of inducing MDSC activation and IL-6 production, and this IL-6 signaling from MDSCs can enhance tumour cell invasiveness [84].

1.2.3.2 Direct targeting of immunosuppressive cells

Another method of enhancing an antitumourigenic effect is by directly targeting immunosuppressive cells. There are now drugs available that specifically impact MDSCs with little impact on other cells. One of the most popular drugs that has been studied is gemcitabine. Gemcitabine in humans has been shown to selectively deplete G-MDSCs [81]. Gemcitabine (120 mg/kg) can cause decreases in MDSCs via apoptosis with no alteration in $CD4^+$ T cells, $CD8^+$ T cells, NK cells, macrophages, or B cells [9]. Gemcitabine treatment, by decreasing MDSC levels, leads to increased NK cell cytolytic activity *in vivo*, and combination of gemcitabine with IFNβ inhibits tumour development [9]. Low dose metronomic doses of gemcitabine and cyclophosphamide (which can target Tregs) can improve antitumour immunity and decrease tumour burden [403]. This impact on MDSCs can also cause a decrease in metastasis [84, 394]. Gemcitabine depletion of

MDSCs have been shown to lead to an increase in TAMs [81, 264], but as there is a decrease on tumour development, it indicates that in the absence of MDSCs, TAMs may not be as protumourigenic. There are other drugs that can impact MDSCs (along with other cells), such as 5-fluorouracil, which can deplete both G-MDSCs and M-MDSCs [404], zoledronic acid, and sunitinib, which is specific to G-MDSCs [341]. Zoledronic acid treatment leads to increased $CD4^+$ and $CD8^+$ T cells in the tumour and decreased Tregs, subsequently leading to smaller tumours [331]. With sunitinib treatment there is increased T cells in the tumour with increased activity, and there is greater survival [341].

There are also attempts of altering TAM numbers. One mechanism of targeting TAMs is by targeting CCL2-CCR2 axis. Blockade of the CCL2-CCR2 axis can inhibit recruitment of monocytes, leading to decreased TAM numbers [28, 81]. This blockade has been shown to lead to an increased T_H1 (both $CD4^+$ and $CD8^+$ T cells) environment in the tumour and decreased Treg recruitment [10, 264]. Furthermore there are also increases in cytotoxic effectors (such as granzyme and IFNγ) in the tumour [10]. Another way of targeting TAMs would be by blockade of CSF1-CSF1R. As CSF1 is important for macrophage survival and for inducing the TAM phenotype [10], it will decrease numbers and potentially alter the protumourigenic phenotype.

1.3 Histamine

1.3.1 Sources, synthesis, and release

Histamine (2-[4-imidazole]-ethylamine) [405] is a low molecular weight amine derived from the decarboxylation of the amino acid L-histidine by the enzyme histidine decarboxylase (HDC). Specifically, HDC expression and activity can be altered by

cytokines such as IL-1 [406, 407], IL-3 [408-410], IL-12 [406, 411], IL-18 [411], CSF2 [408-410], CSF1 [412], and TNF [413]. Reciprocally, IFNγ has been shown to inhibit CSF2 and IL-3-induced HDC expression [410]. LPS has been shown to induce HDC in macrophages [410]. IgE binding to FcεRI on mast cells can also induce HDC activity [414].

Histamine is kept in vesicles which fuse with the plasma membrane upon cell activation. In mast cells and basophils, histamine release is traditionally triggered by the crosslinking of IgE bound to FcεRI [415, 416]. Other compounds that are known to induce histamine release from mast cells include neuropeptides such as substance P, somatostatin, vasoactive intestinal peptide [417], complement factors (such as C3a and C5a) [418], cytokines such as IL-3 and IL-5 [419], morphine, compound 48/80, and poly-L-lysine [417]. HDC is present in a variety of immune cells, most notably mast cells [420] and basophils [421], but also in DCs [422, 423], macrophages [412, 424], neutrophils [425], and platelets [426]. Under certain conditions $CD8^+$ and $CD4^+$ T cells can also be induced to produce histamine [427]. Neurons are also known to express HDC, and histamine can also act as a neurotransmitter on histaminergic neurons [428]. Furthermore, enterochromaffin-like cells [429] in the stomach also synthesize histamine that stimulates parietal cells to secrete acid [430]. HDC can also be found in vascular smooth muscle cells and endothelial cells [431]. Some cancer cells have also been shown to express HDC and synthesize histamine that can act in an autocrine manner to promote tumour growth, such as melanoma [432, 433], small cell lung carcinoma [434, 435], cholangiocarcinomas [436], and breast cancer [437, 438].

Histamine can be broken down by two enzymes: diamine oxidase (DAO) [439] via oxidative deamination, and histamine-*N*-methyltransferase (HNMT) [440]. DAO is a membrane bound enzyme in vesicles in epithelial cells that get released upon activation to scavenge for circulating histamine and metabolizes it into imidazole acetic acid [441, 442]. DAO is secreted by many cells, but major sources include the small intestine [441], kidney [443], and placenta [444]. HNMT is a cytosolic enzyme that metabolizes intracellular histamine into N-methylhistamine and is considered to be important for breakdown of histamine post-synaptically in neurons [445]. DAO has been shown to be altered in some tumour types, including colorectal cancer (decreased) [446], gastric cancer [447], prostate cancer (increased) [448] and breast cancer, where it has been shown to be both increased [449] and decreased [450]. The changes in HDC and DAO levels together with alterations in histamine producing cells would account for the alterations in histamine seen in cancer.

1.3.2 Antagonists vs Inverse Agonists

Some receptors express constitutive activity, meaning in the absence of a ligand, there is still baseline levels of signaling, signifying that the receptor is in an equilibrium where it goes from both the inactive and active state. An agonist preferentially binds to the receptor in its active state and stabilizes this conformation, enhancing the signal. There are two classes of drugs that inhibit signaling: antagonists and inverse agonists. Antagonists do not disturb the equilibrium but prevent agonists and inverse agonists from binding the receptor. Inverse agonists preferentially bind to and stabilize the inactive receptor [451]. Antagonists are used as the "umbrella" term for inhibition of signaling,

but some drugs are incorrectly called antagonists, when in reality they exhibit inverse agonist activity.

1.3.3 Histamine receptors

There are four known histamine receptors, known as histamine receptor 1 through 4 (H1-4) (Table 1.1). They are all G-protein coupled 7 transmembrane receptors with expression that varies on different tissue types; H1, H2, and H4 are found predominantly on immune cells, while H3 is more associated with histaminergic neurons [452]. The effect of histamine depends on the receptor that it is interacting with and the cells that are expressing the receptor.

1.3.3.1 H1

H1 is the primary receptor associated with symptoms of allergies. It is a $G_{\alpha q}$-coupled receptor that stimulates phospholipase C activation which leads to increased diacylglycerol, phosphoinositide and calcium [453]. It has also been shown to have constitutive activity [454]. Its expression is known to be upregulated by IL-4 [455], IL-3 [456], IL-12 [456], insulin [457], and histamine [455, 458]. β_2-agonists, which are used to alleviate bronchoconstriction in asthmatics, can also upregulate H1 on airway smooth muscle cells [459].

H1 “antagonists” as they are referred to, are currently over-the-counter drugs that are primarily used for the treatment of symptoms of allergies. Two of the most common H1 antagonists are cetirizine, the main ingredient in Reactine, and pyrilamine, the main ingredient in Midol [460]. Although these drugs are called antagonists, cetirizine and pyrilamine actually display inverse agonist activity [454]. There are two main groups of

H1 antagonists: first-generation and second-generation H1 antagonists. First-generation H1 antagonists are capable of crossing the blood-brain barrier and causing side effects such as drowsiness [461], while second-generation H1 antagonists (such as cetirizine) are unable to cross the blood-brain barrier and is therefore the preferred treatment for allergies [462]. First-generation H1 antagonists are still available for over-the-counter use and are one of the most commonly used drugs for the treatment of allergic rhinitis, allergic conjunctivitis, and insomnia, due to its side effect [463]. Second-generation H1 antagonists are recommended by WHO for the treatment of allergic rhinitis and conjunctivitis in children and adults [462].

H1 is expressed on most immune cells (Table 1.1). Some immune cells can have altered H1 expression upon activation or differentiation; H1 is downregulated in $CD4^+$ T cells upon TCR activation [464]. Upon differentiation of monocytes into macrophages, H1 is upregulated [465]. During an allergic response when mast cells degranulate and release histamine, histamine acts on endothelial cells and smooth muscle cells, to enhance permeabilization of blood vessels and cause smooth muscle cell contraction, which is what creates many of the acute symptoms associated with allergies [466, 467].

1.3.3.1.1 Adaptive Immune System

The impact histamine has on the immune system by signaling through H1 is primarily thought to be proinflammatory. H1 can enhance expression of CD86 on DCs, which is a costimulatory molecule [468], and blockade of H1 inhibits this maturation of DCs [469]. In a study by Vanbervliet *et al* [470], T cells stimulated by DCs from H1 knockout mice had decreased IFNγ and increased IL-17 production, and the DCs were shown to have decreased activation markers. Furthermore when these DCs activated $CD8^+$ T cells, the

subsequent CTLs had less cytotoxic activity. H1 signaling can also induce CXCL8 production in DCs and endothelial cells [468, 471]. H1 signaling, along with H2 signaling, can also block DC secretion of IL-12 when DC are stimulated with LPS [472].

H1 signaling can also have a direct impact on T cells that is independent of DC activation. In a study by Jutel *et al* [456], histamine enhanced CD3-dependent T_H1 proliferation which is blocked by an H1 antagonist. Furthermore, H1 signaling can have a direct impact on T cell cytokine production and enhance IFNγ by enhancing p38-MAPK activation [456, 464] and decrease IL-4 production [464]. Iida *et al* [473] showed that blocking H1 signaling on T cells causes a decrease in IFNγ secretion, but also a decrease in IL-4 secretion. In H1 knockout mice there is a decrease in IFNγ-producing T cells with enhanced T_H2 cytokine levels, which also leads to increased T_H2-dependent antibody production, including increased IgE and IgG1 [456]. Conversely, H1 signaling has also been shown to upregulate IL-10 and IL-13 secretion in T_H2 cells [474, 475]. H1 signaling can also recruit Tregs [476], but Treg activity can be inhibited through H1 signaling [477, 478]. H1 on keratinocytes can inhibit CCL17 production but enhance CXCL10 production that is induced by TNF and IFNγ [479]. CCL17 can subsequently cause recruitment of Tregs [480, 481], while CXCL10 can recruit activated T cells, particularly T_H1 cells [482-484], therefore H1 signaling can also indirectly impact Treg and T_H1 cell recruitment.

1.3.3.1.2 Innate Immune System

The innate branch of the immune response is also modulated through H1 signaling. H1 signaling can differentially impact migration of immune cells. H1 signaling can directly inhibit neutrophil migration [485]. However, H1 signaling on endothelial cells can cause

upregulation of P-selectin which leads to increased rolling and recruitment of neutrophils [486, 487]. At low doses of histamine, eosinophil migration due to endotoxin-dependent activation is enhanced, which is then blocked by H1 antagonism [488, 489]. Some H1 antagonists can also inhibit IgE-mediated degranulation of mast cells [490], although this effect differs with different H1 antagonists and the location which the mast cells were isolated from. On macrophages and monocytes, H1 signaling impacts the production of a variety of mediators. H1 signaling induces CXCL8 secretion from macrophages [465]. H1 blockade on peripheral blood and monocytes can decrease TNF, IL-1β, CSF2 and IL-6 synthesis. Furthermore H1 blockade can also inhibit NOS2 transcription and nitric oxide synthesis in monocytes [491].

1.3.3.2 H2

H2 is constitutively active [492]. It is coupled to the G_s [493, 494], which leads to activated adenylate cyclase and increased cAMP levels [495] (Table 1.1). H2 has also been shown to couple to G_q [493, 494]. Low concentrations of histamine can cause a decrease in H2 expression, while high levels of histamine can increase H2 [492, 496]. Long term H2 antagonist treatment can also lead to an increase in H2 expression [496].

Parietal cells in the stomach express H2, and stimulation of H2 can induce gastric acid secretion [430]. H2 inverse agonists such as ranitidine (Zantac), famotidine (Pepcid AC), and cimetidine (Tagamet) [460, 497, 498] are used to inhibit gastric acid secretion for the treatment of acid reflux, ulcers, and also to alleviate some of the gastric discomfort associated with chemotherapy [499, 500]. Cimetidine was once the largest selling drug in the world [501], and ranitidine was once in the top 50 drugs prescribed in the United States, with over 12 million prescriptions made in 2007 [502]. A large proportion of H2

antagonist users are over the age of 65 [503]. For the relief of dyspepsia associated with chemotherapy treatment in cancer patients, WHO guidelines suggest the use of H2 antagonists [499]. Recently H2 antagonists have been replaced with the proton pump inhibitors [504, 505] due to their increased potency [506] and due to anecdotal reports of myelosuppressive actions of H2 antagonists [507, 508].

1.3.3.2.1 Adaptive Immune System

H2 signaling on DCs has been studied thoroughly. H2 signaling can induce monocyte differentiation into DCs [509, 510]. Although H2 signaling can induce migration [511, 512], enhance endocytosis of peptide by DCs [513] and expression of CD86 costimulatory molecule [468] and CD40 [423], H2 signaling causes inhibition of cytokine production by monocytes and DCs, such as IL-12, TNF, IL-2, IFNα, and IL-18 [456, 512, 514-522], but does not inhibit the production of, and even stimulates production of anti-inflammatory cytokine IL-10 [474, 514, 518, 521] and CXCL8 [468]. Histamine through H2 can also alter DC stimulation to decrease induction of a T_H1 phenotype [514, 518]. H2 signaling can also induce T_H2 development by inhibiting IL-12 secretion [472, 518, 523] and enhancing IL-10 secretion by monocytes and immature DCs [518, 521], causing polarization of naïve T cells towards a T_H2 phenotype [472, 518]. There have been mixed results in terms of what is altered in H2 knockout mice: it has been shown that there are increased IFNγ-producing T cells [456] and IFNγ- and IL-10-producing macrophages [524], although in another study looking at experimental autoimmune encephalitis, H2 knockout mice showed a delay in disease development and IFNγ and TNF production by T cells were decreased, by a mechanism dependent on APC alterations [525].

Histamine can also have a direct impact on T cells. H2 signaling can directly inhibit T cell cytokine secretion, including IL-4 and IFNγ [526, 527], but does not inhibit IL-10 secretion [474, 528]; some studies have shown that H2 signaling on T_H2 cells can enhance IL-10 production, as well as IL-5 and IL-13 production [474, 475, 529]. H2 signaling can enhance the suppressive functions induced by IL-10 [478] especially on IFNγ production [474]. Histamine can also directly inhibit T cell proliferation [526, 530], but has a greater impact on T_H2 cell proliferation [456]. However, in the presence of TGFβ, histamine can inhibit T_H2 function and T cell proliferation, and can also inhibit IL-4 production [530]. H2 signaling can also impair activation of cytolytic cells by directly inhibiting $CD8^+$ and γδ T cell-mediated cytolysis [531, 532].

With modulation of T cells, T cell-dependent regulation of B cells can also be affected by histamine. Dimaprit, an H2 agonist, suppresses T cell-dependent immunoglobulin secretion by B cells, which can be rescued with the use of an H2 antagonist [533]. In H2 knockout mice, there are also decreased levels of the T_H2 cytokine-dependent antibodies IgE and IgG1 [456]. With H2 blockade, there is an increase in T_H1-dependent antibodies such as IgG2a [534]. Histamine can also directly inhibit secretion of IgG and IgM by B cells, and use of an H2 antagonist alleviates this inhibition [535].

1.3.3.2.2 Innate Immune System

H2 signaling can modulate monocytes in a variety of ways. H2 signaling enhances CCL2 production and expression of CCR2 by monocytes, which would enhance monocyte recruitment [536], and in an H2 knockout model, CCL2 expression was increased in macrophages [525]. Once monocytes are recruited, histamine can further affect monocytes; H2 signaling can alter surface molecules on monocytes, including inhibition

of CD14 and HLA-DR expression [537], and LPS-induced ICAM-1, B7.1, and TNF expression [538]. Production of IL-27 by monocytes, a cytokine which can induce T cell proliferation and synergize with IL-12 to induce IFNγ [539], is inhibited by H2 signaling [540]. H2 activation can also induce IL-1β production by monocytes [541]. H2, in combination with H4 signaling, can inhibit CXCL10 secretion by monocytes [482]. As CXCL10 is involved in T_H1 cell recruitment, H2 could therefore inhibit a local T_H1 environment by inhibiting these cells from being recruited. Histamine signaling through H2 can inhibit the effects IL-18 has on monocytes, including decreasing ICAM-1 expression, decreasing IL-12, TNF, IFNγ, and increasing IL-10 [542]. H2 signaling can also inhibit ADCC by monocytes and granulocytes [543]. One of the most studied aspects of monocytes and histamine is the mechanism by which H2 signaling can inhibit synthesis of ROS [509, 544, 545]. Similarly H2 signaling can inhibit neutrophil synthesis of superoxide formation and degranulation [485, 546, 547]. High levels of histamine can cause apoptosis in neutrophils via H1 and H2 [548]. H2 signaling can also inhibit neutrophil migration [485]. H2 can have indirect effects on neutrophil recruitment, by affecting endothelial cells to enhance neutrophil rolling [486] and inducing CXCL8 secretion [471], which is involved in neutrophil recruitment and activation [549]. H2 blockade can also inhibit expression of Mac-1, i.e. CD11b and CD18, which is an important adhesion molecule on neutrophils [550]. H2 blockade also inhibits release of neutrophil elastase and superoxide production [550].

H2 signaling can also alter other cells in the innate response. When mast cells get activated and degranulate, histamine can act back in a feedback inhibitory mechanism by signaling through H2, inhibiting further degranulation from occurring [551]. Similarly in

basophils, H2 signaling can inhibit basophil activation and IgE-crosslinking degranulation; therefore decreasing histamine release and IL-4 and CXCL8 release [552]. High doses of histamine on eosinophils can inhibit their migration via H2 [488].

1.3.3.2.2.1 H2 and MDSCs

There have been only a few studies on how histamine may modulate MDSCs, but the studies that have been performed indicate that H2 is involved in its effects. Studies revealed that histamine is involved in myeloid cell maturation [553]. Studies by Yang *et al* [554] revealed that histamine signaling is important for MDSC function, and lack of HDC causes G-MDSCs to remain in an immature state while M-MDSCs numbers decrease, which is primarily mediated through H2. Another study revealed that histamine blockade by cimetidine can inhibit NO synthesis and Arg1 expression, and causes MDSC apoptosis [555]. However, cimetidine is not a very specific drug, and has also been shown to inhibit H1 signaling and inhibit some hormone receptors [556]. There have also been several studies that have shown an “H2 antagonistic” effect with cimetidine but not with other H2 antagonists [555, 557-560], suggesting an off-target effect. Histamine has also been shown to be important for inducing proliferation and survival of MDSCs, which is done through H1 and H2 signaling [561]. In this study, histamine increased Arg1 and NOS2 expression in monocytic MDSCs, while it decreased these same mediators in granulocytic MDSCs. Furthermore, as monocytic and granulocytic MDSCs have similar markers as monocytes and neutrophils and similar mediators expressed, there is potential that the previously described effects that histamine has on general monocyte and neutrophil populations could also be applied to MDSCs.

In many clinical studies, it has been suggested that use of ranitidine can have myelosuppressive effects [507, 508], which is primarily characterized by neutropenia [508]. A study by Byron *et al* [562], showed that H2 signaling pushes bone marrow stem cells from G_0 to S phase [563]. IL-3-induced proliferation of hematopoietic stem cells was also inhibited by H2 blockade [564]. Radiation can cause a decrease in the bone marrow population, and histamine can have a protective effect on the bone marrow cells, which is thought to be through H2 signaling [565]. These studies give a molecular mechanism by which H2 blockade causes myelosuppression.

1.3.3.2.3 H2 signaling and the gut microbiome

H2 signaling can have indirect effects on immune function via modulation of acid secretion in the stomach. Alterations in acid secretion can cause alterations in the intestinal microbiome; specifically it can reduce microbial diversity [566]. Alterations in the gut microbiome can alter an immune response. Bacteria break down nutrients from food consumed into metabolites, and some metabolites can have an impact on the immune system. Butyrate, which is produced during starch fermentation, can directly stimulate the Treg population, and also affect DCs to induce Tregs [567]. In germ-free mice, arthritis is attenuated, which was attributed to a decrease in the induction of T_H17 cells [568]. This study by Wu *et al* showed that the monoclonalization of germ-free mice increased T_H17 cells, and that antibiotics on control mice (therefore reducing specific bacteria populations) also led to different effects on the T_H17 population. As H2 antagonists decrease acid secretion, continuous use can lead to alterations in the gut microbiome. With alterations in gut microbiome and subsequent alterations in immune function, there is potential for alterations in tumour development, and even in the potency

of some immunotherapies and chemotherapeutics [569]. One study showed that some colon adenocarcinoma associated samples had higher levels of *Fusobacterium nucleatum* when compared to healthy tissue, and this bacterium in humans can inhibit NK cell activity [570]. In a study by Lakritz *et al* [571], when mice with a genetic predisposition to develop breast cancer were given *Helicobacter hepaticus*, there was an increase in tumour burden compared to control mice, which was dependent on an increase in the neutrophil population. With this knowledge, there is potential that by altering intestinal microbiome through inhibition of acid secretion, H2 blockade can indirectly impact the immune system.

1.3.3.3 H3

H3 also contains constitutive activity [572]. It is coupled to $G_{i/o}$ [573], and signaling through H3 leads to inhibition of adenylate cyclase, leading to decreased cyclic AMP production, therefore leading to decreased cyclic AMP-responsive-element-binding protein activity [452]. H3 can also lead to increased MAPK and phosphatidylinositol 3-kinase [574, 575]. H3, unlike H1 and H2, has a high number of isoforms [575].

Although most H3 signaling studies have involved looking at histaminergic neurons, H3 can also be found in some immune cells (Table 1.1). However, even with expression on some immune cells, function of H3 on these immune cells is unknown.

1.3.3.4 H4

H4 is the most recently discovered histamine receptor [576-578] (Table 1.1). There are three known isomers, two of which cause downregulation of full length signaling H4 receptor from the surface [579]. It is coupled to $G_{i/o}$ and signaling through H4 leads to

decreased cyclic AMP production [578, 580]. H4 signaling can also activate MAP kinase [581] and phospholipase C [582]. H4 can be upregulated by IL-4 [583] and IFNγ [584, 585]. In sepsis, H4 has been shown to be upregulated in many tissues that typically do not express H4, including the lung, liver, heart and kidney [586]. There are currently no H4 antagonists on the market, but there are studies looking at the use of H4 antagonists for the treatment of contact dermatitis [587] and pruritis [588].

The primary function of H4 signaling appears to be immune cell recruitment, including recruitment of eosinophils [589, 590], mast cells [582, 591, 592], neutrophils [592], DCs [512, 593-595], Tregs [476, 596], γδ T cells [532], and NK cells [593]. In a model of contact dermatitis and dermal inflammation, H4 agonists can induce mast cell and eosinophil recruitment, which can be blocked with antagonists [587, 597]. An H4 knockout mouse has been developed, and in an allergy model, H4 knockout mice had decreased recruitment of mast cells, eosinophils, and lymphocytes [598]. In an allergic inflammation model, H4 agonists reduced hypersensitivity and inflammation, and this was shown to be due to a direct effect of H4 signaling enhancing migration of Tregs [476]. Conversely, H4 (and H3) blockade of DCs that were then injected into mice induced an increase in DC and Treg recruitment to the lung [599], indicating H4 signaling directly enhances Treg recruitment, but can indirectly inhibit Treg recruitment through signaling on DCs. H4 knockout mice also show deficiencies in Treg recruitment in a mouse experimental autoimmune encephalomyelitis model [596]. H4 signaling can also indirectly impact migration by altering chemokine release; H4 signaling inhibits CCL2 production in monocytes [585], Langerhans cells [595], and inflammatory dendritic epidermal cells [584], and inhibits CXCL10 production in monocytes [482].

Furthermore, H3/H4 antagonist-treated DCs caused decreased eosinophil and increased $CD8\alpha^+$ DCs recruitment in an allergy model [599].

H4 signaling can also impact cytokines production and activation. H4 signaling is important for DC maturation, and blocking H4 signaling on DCs lead to decreased T cell stimulation and proliferation [469]. H4 agonist treatment can enhance T_H2 cytokines and IgE production, and decrease T_H1 cytokines, which can be blocked with H4 antagonists [512, 597]. H4 signaling on $CD8^+$ T cells can induce proinflammatory IL-16 release [600]. H4-deficient Tregs are shown to have decreased suppressive activity [596]. Gschwandtner *et al* [601] showed that H4 signaling can inhibit secretion of IL-12 and TNF on DCs stimulated by LPS. IL-12 production inhibition by H4 signaling has been shown by other groups as well [512, 584]. Use of H3/H4 antagonist showed increased IL-10 production by DCs [599]. H4-deficient DCs or DCs treated with H4 antagonist had decreased IL-4, IL-6, and IL-17 production [598]. In HDC-deficient mice, natural killer T cells activated by α-galactosylceramide had decreased IL-4 and IFNγ secretion [602], and signaling through H4 can restore this effect.

H4 blockade has also been shown to decrease IgE in serum, [597, 599], but this impact is due to alterations in DCs, which could be due to the impact H4 has on T_H2 cytokines. In a model of allergic rhinitis and allergic contact dermatitis, H4 inhibition can decrease symptoms, which is associated with decreased circulating IgE and decreased IL-4 and IFNγ in nasal lavage fluid [587, 603]. Although these studies never specified the mechanism, based on other studies, it may be due to a direct impact on DCs.

1.4 Histamine and breast cancer

Under normal situations in breast tissue, HDC can be induced by estradiol and histamine through H2 [604]. HDC content and H1 expression in mouse mammary epithelial cells increases with pregnancy and decreases with parturition [605, 606]. There are also alterations in HDC activity and HNMT throughout the estrous cycle [606]. There is H1, H2, and H3 in normal mammary epithelium [605, 607].

Histamine production in tumours appears in many cases is associated primarily with the tumour cells themselves, although the role of mast cells in releasing histamine may also be involved. HDC can be expressed by some cancer types, including breast cancer. HDC activity is higher in tumour tissue compared to healthy mammary tissue [437, 608, 609], correlating with higher histamine concentration in tumour tissue [450, 608, 610] and with lower DAO activity [450, 608] but higher levels of HNMT [450]. Also important to note that healthy tissue directly adjacent to tumour tissue has higher HDC activity compared to healthy tissue from a healthy patient [608]. In the serum of women with breast cancer histamine concentrations are higher compared to healthy women [450, 608]. Histamine levels also increase with metastasis to lymph nodes [450]. H1-4 and intracellular histamine has been shown on some breast cancer cell lines [438, 607]. Another study by this group showed that in human breast carcinomas, there was enhanced H3 expression compared to benign lesions, while H4 can also be expressed in higher levels in carcinomas compared to benign lesions [609]. There are no studies on whether the tumour models previously stated express HDC or whether there is elevated histamine in the mice, but 4T1 does express stem cell factor, which is important for recruitment of

mast cells to the tumour [24]. Whether there is an elevated number of mast cells in 4T1, E0771, or the $STK^{-/-}$/NIC mice has not been analyzed.

1.4.1 Histamine directly affecting cancer cells

Histamine has been shown to have direct effects on cancer cell growth. Blockade of HDC in cancer cells decreases tumour cell growth [611, 612]. Most early studies on the impact of histamine on tumour growth have focused on H1 and H2 signaling. Primarily H1 signaling *in vitro* is associated with inhibition of tumour cell growth, while H2 signaling enhances growth [612]. H1 blockade led to dysregulation in some tumour cell lines, including DNA damage and inhibition of cell cycle progression [613]. H2 antagonism can lead to inhibition *in vitro* and *in vivo* [614, 615] while H1 antagonism can increase tumour growth, although cetirizine was the weakest of the H1 antagonists to have this impact [615, 616]. The mechanism behind H2 antagonism-mediated growth inhibition has been attributed to enhanced ROS levels and decreased catalase activity [438]. Most studies that have shown an impact of H2 antagonist on tumour growth is cimetidine, which has been shown to decrease tumour growth in several mouse models [617]. In gastric cancer cell lines and salivary tumour cells, cimetidine inhibited growth and induced apoptosis, while having no effect on normal gastric cells [618-620]. In a colon cancer model in mice, ranitidine and cimetidine had different effects; ranitidine did not have an impact on growth *in vitro* or *in vivo*, while cimetidine significantly decreased growth in both situations [558].

With the discovery of H3 and H4, the potential impact of histamine on tumour growth expanded. One group has focused their work on the impact of H3 and H4 signaling in breast cancer cells; they have shown that the effect of histamine depends on the

concentration; higher levels of histamine leads to decreased proliferation and eventually apoptosis, while low levels of histamine enhances proliferation [609]. They attribute this effect to the difference in affinity between H1 and H2 receptors versus the H3 and H4 receptors. To specify which histamine receptor was responsible for each effect, they utilized agonists, and showed that an H3 agonist enhanced proliferation while H2 agonists inhibited proliferation [438, 609], contradicting previous results. H3 agonists were also shown to induce migration of tumour cells [609]. An H4 agonist can also inhibit proliferation and cause apoptosis [609]. Similar results have also been shown in other tumour models; loss of H3 expression and increase in H4 expression decreases cholangiocarcinoma growth [621]. Furthermore, H4 signaling was shown to decrease EMT phenotype, and decrease MMP expression. Histamine or clozapine, an H4 agonist, led to decreased tumour volume, decreased neovascularization, and increased survival in melanoma-bearing mice [622]. In colorectal cancer samples, there was decreased H4 expression compared to normal tissue [623]. If H4 was transfected into these cells, there was greater suppression of growth by histamine or clozapine.

Histamine can also have an impact on tumour growth by affecting angiogenesis. HuVEC proliferation, migration, and tube formation can be induced *in vitro* by histamine [624]. *In vivo* there is induction of angiogenesis with histamine, and it is inhibited by H1 and H2 antagonists. HDC-deficient mice had decreased angiogenesis and wound healing compared to wild type mice [625]. HDC deficiency was associated with decreased VEGF, which could be increased by the addition of histamine or dimaprit, an H2 agonist. The presence of histamine in healing tissue leads to induction of VEGF by macrophages, endothelial cells, and fibroblasts [625, 626]. With cimetidine, VEGF production is

decreased, leading to inhibition of angiogenesis [626]. In HDC knockouts, there is decreased mammary adenocarcinoma growth due to lack of angiogenic factors induced by histamine, and supplementation of histamine enhanced tumour growth [627]. In a colon cancer model, H2 antagonist activity led to suppressed tumour growth indirectly by decreasing VEGF in serum, leading to increased necrosis, and decreased density of vessels in tumour tissue [628, 629].

1.4.2 Histamine altering antitumour response

The impact of histamine signaling altering immune cells in terms of an antitumour response depends on the histamine receptor through which histamine is signaling. Other mediators present in the microenvironment could also potentially alter how histamine will impact the immune response, either by synergizing or antagonizing the impact, or potentially being a potent enough mediator that histamine will not impact the cell. However, I will focus on this concept as if only histamine will be impacting the cells directly. As there are very few studies analyzing how immune cell function would be affected in terms of H4 activation, this section will only focus on H1 and H2 signaling on immune cells, and how it will impact them in a tumour setting.

1.4.2.1 Dendritic cells

As previously stated, H1 primarily has a proinflammatory effect, and appears to mainly enhance DC activity. H1 signaling enhances maturation of DCs and CD86 costimulatory molecule expression [468, 469], which can lead to enhanced ability of DCs to activate T cell activation, and potentially skew the T cell towards a T_H1 phenotype, with enhanced IFNγ [470]. These DCs can also activate $CD8^+$ T cells to become potent CTLs [470]. This enhancement of CTLs may potentially be further enhanced by the T_H1 environment

producing IFNγ that is being enhanced by the DCs. This enhanced T_H1 environment can also induce cytolytic activity by NK cells. Therefore it appears that H1 signaling on DCs would lead to enhancement of cytolytic activity by cytolytic cells, potentially leading to clearance of tumour cells. There have been no studies that have analyzed the use of an H1 agonist in combination with a DC-based vaccine, but based on this data, an H1 agonist may be a potent adjuvant to this therapy to induce DC activation and skewing of DCs towards activating an antitumourigenic response.

H2 signaling on DCs can cause activation, but also predominantly causes an anti-inflammatory phenotype. In terms of activation of DCs, H2 signaling may be beneficial, as H2 signaling can induce monocyte differentiation into DCs [509, 510], enhance peptide endocytosis [513], and increase expression of CD86 and CD40 [423, 468] therefore it could be important in increasing the DC population and priming DCs to activate T cells. With upregulation of CD40 on DCs, they can be primed to be activated via CD40-CD40L to cross-present peptide on MHC class I, potentially leading to $CD8^+$ T cell activation. However, in terms of cytokine production, H2 signaling on DCs can decrease several cytokines [456, 512, 514-522], including IL-12, but does not inhibit production of IL-10 [474, 514, 518, 521], therefore even though H2 signaling can enhance DC maturation and activation, activation of T cells by these DCs can skew the T cell phenotype away from a T_H1 response [514, 518], which would lead to decreased cytolytic cell activation. Furthermore if IL-10 production is not being hindered by the histamine signaling, there is potential that the IL-10 will lead to suppression of a T cell response. This IL-10 secretion can also lead to development of inducible Tregs, specifically Tr1 cells, therefore potentially leading to an enhancement of a Treg

population. In one study, famotidine enhanced cytolytic activity of cancer patient blood and tumour infiltrating lymphocytes, but not healthy patients [630]. This study did not specify what cells were being affected, but as H2 inhibition enhanced the effect, there is the possibility that H2 blockade alleviated the inhibition of cytokine secretion, therefore allowing for activation of a T_H1 environment and IFNγ secretion. Therefore, although H2 signaling would enhance DC activation, the phenotype of the activated DC would be more immunosuppressive. In a DC vaccine model, if histamine was used as an adjuvant, an H2 antagonist should be utilized to inhibit this phenotype.

1.4.2.2 NK cells

The direct impact of histamine on NK cells is not very well studied; most of the impact of histamine on NK cell activity is indirect via the impact of histamine on other immune cells. As stated above, H1 signaling on DCs can enhance IFNγ production by T cells, which could activate NK cell activity. The most prominent studies that have looked at the impact of histamine on NK cell activity involve H2 signaling inhibiting ROS synthesis by monocytes [509, 544, 545]. ROS synthesis in monocytes can inhibit NK cell activity, including cytolytic activity [545]. This is why histamine has been used in conjunction with other NK cell activators to enhance NK cell activity. As has been shown in some studies, histamine alone does not impact NK cell killing, but it is able to suppress monocyte suppression dependent on H_2O_2, allowing for cytokine-stimulated NK cells to be more cytolytic towards acute myeloid leukemia (AML) which is dependent on H2 [631]. Histamine injection or dimaprit inhibited B16 metastasis, which was blocked with ranitidine, famotidine, and cimetidine [632]. This effect was NK dependent. NK cell

cytolysis was enhanced with histamine. IL-2 combined with histamine completely eliminated metastasis, which was altered by ranitidine treatment [632].

1.4.2.3 T cells

H1 signaling through DCs can enhance a T_H1 cell development, but H1 signaling can also impact T cells directly. H1 signaling can enhance T_H1 proliferation [456] and IFNγ production [456, 464], while suppressing IL-4 production [464, 473]. H1 signaling can also upregulate T_H2 cytokine secretion [474, 475], but when all cells being impacted by H1 signaling are taken into consideration, DCs via H1 would more likely induce a T_H1 microenvironment, therefore the impact of H1 signaling on T_H2 cytokines would only be relevant if T_H2 cells are already present in the microenvironment. If there are T_H2 cells present, then H1 signaling would enhance T_H2 cytokine secretion, which can then go on to potentially lead to inhibition of an immune response through IL-10, and potentially lead to a Tr1 phenotype. However, H1 signaling can inhibit Treg activity [477, 478]. Whether this occurs on Tr1 cells needs to be analyzed. In general, it appears that H1 signaling would enhance a T_H1 response while dampening T_H2 and Treg activity.

H2 signaling via DCs could lead to T cell proliferation [526, 530] and activation and induction of inducible Tregs. H2 signaling directly on T cells also inhibits cytokine secretion, including IFNγ and IL-4 [526, 527], but enhances IL-10 secretion [474]. Therefore H2 signaling would decrease the numbers of T_H1 cells and inhibit their activity. Furthermore IL-10 production and the presence of histamine signaling through H2 would enhance suppression of T_H1 activity [478] and also induce inducible Tregs. H2 signaling can also directly inhibit CTL cytolytic activity [531] even in the absence of IL-

10. Therefore in general, H2 signaling in T cells appears to inhibit T_H1 phenotype and inhibit CTL activity, leading to a decrease in an antitumourigenic environment.

1.4.2.4 Myeloid cells

Most of the histamine activity on myeloid cells occurs through H2. There is some activity through H1, by causing monocytes to secrete TNF and CSF2 [491]. TNF can lead to tumour cell cytolysis, making it beneficial in a tumour response. TNF can also induce DCs to secrete IL-12, inducing a T_H1 environment. However, CSF2 can enhance MDSC development. Furthermore H1 can enhance NOS2 transcription and NOS synthesis [491], therefore H1 signaling on monocytes could potentially enhance MDSC development and NOS2 expression. Although H1 signaling could induce TNF to directly affect tumour cells, there would also be enhancement of an MDSC microenvironment, leading to immunosuppression.

H2 signaling seems to have a greater effect on myeloid cells, and differ in effect depending on whether the cells are mature or immature. H2 signaling can enhance CCL2 production by monocytes [536], leading to recruitment of monocytes and MDSCs. Once monocytes are recruited, H2 signaling can inhibit maturation markers such as CD14 and HLA-DR [537], suggesting that H2 signaling would keep the monocytes in an immature state, potentially leading to an MDSC phenotype. In another study, histamine signaling was shown to induce maturation of G-MDSCs, and enhance M-MDSCs populations [554]. Other studies have shown that MDSCs have enhanced survival due to H2 signaling [555, 561], suggesting with the previous study that M-MDSC survival is regulated by histamine. H2 signaling can inhibit ROS synthesis by monocytes [509, 544, 545] and superoxide formation by neutrophils [485, 546, 547]. Another study showed that H2 is

important for induction of NOS and Arg1 expression in MDSCs [555], although another study showed that these mediators were enhanced by histamine in M-MDSCs, while it is decreased in G-MDSCs [561]. In summary, for survival, monocytes and M-MDSCs remain immature due to H2 signaling, leading to an enhanced M-MDSC population, while H2 signaling decreases the G-MDSCs population by maturing them to neutrophils. The phenotype of these neutrophils, whether they become N1 or N2 subtype, is unknown, so whether this would be beneficial for an antitumour response is uncertain. Furthermore, remaining G-MDSCs would have decreased NOS and Arg1 expression, therefore G-MDSC function would be inhibited. An increase in M-MDSCs could enhance an immunosuppressive environment. In terms of mediators, although H2 inhibits ROS (which, as stated, is beneficial for enhanced NK cell and CTL activation), there is enhancement of NOS and Arg1, which has been argued to be the more potent mediators of an immune response. Therefore M-MDSC number and functions would be enhanced. It has been suggested that M-MDSCs are more immunosuppressive than G-MDSCs, therefore the increase in M-MDSCs and their potency would override any beneficial effect the decrease in G-MDSCs would have.

1.4.3 Histamine-based therapy

1.4.3.1 Therapy using histamine

There have been several epidemiological studies to determine whether atopic people are at a higher or lower risk of developing cancer. Some studies suggested there is no alteration in risk in developing breast cancer if a woman is atopic [633-635]. Another study suggested that allergies lead to an increased risk of developing breast cancer, and the more allergies a person has, the higher the risk [636]. Another study showed

differences depending on the age of the woman; history of allergies led to reduced risk of breast cancer in women older than 35, but no effect on women younger than 35 [637]. One theory as to why this occurs is that breast cancer risk in older women can be associated with immune anergy that occurs in older people, therefore the presence of allergies may hinder that anergy. Another possibility is that the nature of the disease developing in younger women is more likely to be linked to specific genetic defects and not as readily influenced by immune changes. None of these studies specified what mediator in the allergic response was important for this alteration in risk.

Histamine has been used in several clinical trials for the treatment of different cancers with minimal adverse side effects [638, 639]. Usually histamine treatment is combined with another mediator, such as IL-2 or IFNα. The mechanism behind this treatment is that IL-2 or IFNα enhances T cell and NK cell activation, while histamine inhibits ROS synthesis by monocytes and macrophages, therefore allowing for more efficient T cell and NK cell activity. Interestingly, high doses of IL-2 lead to enhanced histamine release by monocytes, and this study suggests that this may be the reason why high dose IL-2 treatment can lead to toxic effects [640]. Indeed, it has been shown that melanoma patients receiving combination therapy had increased IFNγ production by T cells, and that this was attributed in part to monocyte inhibition by histamine [641]. In melanoma, dual treatment leads to improved overall survival [642].

This combination treatment has been used in leukemias and renal cell carcinoma. IL-2 and histamine treatment led to greater survival in renal cell carcinoma and AML compared to IL-2 treatment by itself [638, 643]. In AML patients there was also enhanced leukemia-free survival in patients treated with histamine and IL-2 [644]. NK

cells in AML patients have decreased NKp46 and NKG2D which was dependent on ROS from myeloid cells, and histamine treatment led to increased NKp46 and NKG2D [645]. There is also decreased downregulation of CD3ζ and decreased apoptosis by NK cells and T cells, and enhanced NK cytotoxicity [646]. In myelomonocytic and monocytic leukemia, the cancer cells are also capable of producing ROS, leading to inhibition of NK cells. These cells also have increased H2 expression, and histamine inhibits ROS synthesis, and histamine combined with IL-2 treatment leads to enhanced remission and survival [647, 648].

1.4.3.2 Therapy using histamine receptor antagonists

There have been epidemiological studies that examined whether histamine antagonist use alters the risk in developing cancer. In terms of H1 antagonists, there has been no link between antihistamine use and breast cancer risk [649-652]. In breast cancer patients, use of antihistamines did not correlate with cancer recurrence or secondary cancer development [653]. Most H2 antagonist studies primarily focused on cimetidine, which does not appear to alter breast cancer development [654-656]. With ranitidine treatment, there is a slight increase in breast cancer risk in elderly patients in one study [657]. In a study involving a variety of cancer patients, ranitidine treatment led to enhancement of survival and some patients had decreased metastasis [658]. Cimetidine use can lead to a decreased risk of lung cancer but interestingly this was only in long-term use of cimetidine, while short term (less than a year) led to an increased risk of tumour development [659]. Famotidine treatment pre-surgery in breast cancer patients leads to enhanced immune cell infiltrates to the tumour and local lymph nodes [660].

The most studied histamine antagonist treatment for cancer is the use of cimetidine in CRC patients. In CRC, HDC and histamine is higher in tumour specimens compared to normal mucosa, and HDC levels increased with metastasis to lymph nodes or distant organs [661, 662]. Most studies look at cimetidine use prior to surgical removal of the tumour. Cimetidine can influence post-tumour resection in CRC patients, with increased survival. This may be due to direct effects on the tumour, and also has been suggested to involve suppression of suppressive T cells [656, 663-665]. There is also a suggestion that cimetidine can enhance tumour infiltrates [656]. Post-surgery, cimetidine helps maintain an immune response in CRC patients compared to untreated patients [666]. Fewer studies have looked at other H2 antagonists for the treatment of CRC patients; ranitidine has been shown to enhance survival in CRC patients under certain conditions, including post-resection [667]. Whether H2 antagonists can impact breast cancer outcome is unknown.

1.5 Rationale and hypothesis

1.5.1 Histamine antagonist treatment and alteration of tumour development

As previously stated, histamine can alter an immune response depending on what histamine receptor is being targeted and what immune cells are involved. As histamine levels can be increased in breast cancer patients, there is potential that by blocking certain histamine receptors, we can inhibit the immunosuppressive effect histamine can have, and therefore skew the histamine effect towards inducing an antitumourigenic response. As H2 signaling primarily induces an immunosuppressive environment, we hypothesize that H2 blockade can enhance an antitumourigenic response, leading to inhibition of breast cancer development.

1.5.2 Histamine antagonist modulation of myeloid cells

H2 antagonist treatment can lead to myelosuppression in humans [507, 508], and although this is one of the reasons cancer patients have been taken off H2 antagonists, there is potential that decreases in myeloid cells can lead to alterations in myeloid populations that are known to inhibit an immune response against the tumour, such as MDSCs. Our hypothesis is that H2 antagonists will lead to myelosuppression associated with monocytic cells, leading to alterations in the M-MDSC population, therefore altering the immunosuppressive environment.

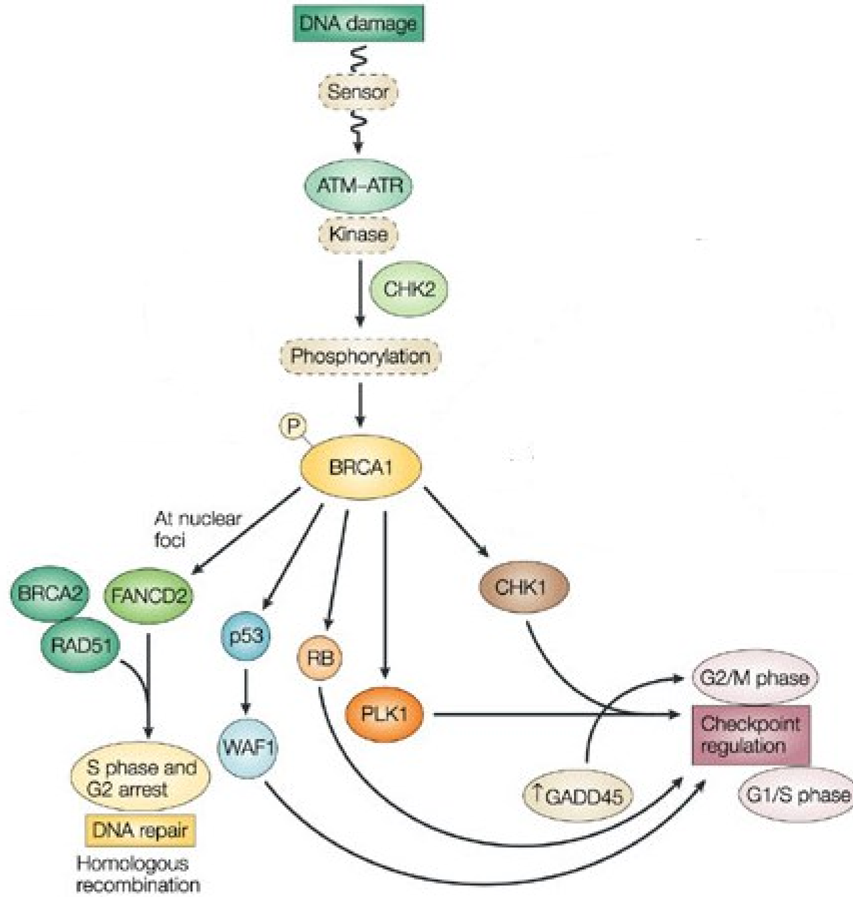

Figure 1.1 Schematic representation of BRCA1-dependent DNA repair and cell cycle arrest.

This figure summarizes some of the major interactions BRCA1 has when activated by DNA damage. This figure is adapted and edited from [668].

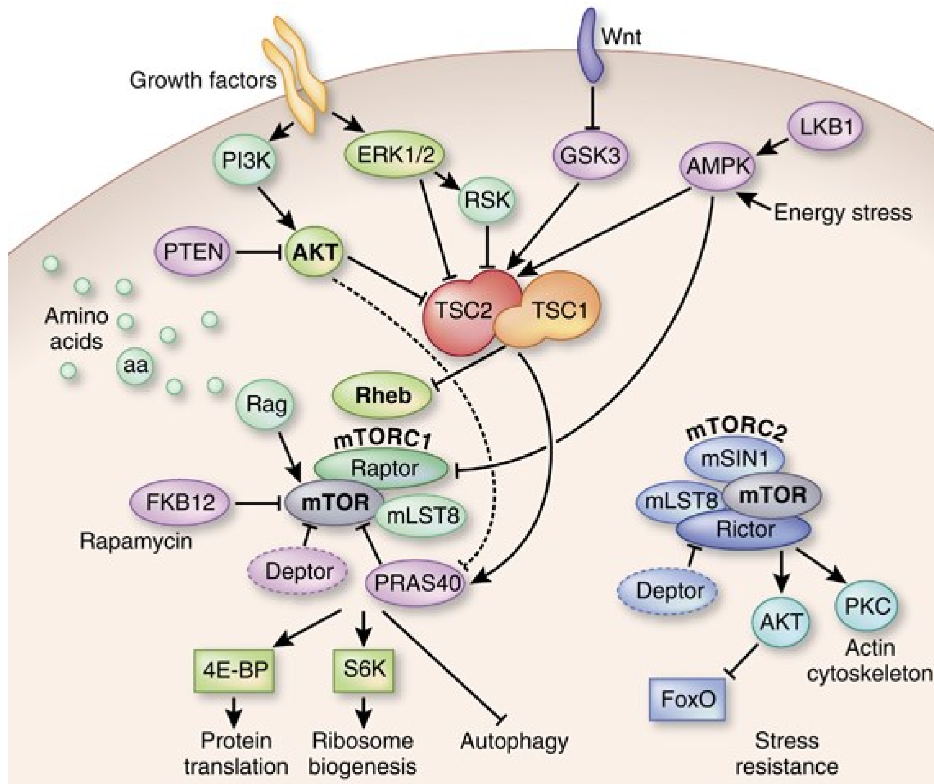

Figure 1.2 Schematic representation of PI3K/AKT/mTOR pathway.

This figure details the signaling pathway from growth factors, leading to activation of PI3K, AKT, and subsequently mTOR activation, leading to enhanced protein translation and inhibition of autophagy. Figure also shows how LKB1 and AMPK can inhibit mTOR signaling. This figure is adapted from [669].

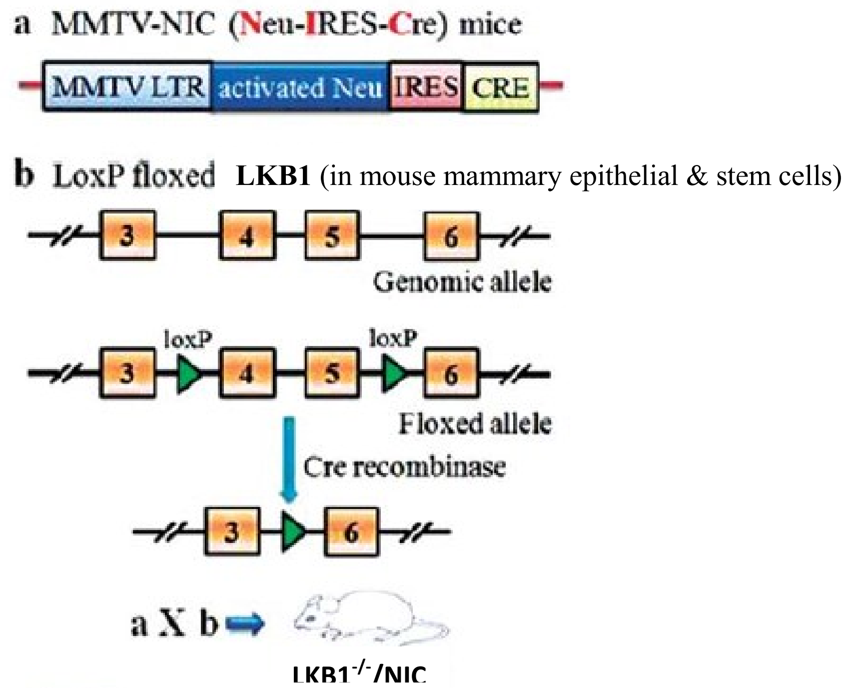

Figure 1.3 Schematic representation of mechanism of Lkb1 excision in Lkb1$^{-/-}$/NIC mice.

This figure details the mechanism floxed Lkb1 gene is excised by Cre enzyme in Lkb1$^{-/-}$/NIC mice. This figure is adapted and edited from [670].

Table 1.1 Summary of histamine receptor characteristics

	pKi	GPCR RECEPTOR	INVERSE AGONIST/ ANTAGONISTS	EXPRESSION ON IMMUNE CELLS	EXPRESSION ON NON-IMMUNE CELLS
H1	4.2 [671]	G_{aq} [453]	Cetirizine, pyrilamine	$CD8^+$ T cells [600, 672], $CD4^+$ T cells [456, 672], Tregs [477], γδ T cells [532], DCs [465, 513, 518, 673], B cells [456], NK cells [593], neutrophils [674], eosinophils [488], mast cells [490], monocytes and macrophages [465, 482, 673, 675], MDSCs [554, 561]	Neurons [676], airway [677] and vascular [678] smooth muscle cells, hepatocytes [679], chondrocytes [680], endothelial cells [681]
H2	4.3 [671]	G_s [493, 494]	Ranitidine, famotidine, cimetidine	Mast cells [682], basophils [552], eosinophils [683], neutrophils [674], monocytes and macrophages [465, 482, 675], MDSCs [555, 561], B cells [535], $CD8^+$ T cells [600], $CD4^+$ T cells [456], γδ T cells [532], DCs [518]	Chondrocytes [680], brain, liver, heart [684], parietal cells in stomach [430]
H3	8.0 [671]	$G_{i/o}$ [573]		DCs [512, 518], MDSCs [561]	Tuberomamillary nucleus in brain [685]
H4	7.8 [671]	$G_{i/o}$ [578, 580]	JNJ7777120	Mast cells [582, 682], basophils [552, 582], neutrophils [578], eosinophils [578, 582, 589, 590], monocytes [578, 585, 593], NK cells [593], natural killer T cells [602], $CD4^+$ T cells [583], $CD8^+$ T cells [600], Tregs [476, 596], γδ T cells [532], inflammatory dendritic epidermal cells [584], monocyte-derived DCs [593]	Small intestines, colon [578]

2 CHAPTER 2 MATERIALS AND METHODS

2.1 Mice

All mouse experiments were pre-approved by the Dalhousie University Committee on Laboratory Animals. Five week old female BALB/c mice, C57BL/6 mice, and athymic nude BALB/c mice were purchased from Charles River Laboratories and housed in specific pathogen-free conditions at the Carleton Animal Care Facility at Dalhousie University. Lkb1$^{-/-}$/NIC female mice were bred at Dalhousie University. CCR2 knockout C57BL/6 mice were bred at the IWK Health Centre animal facility.

2.2 Cancer cells

Mouse melanoma B16-F10 transduced with ovalbumin (generously provided by Dr. John G. Frelinger and Dr. Edith Lord), mouse breast carcinoma 4T1, mouse lymphoma EL4, mouse lung carcinoma LLC1, and mouse breast adenocarcinoma E0771 (ATCC) transduced with GFP were all maintained in a monolayer in Dulbecco's Modified Eagle's Medium (Hyclone) containing 10% fetal bovine serum, and 1% L-glutamine, HEPES, penicillin/streptomycin; for E0771 4 μg/mL of puromycin were added to media for selection of GFP-positive cells, and for B16-F10 500 μg/mL of G418 were added to media for selection of ovalbumin-expressing cells.

2.3 Histamine receptor antagonist drugs

Most histamine antagonists were added to drinking water one day prior to tumour cell injection and were refreshed every other day. These histamine antagonists remain stable in the drinking water during this time [686, 687]. The water bottles at this time were weighed before and after the water was changed, to be able to calculate the amount of

water drunk by each mouse and calculate the amount of drug taken in per mouse. Using this approach the consumption of ranitidine and famotidine per mouse per day ranged 6-8 mg/kg. JNJ7777120 (an H4 antagonist) was injected subcutaneously every two days starting one day prior to tumour cell injection. Amthamine (an H2 agonist) was injected intraperitoneally every day starting one day prior to tumour cell injection. Omeprazole was mixed into mash and given to mice every day, starting one day prior to tumour cell injection. Drug amounts are stated in Table 2.1.

2.4 Antibodies

Antibodies: Rat anti-mouse CD11b-fluorescein isothiocyanate (FITC) (cat. #11-0112, eBioscience), rat anti-mouse Ly6G-biotin (cat. #12760, Biolegend), rat anti-mouse Ly6C-allophycocyanin (APC) (cat. #17-5932, eBioscience), rat anti-mouse CD49d-phycoerythrin (PE) (cat. #12-0492, eBioscience), rat anti-mouse Gr1-FITC (cat. #11-5931, eBioscience), rat anti-mouse CD11b-PE (cat. #12-0112, eBioscience), rat anti-mouse CD11b-PE-Cyanine7 (Cy7) (cat. #25-0112, eBioscience), rat anti-mouse CD45-FITC (cat. #11-0451, eBioscience), rat anti-mouse CD62L-PE (cat. #12-0621, eBioscience), rat anti-mouse Ly6C-PE-Cy7 (cat. #25-5932, eBioscience), rat anti-mouse CX_3CR1-PerCP/Cy5.5 (cat. #149009, BioLegend), rat anti-mouse CCR2-APC (cat. #FAB5538A, R&D Systems), rat anti-mouse CD45-APC (cat. #17-0451, eBioscience), rat anti-mouse CD4-PE (cat. #12-0042, eBioscience). Appropriate isotype matched control antibodies were used in all experiments.

2.5 Injectable tumour models

2.5.1 4T1 tumour model

An adaptation of the protocol of Pulaski and Ostrand-Rosenberg [111] was employed for orthotopic models. 6-8 week old BALB/c or athymic nude BALB/c female mice were anesthetized and 100,000 4T1 cells in 50 µL PBS were injected subcutaneously into the mammary fat pad near the fourth nipple. The volume of the tumour was determined by caliper measurements every second day using the equation volume = length x width^2/2. At day 7 or day 19 – 21 post injection, the mice were sacrificed and the primary tumour, peripheral blood, spleen, femurs, and lungs were collected.

2.5.1.1 Lung metastasis detection

The lungs of tumour-bearing mice were digested for one hour at 37°C in the following enzyme cocktail in HBSS: 300 U/mL collagenase VII (C2139, Sigma Aldrich), 6 U/mL elastase (LE425, Elastin Products Company, Inc.), 100 µg/mL DNAse I (11-284-932-001, Roche), 2.5 mM calcium chloride (Fisher), and 2.5 mM magnesium chloride (M2670, Sigma Aldrich), then pushed through a 100 µm nylon filter. Serial dilutions were plated in media containing 60 µM 6-thioguanine (cat. #154-42-7, Alfa Aesar). After 9-12 days of incubation the 4T1 colonies were fixed and stained with 0.03% methylene blue (M9140, Sigma Aldrich) solution. The plates were scanned using an HP Scanjet G4050 scanner and the colonies were counted using ImageJ software (adapted from [111]).

2.5.1.2 Experimental metastasis model

6-8 week old BALB/c female mice were placed into mouse restrainers and injected via the tail vein 250,000 4T1 cells in 100 μL PBS. At day 14 post injection, the mice were sacrificed and the lungs were collected to measure metastasis (adapted from [688]).

2.5.2 E0771-GFP tumour model

For the E0771 model, 6-8 week old female C57BL/6 and CCR2$^{-/-}$ C57BL/6 mice were anesthetized and 200,000 cells in 100 μL of Matrigel® (Corning) were injected subcutaneously into the mammary fat pad near the fourth nipple (adapted from [114]). Tumour size was tracked as described above.

At day 7, 14, or day 21 post injection, the mice were sacrificed and the primary tumour, peripheral blood, and spleen were collected.

For analysis of circulating monocytes, starting one week prior to tumour cell injection, 100 μL peripheral blood was isolated via facial vein bleed and processed for flow cytometry staining of appropriate cell subsets.

2.5.2.1 Metastasis detection

At day 21 post E0771 injection lungs, local draining inguinal lymph node, and non-draining inguinal lymph node were isolated from mice and DNA was isolated using Qiagen DNeasy extraction kit. 100 ng of DNA was mixed with primers for IFNε (Quantitect Primer Assay, Qiagen) and GFP, and Promega GoTaq® qPCR Master Mix. The mixtures were then read in a Stratagene Mx 3000P using the MxPro program, under the following settings: 95° for 5 minutes; 40 cycles of (95°C for 30 seconds, 60°C for 30 seconds); 95°C for 1 minute; 55°C for 30 seconds; 95°C for 30 seconds. The critical

threshold of each sample was then obtained and used for normalization compared to IFNε, a single copy gene. GFP primers were purchased from Integrated DNA Technologies and are listed in Table 2.2.

2.5.3 LLC1, EL4, and B16-OVA tumour models

6-8 week old C57BL/6 mice were anesthetized and 200,000 LLC1 cells in 50 μL PBS, 100,000 B16-ova cells in 50 μL PBS, or 200,000 EL4 cells in 100 μL PBS were injected subcutaneously in the back. Tumour size was tracked as described above.

At day 7 or day 14-15 (for LLC1 and EL4) or day 20-21 (for B16-ova), the mice were sacrificed and the primary tumour, peripheral blood, and spleen were collected.

2.6 STK$^{-/-}$/NIC spontaneous tumour model

At the time of weaning (approximately 4 weeks of age), female LKB1$^{-/-}$/NIC mice [58] were given either control or ranitidine containing water. Drug-treated water was refreshed three times per week. Mice were examined once a week starting at week 19 for tumours and tumour volume was quantified using calipers in a similar method as mentioned above. A mouse was considered tumour-bearing when a tumour was palpable and measurable by calipers. At week 23-26, mice were sacrificed and the primary tumours were counted and weighed.

2.7 CD8 depletion

Purified anti-CD8 antibody (Clone 53-6.7) was generously provided by Dr. Thomas Issekutz (Dalhousie University). LEAF™ Purified Rat IgG2a κ isotype control was purchased from Biolegend (Cat. #400533).

Two days prior to 4T1 tumour cell injection, 200 μg of either anti-CD8 antibody or the isotype control was injected intraperitoneally in BALB/c mice (adapted from [689]). Eight days post tumour cell injection, 100 μg of antibody was injected intraperitoneally. The efficacy of CD8 depletion was confirmed by flow cytometry of $CD8^+$ cells in peripheral blood samples.

2.8 Depletion of monocytes using gemcitabine *in vivo*

The E0771 model was utilized, as stated above, with ranitidine treatment beginning one day prior to tumour cell injection. Five and thirteen days post tumour cell injection, mice were injected intraperitoneally with gemcitabine hydrochloride (60 mg/kg, G6423, Sigma Aldrich) or vehicle (adapted from [690]). On day 6 and day 14, the efficacy of gemcitabine treatment in depleting monocytes was confirmed by flow cytometry of peripheral blood samples.

2.9 Flow cytometry

2.9.1 Blood, splenocyte, bone marrow, lung and tumour infiltrate

Peripheral blood was isolated either by cardiac puncture or facial vein bleed. Red blood cells were lysed using ACK buffer (0.15 M ammonium chloride [cat. #A4514, Sigma Aldrich], 0.01 M potassium bicarbonate [cat. #P7682, Sigma Aldrich], 0.07 mM EDTA [cat. #15575, Invitrogen]) for 7 minutes at room temperature. Splenocytes were isolated by pushing the spleen through a 100 μm cell strainer and lysed with RBC lysis buffer (0.14 M ammonium chloride, 20 mM Tris base [cat. #604205, Boehringer Ingelheim]) for 3 minutes at room temperature. Both lysis reactions were stopped with the addition of an excess volume of PBS. Bone marrow was isolated from femurs of mice. Lungs were

digested in the same manner as stated above in *4T1 tumour model: lung metastasis detection.* 4T1 tumours were digested for approximately 2 hours in the following enzyme cocktail in 0.05% fetal bovine serum in Dulbecco's Modified Eagle's Medium: 200 U/mL collagenase VII, 2 U/mL Dispase (cat. #17105, Gibco), 100 μg/mL DNAse I. E0771 tumours were digested for 1 hour in the following enzyme cocktail in HBSS: 4.48 U/mL Dispase, 200 μg/mL DNAse I, 10 mM magnesium chloride. All enzyme reactions for lung and tumour digest were neutralized with PBS and then pushed through a 100 μM cell strainer. For E0771 tumour digests, blood was lysed with ACK buffer.

For flow cytometry analysis non-specific binding and Fc receptor interactions on splenocytes and bone marrow cells were blocked by treatment in FACS buffer (2% fetal bovine serum, 0.01 M sodium azide in PBS) containing rat serum for 15 minutes on ice. Samples were then mixed with primary antibodies for 15 minutes on ice, washed, and mixed with streptavidin conjugated to appropriate fluorochrome for 20 minutes at 4°C. Following washing, cells were fixed with 1% paraformaldehyde and acquired for analysis using a Becton Dickinson FACSAria II. Results were analyzed using FCS Express software (De Novo Software).

2.9.2 Cell sorting of MDSCs

Similar protocol as stated above is used. Following the last washing step, cells were resuspended in FACS buffer (1% fetal bovine serum, 1 mM EDTA in PBS). Cell suspensions were then sorted using a Becton Dickinson FACSAria II.

2.10 PCR and qPCR

2.10.1 PCR detection of histamine receptors

DNA was isolated using GenElute™ Mammalian Genomic DNA Miniprep Kit. RNA from cells was isolated using the Qiagen RNeasy Plus Mini kit. Reverse transcription was carried out using the Qiagen Quantitect Reverse Transcription kit. Custom-designed primers for RT-PCR and qPCR reactions were purchased from Integrated DNA Technologies and are listed in Table 2.2.

For RT-PCR and gene detection, cDNA or purified genomic DNA (respectively) were combined with primers and GoTaq Green Master Mix (Promega). The PCR reaction was run at the following settings: 94°C for 2 minutes; 35 cycles of (94°C for 30 seconds, primer annealing temperature for 30 seconds, 72°C extension for 2 minutes); 72°C for 10 minutes. PCR reactions were analyzed on a 1.5% agarose gel containing ethidium bromide.

2.10.2 qPCR detection of immune mediators

RNA from cells was isolated using the Qiagen RNA RNeasy Plus Mini kit or QIAamp RNA Blood Mini Kit. Reverse transcription was carried out using the Qiagen Quantitiect Reverse Transcription kit. For qPCR, cDNA was mixed with primers for GAPDH, HPRT, CCL2, CCL7, CXCL12, H1, H2, NOS2, Arg1, IL-10, IL-12 (Quantitect Primer Assay, Qiagen), and Promega GoTaq® qPCR Master Mix. The mixtures were then read in a Stratagene Mx 3000P using the MxPro program, under the following settings: 95° for 5 minutes; 40 cycles of (95°C for 10 seconds, 60°C for 30 seconds); 95°C for 1 minute;

55°C for 30 seconds; 95°C for 30 seconds. The Ct of each sample was then obtained and used for normalization compared to the average Ct between GAPDH and HPRT.

2.11 ELISA

ELISA plate wells were coated with 50 µL of capturing antibody, either anti-IgG2a or anti-IgG1 in 2.5 µg/mL in 0.02 M borate buffered saline pH 8.2 and incubated overnight at 4ºC. The plates were washed, then blocked with 2% fish gelatin (in PBS). The plates were washed, then diluted E0771-GFP tumour-bearing mouse serum samples were added, and left overnight at 4ºC. Plates were then washed, and 0.125 µg/mL GFP-biotin was added and the plates incubated for 1 hour at room temperature. Plates were then washed, and 0.0625% streptavidin alkaline phosphatase is added to each well and incubated for 25 minutes at room temperature. Plates were then washed and substrate was added to each well and incubated for 25 minutes at room temperature. Plates were then washed and amplifier was added. The plates then incubate for 5 minutes. The plates were read on an Epoch plate reader and results analysed using the Gen5 program [691].

2.12 Luminex assay

Twenty five µL of serum from mice isolated via cardiac puncture was used for a ProcartaPlex™ Mouse Basic Kit (eBioscience) to detect CSF1, CSF2, CSF3, IL-6, IL-10, IL-12, and TNF. Protocol used as designed by eBioscience.

2.13 Calcein-AM proliferation assay

4T1 and E0771-GFP cells were seeded in 96-well plates at 100 cells/well and 2500 cells/well, respectively and cultured overnight prior to addition of drugs (ranitidine (R101, Sigma Aldrich), famotidine (F6889, Sigma Aldrich), pyrilamine (P5514, Sigma

Aldrich), cetirizine (C3618, Sigma Aldrich), and JNJ7777120 (cat. #4021, Tocris Bioscience)) were added to give final concentrations of 100, 10, or 1 μM. Camptothecin (C9911, Sigma Aldrich, 5 μg/mL) was added as a positive control. After incubation for 5 days, Calcein AM (cat. #C1430, Invitrogen), at a concentration of 5 μg/mL, was added to each well. The plates were incubated for 1 hour at 37°C and analyzed using a Fluoroskan Ascent FL fluorometer (excitation 485 nm, emission 537 nm, integration time 500 ms).

2.14 Scratch assay

4T1 cells were seeded in 12-well plates at 125,000 cells/well and allowed to reach confluence before mitomycin C (M4287, Sigma Aldrich) was added at a final concentration of 1 μg/mL. After overnight incubation, the wells were washed with PBS and a consistent scratch was introduced into each monolayer. Pyrilamine, cetirizine, ranitidine, famotidine, and JNJ7777120 were added at concentrations of 100, 10, and 1 μM. An image was taken of the scratch. The plates were incubated for 24 hours, at which point another image was taken. NIH ImageJ software was used to analyze the size of the scratch at both time points [692].

2.15 Effect of Ranitidine treatment on suppressor cells

Mice were treated with ranitidine and injected with 4T1 cells as described above. At day 14 post 4T1 injection peripheral blood was isolated via cardiac puncture. Red blood cells were lysed with ACK buffer and leukocytes were resuspended in complete RPMI (Hyclone) media (10% fetal bovine serum, and 1% L-glutamine, HEPES, and penicillin/streptomycin) to a volume equal to the amount originally isolated from each mouse. Samples were serially diluted in 96-well plates in triplicate, before adding D011.10 splenocytes.

Spleens obtained from D011.10 mice were isolated, red blood cells were lysed with ACK buffer, and leukocytes were counted. Splenocytes were labelled using 1 μM Oregon Green® 488 dye (O-6149, Thermo Fisher Scientific) in RPMI at 37°C for 15 minutes. Cells were washed and 200,000 splenocytes were added per well. To each well 10 μM $OVA^{323-339}$ peptide was added and the cells were cultured. To determine whether the suppressive effect was dependent on NOS2 and Arg1 activity, L-NMMA and/or nor-NOHA (500 μM) was added [366, 384]. At day 3, each well was washed and stained with anti-mouse CD4-PE (cat. #12-004, eBioscience), fixed in 1% paraformaldehyde and acquired using a Becton Dickinson FACS Calibur. Results were analyzed using FCS Express software.

2.16 Statistical analysis

Final tumour weight and volume differences were analyzed with an unpaired Student's t test. Most lung tumour burden data was analyzed with a paired Student's t test, pairing the average of each group within an experiment; the effect of ranitidine at different doses on metastasis was assessed with a one-way ANOVA, followed by a Dunnett's Multiple Comparison test. $LKB1^{-/-}$/NIC tumour latency was assessed by a Log rank test. All FACS data and qPCR data was analyzed with an unpaired Student's t test. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ns denotes not significant.

Table 2.1 Summary of drugs used in *in vivo* studies

	HR	AGONIST/ ANTAGONIST	DOSAGE (MG/KG/DAY)	METHOD OF INTAKE	CAT. #	COMPANY	NOTE
Cetirizine	1	Inverse agonist	10	Drinking water	C3618	Sigma Aldrich	
Pyrilamine	1, 2	Inverse agonist	10	Drinking water	P5514	Sigma Aldrich	Has some H2 antagonist activity
Cimetidine	1, 2	Inverse agonist	100	Drinking water	C4522	Sigma Aldrich	Has some H1 antagonist activity
Ranitidine	2	Inverse agonist	8	Drinking water	R101	Sigma Aldrich	
Famotidine	2	Inverse agonist	8	Drinking water	F6889	Sigma Aldrich	
Amthamine	2	Agonist	0.01	Intraperitoneal injection	A4730	Sigma Aldrich	Stock solution in water, working solution in PBS
JNJ7777120	4	Antagonist	10	Subcutaneous injection	4021	Tocris	Stock solution in DMSO, working solution in PBS:ethanol mixture
Omeprazole	---	Proton pump inhibitor	70	Mash	O104	Sigma Aldrich	

Table 2.2 List of primers used in RT-PCR and qPCR

PRIMER	PRIMER SEQUENCE (5' → 3')	MELTING TEMPERATURE	PRODUCT LENGTH (BP)
H1 Forward	GGGGGTGCAGCCACGGAGAG	63°C	349
H1 Reverse	GGCGGCCCAGGGACCACTTG		
H2 Forward	GACCCCAGAAAGAGTAGCCAGTAG	57°C	535
H2 Reverse	GCCAGCAACAGTGATGAAGATGAG		
H3 Forward	GAGCCTCCGCACCCAGAACAAC	61°C	519
H3 Reverse	GCCCCCATCCAGCCGAAGAC		
H4 Forward	GGATCTCCTGTACTAGCCATCATTTG	55°C	403
H4 Reverse	GAGCCCTATAAGACACAGCATTTG		
GFP Forward	CGACAAGATCATCCGCAGCAAC	60°C	---
GFP Reverse	CTGTCCACCACGGAGCTGTAGTA		

3 CHAPTER 3 RANITIDINE MODIFIES MYELOID CELL POPULATIONS AND INHIBITS BREAST TUMOUR DEVELOPMENT AND SPREAD IN MICE

3.1 Introduction

Selective histamine receptor 2 (H2) antagonists such as ranitidine and famotidine are some of the most frequently used drugs for the treatment of upper gastrointestinal disorders. In recent years, these drugs have frequently been replaced by proton pump inhibitors [504, 505] due to their improved potency and in view of anecdotal reports of myelosuppressive actions of H2 antagonists [507, 508]. However, there remains a large population of people, with and without cancer, that regularly take H2 antagonists. Information on how these drugs impact cancer immunity is limited.

H2 expression has been reported on several tumour cell types [436, 693, 694] including some types of breast cancer (for a review see ref. [695]). Other cells within the tumour microenvironment can also express H2, including immune effector cells, endothelial cells, epithelial cells, and fibroblasts [413, 696]. H2 signaling has been shown to both enhance [612] and inhibit tumour cell growth *in vitro* [438, 693]. H2 antagonists have been reported to be effective in the treatment of certain cancer types, specifically H2-expressing colorectal cancers ([658, 697], for a review see ref. [656]) although the mechanism of these responses remains unclear.

Anti-tumour immune responses, including tumour immune surveillance, are complex and involve both innate and acquired components. Effective host immune function can reduce the incidence of tumours, limiting their growth and subsequent metastasis. NK cells and

$CD8^+$ T cells are recognized as critical effector cells in antitumour immunity and tumour surveillance. Monocytes and macrophage subsets are pivotal in local immune regulation within the tumour microenvironment. Tumour-associated macrophages and a population of cells known as myeloid derived suppressor cells (MDSCs) that includes both immature neutrophilic and monocytic subsets, have been shown to reduce effective antitumour immune function by inhibiting T cells, NK cells, dendritic cells, and cytotoxic macrophages [282, 335, 344, 395, 396, 698, 699], and inducing T regulatory cell development [391]. MDSCs have been found to be a barrier in inducing cancer immunity with immunotherapy, and combination treatments that include drugs such as gemcitabine are used to decrease MDSC levels [9, 403, 404, 700]. In mice, MDSCs can be identified, in part, by the expression of surface markers CD11b, Ly6C and Ly6G, which denote their monocyte and neutrophil lineage [329].

Histamine can modulate multiple immune effector cells via H2. It has been implicated in enhanced mobilization of dendritic cells, and decreased IL-12 secretion [511, 518, 523, 701], which can then alter subsequent antibody generation, and NK and T cell activity. H2 receptors can also modulate the cytolytic activity of NK cells [632, 702], inhibit both TH1 and TH2 cytokine secretion [456] and enhance IL-10 secretion [474]. Conversely, H2 blockade on T cells alleviates suppression of IFNγ secretion [518, 523, 527, 701] and inhibits IL-10 production [474]. H2 signaling on monocytes can block expression of adhesion molecules involved in T cell activation, such as ICAM-1 and CD40. Therefore, in the context of an H2 antagonist, enhanced T cell proliferation and IFNγ secretion can ensue [701]. H2 blockade can also alter the cytolytic activity of NK and $CD8^+$ cells, in general reducing NK activity [702] and enhancing $CD8^+$ activity [531]. Despite these

findings, the role of H2 antagonists as regulators of effective immune responses to breast cancer has not been systematically examined.

There are a few studies looking at the impact of histamine on MDSCs. MDSCs express H1 and H2 and immature myeloid cells express histidine decarboxylase (HDC) which is important for MDSC development. HDC expression by myeloid cells was shown to impact tumour growth in a colon cancer model [554]. Histamine from mast cells can also modify MDSC activity and that symptomatic allergic patients have increased MDSC function although the impact of H2 receptors antagonists was not directly addressed. [554, 555, 561] A study using cimetidine which has both H2 antagonist and a number of off target effects showed evidence of cimetidine-induced MDSC apoptosis. However, the more selective H2 antagonist famotidine did not have similar effects [555].

In the current study, the ability of the widely used selective H2 antagonist ranitidine to modify key myeloid populations in the context of breast tumours was determined. Breast tumour development and metastasis was also studied in three distinct mouse models that highlight different stages of tumour development and spread. Our results demonstrate that ranitidine reduces select populations of monocytic cells consistent with an impact on MDSC, and inhibits initial tumour development, primary tumour growth and metastasis to the lung in breast cancer models.

3.2 Results

3.2.1 Ranitidine treatment reduces $CD11b^{+}Ly6C^{hi}$ cells in the spleen and the bone marrow in both naïve and tumour-bearing mice.

The H2 antagonist ranitidine was orally administered to groups of BALB/c mice at a dose of 8 mg/kg for 8 days and the impact of treatment on splenic and bone marrow myeloid cell populations was determined. Myeloid cells can be identified, in part, by the expression of surface markers CD11b, Ly6C and Ly6G, which denote their monocyte ($CD11b^{+}Ly6C^{hi}$) and neutrophil ($CD11b^{+}Ly6G^{+}Ly6C^{lo}$) lineage. Ranitidine treatment was associated with an increased percentage of $Ly6G^{+}Ly6C^{lo}$ cells and decreased percentage of $Ly6C^{hi}$ cells within $CD11b^{+}$ splenocytes. This was reflected by a decreased frequency of $CD11b^{+}Ly6C^{hi}$ splenocytes (Figure 3.1). Total splenocyte numbers were unaltered by ranitidine treatment ($7.2x10^{7} \pm 8.8x10^{6}$, n = 15 vs. $6.4x10^{7} \pm 7.5x10^{6}$, n = 12 cells in control and ranitidine-treated, respectively). Bone marrow cells from ranitidine-treated mice also showed a decreased percentage of $Ly6C^{hi}$ cells within the $CD11b^{+}$ cells and a decreased frequency of $CD11b^{+}Ly6C^{hi}$ cells (Figure 3.1). Total bone marrow cellularity was not significantly decreased in ranitidine-treated animals ($5.5x10^{7} \pm 4.0x10^{6}$, n=12 vs $5.1x10^{7} \pm 3.6x10^{6}$, n=12 cells in control and ranitidine-treated, respectively).

Certain subsets of myeloid cells, such as MDSCs, are primarily up-regulated in the context of cancer (for a review see ref. [329]), therefore the effect of ranitidine on the splenocyte populations in mice bearing 4T1 breast tumours, associated with modulation of MDSC's [690], was examined. Mice were treated with ranitidine or left untreated for eight days in the context of breast tumour development. Similar to what was observed in

naïve mice, there was an increase in the proportion of $CD11b^{+}Ly6G^{+}Ly6C^{lo}$ cells following ranitidine treatment (Figure 3.2) and a decrease in $CD11b^{+}$ $Ly6C^{hi}$ monocytic cells in the spleen. There was an overall decrease in the numbers of myeloid cells by approximately 40% in the spleen, which could be attributed to a decrease in monocytes with ranitidine treatment. There was no overall change in lymphoid cell populations.

3.2.2 Histamine receptor 2 antagonists decrease lung metastasis in the 4T1 breast cancer model.

Given that ranitidine altered the populations of myeloid cells in the spleen, the ability of ranitidine treatment to alter tumour outcome was examined. Ranitidine and other selected histamine receptor antagonists, including cetirizine (H1), JNJ7777120 (H4), cimetidine and pyrilamine (H1 and H2) and famotidine an alternate H2 antagonist, were examined. None of the drugs showed a significant effect on 4T1 primary tumour endpoint weight (Table 3.1) or in the growth kinetics of the tumour over 19-21 days (Figure 3.3). However, ranitidine had a significant impact on lung tumour metastasis with a mean percent inhibition of 61% compared with control-treated mice (Figure 3.4). Animals given oral famotidine also showed a significant decrease (mean percent inhibition of 58%) in lung metastasis. Pyrilamine showed a trend towards metastasis inhibition (mean percent inhibition of 34%) while cetirizine and JNJ7777120 showed no effect on metastasis. The anti-metastatic effect of ranitidine was dose dependent, with the greatest inhibition at an oral dose of 8 mg/kg. Lung tumour burden was similar to control 4T1 tumour-bearing mice when a dose of 0.125 mg/kg of ranitidine was administered (Figure 3.4).

Figure 3.1 Ranitidine treatment decreases CD11b+Ly6Chi population in the spleen and bone marrow of BALB/c mice.

Composition of total $CD11b^{+}$ cells, $Ly6G^{+}Ly6C^{lo}$ granulocytic cells, and $Ly6C^{hi}$ monocytic cells in spleen (A) and bone marrow (B) of non-tumour-bearing mice with and without 8 days of ranitidine treatment. (C) Representative flow cytometry data showing percentages of $Ly6G^{+}Ly6C^{lo}$ and $Ly6C^{hi}$ in $CD11b^{+}$ splenocytes. Data points represent individual mice and line represents the mean per group. *$p<0.05$, ***$p<0.001$, unpaired t-test.

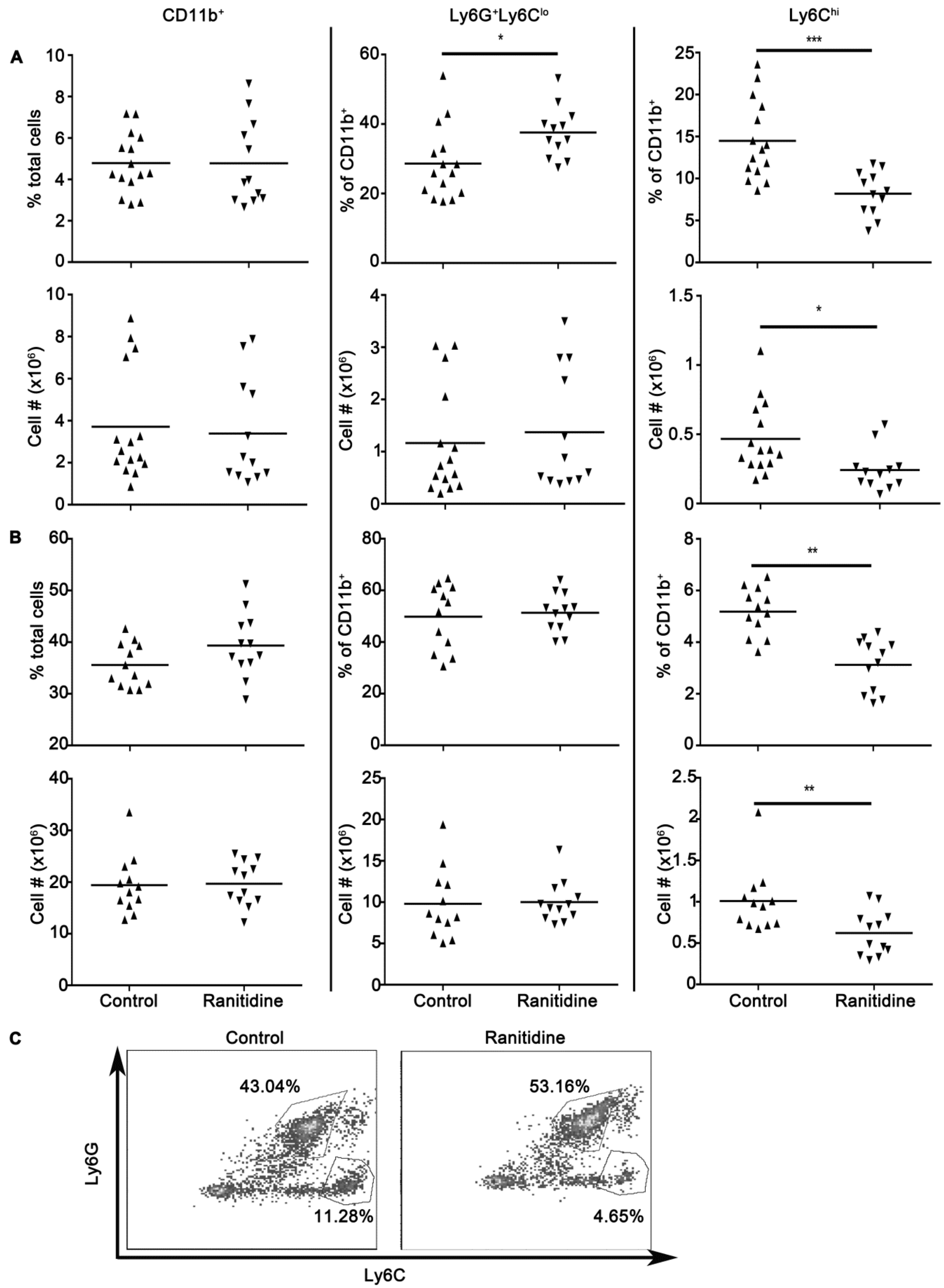

Figure 3.1

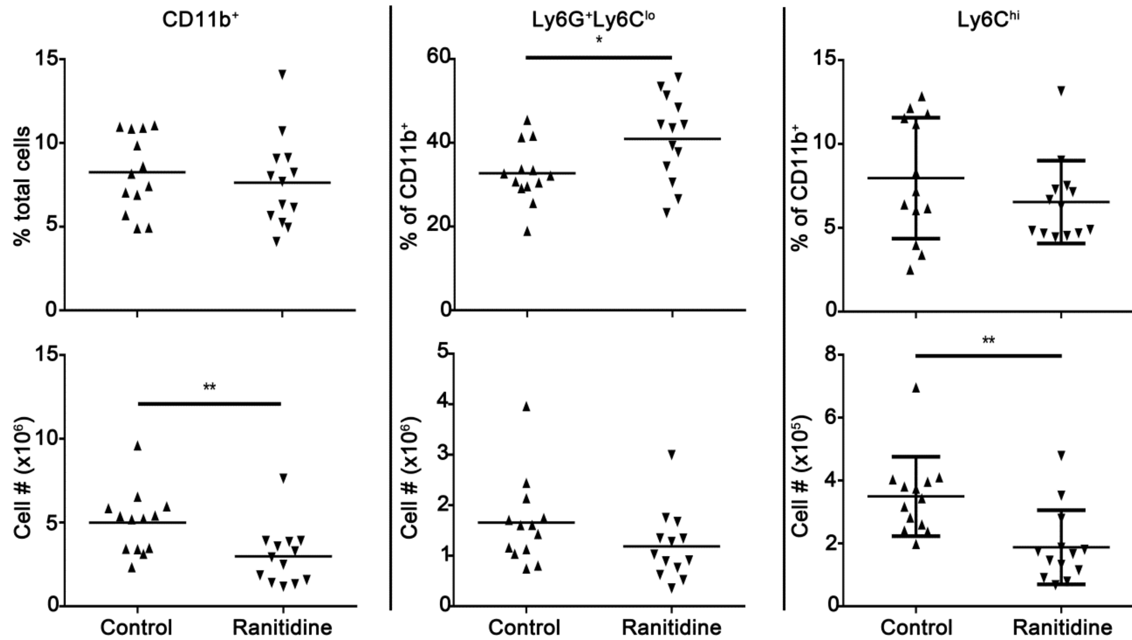

Figure 3.2 Ranitidine treatment decreases CD11b^{+}Ly6C^{hi} population in the spleen of 4T1 tumour-bearing BALB/c mice.

Composition of total CD11b^{+} cells, Ly6G^{+}Ly6C^{lo} granulocytic cells, and Ly6C^{hi} monocytic cells in spleen of 4T1 tumour-bearing mice with and without 8 day ranitidine treatment, starting one day prior to tumour cell injection. Data points represent individual mice and line represents the mean per group. *p<0.05, *p<0.01, unpaired t-test.

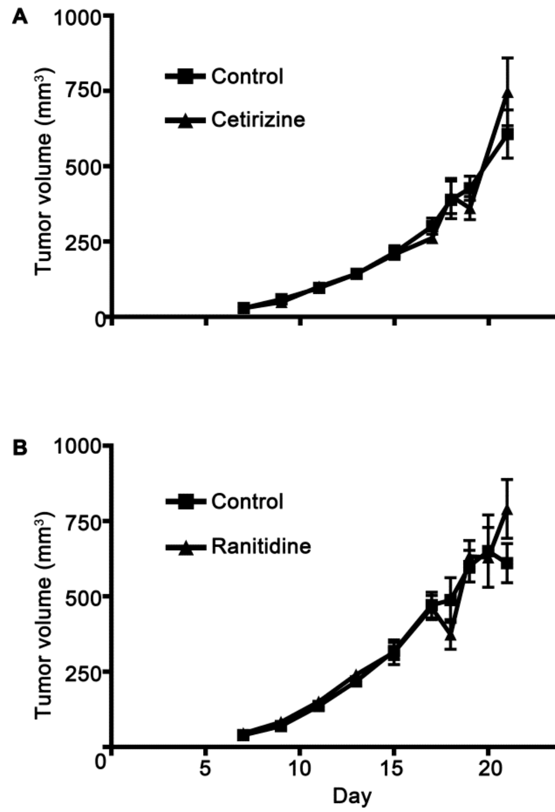

Figure 3.3 4T1 tumour growth *in vivo* is not affected by histamine receptor antagonist treatment.

4T1 tumours were measured using calipers every other day starting on day 7 post-injection. Volume was calculated as tumour length * tumour width^2/2. Each point represents the mean of 15-28 mice/group. ns.

Figure 3.4 Histamine receptor antagonists inhibit 4T1 metastasis.

(A) Average number of 4T1 colonies derived from lungs of tumour-bearing BALB/c mice treated with ranitidine (8 mg/kg), famotidine (□ 8 mg/kg and ☒ 2mg/kg), pyrilamine (10 mg/kg), and cetirizine (10 mg/kg). (B) Number of 4T1 colonies derived from lungs of tumour-bearing mice treated with decreasing doses of ranitidine. Data points in (A) represent mean of 3-4 mice per group per experiment; data in (B) represent mean ± SEM of 3-42 mice. *$p<0.05$, **$p<0.01$, paired t-test (A), ANOVA followed by a Dunnett's test (B).

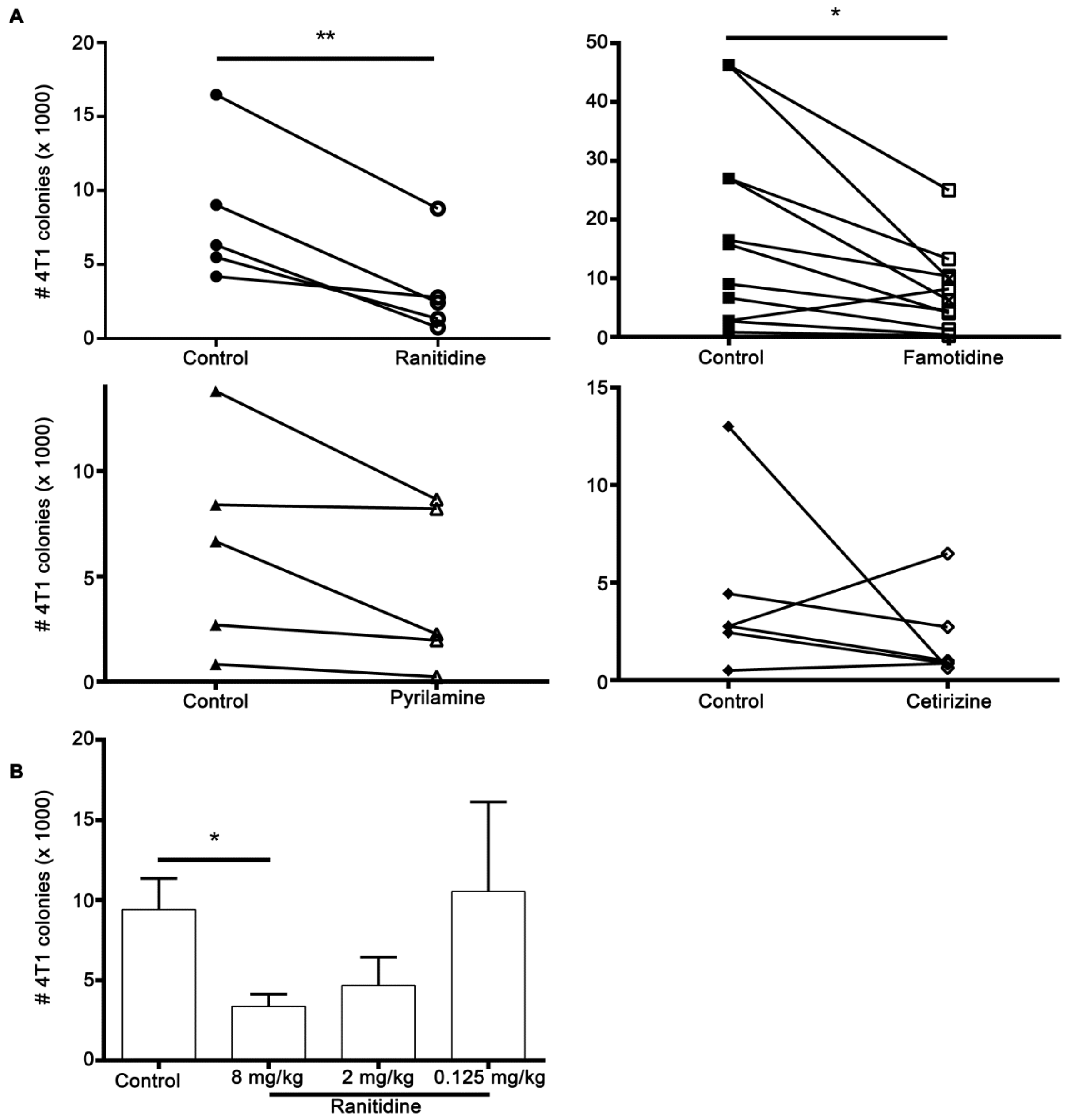

Figure 3.4

Table 3.1 Final tumour weights of histamine receptor antagonist-treated 4T1-bearing mice.

Drug	Target	Dose (mg/kg)	Endpoint weight (g ± SEM)		P-value
			Control	Drug	
Ranitidine	H2	8	0.641±0.07	0.607±0.06	0.70
Famotidine	H2	8	0.537±0.05	0.543±0.08	0.95
Cetirizine	H1	10	0.535±0.06	0.496±0.05	0.62
Pyrilamine	H1, H2	10	0.801±0.15	0.743±0.11	0.76
Cimetidine	H1, H2	100	0.472±0.05	0.544±0.06	0.36
JNJ7777120	H4	10	0.428±0.05	0.385±0.04	0.53

3.2.3 Evaluation of potential direct effects of histamine receptor antagonists on tumour growth.

Some breast cancer cells as well as normal breast tissue can express H2 receptors [703] (for a review see ref. [695]). Neither H1 nor H2 receptor antagonists had a direct effect on 4T1 cell proliferation or ability to migrate *in vitro* (Figure 3.5). E0771 cells were similarly unaffected by histamine receptor antagonist treatment *in vitro* (data not shown). In keeping with these findings, neither 4T1 cells nor E0771 cells expressed H1 or H2 receptors (Figure 3.6). These findings confirmed that H2 antagonists are not directly affecting the tumour cells via H2 receptors.

3.2.4 $CD8^+$ T cells are not essential for ranitidine effects on metastasis.

Previous studies have shown that histamine can inhibit $CD8^+$ T cell activation through a decrease in IL-12 production [518] and an increase in IL-10 [474], which would lead to a decrease in T_H1 cells and IFNγ secretion. Therefore tumour growth and metastasis in the presence or absence of ranitidine following antibody mediated $CD8^+$ T cell depletion within recipient mice was compared. As in fully immunocompetent mice, there was no alteration in final tumour weight and tumour growth kinetics when mice were treated with ranitidine. However, there was still a decrease in lung tumour metastasis following ranitidine treatment, even in the absence of $CD8^+$ T cells (Figure 3.7), with a mean percent inhibition of 57% (3 experiments, 4 mice/group) between control and ranitidine-treated CD8-depleted mice (compared to 61% mean percentage inhibition between control and ranitidine-treated BALB/c, 5 experiments, 3 mice/group). Therefore, $CD8^+$ T cells are not essential for the ranitidine-induced inhibition of metastasis in the 4T1 model.

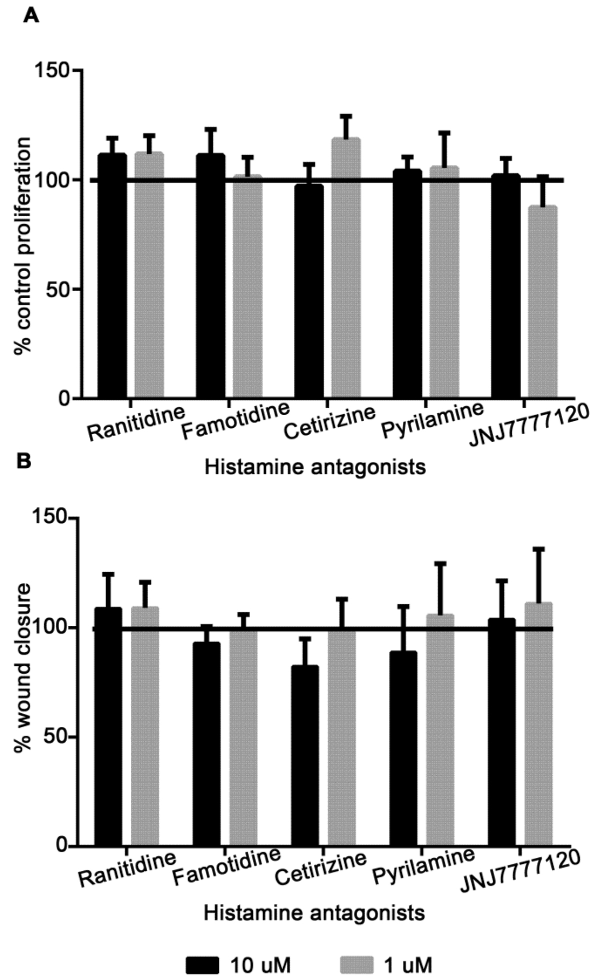

Figure 3.5 Proliferation and migration of 4T1 cells *in vitro* are not affected by histamine receptor antagonists.

(A) Calcein AM assay was used and the average relative fluorescence units (RFU) of 4T1 cells treated with histamine receptor antagonist compared to vehicle control. (B) Average percent closure of wound size with 4T1 cells treated with histamine receptor antagonist, compared to vehicle control. Data in (A) represents mean ± SEM of 3-8 experiments; data in (B) represent mean ± SEM of 4-5 experiments. ns.

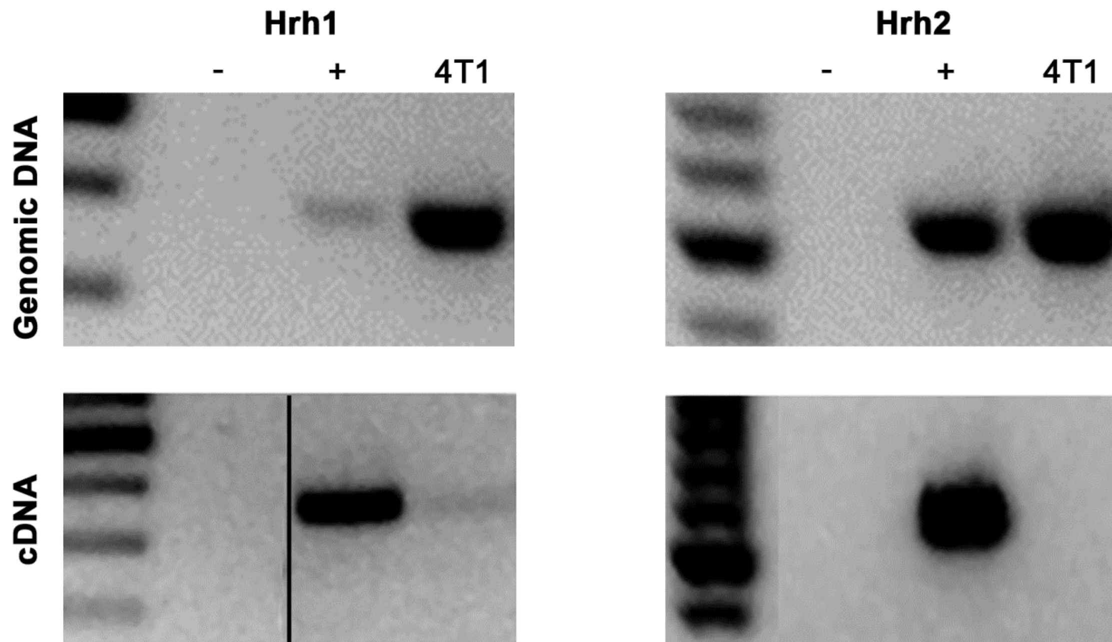

Figure 3.6 Histamine receptor genes are present in the 4T1 genome but are not transcribed *in vitro.*

(A) PCR of 4T1 genomic DNA. Negative control (-) was PCR mixture with no template, and positive control (+) was bone marrow-derived mast cell DNA. (B) RT-PCR of cultured 4T1 RNA. Negative control was RT-PCR mixture with no template, and positive control was whole brain RNA from C57BL/6 $Wsh^{-/-}$ mouse (for Hrh1) and bone marrow-derived mast cell RNA (for Hrh2). Lanes were run on the same gel but were not noncontiguous.

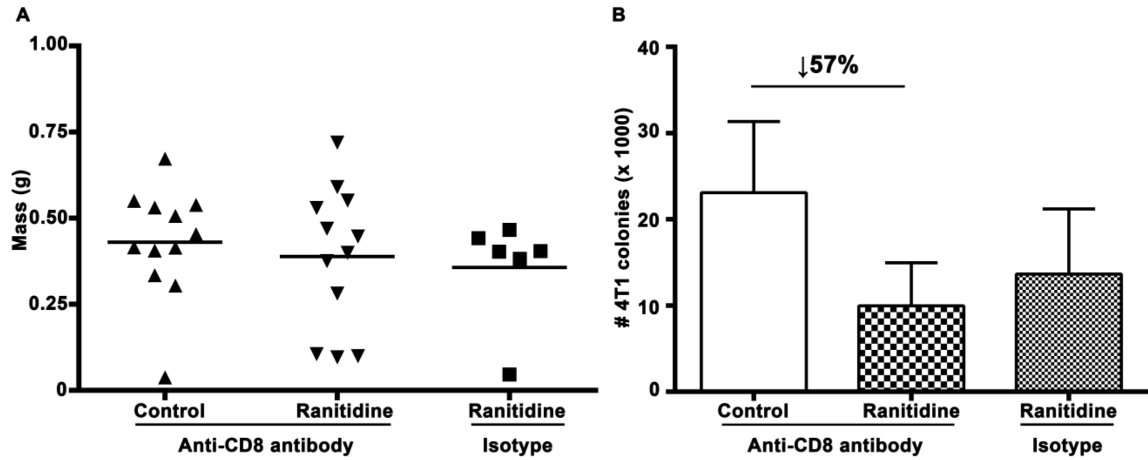

Figure 3.7 Ranitidine does not affect lung metastases by directly affecting CD8$^+$ T cell activity.

(A) Final tumour weight of ranitidine treated mice with and without CD8$^+$ T cell depletion. (B) Number of 4T1 cells isolated from lungs of tumour-bearing BALB/c mice treated with ranitidine with and without CD8 depletion. Data from (A) represent average of individual mice and line represents mean per group. Data in (B) represents mean ± SEM of 6-12 mice per group. ns.

3.2.5 Mice treated with ranitidine demonstrate decreased suppression of T cell function compared with control animals.

In view of the observed changes in myeloid cells bearing the markers associated with MDSCs, the ability of circulating cells from ranitidine-treated mice to modify T cell proliferation was examined. In view of our results which suggested $CD8^+$ T cells are not responsible for the effect ranitidine has on metastasis, the effect of potential MDSCs were analyzed on $CD4^+$ T cell proliferation. Leukocytes derived from ranitidine-treated mice were significantly less able to suppress the $CD4^+$ T cell proliferation in response to antigen ($p < 0.05$) when compared with cells from control mice, with the level of proliferation increasing from 32% to 43% (Figure 3.8). L-NMMA and nor-NOHA were used, at doses shown to be effective in similar systems, to inhibit NOS2 and Arg1 [366, 384]. However neither inhibitor alone was able to significantly reduce the inhibitory activity of MDSCs. There was a trend towards decreased suppressive activity with simultaneous inhibition of NOS2 and Arg1 in animals that did not receive ranitidine treatment (mean 13.4% increase in proliferation, with 5/7 mice showing evidence of inhibited MDSC function) which was not observed in the ranitidine treated group that had been shown to have reduced MDSC function (mean 1.6% decrease in proliferation) following NOS2 and Arg1 blockade , n=7, data not shown). Our results suggest that although there was altered functional activity of MDSCs from mice treated with ranitidine, the main mechanism of suppression of CD4 T cell responses in this model was not dependent on NOS2 or Arg1.

3.2.6 Ranitidine decreases primary tumour growth in a second orthotopic model.

To examine whether the effect of ranitidine was specific to the 4T1 model, a similar experiment using the less metastatic E0771 mouse model of breast cancer in C57BL/6 was performed. Initially, similar tumour growth kinetics was observed with and without ranitidine treatment. However, a decrease in tumour growth occurred in ranitidine-treated animals starting at approximately day 13 post tumour cell injection (Figure 3.9). A similar regression was not observed in animals that received control drinking water. At the time of tumour harvest, a significantly decreased final tumour weight was observed in animals that had received ranitidine treatment compared to the control animals (Figure 3.9). This simpler, short term model was employed for additional studies of the mechanism of ranitidine-dependent tumour inhibition. The impact of omeprazole treatment on tumour growth was examined to control for any impact of reduced stomach acid on microbiome or related immune responses. Omeprazole treatment did not alter E0771 tumour growth when compared to control mice (Figure 3.10). No significant metastasis to the lung was observed in this model within the experimental time frame.

3.2.7 Ranitidine does not alter E0771 development if monocytes are depleted by gemcitabine treatment.

To determine whether the effect ranitidine has on E0771 development was dependent on MDSCs, gemcitabine treatment was used for depletion of MDSCs *in vivo* [9, 403, 690, 704]. Analysis of circulating MDSCs a day after gemcitabine treatment revealed that monocytes were preferentially depleted, with no significant alteration in neutrophils (Figure 3.11). Gemcitabine-treated mice had decreased tumour growth compared to

untreated mice, and gemcitabine combined with ranitidine-treated mice showed similar tumour development as those treated with gemcitabine alone (Figure 3.11).

3.2.8 Long-term ranitidine treatment is associated with increased latency in mammary tumour onset and a decrease in number of tumours in LKB1$^{-/-}$/NIC mice.

A spontaneous breast tumour model provides a more similar scenario to clinical cancer, and allows assessment of the impact of potential treatments on early tumour development. Therefore, experiments were performed in LKB1$^{-/-}$/NIC mice, which normally develop mammary tumours within 20-25 weeks of birth [58]. LKB1$^{-/-}$/NIC mice were given ranitidine in the drinking water, initiated at the time of weaning (21 days after birth). 50% of untreated LKB1$^{-/-}$/NIC mice develop primary mammary tumours by 147 days after birth [58]. However, in ranitidine-treated mice there was a significant increase in latency of tumorigenesis by an additional 24 days; t_{50} of 171 days (Figure 3.12). Furthermore, control mice typically have to be euthanized at week 22 due to large tumours and high tumour burden (average of 8 tumours/mice) to comply with ethical endpoints. However, mice treated with ranitidine had a reduced tumour burden allowing them to survive until the planned end of the experiment, at week 26. On average, tumour burden at this time point was 4 tumours/mice, significantly below that observed in the control animals (Figure 3.12).

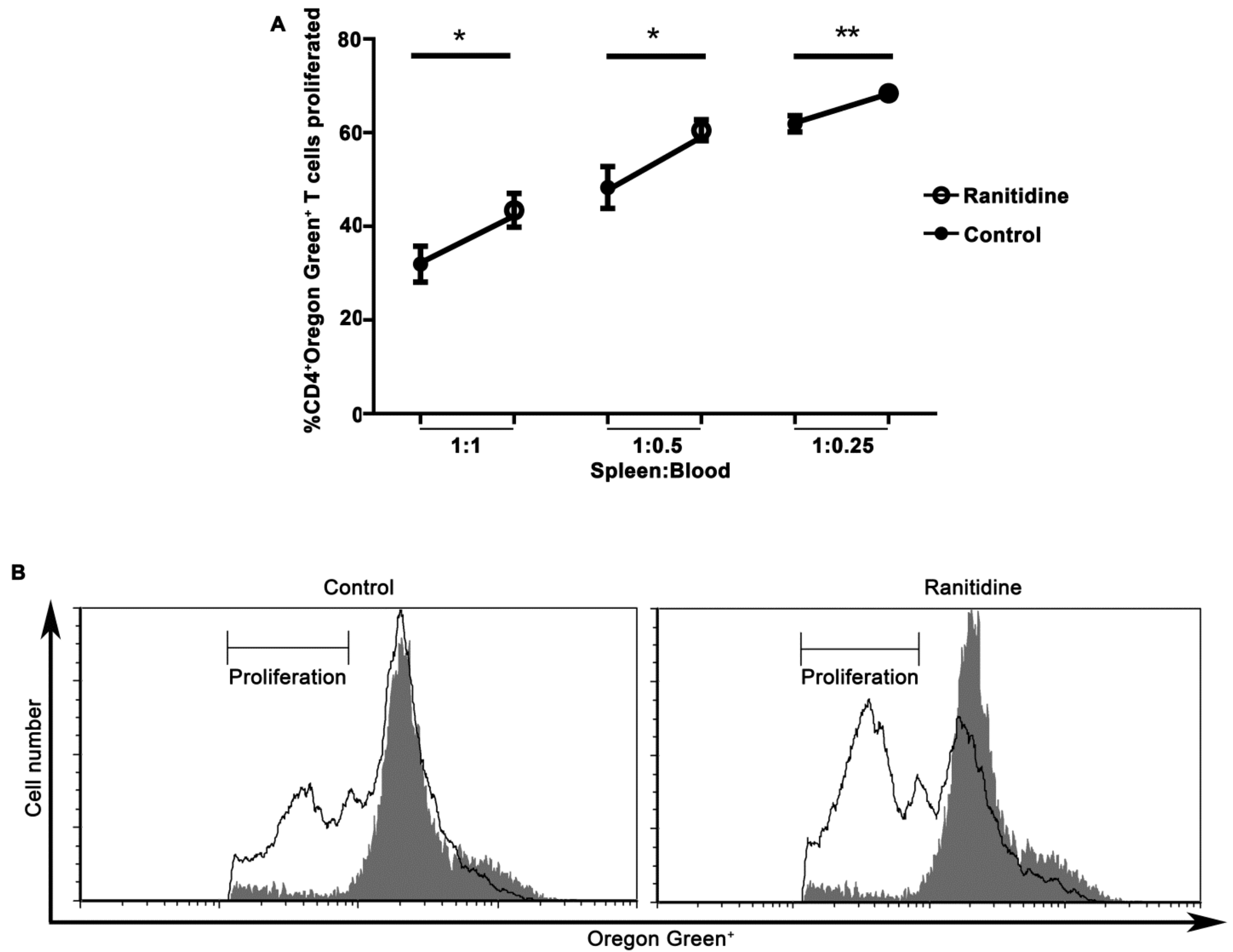

Figure 3.8 Peripheral blood leukocytes from ranitidine-treated tumour-bearing mice have decreased suppressive functions.

Peripheral blood leukocytes from 4T1 tumour-bearing mice with and without treatment were isolated and plated with Oregon Green-labeled D011.10 splenocytes with $ova^{323-339}$. After 3 days incubation proliferation was measured. Data points in (A) represents mean ± SEM of 9 mice. (B) Representative data of one mouse/treatment. Grey histogram represent unstimulated Oregon $Green^{+}CD4^{+}$ cells. *p<0.05, **p<0.01, unpaired t-test.

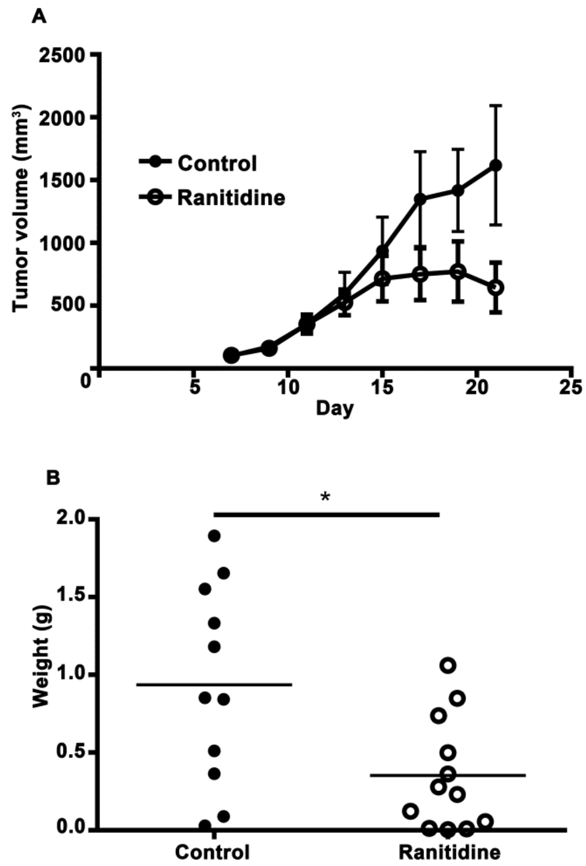

Figure 3.9 Ranitidine treatment decreases E0771-GFP tumour growth.

(A) E0771 tumours in C57BL/6 mice treated with ranitidine (8 mg/kg) were measured every 2 days starting 7 days post E0771-GFP cell injection. (B) At day 21 the primary tumour was excised and weighed. Data in (A) represents the mean ± SEM tumour volume of 11-12 mice/point. Data points in (B) represent final tumour weight of individual mice. *p<0.05, unpaired t-test.

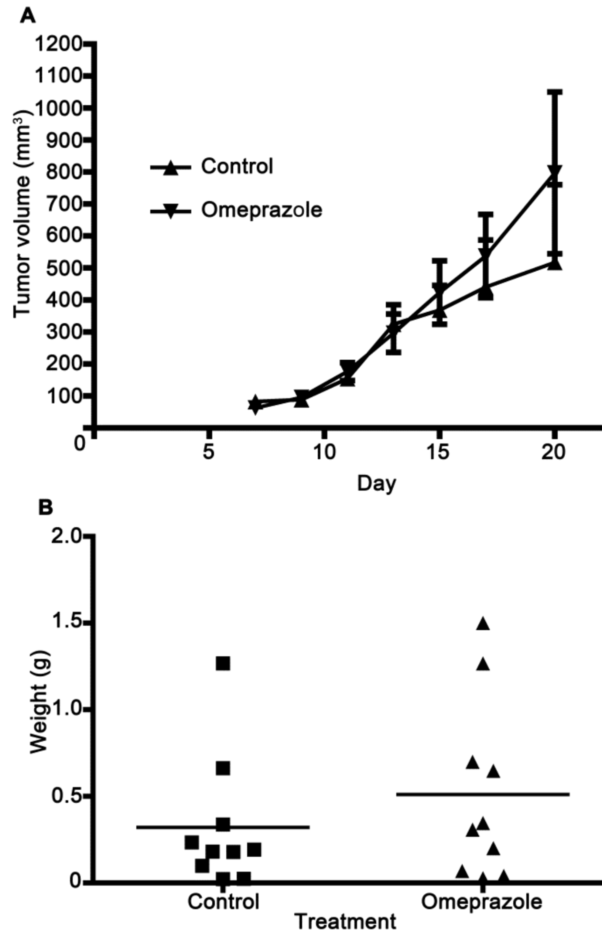

Figure 3.10 Omeprazole treatment does not alter E0771-GFP tumour development. (A) E0771 tumours in C57BL/6 mice treated with omeprazole (1.275 mg/day in mash) were measured every 2 days starting 7 days post E0771-GFP cell injection. (B) At day 20 the primary tumour was excised and weighed. Data in (A) represents the mean ± SEM tumour volume of 10 mice/point. Data points in (B) represent final tumour weight of individual mice. ns.

Figure 3.11 Gemcitabine treatment prevents ranitidine-induced tumour growth inhibition.

(A) Blood samples were taken from each mouse one day after gemcitabine (or vehicle) injection and stained for myeloid cells. (B) E0771 tumours in C57BL/6 mice treated with ranitidine (8 mg/kg) with/without gemcitabine treatment were measured every 2 days starting 7 days post E0771-GFP cell injection. (C-D) At day 20 the primary tumour was measured (C), excised and weighed (D). Data in (A) represents the mean ± SEM of 4-8 mice/group. Data in (B) represents the mean ± SEM tumour volume of 12-24 mice/point. Data points in (C-D) represent the mean ± SEM of 12-24 mice/group. ns.

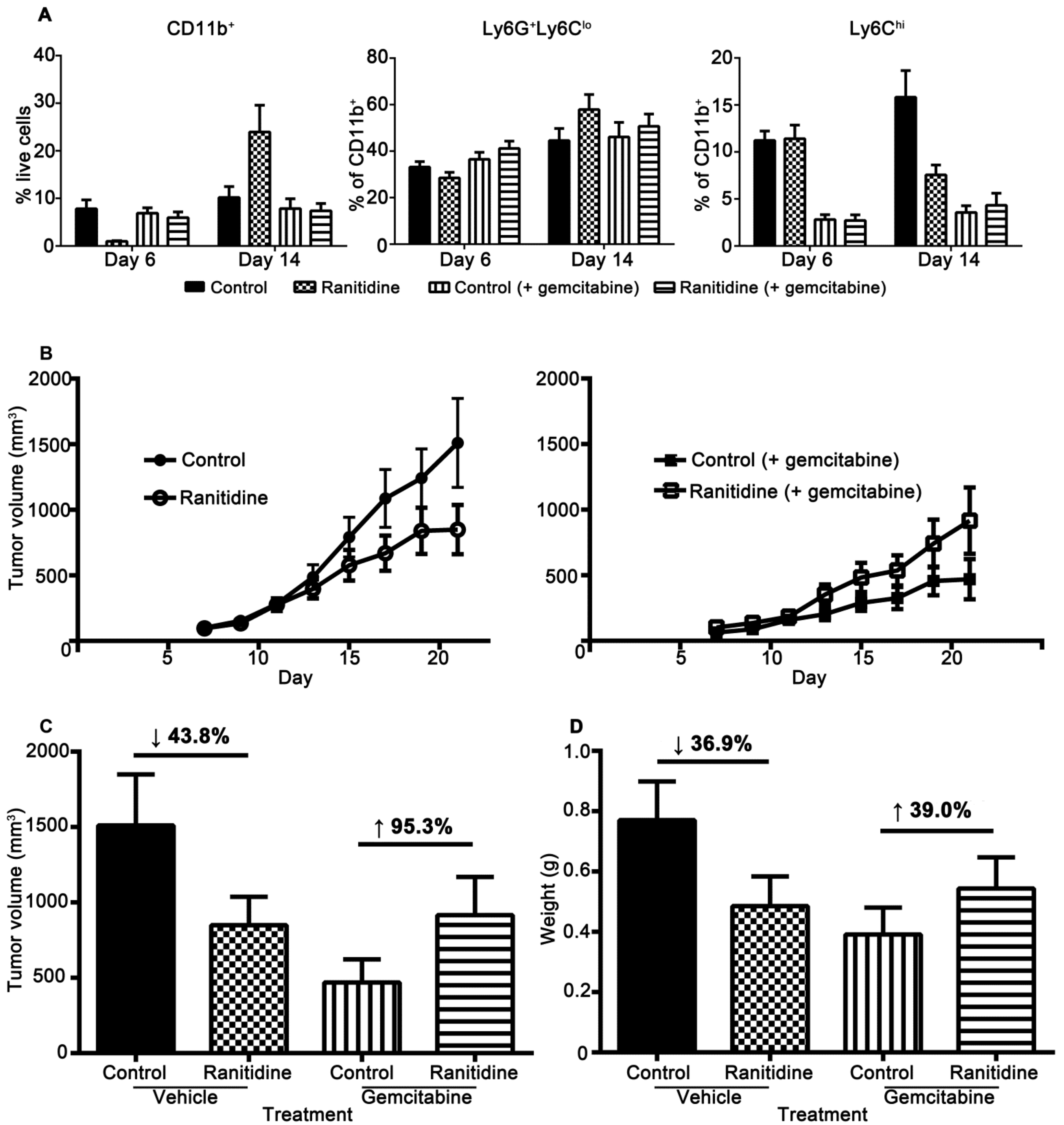

Figure 3.11

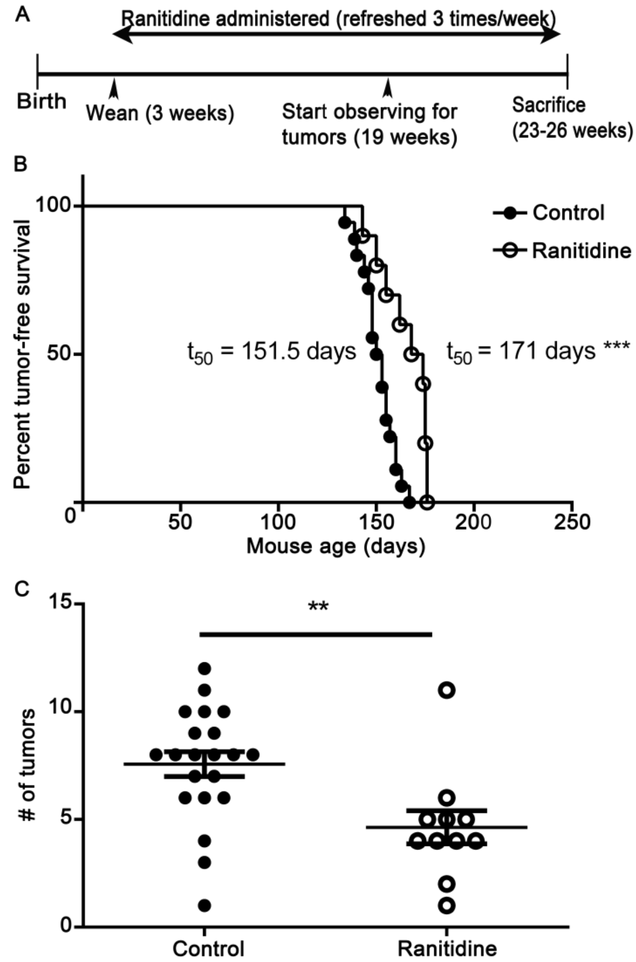

Figure 3.12 Ranitidine increased breast tumour onset latency and decreases final tumour numbers in LKB1$^{-/-}$/NIC mice.

(A) Representative timeline of experiment. (B) Mice were examined weekly for palpable breast tumours. (C) Endpoint number of tumours were counted. (B) Representative tumour-free survival of control (n=15) and ranitidine-treated (n=10) mice. Data points in (C) represent number of tumours in individual mice. ***$p<0.001$, Log rank test (B); **$p<0.01$, unpaired t-test (C).

3.3 Discussion

New approaches to enhancing effective immune responses to breast cancer are urgently required both as an approach to cancer prevention and to improve the effectiveness of other treatments. Modulation of tumour-associated immune suppression and specifically MDSC populations provides a potential approach to enhancing acquired antitumour immunity which could be effective at multiple stages of tumour development and spread. In the current study, ranitidine treatment *in vivo* caused a decrease in select monocyte populations in both the spleen and the bone marrow of both naïve and breast tumour-bearing mice. Blood from ranitidine-treated, tumour-bearing mice also showed a reduced ability to suppress T cell proliferation, and depleting MDSCs through gemcitabine treatment ablated the effect of ranitidine on tumour growth. H2 antagonists, when administered in drinking water at doses within the clinical therapeutic range, inhibited lung metastasis in the 4T1 breast tumour model and led to inhibition of initial tumour growth in the E0771 breast cancer model. H2 antagonist treatment also increased the time before tumour development and reduced the number of tumours developing in the $LKB1^{-/-}$/NIC mouse model of spontaneous breast tumour development. Overall, H2 blockade was beneficial in these breast tumour models. These results are surprising considering that previous studies report that ranitidine decreases the cytotoxic activity of NK cells [702] and that histamine signaling through H2 inhibits reactive oxygen species synthesis by monocytes, thereby enhancing NK cell activity [544, 545, 642]. These findings demonstrate that ranitidine treatment can function at a variety of stages during tumour development and in multiple breast tumour settings, consistent with an impact on tumour-associated immune suppression.

One previous study has analyzed whether treatment with H2 antagonists can alter breast tumour outcome [654], but in these patients, treatment was provided only at very late stages of disease. In keeping with our findings, another study has suggested a decreased risk of developing lung cancer with long term H2 antagonist treatment [659]. Our results suggest that H2 antagonists could have a potentially beneficial effect if administered either before tumour development or at an early point in tumour development, particularly in those tumour settings where limiting MDSC function would be beneficial. The beneficial effects of ranitidine treatment are not dependent upon H2 expression by the tumour cells and neither of the injectable tumour models we used expressed this receptor.

As H2 antagonists block acid secretion in the stomach, this can cause alterations in the intestinal microbiome [566] which could then alter the immune response [567, 568]. A recent study revealed that altering the gut microbiome in mice with a predisposition to developing breast tumours increased the number of tumours the mice had, a change that was related to neutrophil activity [571]. Omeprazole is a proton pump inhibitor that is capable of inhibiting acid secretion without binding H2 receptors. Other studies have shown that omeprazole can inhibit breast cancer cell proliferation *in vitro* and experimental metastasis *in vivo* [705, 706], but our results show that, unlike ranitidine, omeprazole treatment does not inhibit E0771 tumour growth.

Eight days of ranitidine treatment were associated with a significant decrease in monocytes in the spleen and bone marrow. This is consistent with the known myelosuppressive effect of ranitidine [507, 508]. In humans it is thought that such myelodepletion is primarily associated with neutropenia [508]. In mice, the ranitidine–

induced depletion we observed was monocyte specific. Previous results have shown that the less selective H2 antagonist cimetidine can cause apoptosis of MDSCs [555], but these results were not replicated by the alternate H2 antagonist famotidine, and were not reversed by histamine, suggesting an “off target” mechanism of cimetidine action.

In general, monocytes and macrophages have several protumourigenic effects. Tumour-associated macrophages are important for angiogenesis and microenvironment modulation (for a review see ref. [259]). Decreasing the monocyte populations could thereby inhibit tumour growth and development [86, 707]. The monocyte population of MDSCs is also considered more immunosuppressive than the neutrophil population [334]. Ranitidine has previously been demonstrated to have a number of effects on monocytes including modulation of adhesion molecule expression, in keeping with the known functions of H2 [701]. However, it remains possible that the impact of ranitidine on monocyte populations may be indirect.

MDSCs are a key cell type involved in immune suppression in cancer and can also directly impact tumour cells to induce growth and metastasis [84, 339]. Increases in circulating MDSCs correlate with disease progression in breast cancer patients [332] and circulating MDSCs in cancer patients are shown to inhibit T cell proliferation [332, 339]. In our experiments, blood leukocytes from ranitidine-treated 4T1 tumour-bearing mice had less suppressive activity on antigen-driven T cell proliferation than those from control tumour-bearing mice. These findings confirm that ranitidine treatment causes an alteration in the functional MDSCs in the blood. Both neutrophilic and monocytic MDSCs could contribute to this response. In keeping with these findings histamine and histamine-producing cells have previously been demonstrated to have a key role in

MDSC regulation in mice, although the most profound effects were observed on granulocytic MDSC's [353, 561]. This study by Martin *et al* [561] stated that granulocytic MDSCs can be impacted by both H1 and H2 antagonists, but our studies showed no effect by H1 antagonists. This may be in part due to the impact H1 has on other immune cells, which could offset the positive effects H1 blockade has on MDSC inhibition.

NOS2 and Arg1 are often thought of as the predominant mediators in MDSC-mediated suppression but inhibition of these pathways did not significantly modify the suppression of CD4 T cell responses by peripheral blood cells from 4T1 tumour bearing mice. However, some populations of MDSCs have been shown to cause suppression via NOS2- and Arg1-independent mechanisms. MDSCs also synthesize reactive oxygen species (ROS), and in some cases have been shown to be the primary mediator in T cell suppression [297, 390, 398]. In a study by Nagaraj *et al,* ROS synthesis was the primary mediator of suppression by MDSCs, when gp91 was knocked out, therefore leading to an inability to produce ROS, MDSC function was lost, while MDSCs deficient in NOS2 still had suppressive activity [385]. Another study showed that production of TGFβ by MDSCs was very important for hindering tumor immunosurveillance [392, 393, 395], and that TGFβ in humans is important for immune suppression with no contribution by NOS2 and Arg1 activity [327]. Overall, our data may signify that the impact ranitidine had on alterations in immune suppression was dependent on an alteration in the number of MDSCs present in circulation, not due to ranitidine altering expression of NOS2 and Arg1 and also suggests that TGF-β, ROS or a combination of such pathways mediate MDSC function in the blood, in this model.

Gemcitabine is widely used as an inhibitor of MDSCs [9] and was utilized to investigate the role of such MDSCs in the E0771 model of ranitidine-inhibited primary tumour growth. Gemcitabine can selectively cause depletion of MDSCs, while not altering other immune cell numbers [403, 704]. This depletion was specific to monocytic MDSCs in the E0771 model. Following gemcitabine treatment, ranitidine did not have an effect on tumour development. Notably, other cells from the monocytic lineage, including mature macrophages and tumour-associated macrophages (TAMs) are not impacted by short term gemcitabine treatment [708]. However, it remains possible that long term ranitidine treatment could alter TAM populations by altering monocytes that would otherwise be recruited and differentiated into TAMs.

H2 blockade has been shown to alleviate inhibition of IFNγ function [456] and modify DC function and migration [511, 518]. Notably, $CD8^+$ T cell depletion did not alter ranitidine's effect on 4T1 metastasis. Although $CD8^+$ T cells are important in tumour clearance, they are not the only cell involved [709]. MDSCs have been reported to have inhibitory effects on several immune effector cell types including NK cells [395, 396] and $CD4^+$ T cells [710, 711] directly [344], or via inhibition of DC function [699] or induction of regulatory T cell development [328]. In addition, antibody mediated processes such as complement mediated lysis and antibody-dependent cell-mediated cytotoxicity can also be affected by MDSCs.

In this study, ranitidine had an impact on three distinct models of breast cancer. We have confirmed that this is not due to a direct effect on the tumour cells in the two orthotopic models used. The different degrees and levels of tumour inhibition may be attributed to the differences in mouse genetic backgrounds [107, 712] and/or the differences in the

biology of the tumours themselves as they interact with the immune system. 4T1 cells are not highly immunogenic [713]. Modification of monocyte populations, such as MDSCs, has previously been shown to profoundly affect the ability of the 4T1 tumour model to metastasize [82, 84, 690]. The E0771 cell line appeared to be more vulnerable to immune changes mediated by ranitidine at the primary tumour site. In the LKB1$^{-/-}$/NIC model of spontaneous breast cancer, ranitidine is given over a longer period of time, which allows the ranitidine to modify immune effector cell populations prior to tumour development. The impact of ranitidine-induced MDSC changes on tumour growth and spread will likely vary extensively between tumours and also be related to other effects of ranitidine on immune function of importance in regulating tumour growth or metastasis. Overall, these studies highlight the profound impact that widely used H2 antagonists can have on antitumour immune function and suggest the use of these agents may provide opportunities to reduce tumour-associated immune suppression in breast cancer or reduce breast cancer development in those at high risk.

In conclusion, we have shown that the commonly used histamine receptor 2 antagonist ranitidine can affect monocyte populations in both normal and tumour-bearing animals. Consistent with an inhibition of MDSC populations, ranitidine treatment can also inhibit breast tumour development and spread in three separate breast tumour models including a model of spontaneous breast tumour development. These results suggest that it may be beneficial to consider including a histamine receptor 2 antagonists, as opposed to other regulators of gastric acid secretion, in the context of breast cancer therapy or prevention. Clinical studies are urgently required to address these issues.

4 CHAPTER 4 THE IMPACT OF RANITIDINE ON MONOCYTE RESPONSES IN THE CONTEXT OF SOLID TUMOUR.

4.1 Introduction

Monocyte recruitment is important for tumor progression [81-83, 272]. A subset of monocytes will develop into tumor associated macrophages; such cells can enhance tumor cell progression, angiogenesis, extravasation, metastasis, and resistance to chemotherapeutics [28, 83, 262, 263]. Therefore a potential method to limit tumor progression would be to target monocyte development. Studies in mice have shown that there are alterations in tumor development when monocytes are depleted or their recruitment is inhibited [10, 82, 264, 714, 715]. In humans, treatments targeting monocytes are undergoing clinical trials [259, 715].

Monocyte development in the bone marrow of mice is dependent on monocyte colony stimulating factors, such as CSF1 [197, 198]. Hematopoietic stem cells can develop into common myeloid progenitors (CMPs; $Lin^-Thy1^-IL\text{-}7R\alpha^-Sca1^-c\text{-}Kit^+Fc\gamma R1^{lo}CD34^+$) which have the potential to develop into granulocyte-macrophage progenitors (GMPs; $Lin^-Thy1^-IL\text{-}7R\alpha^-Sca1^-c\text{-}Kit^+Fc\gamma R1^{hi}CD34^+$) [203]. Subsequently such cells can go on to develop into macrophage and dendritic cell precursors ($Lin^-c\text{-}Kit^+CD115^+CD135^+$ $Ly6C^-$ $CD11b^-$) [204, 205], and then common monocyte progenitors ($Lin^-c\text{-}Kit^+CD115^+$ $CD135^-$ $Ly6C^+CD11b^-$) [205]. These common monocyte progenitors may then develop into monocytes [205] which are $CD11b^+Ly6C^{hi}$ [204, 206]. The $Ly6C^{hi}$ monocytes then leave the bone marrow to be part of the peripheral blood monocyte population [204].

Once recruited into the circulation, the Ly6C^{hi} population can differentiate into Ly6C^{low} circulating monocyte population [204, 206], or be recruited to sites of inflammation where they can differentiate into monocyte-derived dendritic cells or macrophages [188]. These inflammatory monocytes are usually CCR2^{hi}CX$_3$CR1^{low}. The Ly6C^{low} population is thought to "patrol" the endothelium and is involved in endothelial repair [227]. These cells are also required for the extravasation and tissue invasion of inflammatory monocytes during infection [226] and usually are CX$_3$CR1^{hi}. The inflammatory monocyte population is the predominant target for monocyte depletion with the aim of reducing tumor progression.

A subset of monocytes make up part of a group of cells noted as myeloid-derived suppressor cells (MDSCs). MDSCs are a population of immature monocytic and granulocytic cells that have immunosuppressive functions. With cancer progression, there are elevated levels of cytokines such as CSF1-3 and stem cell factor, which leads to abnormal myelopoiesis [191, 225] and an increase in immature myeloid cells in circulation [191], which can develop into MDSCs. Suppressive functions of MDSCs include inhibition of cytotoxic and helper T cell activation and proliferation [354], induction of T regulatory cells [391], reduction of NK cell activity [396], and induction of immunosuppressive macrophage phenotypes [329, 394]. MDSC suppressive activity occurs through a number of mechanisms. MDSCs produce mediators including IL-10 and TGFβ [391, 394], as well as nitric oxide and reactive oxygen species [390, 716] that are immunosuppressive. Furthermore, MDSCs can deplete arginine, an essential amino acid for T cell proliferation [378], from the environment with enzymes such as inducible nitric oxide synthase (NOS2) and arginase [342, 716]. MDSCs have been found to be a barrier

to inducing an effective immune response against tumors, even in the context of immunotherapy.

Histamine is increased in concentration within tumors and regulates immunity [438, 608]. Histamine signals through four known histamine receptors (H1-4) which are differentially expressed on all immune cells, including monocytes and MDSCs. Monocytes can express H1, H2, and H4 [465, 482, 578, 585, 593, 673, 675], and MDSCs express H1-3 [554, 555, 561]. H2 signaling has been implicated in the regulation of monocytes since it enhances CCL2 production and their expression of CCR2, which would enhance monocyte recruitment [536]. H2 signaling can inhibit production of cytokines such as TNF [538] and IL-27 [540] and also induces IL-1β production by monocytes [541]. H2 signaling also inhibits synthesis of reactive oxygen species in monocytes [509, 544, 545]. Yang *et al* [554] revealed that histamine signaling, primarily via H2 receptors, was important for MDSC function and that lack of HDC caused myeloid cells to remain in an immature state. Another study revealed that cimetidine, an H2 antagonist, inhibited nitric oxide synthesis and arginase I expression in monocytic MDSCs [555, 561], and caused MDSC apoptosis [555]. Histamine has also been shown to be important for inducing proliferation and survival of monocytic MDSCs through H1 and H2 signaling [561]. While functional aspects of MDSC have been investigated, there has been little focus on how H2 signaling can impact monocyte and MDSCs development.

H2 antagonist treatment can inhibit breast cancer development (Vila-Leahey *et al,* under revision [717]). This is associated with a decrease in monocytes in the spleen and bone marrow. In the current study, we examined a variety of tumors and the impact of

ranitidine on their development. Notably, ranitidine did not reduce tumor growth in several non-breast cancer models although it selectively reduced E0771 primary tumor growth and 4T1 metastasis. Using the orthotopic E0771 breast tumor model the impact of H2 antagonists was not observed in CCR2-deficient mice with defective monocyte recruitment. Further analysis revealed a difference in monocyte histamine receptor expression in tumor bearing compared with naïve mice. Monocyte progenitors were decreased in non-tumor bearing mice following ranitidine treatment. Populations of monocytes in tumor-bearing mice were also altered in the presence of ranitidine. These results reveal that enhanced tumor immunity in the presence of ranitidine is associated with changes in monocytic cell populations and is CCR2-dependent.

4.2 Results

4.2.1 Ranitidine does not alter tumour development in the absence of CCR2

In previous studies we demonstrated that ranitidine treatment decreased 4T1 lung metastasis by 61% compared to control mice and reduced the growth of primary E0771 breast tumours in an orthotopic model (Vila-Leahey *et al,* under revision [717]). The impact of ranitidine treatment on tumor growth was further investigated using a panel of five tumor models; only E0771 primary tumor growth was significantly altered by ranitidine treatment (Table 4.1). Monocytic MDSCs have been implicated as important for the impact of ranitidine on breast tumour progression. We therefore analyzed the myeloid cell population in tumor-bearing mice 7 days after tumor cell injection. The total number of monocytes in ranitidine-treated 4T1 tumor-bearing mice was previously found to be decreased by 46.3% ($p<0.005$) (Vila-Leahey *et al,*under revision [717]). The percentage of myeloid cell subsets in the spleen were unaltered in LLC-1, B16-OVA, and EL4 following ranitidine treatment, while there were increased CD11b$^+$ myeloid cells in the spleen of ranitidine-treated E0771 tumor-bearing mice, and increased neutrophils in ranitidine-treated 4T1 tumor-bearing mice compared to control mice (Table 4.2). As ranitidine selectively decreased primary E0771 tumor growth and this was associated with myeloid cell changes we further analyzed the relationship between ranitidine treatment and monocytes in tumor development utilizing this model.

E0771 cells were injected into CCR2$^{-/-}$ C57BL/6 mice. Analysis of blood from these mice showed decreased levels of monocytes in the CCR2 knockout mice compared to wild type mice. In control C57BL/6 mice, ranitidine caused inhibition of tumor development, starting at approximately day 13 of tumor development. In the CCR2$^{-/-}$

mice, there was no difference in tumor growth or final tumor weight between ranitidine treated and control groups (Figure 4.1). These results demonstrate a critical role for CCR2 in the mechanism of action of ranitidine and suggest monocytes and recruitment of monocytes to the tumour may be important for the effect ranitidine has on tumour progression.

4.2.2 Analysis of monocytes in E0771-bearing C57BL/6 mice

The nature of the monocyte population in the E0771 tumour-bearing mice with or without ranitidine treatment was further analyzed by flow cytometry. In naïve mice, the monocyte population were not altered in either the spleen or the bone marrow following ranitidine treatment (Figure 4.2). At 7 days post tumour cell injection there was a small but significant increase in overall numbers of myeloid cells in the spleen of ranitidine-treated tumour-bearing mice, compared with control tumour-bearing mice that was not due to alterations in identified monocytes or neutrophils (Figure 4.2). We also analyzed the spleens and tumours 14 days post tumour cell injection. At day 14, which is where the tumour growth starts to slow down with ranitidine treatment, an increase in myeloid cells is seen at the tumour site, with this increase being associated with neutrophil recruitment (Figure 4.2). There was no significant difference in the spleen at this time point, and there was no alteration in the spleen or in the tumour at the end point of the experiment (day 20) with ranitidine treatment.

4.2.3 Ranitidine does not impact circulating monocytes.

Circulating monocytes were analyzed during tumour development to see if there were alterations in surface markers. We also analyzed whether there were any differences in CCR2 and CX3CR1 to determine if there were alterations in inflammatory monocyte

numbers in circulation in the context of ranitidine treatment. For these studies, mice were treated orally, beginning ranitidine seven days prior to tumour cell injection.

Starting ranitidine treatment one week prior to tumour cell injection caused tumour growth to slow, to an equivalent extent as starting treatment one day prior to tumour cell injection (Figure 4.3). Over the course of the experiment there was an increase in myeloid cells in circulation, but no significant alterations were seen in the total monocytic cells or in inflammatory monocytes in the circulation. However, at the end point of the experiment, there was a significant decrease in monocytes in the spleen in tumour-bearing mice treated with ranitidine.

4.2.4 Monocytes decrease H1 expression in the presence of a tumour.

Histamine receptors are known to be expressed on monocytes [465, 482, 578, 585, 593, 673, 675], and the ability of ranitidine and histamine to modulate MDSC function is highly dependent on histamine receptor expression. Therefore we examined the expression of histamine receptors on monocytes, with characteristics associated with MDSC, during tumour progression.

Haile *et al* [334] describe that monocytic cells expressing CD49d are suppressive, therefore we sorted for $CD11b^{+}Ly6C^{hi}CD49d^{+}$ monocytic MDSCs. Splenic monocytic MDSCs from tumour-bearing mice had a higher ratio of H2 to H1 compared to monocytic MDSCs from naïve mice (10.6 vs 1.7, respectively) (Figure 4.4). The ratio of H2 to H1 in tumour-associated monocytic MDSCs was also higher compared to naïve mice (13.4 vs 1.7, respectively).

Table 4.1 Final tumour weights of histamine receptor antagonist-treated tumour-bearing mice. *p<0.05

	Control		Ranitidine		N = (/group)
	Mean (g)	± SEM	Mean (g)	± SEM	
B16-OVA	1.33	0.45	1.74	0.4	7-8
LLC-1	0.20	0.05	0.16	0.03	12
EL4	0.55	0.08	0.66	0.08	8
E0771	0.94	0.19	0.35*	0.10	11-12
4T1	0.64	0.07	0.61	0.06	15

*p<0.05

Table 4.2 Summary of the splenic myeloid population of histamine receptor antagonist-treated tumour-bearing mice 7 days after tumour cell injection.

		% $CD11b^+$ of live		% $Ly6C^{hi}$ of $CD11b^+$		% $Ly6G^+$Ly6Clow of $CD11b^+$		
		Mean	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**N = (/group)**
B16-OVA	**Control**	10.78	0.82	7.42	0.38	19.5	1.36	14
	Ranitidine	12.67	1.64	7.52	0.71	17.37	2.73	14
LLC-1	**Control**	10.67	0.51	7.48	0.91	29.39	2.03	9
	Ranitidine	13.45	2.67	7.16	0.67	28.38	3.09	9
EL4	**Control**	15.35	1.88	6.54	0.88	19.2	2.40	9
	Ranitidine	16.3	1.30	6.44	1.04	16.56	1.86	9
E0771	**Control**	6.90	0.25	7.79	0.25	21.62	1.42	12
	Ranitidine	8.92**	0.65	7.50	0.60	24.44	1.83	12
4T1	**Control**	8.26	0.64	7.96	1.00	32.70	1.94	13
	Ranitidine	7.63	0.76	6.54	0.68	40.94*	2.83	13

*p<0.05, **p<0.01

Figure 4.1 The impact of ranitidine on E0771 tumour progression is associated with changes in circulating monocytes.

(A) Composition of blood $CD11b^{+}$ cells, $Ly6G^{+}Ly6C^{low}$ granulocytic cells, and $Ly6C^{hi}$ monocytic cells of E0771-GFP tumour-bearing C57BL/6 (WT) and $CCR2^{-/-}$ C57BL/6 mice at day 21. E0771-GFP tumours in C57BL/6 (B) and $CCR2^{-/-}$ C57BL/6 (C) mice treated with ranitidine (8 mg/kg) were measured every 2 days starting 7 days post E0771-GFP cell injection. (D) At day 21, the primary tumour was excised and weighed. Data in (A) represents individual mice and the line represents mean ± SEM per group. Data points in (B-C) represent the mean ± SEM tumour volume of 12-20 mice. Data points in (D) represent final tumour weight of individual mice and line represents the average per group. $*p<0.05$, $**p<0.01$, $***p<0.001$, unpaired t-test.

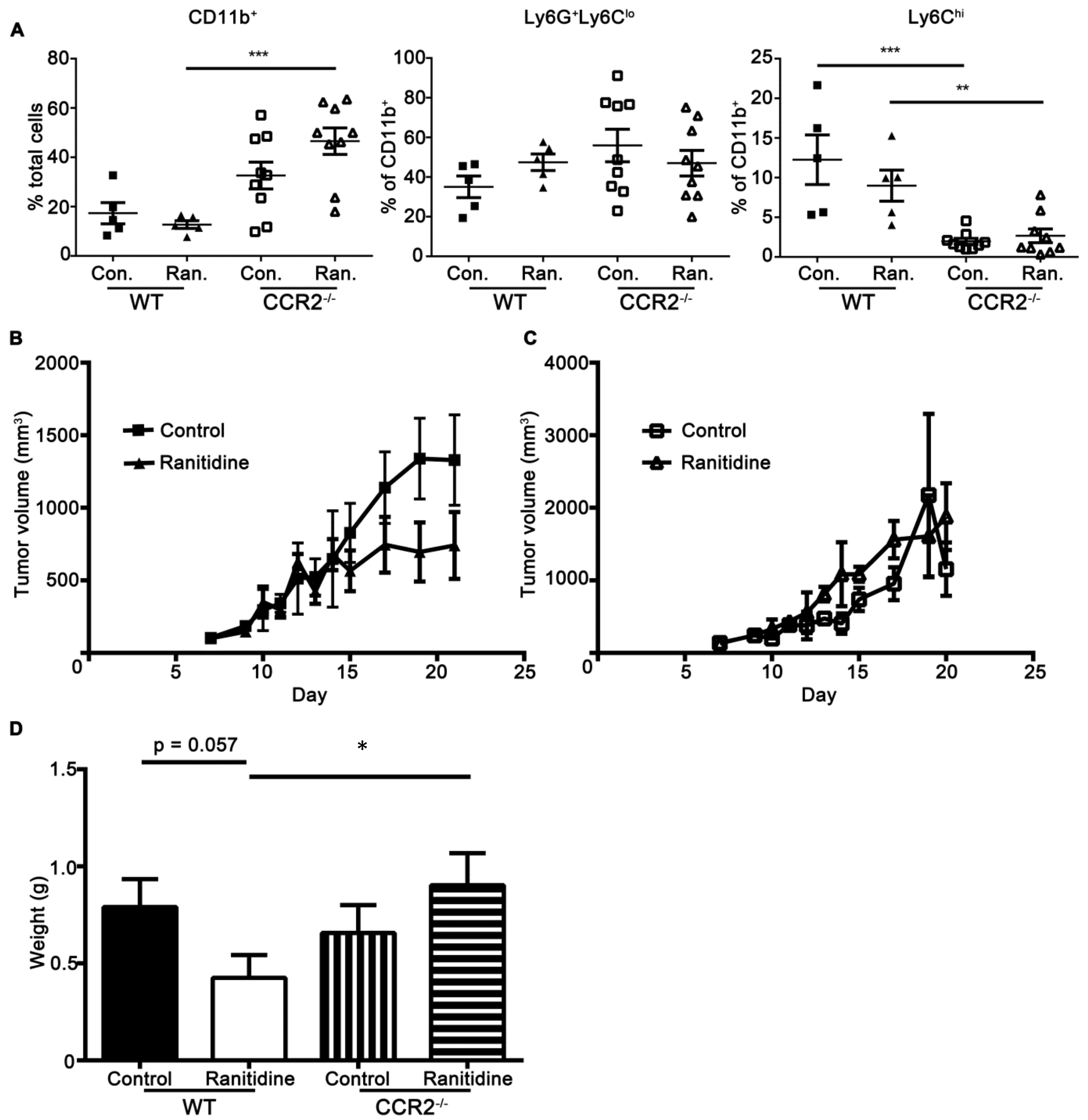

Figure 4.1

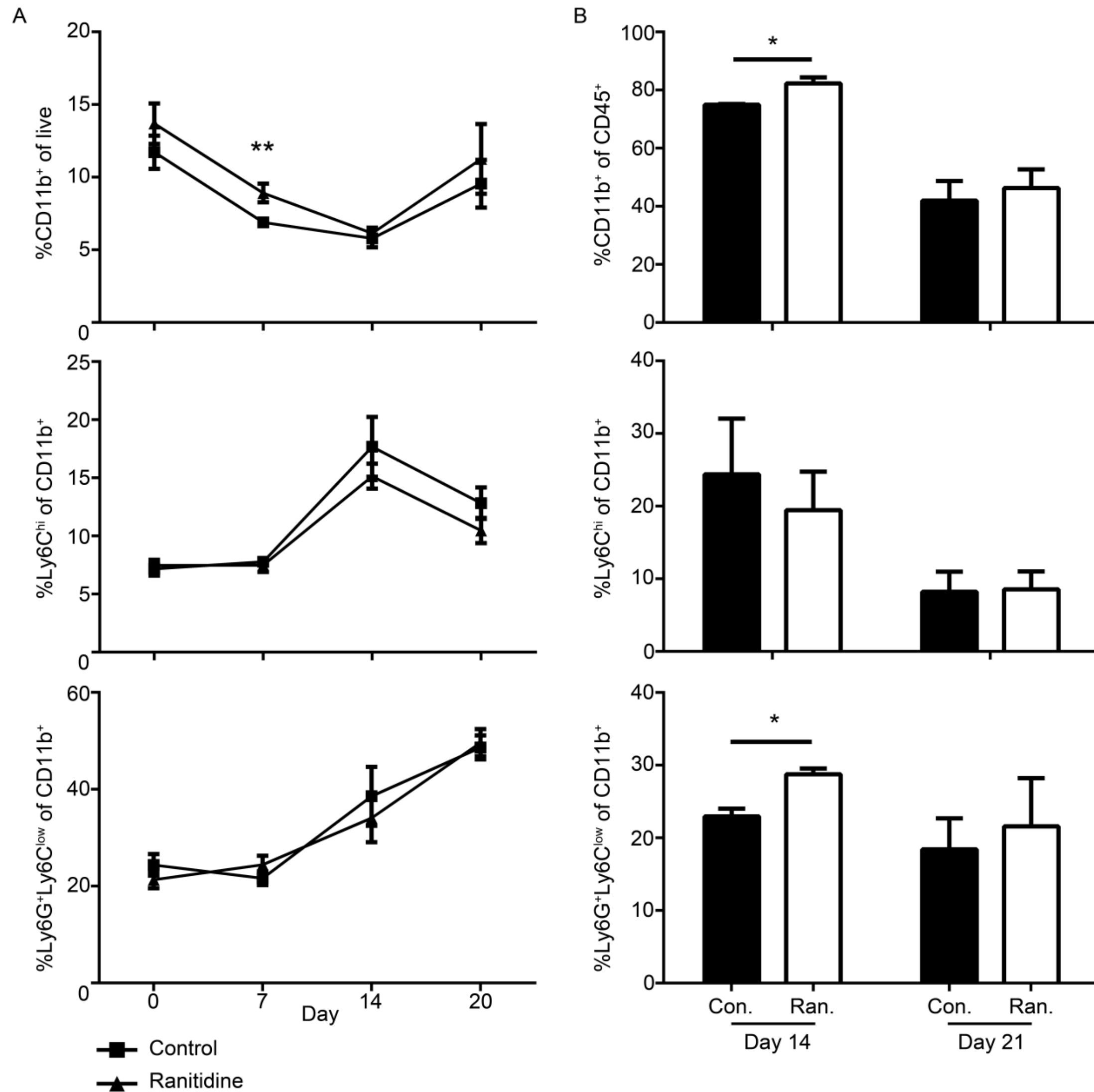

Figure 4.2 Alterations in myeloid cells at day 14 post E0771 cell injection.
Composition of $CD11b^{+}$ cells, $Ly6G^{+}Ly6C^{low}$ granulocytic cells, and $Ly6C^{hi}$ monocytic cells of splenic cells (A), and isolated from the tumour (B) from E0771-GFP tumour-bearing mice over time. Day 0 in (A) represent non-tumour-bearing C57BL/6 mice after 8 days of treatment, while the rest represent days after tumour cell injection. Data in (A-B) represents mean ± SEM of 3-17 mice/group. $^*p<0.05$, $^{**}p<0.01$, unpaired t-test.

Figure 4.3 Ranitidine treatment, initiated 7 days prior to tumour cell injection, does not impact circulating monocytes but decreases splenic monocytes.

(A) E0771-GFP tumours on C57BL/6 mice treated with ranitidine (8 mg/kg) were measured every 2 days starting 7 days after tumour cell injection. (B) Composition of blood $CD11b^{+}$ cells, $Ly6C^{hi}$ monocytic cells, and $CCR2^{+}$ inflammatory cells were measured starting 7 days pre-E0771-GFP injection and measured every 7 days. (C) Composition of splenic $CD11b^{+}$ cells, $Ly6G^{+}Ly6C^{low}$ granulocytic cells, and $Ly6C^{hi}$ monocytic cells were measured at day 21. Data in (A) represents the mean ± SEM tumour volume of 12 mice. Data points in (B) represents individual mice and the line represents mean ± SEM per group. *$p<0.05$, unpaired t-test.

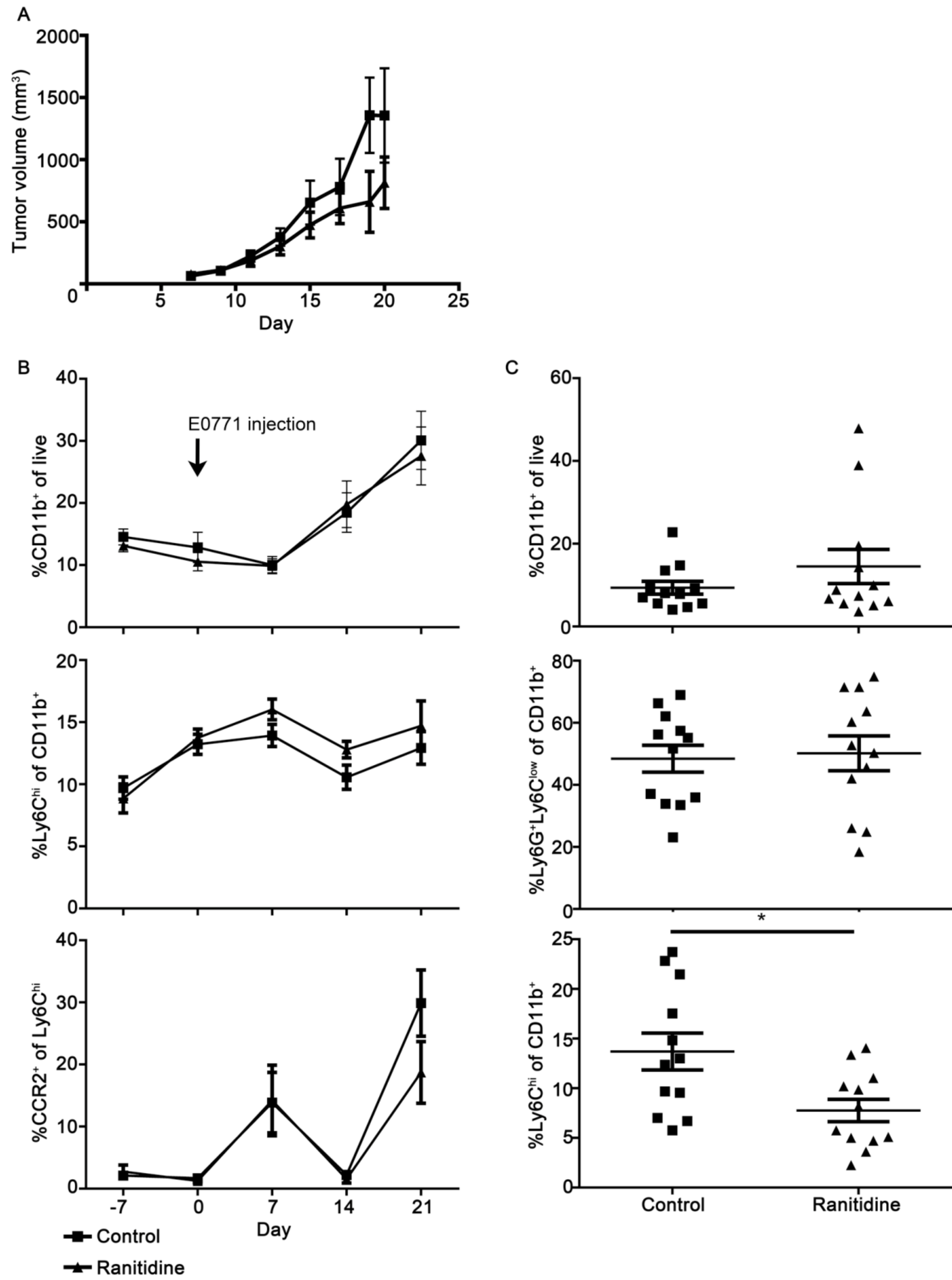

Figure 4.3

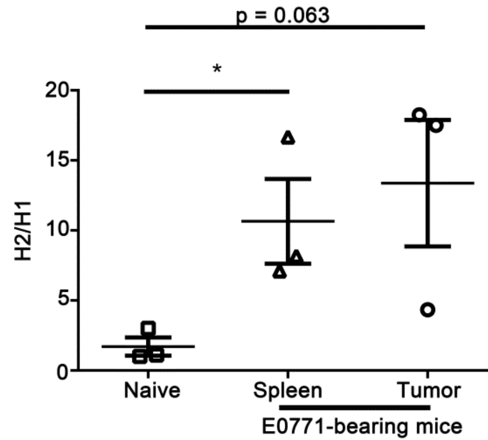

Figure 4.4 H2 levels are increased compared to H1 in monocytic MDSCs from E0771-bearing mice compared to naïve mice.

qPCR on CD11b^{+}Ly6C^{+}CD49d^{+} isolated from E0771-GFP tumours, spleens from E0771-GFP tumour-bearing mice, and spleens from naïve C57BL/6 mice was performed for detection of H1 and H2, and a ratio of H2 to H1 expression was calculated. Data points represent individual mice and line represents the mean ± SEM per group. *$p<0.05$, unpaired t-test.

4.2.5 Ranitidine does not alter tumour-associated monocytes.

To analyze whether ranitidine alters monocyte in the spleen or tumour of E0771 tumour-bearing mice, monocytes were sorted by FACS in a similar manner as previously stated using the markers $CD11b^{+}Ly6C^{hi}CD49d^{+}$ for monocytic MDSCs, and expression of key mediators was assessed by qPCR. There was no alteration in any of the measured mediators between the control and ranitidine-treated groups (Figure 4.5).

4.2.6 Analysis of mediators involved in monocyte differentiation and recruitment.

As monocytes are pivotal for tumour development and ranitidine was shown to impact monocyte populations most strongly using the 4T1 tumour model, we utilized this model to further analyze the mechanism of monocyte modulation by ranitidine. Levels of mRNA expression and presence of mediators in the plasma that can alter monocyte differentiation and recruitment were determined. Analysis of plasma samples from BALB/c mice with and without ranitidine treatment for the presence of colony stimulating factors (CSF1-3) that are involved in myeloid differentiation showed no alterations with ranitidine in naïve mice, but in 4T1 tumour-bearing mice there was a significant decrease in CSF3 at day 7, that disappeared after 21 days (Figure 4.6). Levels of mRNA expression for chemokines that are important for recruitment of monocytes were also examined. Expression of CCL2, CCL7, and CXCL12 in the spleen and bone marrow in BALB/c mice treated with ranitidine for 6-9 weeks was determined. CCL2 mRNA levels were not altered in either area as a result of ranitidine treatment while CCL7 showed a trend towards a decrease in ranitidine-treated animals in the spleen, and significantly decreased in the bone marrow (Figure 4.6). CXCL12 trended towards a decrease with ranitidine treatment but this was not statistically significant (Figure 4.6).

4.2.7 Long term ranitidine use alters splenic and bone marrow monocytes and progenitor cells.

The impact of ranitidine treatment on monocyte and monocyte-related progenitor cell populations in the bone marrow was also determined. Our previous work has shown that after 7 days of ranitidine treatment, BALB/c mice have decreased monocytes in the spleen and bone marrow (Vila-Leahey *et al*, under revision [717]) but longer periods of ranitidine treatment more likely in a clinical setting were not examined. The spleen, blood, and bone marrow of BALB/c mice that were treated with ranitidine for 6-9 weeks were examined in comparison with control mice. Long term ranitidine use led to decreased monocyte populations in the spleen (Figure 6A). Analysis of peripheral blood showed no significant alterations in myeloid cells, although there was a trend toward an increase in myeloid cells in the blood. Surprisingly the numbers of myeloid cells was not decreased in the bone marrow as it was with short term ranitidine treatment. When analyzing progenitor cells in the bone marrow, there was a significant decrease in GMPs and CMPs following ranitidine treatment (Figure 6D). There was no significant alteration in monocyte progenitors downstream of CMPs. There were no significant alterations in total splenocyte, bone marrow cell, and peripheral blood cell numbers.

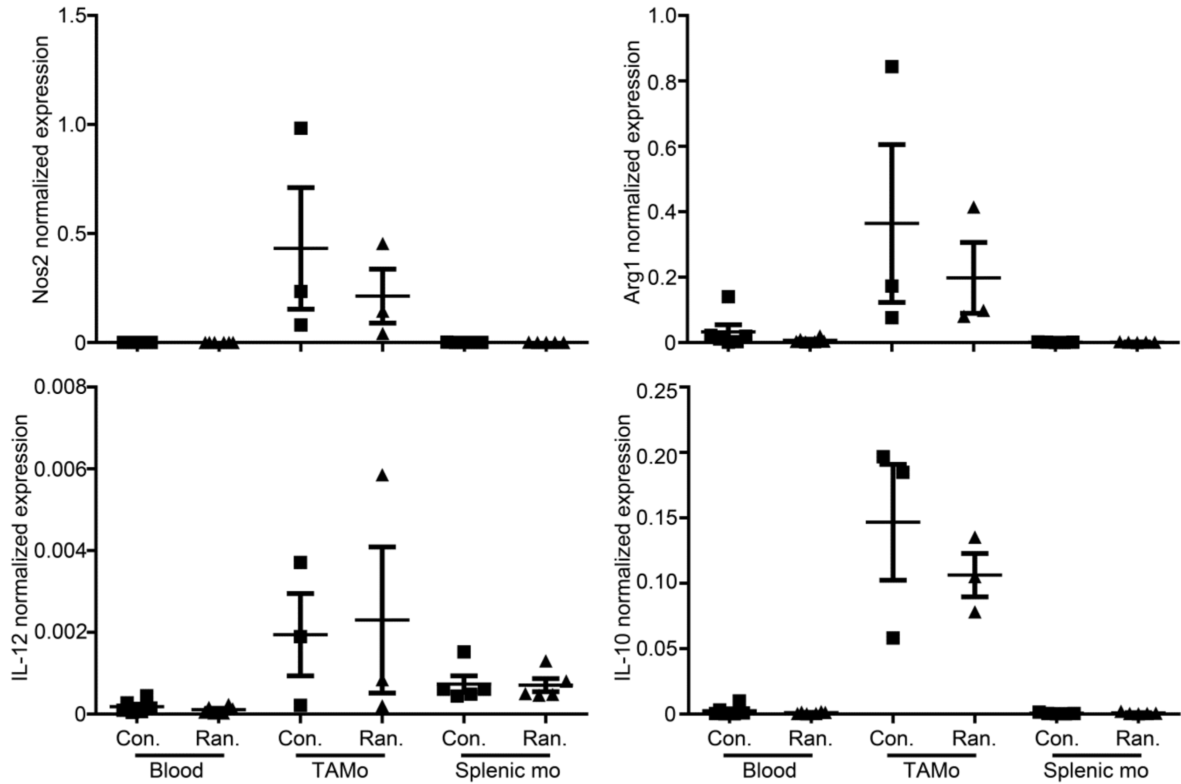

Figure 4.5 Ranitidine treatment does not alter mediator expression in monocytic MDSCs.

qPCR on whole blood leukocytes and isolated $CD11b^{+}Ly6C^{+}CD49d^{+}$ monocytic MDSCs from E0771-GFP tumours (tumour-associated monocytes [TAMo]) and spleens (splenic mo) from E0771-GFP-bearing mice 14 days post tumour cell injection were performed for the detection of NOS2, Arg1, IL-12, and IL-10. Data points represent individual mice and line represents the mean ± SEM per group. ns.

Figure 4.6 Ranitidine alters CSF3 in 4T1 tumour-bearing BALB/c mice after 7 days.

(A) CSF1, CSF2, and CSF3 levels in naïve BALB/c mice, and 4T1 tumour-bearing mice (7 days or 21 days after tumour cell injection) with and without ranitidine treatment was analyzed using Luminex. (B) qPCR of splenocytes and bone marrow cells was performed for detection of CCL2, CCL7, and CXCL12 in naïve BALB/c mice. Data in (A-B) represent individual mice and line represents the mean ± SEM per group. *$p<0.05$, unpaired t-test.

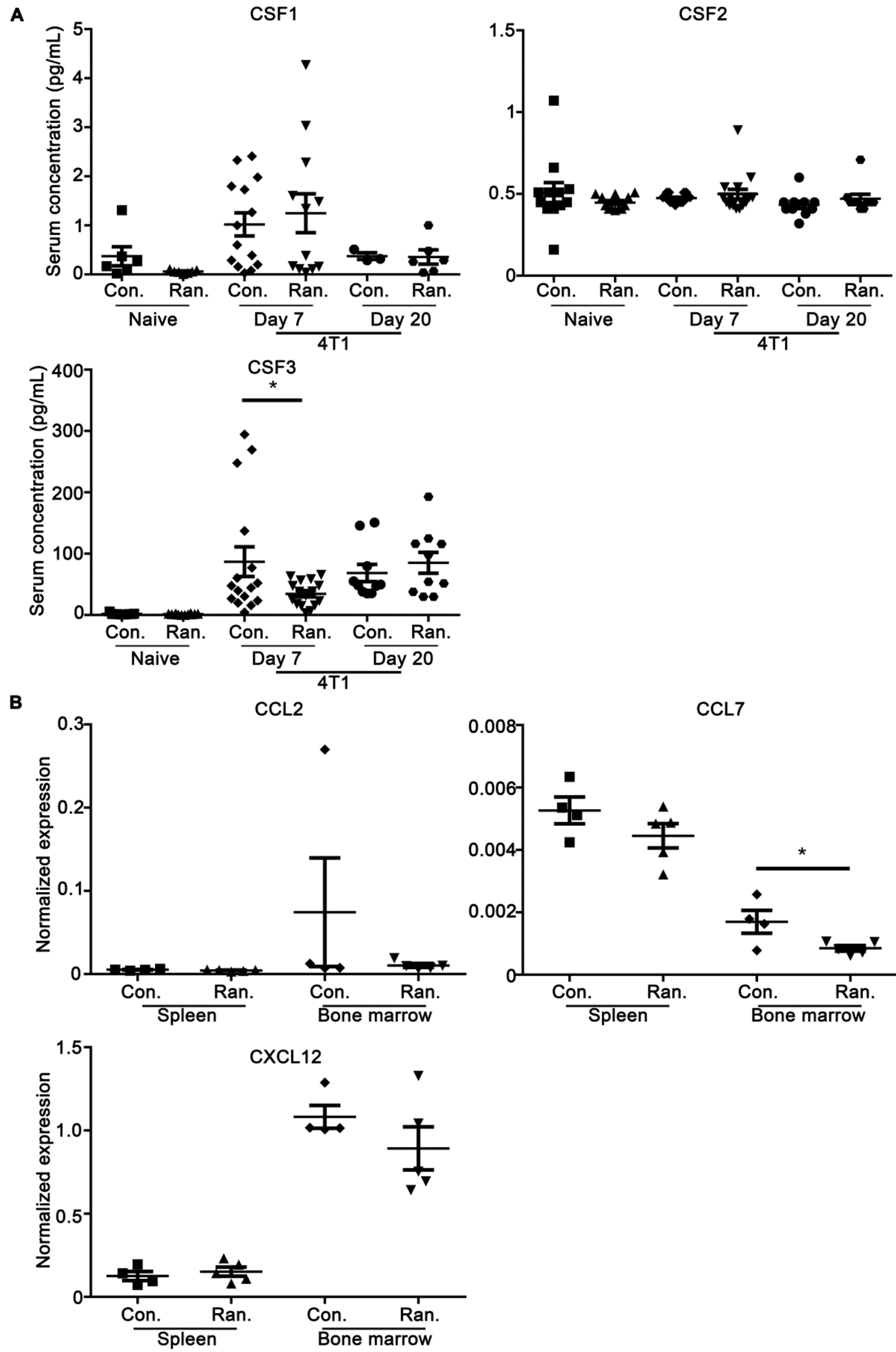

Figure 4.6

Figure 4.7 Ranitidine treatment causes a decrease in CMPs and GMPs in naïve BALB/c mice.

(A-C) Composition of total $CD11b^{+}$ cells, $Ly6G^{+}Ly6C^{lo}$ granulocytic cells, and $Ly6C^{hi}$ monocytic cells in spleen (A), bone marrow (B), and peripheral blood cells (C) of non-tumour-bearing mice with and without 6-9 weeks of ranitidine treatment. (D) Composition of total CMP, GMP, and MDP cells in bone marrow. Data points represent individual mice and line represents the mean ± SEM per group. *$p<0.05$, **$p<0.01$, unpaired t-test.

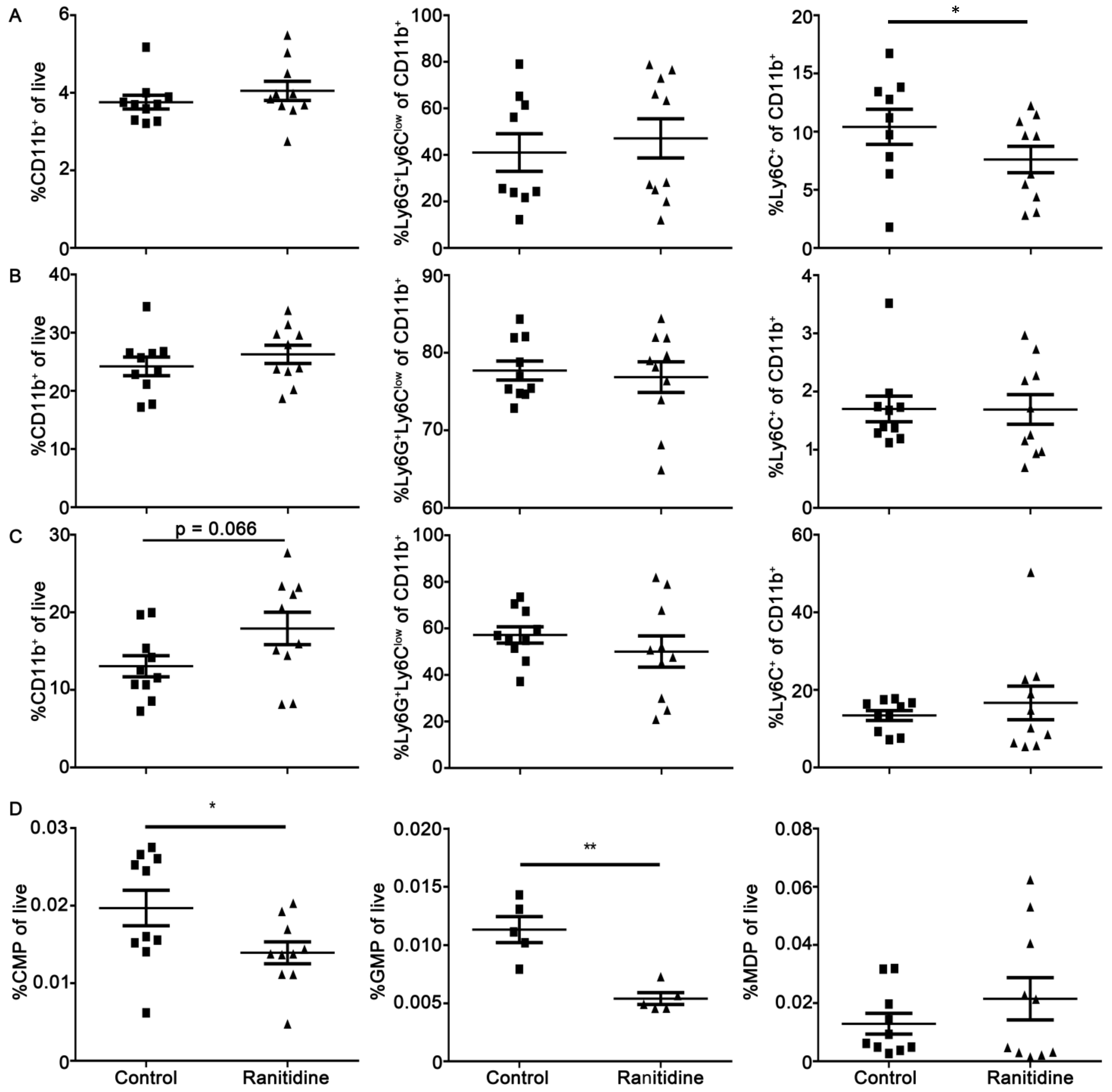

Figure 4.7

4.3 Discussion

Ranitidine is a widely used drug for the treatment of acid reflux, but also has an impact on immune cells. Although considered to be a safe drug with few side effects, the impact of consistent oral ranitidine on the immune system in a cancer setting has not been fully analyzed. Ranitidine treatment is recommended for the treatment of the gastric side effects associated with chemotherapy [499]. Therefore understanding how clinically-relevant dosage of ranitidine may impact the immune system in a tumor-bearing host is important to study. In our study long term ranitidine treatment was associated with a decrease in splenic monocytes and monocyte progenitors in the bone marrow. In breast tumor models, ranitidine limited tumor growth or metastasis, where altered monocyte populations were also observed. The impact ranitidine had in decreasing E0771 tumor growth was CCR2-dependent and therefore potentially linked to monocyte recruitment. Monocytic MDSCs from E0771 tumor-bearing mice had an increased ratio of H2 to H1 compared to naïve mice. These alterations in the monocyte populations following ranitidine treatment have implications beyond breast cancer immunity.

After 8 days of ranitidine treatment, there were decreased monocytes in naïve and tumor-bearing BALB/c mice; with long term ranitidine treatment there were significant differences in the monocyte population in the spleen, which were not seen in the bone marrow. Extramedullary hematopoiesis occurs in the spleen to create a monocyte reserve [215]; under steady state conditions monocytes can migrate back into the bone marrow and contribute to the monocyte pool [204]. There is therefore potential for ranitidine to have an impact on splenic progenitor cells in the spleen or in the bone marrow.

In humans ranitidine treatment associated myelo-depletion is primarily associated with neutropenia [508] while in mice our studies suggest that ranitidine–induced depletion was monocyte specific. A previous study by Byron *et al* [562], showed that H2 signaling pushes bone marrow stem cells from G_0 to S phase, therefore allowing for stem cell proliferation to occur. Our experiments support this data, but further shows that this is specific to CMPs and GMPs. These results also support our data showing alterations in mature monocyte numbers, suggesting the mechanism is by decreasing the number of monocyte progenitor cells. There is also potential that CMPs and GMPs are decreasing in numbers because they are being mobilized into circulation. Our data showed that ranitidine causes a decrease in CCL7 and possibly also in CXCL12 (Figure 4.6). CXCL12 is important for retention of stem cells in the bone marrow [218].

The impact of ranitidine on monocyte progenitors and mature monocytes has clinical implications, although there are very few studies that have directly examined these issues in humans. Decreases in monocyte numbers can further impact multiple different disease states. In a tumor setting, these alterations in monocyte numbers and localization can lead to alterations in tumor infiltrate populations, including tumor-associated macrophages [28]. H2 antagonists are often prescribed to patients that are going through chemotherapy, therefore there is potential that these patients have better survival post-treatment because they were on H2 antagonists. Alternatively, in patients with chronic inflammatory diseases where monocytes and MDSCs are important for regulating the immune response may also be impacted by H2 antagonists [718, 719]; with potential that disease may be exacerbated.

To our knowledge this is the first time differences in histamine receptor expression has been shown to be altered in monocytes in a tumor-bearing animal versus a naïve animal. High levels of histamine can increase H2 expression [492, 496] and there are elevated levels of histamine in breast cancer patients [438, 608]. H2 signaling is considered to create an immunosuppressive state, including enhancing MDSC survival [555, 561] and induction of mediators such as NOS2 and arginase [561]. H2 antagonists may also impact monocyte survival or the activation of MDSCs. Although MDSCs were isolated from both naïve and tumor-bearing mice, there were differences in receptor expression, suggesting that in a tumor setting there is an increased expression of receptors that may help promote the survival of MDSCs, showing another mechanism by which tumors promote an immunosuppressive environment.

The E0771 tumor model in $CCR2^{-/-}$ mice revealed that the impact ranitidine has on tumor development is CCR2-dependent. The CCL2-CCR2 axis is important for recruitment of monocytes to the tumor [28]. Although CCR2 is found on other immune cells, the splenic monocyte and monocyte progenitor data supports that the CCR2-dependent effect is due to monocytes. In humans, there is potential H2 antagonists can directly impact the accumulation of neutrophils, or that neutrophil numbers are impacted indirectly by altered monocytes, as lack of monocyte recruitment can lead to enhanced neutrophil numbers in a tumor [264]. However, our data strongly implicates monocytes are the key cells in the ranitidine-dependent effect on breast tumor growth and spread.

In conclusion, we show that the impact of ranitidine on tumor development is associated with alterations in the monocyte population and associated progenitor cells. H2 blockade leads to a decrease in monocyte progenitors and alterations in myeloid cell numbers in

the tumor. Inhibition of monocyte recruitment, through CCR2 deficiency, prevents the action of ranitidine in reducing tumor growth. These results suggest a mechanism by which H2 blockade can cause a decrease in tumor development. In the context of a tumor, there are alterations in myeloid cells in the tumor, which can then impact tumor outcome. The alteration in H2 expression in monocytic MDSCs suggests that upregulation of H2 expression may support their function or survival, and that specific blockade of H2 signaling in monocytes and MDSCs can inhibit their development. These data suggest that ranitidine usage may have effects on monocyte populations with implications for the development and outcome of both breast cancer and inflammatory diseases.

5 CHAPTER 5 DISCUSSION

5.1 Summary of major findings

The research reported in this thesis has shown that continuous ranitidine intake, at doses similar to what is used clinically in humans, can cause alterations in mammary carcinoma development. These include an increase in tumour latency, a decrease in tumour growth, and a decrease in metastasis in different experimental models. The anti-metastatic properties of ranitidine were shared by an alternate H2 antagonist, famotidine, but not by selective H1 or H4 antagonists. This anti-cancer effect of ranitidine was specific to the breast tumour models and appears to not be due to a direct effect of the H2 antagonist on the breast tumour cells, which do not express H2 receptors, but due to alterations in the immune response. In some but not all injectable tumour models there were substantial alterations in monocyte subsets, associated with a decrease in the overall monocyte population in the spleen. When monocyte recruitment was inhibited or monocytes are depleted, ranitidine did not alter tumour development in susceptible models. Furthermore in such ranitidine-treated tumour-bearing mice, there was a decrease in the suppressive capabilities of peripheral blood leukocytes, attributed to alterations in MDSCs. Analysis of M-MDSCs showed that there were no alterations in the expression of selected mediators, but there were alterations in *in vivo* levels of some mediators, such as CSFs and chemokines that impact myeloid numbers and recruitment following ranitidine treatment. There were also alterations in the monocyte population in the bone marrow of ranitidine-treated mice even in the absence of a tumour. Long term ranitidine treatment led to a decrease in progenitor cells that would subsequently decrease monocyte levels.

5.1.1 Overall conceptual model

Based on the research findings reported in this thesis and the current literature, I propose that the mechanism by which ranitidine is impacting tumour development is through direct alteration of monocyte development (Figure 5.1). Ranitidine inhibition of H2 signaling causes a decrease in the number of CMPs, leading to a downstream decrease in monocytes in the bone marrow and subsequently a decrease in the monocytes in the spleen. When a tumour develops in a mouse that is being treated with ranitidine, there are less monocytes present at the site of the tumour than in an untreated animal. A decrease in monocytes can impact the tumour development in three ways: 1) a decrease in monocytes leads to a decrease in the number of M-MDSCs present at the tumour and in circulation, leading to a decrease in an immunosuppressive environment, allowing for a more effective T cell or NK cell-mediated inhibition in tumour growth (as seen in the E0771 model) or 2) an increase in immune surveillance that can then inhibit tumour development (as seen in the $LKB1^{-/-}$/NIC model); and 3) a decrease in monocytes and MDSCs in the tumour that can lead to decreases in mediators that impact tumour invasion and metastasis (as seen in the 4T1 model).

5.2 Implications and relevance of major findings

5.2.1 Immunological implications

The research I report has demonstrated that H2 signaling is important for monocyte and M-MDSC development and survival. Most studies analyzing histamine's impact on monocytes focus on ROS synthesis. This is the first instance where monocyte numbers and survival have been analyzed in tumour-bearing animals treated with H2 antagonists. The findings of these experiments suggests that histamine receptor signaling is more

important for monocyte numbers and survival than previously recognized. However, in one previous study lack of histamine signaling through H1 and H2 was shown to lead to decreased levels of M-MDSC [554]. In our experiments, H2 blockade alone was shown to not be sufficient to alter the expression of several monocyte mediators involved in the suppressive activity of MDSCs. These observations differ from a previous study [555], although this study used cimetidine as the sole H2 antagonist, which as has already been stated, has several off-target effects. There remains the potential that H2 blockade can alter aspects of MDSCs and/or monocytes function that were not evaluated in this thesis. The data reported herein from the analysis of bone marrow cells in animals treated with ranitidine demonstrated that H2 blockade had an impact on monocyte progenitors. These changes correlate with the reported myelosuppression in patients being treated with H2 antagonist. Whether the impact on monocytes in our studies, and clinically, is due to a direct impact on monocytes or due to decreased progenitor cells, or possibly both scenarios, needs to be further investigated.

A further indication of the potential importance of histamine in regulating monocytic cell responses to tumours was alteration in the nature of histamine receptor expression when there is a tumour burden. As shown in Figure 4.4, H1 expression decreases on monocytes when the mouse has a breast tumour. Previous research has shown that H1 expression can be enhanced by the presence of IL-4, IL-3, IL-12, insulin, and histamine [455-458]. There may be decreases in one or several of these mediators in the tumour environment that may cause H1 expression alteration, or another mediator that is being secreted by the tumour may also impact H1 expression in a manner that has not yet been documented. Modifications of histamine receptor expression may potentially provide a novel

mechanism of inducing immunosuppression; in the context of decreased H1 expression, H2 would likely become the predominant histamine receptor on the cell surface. With such a skew towards H2 expression by the cells, histamine in the microenvironment would then signal more through H2, which as stated in section 1.3.3.2, predominantly causes an immunosuppressive effect. Whether this modification of histamine receptor expression also occurs in other immune cells or structural cells would be of interest to investigate in future experiments.

5.2.2 Clinical implications

As previously stated, H2 antagonists were once the most popular over the counter drugs, but over recent years many patients have been put on proton pump inhibitors instead due to their increased potency in the treatment of gastroesophageal reflux and to avoid the side effect of myelosuppression associated with H2 antagonist use. My studies suggest that H2 antagonist may have previously unrecognized benefits by altering tumour immunity and tumour surveillance. The myelosuppression that may occur with these drugs may be beneficial by decreasing circulating monocytes, therefore decreasing the M-MDSC and TAM populations. Given the importance of monocytes to the regulation of chronic inflammatory disorders, the impact of H2 blockade would also be of interest to study in the context of disease processes such as atherosclerosis or arthritis.

Histamine signaling, through H2 on monocytes, has been shown to be effective in decreasing the ROS production that is known to inhibit NK cell function. This process may occur in the breast tumour models that I utilized; however, it appears that other impacts of H2 blockade outweigh the potential beneficial consequences of histamine-mediated inhibition of ROS synthesis *in vivo*. H2 signaling has been described to have

multiple immunosuppressive consequences, so blockade of H2 signaling although it would allow for ROS synthesis by monocytes, would also alleviate the inhibitory effects histamine has on T_H1 and CTL activity, and alter MDSC numbers. H2 antagonists are generally benign drugs that are well studied in terms of their safety and efficacy, therefore it could be relatively straightforward (compared to studies involving a novel drug) to perform a cohort study in women with a predisposition towards breast cancer (such as women with a BRCA1/2 mutation) or women that have been newly diagnosed with breast cancer to determine whether chronic use of H2 antagonists would benefit them.

H2 antagonists are prescribed to patients who are going through chemotherapy to alleviate the side effects that chemotherapy can have on the gut, but it is also possible that these patients have better survival post-treatment because they were on H2 antagonists. With some chemotherapeutic treatments, there is a decrease in tumour burden followed by increased immune cell activation. Therefore there is the potential that the H2 antagonists being prescribed during cancer therapy are doing more than just alleviating the gastric side effects of chemotherapy. For patients who are being treated with immunotherapeutics, H2 antagonists may provide a useful adjuvant effect with negligible side effects.

The current studies also suggest that perhaps more caution should be taken when certain patients are taking H2 antagonists. Models of autoimmune disease in mice have been shown to include induction of MDSCs, and depletion of these cells can further exacerbate inflammatory disease processes [720]. For example, patients with multiple sclerosis have MDSCs present in circulation [718] which could play a role in regulating disease. In a

study by Shi *et al* [719], MDSCs were important regulators in asthma, and a decrease in their numbers led to increased lung inflammation and an increased T_H2 response. In my studies in BALB/c mice, which have a predisposition towards a T_H2 response, there was an alteration in monocytes with ranitidine even in the absence of tumour. Whether these mice would also be at an increased risk of developing worse inflammation is unknown. It would be important to know whether patients with asthma or an autoimmune disease would have worse symptoms if they were to take H2 antagonists. However, these issues have been largely ignored in the literature.

5.3 Limitations of the experimental systems

As has been previously stated, there are no clear surface marker differences that fully distinguish between mouse monocytes and M-MDSCs. Although CD49d has been presented as a marker of immunosuppressive ability in MDSCs [334], the variation in CD49d expression can vary substantially between experiments. Furthermore, when analyzing the CD49d expression there was no distinct $CD49d^+$ population, but a gradient going from fully $CD49d^-$ to a range of $CD49d^+$ cells. When determining whether these "MDSCs" were suppressive, the suppression assay itself was very delicate, whereby if it took too long to set up the experiment, the MDSCs lose their function *in vitro*. As a result I had to perform several experiments with 2 mice per group, as opposed to fewer experiments with several mice per group.

My data on 4T1 metastasis only focuses on the metastatic burden at the end point of the experiment. The mouse has to be sacrificed to be able to detect the metastasis, therefore it is not possible to see the differences in tumour burden in the lung over time. Furthermore, the assay which I utilized to determine the number of metastatic lesions in the lung does

not differentiate between cells being isolated from different metastatic lesions, or a similar number of tumour cells from one or two larger lesions. This makes a significant difference, because if there is a decrease in metastasis to the lung, but the cells that do reach the lung can proliferate rapidly, then the digestion of the lung and subsequent culturing of the cells will make it appear that there was extensive metastasis. The optimal method for detection of metastasis that would allow tracking of the alterations in metastasis over time and to determine the number of lesions, would be to use *in vivo* imaging techniques. There are mouse imagers that are now available that can detect luciferase activity, therefore by transducing 4T1 cells with luciferase, the tumour and any site where there is metastasis can be visualized. The mouse can be scanned several times to visualize the difference over time, and depending on the imager, individual metastatic nodes can be observed, therefore giving a more accurate and detailed analysis of the metastatic process.

Similar to the problem with measuring metastasis, alterations in monocytes and MDSCs in sites such as the tumour, spleen, or lung can only be performed at the end point of the experiment. In particular, in the tumour, it would be ideal to see if it is recruitment of monocytic cells that is being altered, or site-dependent changes in the monocytic cells that are reaching the tumour that leads to the observed decrease in numbers. In the lung, especially in the context of a pre-metastatic niche, it would be vital to measure whether there are alterations in recruitment or alterations in the microenvironment at the metastatic site. Although the CCR2-deficient model, together with the results of the gemcitabine treatment study, does give some insight into the mechanism of ranitidine action, it merely addresses that in the absence of monocytes, ranitidine does not alter

tumour development. Improved mouse imaging would help determine whether the ranitidine is impacting recruitment or affecting cells at the site, with the use of a fluorescent marker specific to monocytes.

The biggest limitation with this study, that could potentially also impact whether the results can translate to humans, is that the mice are constantly being exposed to the drug. The mice that are treated with ranitidine have the drug in their drinking water, which is not changed, therefore the drug is taken in by the mouse every time they drank from the bottle. We decided against single bolus dose to avoid stressing the mouse with daily injections or daily oral gavage. By either the gavage method or by having the drug in their drinking water, the mouse is getting the same dosage of drug per day, but a bolus of drug may have a different impact on the mice rather than continuous drug intake. In humans, bolus doses of ranitidine would be taken, therefore in addition to the obvious species differences, there is potential that there may be other differences in the effect ranitidine has on humans. Although in one day the same amount of drug will be taken in by the mouse by either a single bolus or continuous drug intake by drinking water, with a single bolus, the drug will be cleared out of the system, and for the rest of the day the immune cells will not be affected; with continuous drug intake, although it is taken in small amounts, it allows for a constant amount of drug to be present in the mouse. Whether the immune cells will revert to their normal state once the drug has been cleared from the system is unknown. This may suggest that the best way for treatment in a human is by smaller doses but several times a day, as opposed to the single large dose. This difference in intake of drug would also be important to study in future work.

5.4 Future studies

If my program of research was extended, future studies would involve studying the direct impact of H2 antagonists on monocytes and MDSCs, and monocyte progenitors. I would isolate MDSCs from tumour-bearing mice, and treat them with an H2 antagonist *in vitro.* These cells would then be analyzed for differences in Arg1 and NOS2 activity, as well as for the presence of ROS. An *in vitro* T cell suppression assay would also be performed to determine whether these cells have altered suppressive activity. Similarly, I would isolate monocytes from mice and potentially from humans, and treat them with H2 antagonists *in vitro*, and measure alterations in cell number and apoptosis. Alterations in monocyte progenitors would be trickier to analyze, as the population in the bone marrow is very low. The best option would be isolating whole bone marrow from mice or stem cells from umbilical cord blood in humans, and culturing them in different concentrations of H2 antagonists and in the presence of mediators such as CSF1 to induce monocyte development.

One major feature that should be studied in the future is the presence of histamine in these mouse models. We have been presuming that similar to the increased levels of histamine that has been observed in breast cancer patients, there is increased histamine in the tumour microenvironment. To definitively prove this, we would need to analyze the mice for the presence of histamine and HDC. This would include use of qPCR to detect HDC expression in the tumour cells, and detection of histamine in the supernatant of cultured tumour cells. Immunostaining can also be utilized to detect histamine in tumours *ex vivo* [609]. Analysis of histamine in circulation can also be performed with an ELISA [608].

Future studies would also involve analysis of different immune cells and how they are impacted with H2 antagonists, such as macrophages, neutrophils, and NK cells. As there are alterations in monocytes, it is assumed that there will be alterations in the macrophage population as well, specifically in TAMs where circulating monocytes can differentiate into TAMs [237]. Future experiments will include similar experiments to those that were performed with monocytes, except macrophages will be isolated. Macrophage expression of mediators such as IL-12 and IL-10 could also be measured to determine whether there is skewing of the TAM population towards an M1 or M2 population. A cytotoxic assay could also be utilized to determine whether ranitidine causes enhancement of macrophage cytolytic activity. In some experiments there was an increase in neutrophils in some compartments, therefore future experiments would include analysis of these neutrophils to determine whether there are alterations in these neutrophils, including skewing of neutrophils to N1 or N2, or whether they have altered cytolytic activity. I showed previously that $CD8^+$ T cells were not involved in the ranitidine-dependent impact on metastasis (Figure 3.7), but this type of analysis was not performed in the E0771 model where ranitidine impacted primary tumour growth. I also attempted to perform an NK depletion *in vivo* to determine whether they were involved in the mechanism by which ranitidine impacts tumour development. Unfortunately the NK depletion was not as thorough as the $CD8^+$ T cell depletion and so could not be used to address our research question. Recently improved models of NK deficient mice have become available which could be utilized to better address these questions.

In terms of future clinical studies, analysis of whether humans that are on H2 antagonists would have altered monocyte numbers in their circulation would be of clinical relevance.

As I showed previously, the alterations in monocytes was not limited to mice bearing tumours, therefore studies in healthy humans would be required; if ranitidine is impacting monocytes in humans, it would be important to note as this may impact an inflammatory response in the future. It may be difficult to perform this similar study in breast cancer patients, but as many cancer patients are already on H2 antagonists for treatment of side effects of chemotherapy, it may be simpler to get blood samples from these patients to determine whether there are alterations in circulating monocytes or MDSCs.

Another important study would be to determine whether people who take H2 antagonists consistently have an altered risk of developing cancer. Starting a study of this type would take several years and include the patients thoroughly reporting the use of H2 antagonists and how often it is used. We have attempted to find a long term study that is looking at cancer risk, to see if these studies also record the amount of H2 antagonists these people have taken, but have yet to find a study that does. This is due to H2 antagonists being an over-the-counter drug that is mostly benign, therefore it is difficult to keep track, and in most cases, not relevant to the study. However, with my study, it could potentially be important to note whether people are on these drugs, as it could be an outlier that impacts other risk factors.

5.5 Conclusion

In summary, H2 antagonist can decrease tumour development in three different models of breast cancer. Our findings indicate that this effect is attributed to ranitidine decreasing monocyte numbers, via decreasing numbers of monocyte progenitors in the bone marrow and potentially also directly impacting monocyte survival. This decrease in monocytes in the bone marrow and spleen also leads to decreases in monocytes in the tumour. With a

decrease in monocytes there are reductions in effective M-MDSCs, leading to decreased immune suppression. This allows for increased tumour latency, decreased tumour growth, and decreased metastasis.

Novel cancer treatments are being developed that do not directly target the cancer, but target immune cells to better enhance an antitumour response. Unfortunately the tumour has evolved ways of suppressing immune cells; therefore novel immunotherapeutics will need to include drugs or antibodies that will directly target these immunosuppressive cells for an optimal response. As H2 antagonists have been extensively studied for their safety, H2 antagonists can be more easily incorporated into a treatment regimen that could enhance the positive impact of immunotherapy than a novel drug.

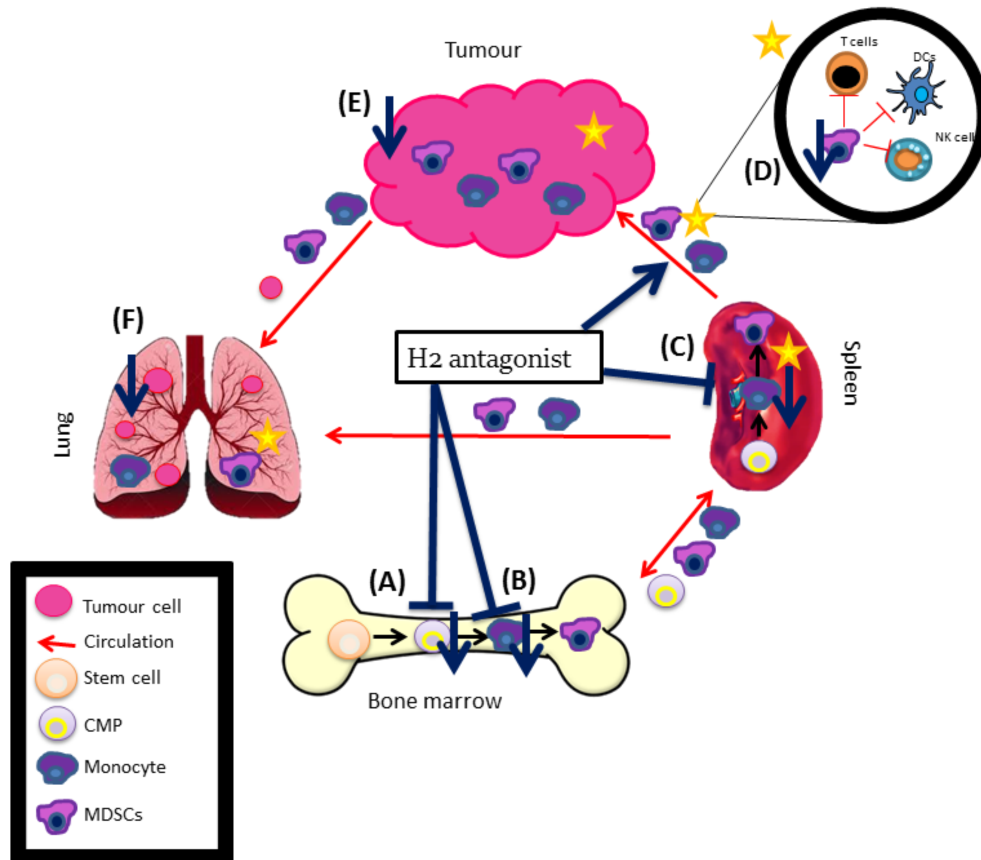

Figure 5.1 Model of the impact H2 antagonist has on monocytes in tumour development.

H2 antagonists causes a decrease in monocyte progenitor cells (A), which can then lead to a decrease in monocytes. H2 antagonists also cause a decrease in monocytes in the bone marrow (B) and spleen (C). With decreases in the spleen and bone marrow, this can lead to decreased monocytes in the tumour, and a decrease in M-MDSCs. A decrease in M-MDSCs can lead to decreased immunosuppression (D) of DCs, $CD4^{+}$ T cells, and NK cells that can alter an antitumour response (which can occur at sites indicated with a star). A decrease in these monocytes and M-MDSCs can also cause a decrease in tumour growth and development (E), and decrease metastasis (F).

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Appendix 1: The impact of histamine antagonists and agonist on 4T1 tumour development

Initially I tested a variety of histamine antagonists on 4T1 tumour development; what was not included in manuscript one was cimetidine (an H2 antagonist) and JNJ7777120 (an H4 antagonist). Due to JNJ7777120's low water solubility, it was injected subcutaneously every 2 days. I also attempted to determine whether an H2 agonist, such as amthamine, would have a tumour metastasis-enhancing effect. This drug was also administered subcutaneously, once per day. The same method as was described in section 2.5.1 was utilized.

Cimetidine did not have a significant effect on metastasis like ranitidine did (Figure 0.1). This is counter to other studies where there was an effect shown on tumour development with cimetidine, but not with ranitidine [555, 557-560]. Cimetidine is not an H2-specific drug; experiments in our lab have shown that there is some H1 antagonism effect with cimetidine, and cimetidine can also antagonize androgen receptors [556], therefore there are other effects that need to be taken into consideration aside from the impact of H2 antagonism.

There was no significant impact on metastasis by JNJ7777120 or amthamine (Figure 0.1). For JNJ7777120, it may be that H4 blockade may not have a significant impact on tumour immunity. There is also the possibility that a single bolus injection of JNJ7777120 every other day is not sufficient enough to have an impact on immune function. This may also apply to why amthamine did not impact tumour development. With the other drugs that were administered via drinking water, the drug was being administered continuously throughout the day, allowing for constant histamine receptor

antagonism. With single injections, and a half-life of a few hours, there may be an impact initially, but once the drug is out of the mouse's system, the alteration on the immune cells may be lost. In the future, a better way of analyzing these drugs may be through the use of an osmotic pump, which will allow for continuous exposure to the drug.

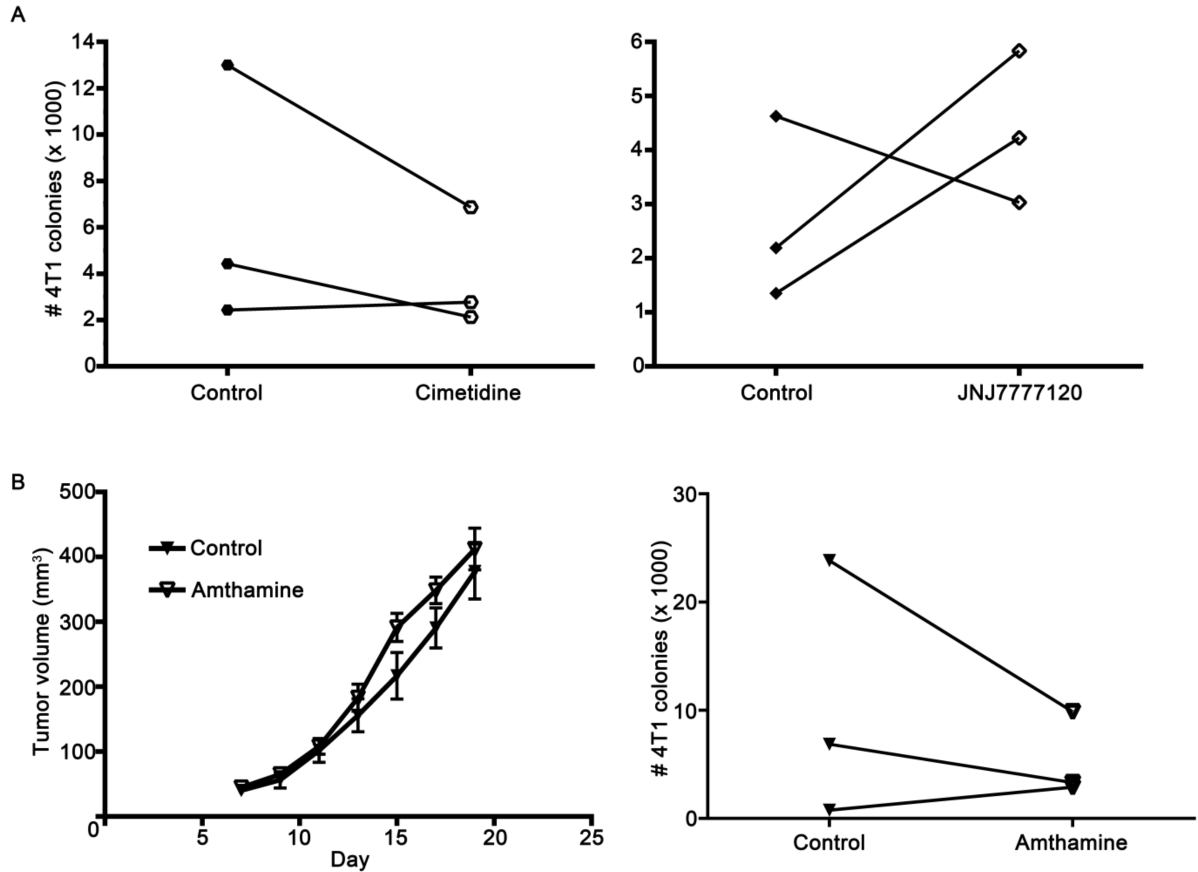

Figure 0.1 The effect of H2 agonist, antagonist, and H4 antagonist on 4T1 metastasis.

(A) Average number of 4T1 colonies derived from lungs of tumour-bearing BALB/c mice treated with cimetidine (100 mg/kg) and JNJ7777120 (10 mg/kg). (B) 4T1 tumours were measured using calipers every other day starting on day 7 post-injection, and the average number of 4T1 colonies derived from lungs of tumour-bearing mice treated with amthamine (0.01 mg/kg) were counted. Tumour volume was calculated as tumour length * tumour width^2/2. Each point represents the mean ± SEM per group. ns.

Appendix 2: The impact of ranitidine on metastasis in an experimental model of metastasis and in nude BALB/c mice.

When initially trying to elucidate the mechanism by which ranitidine was altering metastasis in the 4T1 model, I utilized the experimental metastasis model, whereby 4T1 cells were injected intravenously as described in section 2.5.1.2. Using this model would help determine whether the mechanism by which ranitidine was impacting metastasis was due to alterations in the lung microenvironment that can decrease the survival of the tumour or impact the metastatic niche, including recruitment of immune cells that would support tumour growth in the lung.

To determine whether the impact of ranitidine was dependent on an adaptive immune response, I performed the 4T1 experiment as described in section 2.5.1 in nude BALB/c mice. Nude BALB/c mice do not have a thymus, and therefore do not have functional $CD4^+$ or $CD8^+$ T cells. If ranitidine still had an impact on metastasis in nude BALB/c mice injected with 4T1 cells, then the mechanism ranitidine is having on metastasis is independent of an adaptive immune response.

Issues arose with the experimental metastasis model, whereby at the time of harvest, several of the mice had macrometastatic lesions in the lung, visible to the naked eye. If these metastatic lesions are visible, they are made up of thousands of cells, therefore the lung digest protocol stated in section 2.5.1.1 would not give us a number of actual cells that seeded in the lung, but the number of 4T1 cells that seeded in the lung and subsequently proliferated. Another issue with this protocol is that the lung is the first site in circulation post-intravenous injection that is rich in capillary beds, therefore the 4T1 cells may be getting trapped in the capillaries, and therefore not necessarily be

extravasating into the lung. The way to optimize this procedure would be to measure the number of macro and micro 4T1 colonies individually, by doing an *in vivo* stain of the lungs for 4T1 colonies, or performing histological analysis of whole lung tissue. The results did not conclusively show whether ranitidine impacted experimental metastasis (Figure 0.1).

With the 4T1 experiment in nude mice, I saw an impact on primary tumour volume and weight which I did not see in wild type BALB/c mice (Figure 0.2). Nude mice had smaller tumours compared to wild type mice, and nude mice treated with ranitidine treatment had a final tumour weight comparable to wild type mice. With this alteration in the primary tumour, it was difficult to assess whether alterations in metastasis was due to a specific effect on metastasis, or due to alterations in the primary tumour. The theory as to why the primary tumour was affected is that it is known that nude mice have NK cells with greater cytolytic activity than in wild type mice. Therefore if there is increased NK cell activity in nude mice there may be enhanced clearance of tumour in the control nude mice. With ranitidine treatment, there is alleviation of the inhibition histamine may have on monocyte ROS production; therefore with enhanced ROS production, there is inhibition of NK cell activity, allowing for tumour growth. Whether this was the precise mechanism was not further studied.

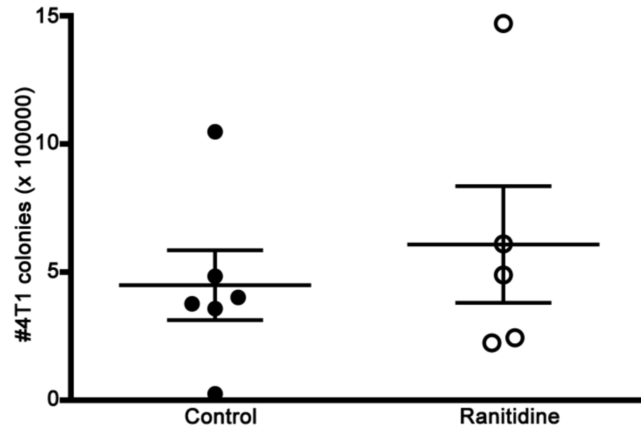

Figure 0.1 Ranitidine does not impact 4T1 experimental metastasis.

Average number of 4T1 colonies derived from lungs of BALB/c mice injected intravenously with 4T1 cells, treated with ranitidine (8 mg/kg). Data points represent individual mice and line represents the mean ± SEM per group. ns.

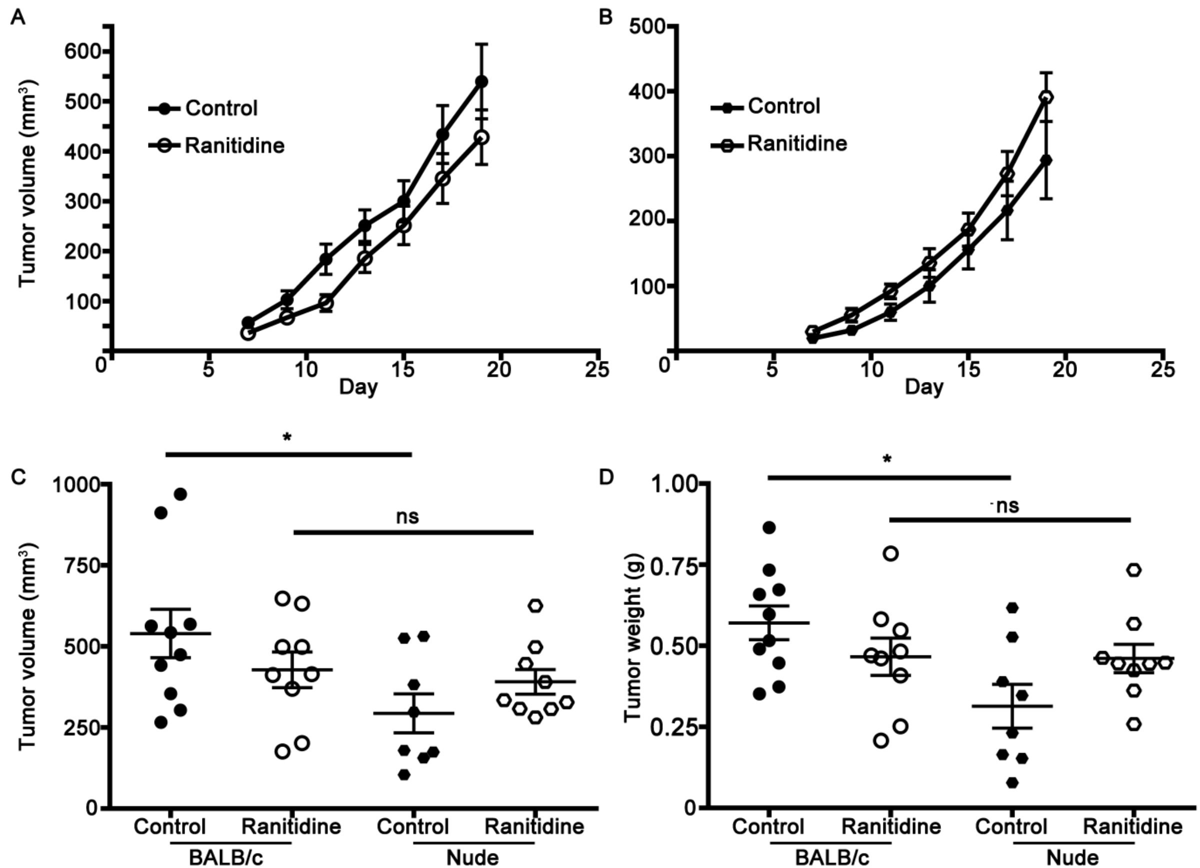

Figure 0.2 The impact of ranitidine on 4T1 tumour development in BALB/c and nude BALB/c mice.

(A-B) 4T1 tumours in BALB/c (A) and nude BALB/c (B) mice treated with ranitidine (8 mg/kg) were measured every 2 days starting 7 days post 4T1 cell injection. (C-D) At day 21 the primary tumour volume was measured (C), and the tumour was excised and weighed (D). Data in (A-B) represents the mean ± SEM tumour volume of 9-10 mice/point. Data points in (C-D) represent final tumour volume and weight of individual mice and line represents mean ± SEM of group. *p<0.05, ns = not significant, unpaired t-test.

Appendix 3: A summary of the impact of ranitidine on immune cell populations in 4T1 tumour-bearing mice.

At day 21 of the 4T1 *in vivo* experiment, I also harvested the lungs and spleen of the mice to perform flow cytometry to determine whether there were differences in the myeloid cell population at these two sites (protocol as described in section 2.9.1). Although as was shown in Figure 3.2 that at day 7 there was a decrease in the spleen, at the endpoint of the experiment there was no difference between the spleens of ranitidine-treated and control mice (Table 0.1). The theory as to why there was no difference was that with no differences in primary tumour size with ranitidine treatment, the splenomegaly that was induced by the tumour was strong enough to overcome any effect ranitidine may have had on immune cells in the spleen. In the lungs there was also no difference in myeloid cell composition, even though there was a difference in metastasis with ranitidine treatment (Figure 3.4). Similarly to the spleen, as there is no difference in primary tumour size, recruitment of immune cells to lung may be more strongly impacted by the primary tumour, that it overcame any impact ranitidine had. However, there is potential that although there are less metastatic lesions with ranitidine treatment, the lesions that are present may be bigger, and have higher infiltrate of immune cells that would make it appear as if there was no difference between the two groups. A better measure of alterations of infiltrate into the lung would be a histological analysis of the whole lung to determine whether there are differences in size and number of metastatic lesions. There is also the possibility that the tumour is still capable of inducing a premetastatic niche even in the presence of ranitidine, and that ranitidine is impacting cells at the site of the primary tumour that impacts tumour cell invasion and EMT. As monocytes can induce

tumour cell invasion and metastasis, it is possible that ranitidine is impacting the monocyte population at the tumour which then impacts metastasis.

I also analyzed lung and spleen, and isolated monocytes from the spleen 7 days after 4T1 injection, to analyze whether there were differences in any mediators with ranitidine treatment. I looked at differences in CSFs since I saw differences in monocytes; I also analyzed expression of NOS2 and Arg1, and IL-12 and IL-10 in the whole spleen and monocytes. I had previously performed a qPCR screen on lung samples of various mediators involved in metastasis to see if there were differences with ranitidine treatment; this initial screen showed some differences with PGF, TIMP2, and VEGF, so I repeated this with more lung samples to confirm this. As is summarized in Table 0.2, there were no differences in the monocytes, spleens or lungs of mice with ranitidine treatment. The data from the lung tissue corroborate with the data in Table 0.1, where there was no difference in immune infiltrates at the end point of the experiment. Day 7 may have been too early of a time point for the upregulation of cytokines by an adaptive immune response that would then induce mediators such as NOS2 and Arg1. As the hypothesis is that the impact ranitidine has is on monocyte numbers and not function, since the qPCR data is normalized, this data supports the idea that ranitidine is mostly impacting monocyte numbers rather than function.

Table 0.1 Summary of splenic cells and lung infiltrates at day 21 post-4T1 tumour cell injection.

Site		Control		Ranitidine		N= (/group)
		Mean	± SEM	Mean	± SEM	
Spleen	% $CD11b^+$ of live	24.69	1.90	25.84	2.04	19
Spleen	% $Ly6C^{hi}$ of $CD11b^+$	4.05	0.30	3.93	0.27	19
Spleen	% $Ly6G^+Ly6C^{low}$ of $CD11b^+$	33.91	2.88	37.38	2.63	19
Lung	% $CD11b^+$ of live	37.63	3.10	30.06	3.61	15
Lung	% $Ly6C^{hi}$ of $CD11b^+$	3.65	0.39	4.56	0.55	15
Lung	% $Ly6G^+Ly6C^{low}$ of $CD11b^+$	46.83	4.27	48.73	4.10	15

Table 0.2 Summary of expression of genes at day 7 post 4T1 injection.

Cell type	Gene of interest	Control		Ranitidine		N= (/group)
		Mean	SEM	Mean	SEM	
Whole lung	**CSF1**	0.680	0.231	1.378	0.455	10
Whole spleen	**CSF1**	0.163	0.095	0.308	0.125	3
Whole lung	**CSF2**	0.069	0.034	0.093	0.044	10
Whole spleen	**CSF2**	0.001	0.001	0.002	0.000	3
Whole lung	**CSF3**	0.011	0.019	0.015	0.019	7
Whole spleen	**CSF3**	0.001	0.001	0.004	0.001	3
Whole lung	**NOS2**	0.154	0.031	0.126	0.038	10
Whole spleen	**NOS2**	0.015	0.003	0.014	0.003	15
Monocytes	**NOS2**	0.012	0.003	0.007	0.002	12
Whole lung	**Arg1**	0.109	0.025	0.103	0.037	7
Whole spleen	**Arg1**	0.039	0.005	0.050	0.007	15
Monocytes	**Arg1**	0.059	0.018	0.032	0.006	12
Whole lung	**IL-12**	0.002	0.000	0.002	0.001	4
Whole spleen	**IL-12**	0.008	0.003	0.039	0.010	15
Monocytes	**IL-12**	0.005	0.003	0.003	0.001	12
Whole lung	**IL-10**	0.002	0.001	0.002	0.001	4
Whole spleen	**IL-10**	0.006	0.001	0.007	0.001	15
Monocytes	**IL-10**	0.007	0.003	0.003	0.000	12
Whole lung	**PGF**	0.020	0.002	0.017	0.002	4
Whole lung	**TIMP2**	9.144	0.780	7.508	1.730	10
Whole lung	**VEGF**	8.996	0.741	9.830	1.113	4

Appendix 4: Alterations in antibody production against E0771-GFP tumour antigen with ranitidine treatment.

To further analyze the impact ranitidine is having on immune function against the tumour, we analyzed the serum from C57BL/6 mice bearing E0771-GFP tumour to detect the presence of antibodies against tumour antigens. The protocol as described in section 2.11 uses antibodies against mouse IgG1 or mouse IgG2a to isolate these antibodies from the serum, and then uses biotinylated GFP, allowing for detection of mouse antibodies specific to GFP. Our preliminary results show that while ranitidine had no impact on IgG1 specific for GFP, ranitidine significantly increased GFP-specific IgG2a levels (Figure 0.1). Furthermore when gemcitabine was used, this impact ranitidine had on IgG2a was lost.

Previous work has shown that with cimetidine treatment, there is an increase in IgG2a [534]. But there are several different nodes of the immune system that can impact antibody production. Whether H2 directly impacts B cells is unknown. As previously stated, H2 signaling can decrease T_H1 cytokine production both directly and indirectly, therefore blockade of H2 signaling would alleviate this inhibition, and potentially lead to an enhanced IgG2a production. With the hypothesis that ranitidine impacts the MDSC population, with decreased immunosuppression, there can be an enhanced immune response. However with depletion of M-MDSCs there is decreased IgG2a compared to mice that did not receive gemcitabine. Although previous literature suggests that gemcitabine does not impact B cells at immunotherapeutic doses [9], there is still potential that there is an impact on B cells, even though there was no difference on IgG1 production with and without gemcitabine treatment. The mice with gemcitabine treatment

did have smaller tumours (Figure 3.11), therefore perhaps due to decreased antigens, there was a decrease in induction of IgG2a production. Future research will include analysis of B cells with and without ranitidine treatment, and also whether these antibodies are capable of directly binding to antigen on tumour cells to cause ADCC.

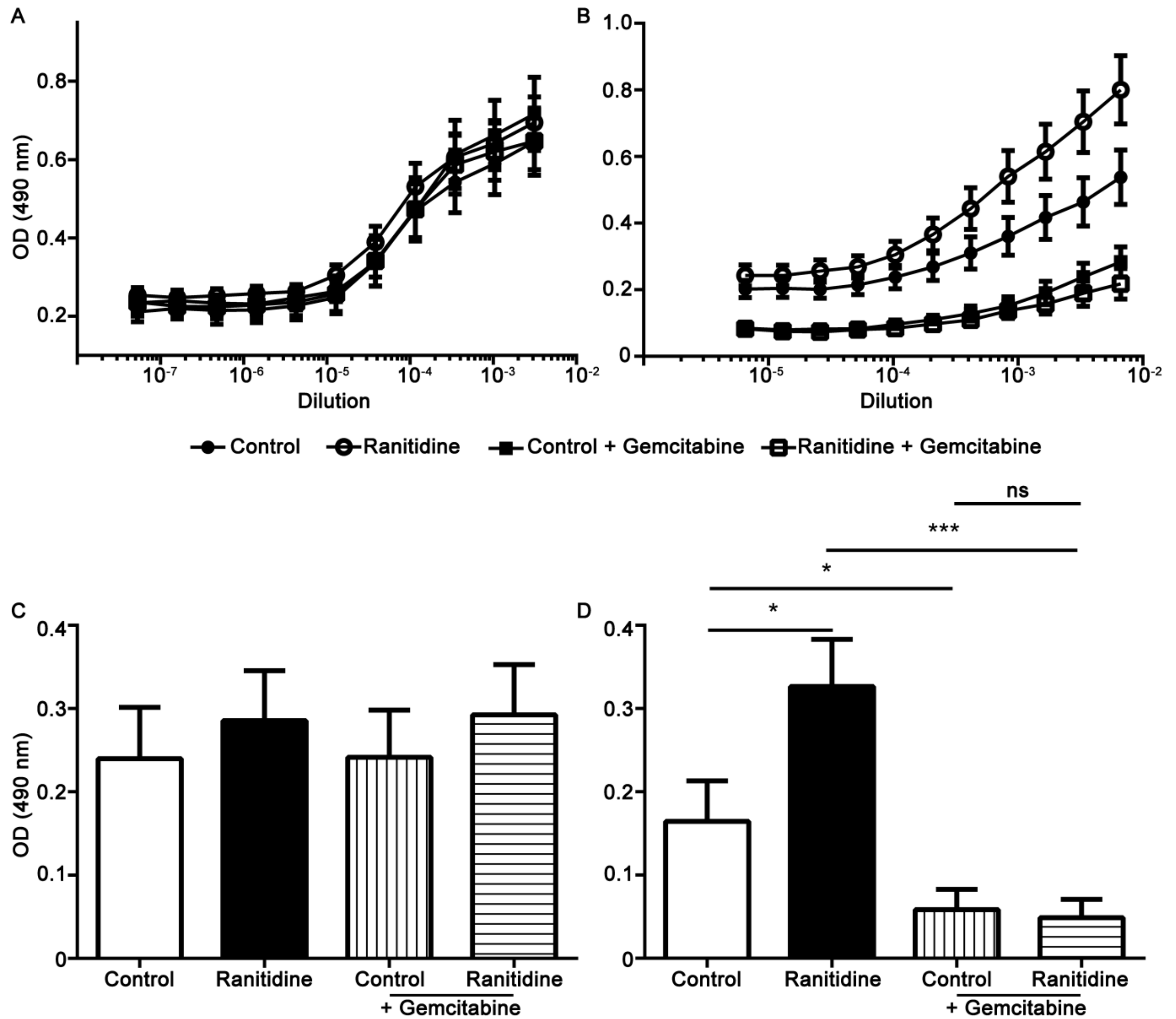

Figure 0.1 Ranitidine increases antitumour GFP IgG2a production.

(A-D) Serum from C57BL/6 mice bearing E0771-GFP tumour was analyzed for the presence of IgG1 (A, C) and IgG2a (B, D) specific for GFP. Data represents mean ± SEM of optical density (OD) in 12-24 mice. *$p<0.05$, ***$p<0.001$, ns = not significant, unpaired t-test.