INVESTIGATION OF THE ROLE OF EOSINOPHILS IN PULMONARY METASTASIS
AND PRIMARY TUMOURS IN MOUSE MODELS OF BREAST CANCER

by

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Abstract

Eosinophils are multifunctional granulocytes with potent immune modulatory and cytotoxic capabilities. Despite the presence of eosinophils in various solid tumours and eosinophilia being a prognostic indicator in some cancers, the role of this innate immune cell has been largely overlooked in the context of cancer. Specifically, the role of eosinophils in pulmonary metastasis is poorly described despite their prevalence in the lung and association with various pulmonary diseases. I sought to delineate the role of eosinophils in murine models of metastatic breast cancer using novel mouse models of genetic eosinophil deficiency (ddGATA) and by immunodepletion in orthotopic mouse models of cancer using an anti-IL-5 antibody. I hypothesize that eosinophils enhance pulmonary metastasis in mouse models of breast cancer, but do not affect the vascularization of the primary tumour.

Eosinophils are increased 5.8-fold in the lungs of mice bearing 4T1 metastatic mammary tumours (p<0.001) which may be due to an observed 4-fold increase in the expression of CCL24 (p<0.0001) that is produced by mMDSCs. Moreover, some resident eosinophils appeared to differentiate into a novel CD11b^hi^Gr-1^hi^ subset in the pre-metastatic lungs. Immunological depletion of eosinophils using anti-IL-5 antibody did not affect the growth or vascularization of 4T1 tumours, or metastatic development by 3 weeks post-implant of the primary tumour. Surprisingly, there was a trend towards an increase in tumour cell colonization of the lungs by IV injected LLC—but not E0771-LMB—tumour cells in ddGATA mice relative to WT controls; indicating that eosinophils may exert anti-tumour effects in this model. Furthermore, IL-5Tg mice exhibiting eosinophilia had a 5-fold decrease in the number of lung Tregs (p<0.001) and were significantly protected from tumour cell colonization of the lungs.
Our data suggests that a pro-inflammatory subset of CD11b<sup>hi</sup>Gr-1<sup>hi</sup> eosinophils may be present in the pre-metastatic lungs and additional eosinophils are recruited to the metastatic lung in response to CCL24 produced by mMDSCs. However, eosinophils do not significantly influence pulmonary metastasis or primary tumour growth or vascularization in mouse models of breast cancer. Furthermore, our data indicates that IL-5 may be exerting pleiotropic effects on other immune cell populations.
Lay summary

Eosinophils—immune cells in the lungs that are most commonly associated with allergies—are capable of a variety of important functions, including the destruction of parasites and the development of new blood vessels in wounds. It is thought that eosinophils may create new blood vessels in tumours that promote tumour growth. In addition, eosinophils are thought to encourage the spread of cancer to the lung (metastasis) by recruiting other suppressive immune cells that allow tumour cells to grow uncontrolled in the lung. Using genetically modified mice that lack eosinophils, or by depleting eosinophils in mice using a drug approved for the treatment of eosinophilic disorders, I found that eosinophils do not seem to have any effect on metastasis to the lung in mouse models of breast cancer. Furthermore, eosinophils do not appear to affect the rate of growth or the number of blood vessels in the tumour.
Preface

I developed the hypothesis, the aims of this study and the experimental design with guidance from Dr. Kevin Bennewith.

Antibody conjugation for mass cytometry was performed by Dr. Michael Williams at the University of British Columbia Antibody Lab (AbLab). Mass cytometry was performed by William Kennedy and the resultant data was analyzed by myself.

Immunohistochemistry of patient tissues was performed by Sylvia Lee at the Centre for Translational and Applied Genomics (CTAG; Vancouver, BC, Canada) and the resultant data was analyzed by myself.

All remaining \textit{in vitro} and \textit{in vivo} experiments were performed by myself. Liz Halvorsen, Brennan Wadsworth, Natalie Firmino, Ada Young, and Dr. Elizabeth Franks assisted with the processing of murine tissues. Knockout mice were bred, genotyped, and distributed by Michael Hughes of Dr. Kelly McNagny’s lab in the Biomedical Research Centre. I was responsible for all of the data collection, analysis, and thesis composition.

All mouse work and methods were approved by the University of British Columbia’s Committee on Animal Care; project title: Promotion of Metastasis by Tumour Hypoxia and Myeloid Cells. Certificate # A13-0223 and B13-0046.
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<td>B16-F10</td>
<td>Melanoma cancer cell line (syngeneic to C57/bl6 mice)</td>
</tr>
<tr>
<td>BMDC</td>
<td>Bone marrow-derived cell</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>Breg</td>
<td>Regulatory B cell</td>
</tr>
<tr>
<td>C5a</td>
<td>Complement component 5a</td>
</tr>
<tr>
<td>CCL</td>
<td>Chemokine ligand</td>
</tr>
<tr>
<td>CCR</td>
<td>Chemokine receptor</td>
</tr>
<tr>
<td>CT26</td>
<td>Colorectal cancer cell line (syngeneic to Balb/C mice)</td>
</tr>
<tr>
<td>CXCL</td>
<td>CXC chemokine ligand</td>
</tr>
<tr>
<td>DAMP</td>
<td>Danger-associated molecular pattern</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>ddGATA</td>
<td>Double-GATA knockout mouse</td>
</tr>
<tr>
<td>ddGATA/IL-5Tg</td>
<td>Double-GATA knockout mouse expressing IL-5 transgene</td>
</tr>
<tr>
<td>dH2O</td>
<td>Distilled water</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s minimal essential medium</td>
</tr>
<tr>
<td>E0771-LMB</td>
<td>Breast cancer cell line (syngeneic to C57/bl6 mice)</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-to-mesenchymal transition</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
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<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridization</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony stimulating factor</td>
</tr>
<tr>
<td>gMDSC</td>
<td>Granulocytic myeloid-derived suppressor cell</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin staining</td>
</tr>
<tr>
<td>H33342</td>
<td>Hoescht 33342</td>
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<tr>
<td>IF</td>
<td>Immunofluorescence</td>
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<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IL-5Tg</td>
<td>IL-5 transgenic mouse</td>
</tr>
<tr>
<td>ILC</td>
<td>Innate lymphoid cell</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
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<tr>
<td>LLC</td>
<td>Lewis Lung Carcinoma cancer cell line (syngeneic to C57/bl6 mice)</td>
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<td>LTB4</td>
<td>Leukotriene-B4</td>
</tr>
<tr>
<td>MC38</td>
<td>Colorectal cancer cell line (syngeneic to C57/bl6 mice)</td>
</tr>
<tr>
<td>MCP-3</td>
<td>Macrophage chemotactic protein-3 (CCL7)</td>
</tr>
<tr>
<td>MDSC</td>
<td>Myeloid-derived suppressor cell</td>
</tr>
<tr>
<td>MET</td>
<td>Mesenchymal-to-epithelial transition</td>
</tr>
<tr>
<td>MHC I</td>
<td>Major histocompatibility complex class I</td>
</tr>
<tr>
<td>MHC II</td>
<td>Major histocompatibility complex class II</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>Macrophage inflammatory protein-1α</td>
</tr>
<tr>
<td>mMDSC</td>
<td>Monocytic myeloid-derived suppressor cell</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimal cutting temperature compound</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
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</tr>
<tr>
<td>OVA</td>
<td>Chicken ovalbumin (commonly used as an allergen)</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PgR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium (tissue culture media)</td>
</tr>
<tr>
<td>Tconv</td>
<td>Conventional T cell (CD4+FoxP3-)</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>TMA</td>
<td>Tumour microarray</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Transforming growth factor-α</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cell (CD4+CD25+FoxP3+)</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
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</table>
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To my family, especially my father.

You will always be an inspiration.
Chapter 1: Introduction

1.1 Cancer

Humans are comprised of highly organized structures defined by a network of 37.2 trillion individual cells varying in number, shape, and function [1]. Normally, deteriorating cells undergo apoptosis and are replaced as needed throughout the lifetime of an organism in a tightly regulated process of cellular renewal. When this process fails, allowing aged dysfunctional cells to avoid death and instead proliferate inappropriately, the result is cancer: a term used to describe a broad collection of diseases that involve uncontrolled cellular growth that may give rise to neoplasia. The accumulation of a unique signature of mutations by rapidly proliferating cells facilitates their expansion into tumours. Tumours consisting of cells that are able to invade nearby tissues and metastasize to distant sites are classified as malignant, while tumours lacking these capabilities are deemed benign.

In 2016, it is expected that 202,400 new cases of cancer and 78,800 deaths from cancer will occur in Canada [2]. This presents a severe economic burden on Canadians, which was estimated in 2008 to be over $3.8 billion in healthcare costs and a cost of over $586 million due to a loss of productivity resulting from illness or death [3]. These figures are expected to continue to rise as the life expectancy of the average Canadian steadily increases, highlighting the critical need for research into the mechanisms of cancer development that will form the basis of innovative therapies for patients in the future.
1.1.1 A brief overview of the “Hallmarks of Cancer”

Cancer research is an expansive field describing a wide variety of cancer types that each present with distinct features and challenges. As improved technology allows a greater understanding of the underlying molecular and genetic mechanisms of cancer development, the significance of individual characteristics of each tumour in patient outcome and therapeutic response is becoming increasingly appreciated. Despite the recent push towards individualized medicine that highlights the uniqueness of cancer types, there remain a defined set of acquired capabilities that describe the development of neoplastic cells: these are the “Hallmarks of Cancer” first described by Hanahan and Weinberg in 2000.

The first six hallmarks describe the intrinsic qualities that define neoplastic cells. Firstly, neoplastic cells giving rise to cancer proliferate uncontrollably due to autonomy from the exogenous growth factors that are normally required for cellular replication. This increased rate of proliferation is sustained through the increased maintenance of telomeres that normally degrade with each cell cycle and contribute to eventual cell death. In concert with increased proliferation, neoplastic cells also have decreased rates of cellular attrition: cancer cells evade anti-proliferative signals in the environment and resist apoptosis through the disruption of tumour suppressor pathways and modification of the apoptotic machinery. To survive the increased metabolic demand associated with the expansion of neoplastic cells in a tumour, neoplastic cells must also develop the ability to facilitate angiogenesis. Finally, the defining characteristic of malignant tumours from their benign counterparts is the ability to invade nearby tissues and metastasize to distant sites; a complex process described in section 1.2 [4]. While these initial hallmarks described the capacity of cancer cells to manipulate and function
independently of signals within the environment, over a decade of subsequent research began to reveal additional qualities that are crucial in the pathogenesis of cancer.

Four additional hallmarks of cancer were described by Hanahan and Weinberg in 2011. The first hallmark describes the ability of neoplastic cells to deregulate energetic metabolism to fuel rapid proliferation and persistence under hypoxic conditions within solid tumours. Secondly, the genomic instability of carcinoma cells gives rise to mutations that are subject to Darwinian selection such that tumours become dominated by clones with the greatest fitness. The final two hallmarks pertain to the significant role of the immune system in the development of cancer: the ability of tumour cells to evade immune destruction and promote pro-tumour inflammation [5]. With the relatively recent introduction of immunotherapy in the clinic, the role of the immune system in cancer has become a field of active study. Collectively, these distinct hallmarks of cancer have complementary roles in facilitating the persistence of neoplastic cells. While the hallmarks clearly outline intrinsic characteristics allowing cancer cells to proliferate independently from prohibitive signals in their environment, they also profile the abilities of neoplastic cells to influence their environment and ensure survival by manipulating the immune system of the host; this represents a paradigm shift in the way cancer is viewed.

1.1.2 The tumour microenvironment

The modified energetic metabolism of tumour cells, irregular vasculature, prominence of neoantigens, and immune cell infiltrate all contribute to the heterogenous landscape of a tumour described by the hallmarks of cancer. The tumour and the immediate local region surrounding it is referred to as the tumour microenvironment. This unique setting consists of a myriad of
interconnected components in addition to the carcinoma cells: the extracellular matrix that maintains the tissue architecture and facilitates cellular movement— including mesenchymal cells such as fibroblasts, adipocytes, and endothelial cells—as well as a consortium of resident and recruited innate and adaptive immune cells. The hallmarks of cancer describing the ability of tumour cells to evade immune destruction and promote pro-tumour inflammation highlights the critical role of both the innate and adaptive immune systems in the development of cancer [5]. The immune system eliminates incipient cancer cells through a rigorous process known as immunosurveillance. However, the rapid accumulation of mutations by proliferating cancer cells results in a variety of clones available for selection by the immune system—cancer cells that manipulate the immune system dominate the population that gives rise to tumours.

There is a diversity of mechanisms of immune evasion. The immune cells primarily associated with cellular immunity that persistent cancer cells evade include natural killer (NK) cells and cytotoxic CD8+ T cells: CD8+ T cells destroy cells presenting neo-antigens within the context of MHC I and NK cells destroy cells that have downregulated MHC I and are therefore unable to be detected by CD8+ T cells. Tumour cells often have downregulated MHC I to successfully avoid detection by CD8+ T cells, however cultivating a highly immunosuppressive tumour microenvironment remains critical for immune escape [6]. The immunosuppressive cells in tumours may include resident macrophages characteristic of the particular site—such as Kupffer cells in the liver or alveolar macrophages in the lung—as well as recruited myeloid cells such as monocytes, neutrophils, macrophages (tumour-associated macrophages; TAMs), and myeloid-derived suppressor cells (MDSCs) recruited from the bone marrow, dendritic cells, NK cells, antigen-presenting cells (APCs) such as dendritic cells (DCs), CD8+ T cells, and regulatory T cells (Tregs) [7]. In an immunogenic or well-controlled tumour, the anti-tumour
immune cells such as NK cells, CD8+ T cells, and macrophages secreting pro-inflammatory type I cytokines—M1 macrophages—are in high abundance. In contrast, a poorly immunogenic tumour consists of primarily alternatively-activated (M2) macrophages secreting anti-inflammatory type II cytokines in addition to other suppressive immune cells such as regulatory Tregs and MDSCs (Figure 1.1).

![Figure 1.1: The tumour microenvironment.](image)

*The tumour microenvironment.** Various structural, stromal, and immune components constitute the tumour microenvironment within and surrounding well controlled and poorly controlled tumours pictured left and right, respectively. Used with permission from Quail, D.F. and J.A. Joyce, Microenvironmental regulation of tumor progression and metastasis. Nature medicine, 2013.

The rapid development of blood vessels to sustain tumour growth leads to various aberrations in the structure of the tumour vasculature causing hypoxia: abrupt dead-ends in the vascular network, excessive vessel diameter leading to collapse, insufficient vessel diameter leading to blockages, and variations in vascular permeability [8]. Hypoxia is a critical component of the tumour microenvironment for a multitude of reasons. Firstly, intratumoural hypoxia is correlated to an aggressive phenotype and poor outcome [9], poor response to radiotherapy [10],
and an increase in metastasis [11]. Moreover, T cells tend to be excluded from hypoxic regions [12] while immunosuppressive cells such as macrophages are actively recruited to such areas [13]. Hypoxia occurs as a gradient of oxygen tensions ranging from 4% O\textsubscript{2} (30 mmHg) near well-perfused blood vessels to 1% O\textsubscript{2} (8-10 mmHg) in hypoxic regions and 0% O\textsubscript{2} in anoxic regions [8, 14]. In comparison, the oxygen tension in normal tissue ranges from 4-11% (30-83.6 mmHg) [15, 16]. The inadequate supply of nutrients combined with the highly glycolytic signature of tumour cells results in hypoxic regions that may progress to necrosis [5, 8, 14]. The study of hypoxia in tumours is critical to patient prognosis and therapeutic response.

1.2 Metastasis

It is estimated that over 90% of cancer-related deaths are associated with the development of metastatic disease [17]. Metastasis occurs when tumour cells disseminate from the site of the primary tumour and colonize distant regions of the host where they form secondary masses. Two fundamental models describe the progression of metastasis: linear progression and parallel progression. Linear progression describes a model in which successive generations of mutations and selection occur before the most malignant cancer cells finally egress to colonize distant metastases at the most advanced stages of the disease. In contrast, the parallel progression model suggests that metastatic tumour cells disseminate from the primary tumour during the early stages of development and may continue to do so throughout the course of the disease [18]. Physiologically distant metastases have been shown to be more genetically divergent than local lymph node metastases in breast cancer, which may describe one of the factors contributing to increased therapeutic resistance of advanced disease [19]. Not only do
metastases differ genetically, but metastases are located in distinct tumour microenvironment with unique immune and stromal cell components. These non-malignant contributors develop a permissive environment—the “pre-metastatic niche”—in metastatic sites before tumour cells arrive and are maintained to facilitate cancer cell colonization and outgrowth.

1.2.1 The metastatic process

There are five main steps in the metastatic cascade: local invasion at the primary site, intravasation into the vasculature or lymphatics, survival in the circulation, extravasation from the circulation into the tissue of a new site, and finally colonization (Figure 1.2). A subpopulation of cells in the primary tumour downregulates expression of tight- and adherens junction proteins, such as E-cadherin and α-catenin, and concomitantly upregulate their expression of mesenchymal markers such as N-cadherin, vimentin, and fibronectin: these collective cellular changes are known as epithelial-to-mesenchymal transition (EMT). This process is enhanced by factors produced by components of the tumour microenvironment, such as transforming growth factor-β (TGF-β) that is produced by TAMs and fibroblasts [7, 20]. Unlike epithelial cells, mesenchymal cells lack apical-basal polarity and exhibit an enhanced migratory phenotype and a superior capacity to resist apoptosis in the circulation. These cells invade the local tissue surrounding the tumour and intravasate into the lymphatics or pulmonary circulation where they evade immune surveillance [7, 20, 21]. They then extravasate into the tissue of a permissive pre-metastatic niche such as the lung where they undergo a mesenchymal-to-epithelial transition (MET), which allows the tumour cells regain their sustained proliferative capacity [22]. Although the process of EMT and MET has long been accepted as indispensable
for metastasis, cogent evidence presented in 2015 illustrates that epithelial cells are also capable of metastasis [23, 24].

![Image](image_url)

**Figure 1.2: The metastatic process.** Various structural, stromal, and immune components contribute to metastatic dissemination and the colonization of distant pre-metastatic niches. Used with permission from Quail, D.F. and J.A. Joyce, Microenvironmental regulation of tumor progression and metastasis. Nature medicine, 2013.

### 1.2.2 The “pre-metastatic niche”

The “seed and soil” hypothesis describing pre-metastatic niche development was first coined in 1889: it was suggested that particular organs such as the lung and liver—the “soil”—are inherently more susceptible to metastatic colonization by cancer cell “seeds” [25]. The pre-metastatic niche, located at a distance from the primary tumour, consists of stromal cells, extracellular matrix, vascular changes, immune cells, and the various factors that describe an environment permissive to cancer cell colonization and development [26]. In the lung, the
initiation of the pre-metastatic niche is thought to begin with an increase in vascular permeability. Secreted factors such as VEGF and angiopoi etin-like 4 (ANGPTL4) from the primary tumour and local immune cells results in a loss of vascular integrity through endothelial cell production of matrix metalloproteinases (MMP): MMP3, MMP9, and MMP10 [27, 28]. Fibroblasts become activated by factors such as VEGF [29] and deposit fibronectin to facilitate colonization of metastatic cancer cells and immune cells in the lung [30]. Non-resident immune cells are subsequently recruited that suppress local immune cells: bone marrow-derived cells (BMDCs) migrate towards the lung in response to VEGF and CCL2 produced by local stromal components while the distant primary tumour contributes to the recruitment of MDSCs [31, 32]. Both resident alveolar macrophages [33] and MDSCs secrete immunosuppressive cytokines to abet the immune escape of metastatic cancer cells through the local suppression of CD8+ and NK cells [34]. The consortium of immune cells and stromal cell components secrete factors that contribute to pre-metastatic niche maturation, including cytokines such as TGF-β that mobilize Tregs and additional BMDCs [7], in addition to chemokines such as S100A8 and S100A9 that attract macrophages and tumour cells [35]. Throughout this process, exosomes produced by the primary tumour consistently contribute to niche development [36]. As it is suspected that secreted angiogenic factors from the primary tumour incites the development of the pre-metastatic niche through increasing vascular permeability in the lung, evidence suggests that intratumoural hypoxia contributes to metastatic dissemination [11].
1.3 Eosinophils

Eosinophils are multifunctional granulocytes of the innate immune system that were initially defined by their eosinophilic granules of various cytotoxic proteins, cytokines, chemokines, and regulatory molecules. Eosinophils develop from myeloid progenitors within the bone marrow: a process that relies on regulation by various transcription factors—notably the transcription factor GATA-1—and prevalence of the cytokines interleukin-3 (IL-3), IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF) [37]. Mature eosinophils exit the bone marrow and localize primarily to the gastrointestinal tract (GI), spleen, lymph nodes, thymus, mammary glands, and uterus where they assist in the maintenance of homeostasis [38].

Eosinophils express a multitude of receptors responsible for chemotaxis towards sites of cellular stress and pattern recognition receptors (PRRs) that facilitate activation in response to pathogens (Figure 1.3). Eosinophils are traditionally known for their role as cytotoxic effectors, but they are also capable of producing various cytokines and exert immunomodulatory effects on other immune cells [39]. It is therefore unsurprising that these granulocytes have a variety of effector functions in both homeostasis and disease. Although traditionally associated with the type II immune response, eosinophils are capable of secreting type I and type II cytokines and chemokines rapidly and differentially [40]. Although mechanisms have not been clearly elucidated, eosinophils appear to be important for the maintenance of homeostasis within the reproductive tract: studies in eotaxin-1 (CCL11)-deficient mice have shown that these cells are important for the onset of estrus in the uterus [41]. Furthermore, eosinophils have also been shown to have a critical role in the proper development of the mammary gland—for which CCL11 is necessary—possibly due to promoting angiogenesis [42, 43]. Within the thymus,
Eosinophils have been suggested to act as APCs and are involved in double-positive thymocyte deletion [44]. In addition to their varied roles in tissue homeostasis, eosinophils have also been implicated in disease.

Figure 1.3: Cellular features of eosinophils. The multitude of receptors expressed by eosinophils and the various contents contained in these cells facilitate various effector functions in both homeostasis and disease. Used with permission from Rosenberg, H.F., Dyer, K.D., and Foster, P.S. Eosinophils: changing perspectives in health and disease. Nature reviews immunology, 2013.

In asthmatic airways, eosinophils have been shown to secrete VEGF [45] and other angiogenic molecules to promote neovascularization and contribute to tissue remodeling [46]. Eosinophils express MHC II and are capable of acting as APCs during both infection [47] and allergy [48]. Interestingly, eosinophils may have a role in autoimmunity: the induction of
eosinophils and IL-5 due to the injection of helminth products has been shown to be important for conferring protection against experimental autoimmune encephalitis independent of the recruitment of Tregs, though the exact mechanism of this effect remains unclear [49].

There are a variety of tools that can be leveraged for the specific study of eosinophils in both human and murine tissues. Conventionally, murine and human eosinophils can be identified by their characteristic eosinophilic granules by H&E staining. The cells between the two species can be differentiated based upon the shape of their nucleus: murine eosinophils exhibit an annular nucleus in contrast to the bi-lobed nucleus of human eosinophils. Alternatively, antibodies targeting eosinophils can be used to detect both the cells and their degranulation products. One example is the anti-major basic protein antibody, which is specific for one of the cytotoxic proteins specific to eosinophils. Alternatively, anti-eosinophil peroxidase antibodies may be used in immunohistochemistry staining procedures, but caution must be used to avoid detection of endogenous peroxidases [38, 39]. By flow cytometry, murine eosinophils can be identified as Siglec-F+ granulocytes that autofluoresce in an excitation wavelength of 530 nm and exhibit characteristically high side-scatter. In the murine lungs, eosinophils are differentiated from alveolar macrophages, which express equivalent levels of Siglec-F, by their lack of expression of CD11c and high expression of CD11b [50, 51].

1.3.1 The role of eosinophils in cancer

Despite long-standing evidence suggesting that a variety of solid tumours in humans are infiltrated by eosinophils, there remains a paucity of research investigating the role of eosinophils in cancer. The prevalence of eosinophils in a variety of solid human tumours was
initially described two decades ago [52]. Tumour-infiltrating eosinophils have also been described in mouse models of cancer, including lung adenocarcinoma, melanoma, and colorectal cancer [53, 54]. Furthermore, eosinophilia has been shown to be a biomarker of response to immunotherapy: an increase in the number of eosinophils in the peripheral blood of patients taking ipilmumab or nivolumab for the treatment of metastatic melanoma is an indicator of improved outcome and survival, but also toxicity and serious immune-related adverse events (irAEs) [55-58]. Furthermore, low eosinophil counts in the peripheral blood of breast cancer patients was indicative of a significantly higher risk of recurrent disease [59].

Although blood eosinophilia may be used as a prognostic factor in the clinic, the role of infiltrating eosinophils in solid tumours remains unclear. This is surprising, considering the vast immunomodulatory functions for which eosinophils are capable, including: DC maturation [60], the recruitment [61] and activation of effector T cells [48], and activation of neutrophils [62]. The presence of tumour-infiltrating eosinophils has been shown to correlate to a positive prognosis in head and neck cancer [63], gastric cancer [64], rectal cancer [65], and colorectal cancer [53, 66], while a poor prognosis in Hodgkin’s lymphoma [67] and cervical cancer [68]. For some types of cancer, the prognostic utility of eosinophils is less clear. There are significant numbers of studies that suggest that tumour-infiltrating eosinophilia predicts a positive prognosis in oral squamous cancer, while there are many other studies that suggest the opposite or that the presence of eosinophils in tumours is of no significance [69]. The discrepancies may be a result of differences in eosinophil quantification, as well as functional heterogeneity due to the activation state of the eosinophils in response to polarizing factors within the particular tumour microenvironment (Figure 1.4) [40].
Figure 1.4: The potential roles of eosinophils in the tumour microenvironment. In the tumour microenvironment, eosinophils are capable of exerting differential effects that may either support or hinder tumour growth. Used with permission from Reichman, H., Karo-Atar, D., and Munitz, A. Emerging Roles for Eosinophils in the Tumor Microenvironment. Trends in cancer, 2016.

1.3.1.1 Eosinophil recruitment to the tumour microenvironment

The mechanisms of eosinophil recruitment to the allergic and asthmatic lungs have been well documented. Cytokines such as IL-13 [70] and IL-33 in models of airway inflammation induces mTOR signaling in Th2 T cells and innate lymphoid cells (ILCs) that produce IL-5 and CCL11 that promote the recruitment of eosinophils to the lungs [71]. However, the mechanisms of eosinophil recruitment to the tumour microenvironment are not clearly understood. While the infiltration of eosinophils into B16-F10 primary tumours is thought to be primarily mediated by...
the release of danger-associated molecular patterns (DAMPs) such as high-mobility group box protein 1 (HMGB1) from necrotic cells [72-74], the infiltration of eosinophils into human colorectal neoplasms was correlated with the expression of CCL11 and CCL24 [75, 76]. Human breast cancer cells stimulated with TNF-α—which may be present within the tumour microenvironment—have been shown to upregulate both cell adhesion molecules and secrete CCL5 (RANTES) that enhances the adhesion of eosinophils and promotes their activation, respectively [77]. However, CCL5 was not prevalent in the metastatic lungs of the 4T1 or 4T07 tumour-bearing mouse models of breast cancer used in our studies [78]. In addition to eotaxin-1, 2, and 3—CCL11, CCL24, and CCL26, respectively—there are a variety of chemokines, cytokines, factors that influence eosinophil migration in accordance with the diverse number of receptors expressed by these granulocytes (Figure 1.3). The eotaxins, CCL5, CCL7 (MCP-3), and CCL3 (MIP-1α), complement such as C5a, and lipid mediators such as leukotriene-B4 (LTB4) act as chemoattractants, while cytokines such as IL-3, GM-CSF, and IL-5 act as chemokinetic factors of eosinophils [79]. Eosinophils express the receptor for advanced glycation end products (RAGE), the receptor for C5a (C5aR), CCR3, and CCR1 that facilitates their chemotaxis in response to HMGB1 [74], C5a [80], eotaxins, as well as CCL5 and CCL3, respectively [38]. C5a [81] and LTB4 [82] have been implicated in pulmonary metastasis in mouse models of breast cancer. Furthermore, a study has shown that alternatively-activated alveolar macrophages secrete high levels of CCL24 in the presence of IL-33 and the Th2 cytokines IL-4 or IL-13—all of which would be expected to be present in the immuosuppressive tumour microenvironment [83]. Although the mRNA encoding CCL24 has been shown to be upregulated by monocytic MDSCs (mMDSCs), it is not highly expressed by granulocytic MDSCs (gMDSCs)—the population that is highly prevalent in the lungs of mice bearing metastatic mammary tumours [84, 85]. Various
chemoattractants—C5a, LTB4, CCL24, and/or HMGB1—may also contribute to the selective recruitment of eosinophils to the metastatic lungs, which may be dependent on the stage of metastatic development: CCL24 from Th2-polarized alveolar macrophages may be responsible for initial eosinophil recruitment to the pre-metastatic niche in the lungs, while at later stages of higher metastatic burden, DAMPs such as HMGB1 may be more significant. Identification of chemoattractants responsible for the migration of eosinophils into the metastatic lungs may reveal potential strategies to modulate the effects of these granulocytes on metastasis.

1.3.1.2 The role of eosinophils in primary tumours

Evidence suggests that eosinophils rarely infiltrate breast tumours in humans [86, 87], however a small study suggested that eosinophil peroxidase deposits can be identified in the majority of breast cancer specimens—if diligently sought—which are absent from normal breast tissue [88]. Peripheral blood eosinophil counts are indicators of prognosis in primary breast cancer [59] and chronic eosinophilic pneumonia has been observed in some breast cancer patients following radiation therapy [89]. Moreover, eosinophils have been shown to be recruited to the edge of breast cancer biopsy sites where increased tumour cell proliferation was noted, suggesting that they may promote the proliferation of breast cancer cells and/or facilitate metastatic dissemination [90].

The current literature describing the role of eosinophils in primary tumours is largely based on observations from the B16-F10 mouse model of melanoma. In this model, it has been shown that eosinophils accumulate in the capsule of the primary tumour and localize to necrotic regions where they actively degranulate; chemotactic factors that may be responsible for this
effect are described in section 1.3.1.1 [72]. It is hypothesized that necrotic material enhances the respiratory burst of eosinophils which oxidizes—and therefore inactivates—danger-associated molecular patterns (DAMPs) such as HMGB1, S100, heat-shock proteins, and adenosine triphosphate that are released from stressed cells. The inactivation of DAMPs is thought to temper immune recruitment to the site [74]. Moreover, the contents of eosinophils have been shown to be cytotoxic towards B16-F10 melanoma in vitro [91] and human eosinophils have been shown to degranulate in response to necrotic cells [73].

A variety of reports have suggested that eosinophils influence primary tumour neovascularization and hypoxia, however there is no consensus on whether endogenous eosinophils tend to promote vascular normalization within the tumour or disorganized angiogenesis contributing to intratumoural hypoxia. In one study, there was no significant difference in the vascular permeability of progressive fibrosarcomas in WT mice compared to non-progressive tumours in IL-5 Tg Balb/C mice exhibiting eosinophilia [92]. However, more in-depth measurements of vascular density or intratumoral hypoxia were not examined in this study. An alternate study suggested that the recruitment of eosinophils into murine sarcoma tumours overexpressing CCL11 resulted in an inhibition of neovascularization and contributed to necrosis, as the eosinophils activated by CCL11 were capable of damaging both tumour cells and endothelial cells in vitro. The use of a small molecule antagonist of CCR3 decreased the infiltration of eosinophils and concomitantly restored angiogenesis within the tumours [93]. In contrast, another study of CCL11-secreting B16-F1 melanoma tumours infiltrated by eosinophils exhibited increased microvascular density and blood clotting [94]. These studies suggest that eosinophils may influence the tumour vasculature in different ways, however the effects of eosinophils cannot be distinguished from the direct effects of increased CCL11 secretion by the
tumours examined in these particular studies: CCL11 and CCR3 antagonists are well known to have direct effects on endothelial cell recruitment and angiogenesis in both humans and mice [95-98]. Nonetheless, there remains significant evidence suggesting that eosinophils are capable of contributing to angiogenesis and vascular permeability in both humans [46] and hamsters [99, 100]. One notable study demonstrated that adoptive transfer of eosinophils skewed towards a type I phenotype *ex vivo* resulted in dramatic normalization of the vasculature of B16-F10 melanoma tumours, decreased hypoxia, increased CD8⁺ T cell infiltration, and the polarization of macrophages toward a pro-inflammatory M1 phenotype [100]. However, adoptive transfer of eosinophils that were not polarized with type I cytokines *ex vivo* did not have this effect *in vivo* and it is questionable as to whether endogenous eosinophils are capable of sustaining such a pro-inflammatory phenotype within the immunosuppressive tumour microenvironment detailed in section 1.1.2.

Comparatively, *in vivo* studies describing the effector function of eosinophils in primary tumours have come to conflicting conclusions indicating that eosinophils may have opposing roles depending on the tumour type and microenvironment. Many of the studies examine tumours that arise from syngeneic cell lines that have been genetically modified to overexpress cytokines or chemokines such as IL-4, IL-5, or CCL11, to enhance eosinophil infiltration. Transduction of the mammary adenocarcinoma TS/A and plasmocytoma cell line J558L with IL-5 resulted in a significant increase in eosinophil infiltration, but no effect on subcutaneous primary tumour growth in Balb/C mice [101]. Similarly, the rate of growth of tumours transduced to express CCL11 did not significantly differ from that of WT controls [94]. In contrast, transduction of CT26 colorectal cancer cells with the IL-5 gene resulted in significant inhibition of subcutaneous tumour growth due to increased infiltration of cytotoxic eosinophils.
In contrast, inhibition of the growth of IL-4 transduced tumours was suggested to be due to an increase in neutrophil infiltration—not due to the increase in eosinophil infiltration [103]. These data appear to suggest that the effect of eosinophils on primary tumour growth is dependent on the tumour type—and likely the tumour microenvironment. However, the use of tumour cell lines expressing increased levels of cytokines confounds these observations, as both eotaxins such as CCL11 [104] and the cytokine IL-5 [105] have been shown not only to impact eosinophil migration, but also strongly influence the effector function and survival of eosinophils. Furthermore, there have not been any significant studies suggesting that eotaxins or IL-5 are particularly upregulated in human tumours relative to normal tissue, suggesting that these models may not be representative of the human disease. The immunodepletion of eosinophils had no effect on the growth of a subcutaneous LLC tumour in mice, however this is not an orthotopic site and would not be expected to accurately represent the native tumour microenvironment. The study of the role of endogenous eosinophils in models of cancer that accurately recapitulate the tumour microenvironment in the absence of genetic manipulation is necessitated.

1.3.1.3 The role of eosinophils in breast cancer metastasis

Comparable to reports on the role of eosinophils in neovascularization within primary tumours, the role of eosinophils in metastasis also remains controversial. The existing literature that intends to define the role of eosinophils in metastasis is largely dependent upon mouse models of metastasis in which tumour cells are injected intravenously (IV) directly into the circulation, which results in cancer cells becoming trapped within the dense vasculature of the
lungs where they develop into tumour foci. However, this model bypasses the complex metastatic process described in section 1.2.1 that must be accomplished by tumour cells. In addition, IV injection of tumour cells fails to account for the priming of the pre-metastatic niche by the primary tumour, as outlined in section 1.2.2.

Like many of the studies investigating the functional influence of eosinophils on primary tumour growth, transduced tumour cells have also been used to investigate the role of eosinophils in metastasis. IV injection of CT26 colorectal cancer cells—syngeneic to the Balb/C background—transduced with the IL-5 gene exhibited over a 16-fold decrease (p<0.01) in tumour foci visually counted in the lung [102]. A study showed that IL-5 increased in the lungs of mice within 24 hours of injection with intravenous LLC tumour cells and eosinophils actively infiltrated the periphery of tumour lesions in the lung. Preemptive systemic immunodepletion of eosinophils using an anti-IL-5 antibody, clone TRFK5, resulted in a dramatic decline in the number of surface tumour nodules in mice IV injected with LLC and IL-5KO mice injected with LLC, B16-F10, or MC38 tumour cells two weeks following injection. Although the number of lung surface colonies was affected, the diameter of the nodules was not. Further investigation revealed that the promotion of metastatic colonization by eosinophils was due to the recruitment of Tregs through the secretion of CCL22, which suppressed IFN-γ production by NK cells and skewed macrophages towards a more anti-inflammatory phenotype [50]. In contrast, preemptive immunodepletion of eosinophils using a different anti-IL-5 antibody, clone NC17, resulted in an increase in the number of B16-F10 tumour nodules at day 7 post-injection in a separate study [106]. Furthermore, priming the lung by intranasal administration of IL-33 recruited activated eosinophils expressing high levels of granzyme B and IFN-γ that inhibited lung colonization by IV B16-F10 melanoma as determined by clonogenic assay [107].
These data suggest a nuanced and temporal role of eosinophils in pulmonary metastasis that necessitates thoughtful investigation with consideration of the pre-metastatic niche that develops in the lung within the context of metastatic disease. The initial increase in pulmonary colonization at 7 days post-IV injection of B16-F10 may be due to the role of resident eosinophils in immunosurveillance of the lung. This is substantiated by evidence suggesting the eosinophils are important in reducing the incidence of methylcholanthrene (MCA)-induced fibrosarcomas in mice [92] and the finding that depletion of eosinophils decreased the number of colonies in the lung, but not the diameter or growth of the tumours [50]. However, to reconcile the seemingly conflicting results suggesting that eosinophils contribute to an increase in pulmonary metastasis at the later stages of metastatic disease, one must consider the plasticity of these multifunctional granulocytes. Comparable to the Th1/Th2 and M1/M2 paradigm describing T cells and macrophages, respectively, eosinophils may also develop different phenotypes depending on their activation state and environment. While dormant in the lung, eosinophils may not be favorably skewed toward a pro-inflammatory or anti-inflammatory phenotype initially, but the influx of immunosuppressive cells into the pre-metastatic niche may drive eosinophils towards a type II phenotype; thus explaining why these cells secrete CCL22 to attract Tregs at 14 days post-injection [50]. Adoptive transfer of CD4+ T cells skewed \textit{ex vivo} towards a Th2 phenotype at 7 days post-IV injection of B16-F10 tumour cells into mice resulted in an influx of cytotoxic eosinophils that may have developed a pro-inflammatory phenotype at this earlier time-point before the lung became a more immunosuppressive environment at later time-points [91]. These immunosuppressive eosinophils in the metastatic lung clearly exhibit a dramatically distinct phenotype from adoptively transferred eosinophils stimulated \textit{ex vivo} that produced pro-inflammatory cytokines, mediated CD8+ T cell infiltration into primary tumours, and normalized
the tumour vasculature [100]. To further complicate these conclusions, many of these studies rely on eosinophil depletion with the use of IL-5KO mice or immunodepletion using anti-IL-5 antibody, however there may be pleiotropic effects on both Tregs and regulatory B cells (Bregs) that will be outlined in section 1.3.1.4. Nonetheless, these data suggest that resident eosinophils in the lung—and perhaps those initially recruited to this site—are involved in protective immunosurveillance, but may exert pro-tumour effects in later stages of metastatic disease. Indeed, the role of eosinophils in the pre-metastatic niche remains to be elucidated.

1.3.1.4 Potential effects of IL-5 on regulatory B and T cells in cancer

The IL-5KO and IL-5Tg mouse models of eosinophil deficiency, as well as neutralizing antibodies targeting IL-5 are relied upon for the study of eosinophils in the context of various diseases. IL-5 is a cytokine secreted by CD4+ Th2 cells [108]. Historically, IL-5 was initially discovered as an eosinophil differentiation factor in 1986 and was described as having remarkably specific functions towards eosinophils [109, 110]. However, the generation of the first IL-5KO mouse in 1996 revealed that IL-5 had pleiotropic effects on a subset of B cells, CD5+B220loIgDlo B-1a cells, that express the IL-5 receptor (IL-5R) and rely on IL-5 for their survival, proliferation, and maturation into antibody-secreting plasma B cells [108]. B-1a cells secrete natural antibodies and have been implicated in autoimmunity [111]. Indeed, the effects of IL-5 is not specific to eosinophils: transgenic mice expressing IL-5 under the control of a CD3δ promoter exhibit a >30-fold increase in the number of circulating B220+ B lymphocytes, while mice expressing IL-5 under the control of a metallothionein promoter have increased numbers of CD5+ (B-1) B cells, elevated IgM levels, and exhibit autoimmunity [112, 113]. Conversely,
mice deficient in IL-5R are deficient in B-1a cells [114] and IL-5KO mice exhibit delayed B-1a B cell development at 2 weeks of age [115]. The pleiotropic effects of IL-5 on B cells are particularly nontrivial in the context of cancer, as it implicates a subset of B cells with immunomodulatory effects.

As the number of markers used to differentiate particular Breg subsets has expanded in response to a greater appreciation of the nuanced roles of these cells, the broad use of the term B-1a has become outdated. B-1a cells have been shown to be the main producers of B cell-derived IL-10: a cytokine known to be immunosuppressive in the tumour microenvironment [116, 117]. There is some evidence that B-1a cells may promote the differentiation of FoxP3- T cells with regulatory activity [116] via an IL-10 independent pathway and reduce pulmonary inflammation in a mouse model of allergy [118]. B-1a cells secreting IL-10 may therefore fall under the definition of modern B10 cells. B10 cells exclusively produce IL-10; however minor populations of other B cell subsets are also capable of producing IL-10. B10 cells are defined as CD5+CD1d+ cells, however this phenotype also overlaps with that used to describe B-1a cells, spleen marginal zone B cells, and T2-marginal zone B cell precursors [117, 119-121]. B10 cells represent a critical subset of B cells that are important regulators of the T cell-dependent inflammatory response [122], autoimmunity [120], rheumatoid arthritis [123], and other diseases among cancer [119, 124]. Importantly, there is a B10 subset in present in humans [117]. In vitro studies have shown that IL-5 supports the expansion of Fas ligand (FasL)-expressing B10 cells that induce antigen-specific apoptosis of CD4+ T cells [125]. However, it remains unclear the role of B10 cells in pulmonary metastasis, or whether these immune cells are present or recruited to the metastatic lungs. It is clear that the effects of IL-5 on B10 Bregs may confound conclusions drawn from the study of breast cancer metastasis in IL-5KO mice.
The role of IL-5 may also extend to the maintenance of antigen-specific regulatory T cells (Tregs) as well: CD4+CD25+FoxP3+ Tregs cultured in vitro with autoantigen and IL-4 upregulated IL-5Rα and expanded more robustly in response to autoantigen in the presence of recombinant IL-5 (rIL-5) [126]. Antigen-specific Tregs have been identified to be associated with colorectal cancer [127] and advanced melanoma [128], but it is unknown whether these cells express IL-5R or the consequences of IL-5 deficiency on Tregs in the context of cancer.

### 1.3.2 Murine models of eosinophilia and eosinophil deficiency

Murine models of eosinophilia and eosinophil deficiency have facilitated the study of the role of these immune cells in the context of disease such as allergy and cancer. Most of these models have been developed by manipulating the expression of IL-5, which is thought to be necessary for eosinophil development in the bone marrow [38]. There are three mouse models commonly used for the study of eosinophils in disease that will be described: the IL-5 transgenic mouse (IL-5Tg), the IL-5 deficient mouse (IL-5KO), and the GATA-1 deficient mouse (ddGATA).

The IL-5Tg mouse was initially developed in 1990 and a second mouse was described soon thereafter in 1991 [113, 129]. However, the IL-5Tg mouse (NJ.1638 CD3^{IL-5+}) on the C57/bl6 background that was used in our studies was first described by Nancy and James Lee in 1997. To generate this mouse, a 170 bp fragment from the 5’ end of murine IL-5 cDNA was ligated with the downstream portion of the IL-5 gene to produce a resulting minigene devoid of 5’-flanking sequence, but containing all IL-5 introns and 1.2 kb of 3’ downstream sequence. The expression of IL-5 was directed specifically to T lymphocytes by the inclusion of the murine
promoter and enhancer of CD3δ. Transgenic mice contained high levels of circulating IL-5 and systemic eosinophilia due to the establishment of extramedullary sites of eosinophilopoiesis, which results in a 1000-fold increase in the absolute number of eosinophils in the blood. Mice are prone to premature death by 12 months of age, exhibit splenomegaly, and may suffer from various histopathologies common to hypereosinophilic syndrome patients, such as cardiomyopathy and ulcerations of the skin [112].

Conversely, the IL-5 deficient mouse (C57BL/6-II5\textsuperscript{tm1Kopf/J}; IL-5KO) is characterized by very low levels of eosinophils in both the bone marrow and peripheral blood. This model was generated using a construct consisting of a fragment of HSV-Tk and the neo\textsuperscript{R} gene within a 3.7 kb fragment of the IL-5 gene targeting the first cysteine codon of exon 3. C57/bl6 embryonic stem cells were electroporated with the linearized construct and homologous recombinants were isolated by positive-negative selection. The splenocytes of the resultant homozygotes expressed very low levels of IL-5 [115]. Although the pulmonary eosinophilia normally associated with allergen challenge is abolished in IL-5-deficient mice, there is evidence that eosinophil differentiation may be induced in IL-5 deficient mice in the context of cancer and other inflammatory conditions [70, 130]. OVA-specific CD4+ T cells cultured \textit{ex vivo} under Th2-polarizing conditions were capable of clearing established tumour cells in a mouse model of intravenous B16-OVA lung metastasis—an effect thought to be due to an increase in the recruitment of degranulating eosinophils. When CD4+ T cells were adoptively transferred from IL-5KO donors to IL-5KO recipients, the inhibitory effects on metastatic development were greatly diminished, but eosinophils still localized to the lungs in significant numbers [91]. This data suggests that the loss of IL-5 alone is insufficient to ensure eosinophil deficiency within the context of cancer. An additional model of eosinophil deficiency includes CCL11-deficient mice.
(CCL11KO) that exhibit normal levels of eosinophil differentiation in the bone marrow, but diminished levels in the peripheral blood and lung tissue [131]. This model was avoided for our studies, as CCL11 is known to be a potent CCR3 ligand, which is expressed by numerous immune cell populations—eosinophils, mast cells, Th2 lymphocytes, basophils—as well as pulmonary epithelial cells, airway smooth muscle cells, and endothelial cells [132].

Unlike the eosinophil deficient mouse models that rely on the genetic modification of cytokines and chemokines critical to the development and trafficking of eosinophils and other cells, the phenotype of ddGATA mice is due to a deficiency of the GATA-1 transcription factor necessary for eosinophil differentiation [37]. The GATA-1 transcription factor is expressed in four haematopoetic lineages: erythroid cells, megakaryocytes, mast cells, and eosinophils. The GATA-1 locus contains heterogenous upstream (IT) and downstream (IE) promoters as well as two enhancers: one of which is a palindromic (or “double”) enhancer site that binds GATA-1 and is necessary for full promoter activity through autoregulation [133]. In contrast to GATA-1-null mice that perish in utero, deletion of this enhancer in mice results in an inability to produce eosinophils with no significant defects in the development of mast cells, platelets, or red blood cells. Mice were generated through homologous recombination of a floxed PGK-neo cassette into the double-GATA-1 (ddGATA) site of CJ7 embryonic stem cells that was subsequently excised upon electroporation of a vector containing cre recombinase [37]. ddGATA mice have been used to demonstrate the role of eosinophils in glucose homeostasis, sustaining adipose M2 macrophages [134], protecting against respiratory viral infection [135], enabling allergic airway remodeling [136], and mediating arthritis [137]. More recently, it has been shown that ddGATA mice have lower levels of basophils in both the bone marrow and periphery relative to wild-type mice [138]. There is a paucity of data on the role of basophils in metastasis, however, lung
cancer patients have been shown to have elevated levels of blood basophils [139]; the accumulation of basophils in the tumour-draining lymph nodes of pancreatic cancer patients is correlated to reduced survival [140]; and basophils appear to have an anti-tumour role in a mouse model of FoxP3-DTR Treg depletion [141]. Furthermore, ddGATA mice have been shown to have augmented B-1a expansion in the pleural cavity of mice in response to acute lung fungal infection—likely as a result of an increased bioavailability of IL-5 due to the absence of eosinophils [142]. Although the ddGATA mice have defects in basophil development and may have slightly enhanced B-1a expansion under some conditions, we considered the potential confounding effects of abolition of IL-5 on Bregs and Tregs to be of greater concern for the study of pulmonary metastasis. Therefore, ddGATA mice were used for the studies described in this thesis rather than IL-5KO mice.

1.3.3 Targeting eosinophils

Despite significant technological and scientific advances in the field of target discovery and pharmaceutical development, the overall efficacy of research and development is in decline. The number of new drugs approved per billion US dollar expenditures on R&D has halved every nine years since 1950 and the rate of approval by the Food and Drug Administration (FDA) of new antineoplastic drugs from 2003 to 2011 was 6.7%; about half of the rate of approval of non-oncological drugs [143, 144]. This decrease in new pharmaceutical output is occurring at a time when clinicians are seeking to rapidly diversify the armamentarium of drugs that can be used successfully with combination immunotherapy. Combinations of synergistic immunotherapies, such as ipilimumab and nivolumab, have shown unprecedented durable responses for the
treatment of melanoma [145]. Application of immunotherapies in combination with other small molecule inhibitors, traditional therapies such as chemotherapy, and other biologics, are being heavily investigated in many other types of cancers—some of which have shown promising outcomes. Despite the success of some combination immunotherapy regimens, more successful combinations are needed to expand the application of these treatments to a greater subset of cancer types and increase the number of patients that respond positively to treatment. An effective strategy to rapidly meet this demand is to repurpose existing drugs that have already been approved for alternate indications. This curtails the lengthy process of establishing dosing protocols, toxicities, pharmacokinetics, and other basic biochemical data necessary for drug approval preceding cumbersome clinical trials [146].

Defining the role of eosinophils in cancer is critical at this time when two antibodies targeting IL-5 have been recently approved by Health Canada for the treatment of late-onset eosinophilic asthma: a relatively rare condition [147]. The first antibody to be approved for this purpose by Health Canada in 2015, mepolizumab (trade name Nucala), is a humanized monoclonal antibody that effectively reduced the rate of clinically significant asthmatic exacerbations (p<0.001) and improved lung function measured by forced expiratory volume (p=0.03, n=161) of patients suffering from eosinophilic asthma compared to placebo (n=161) [148, 149]. A meta-analysis of studies confirmed that mepolizumab approximately halved the number of exacerbations requiring the hospitalization of patients [150]. Approved more recently in 2016, reslizumab (trade name Cinqair), is a similar antibody that has been shown to effectively decrease blood eosinophils and improve lung function, but exhibited more modest effects on asthma control [151, 152]. Moreover, in late 2016, benralizumab, targeting the IL-5 receptor α subunit, was shown to be effective in a randomized, double-blind phase III clinical
trial in reducing the annual rates of asthma exacerbations by 51% (p=0·0008, n=95) compared to placebo (n=97) in patients suffering from uncontrolled eosinophilic asthma [153]. These antibodies were generally well tolerated with minimal side effects. Should eosinophils be established as promoting metastatic development in the lungs, it would be beneficial to deplete these cells in cancer patients by repurposing mepolizumab or benralizumab.

1.3.3.1 Anti-IL-5 antibody clone TRFK5

Unlike mepolizumab, reslizumab has been shown to be cross-reactive to mouse IL-5 in vivo [154, 155]. To facilitate the study of eosinophils in tumour models, an established commercially available monoclonal antibody targeting IL-5, clone TRFK5, was used. In response to a single intraperitoneal injection of this antibody at 1 mg/kg in mice, durable depletion of eosinophil recruitment to the bronchoalveolar lavage fluid (BALF) in response to OVA allergen sensitization/challenge was reduced to about 25% of the control (p ≤ 0.05); an effect lasting for up to 12 weeks [156]. At 24 weeks following the dose of antibody, the number of eosinophils recruited to the BALF of allergen-challenged mice given TRFK5 rebounded to levels equivalent to controls [156]. Interestingly, allergic mice that received TRFK5 experienced an approximately 7.5-fold increase in the absolute number of T cell recruitment to the BALF relative to controls (statistics were not reported) which were described as being primarily CD44⁺CD45Blo memory CD4⁺ T cells [156]. This antibody has also been validated in numerous murine models of cancer and metastasis, as described in section 1.3.1. Nonetheless, anti-IL-5 therapy may represent an effective strategy for the depletion of eosinophils for the treatment of pulmonary metastasis.
An alternative antibody targeting Siglec-F was briefly considered for use in these studies, as it effectively results in the systemic depletion of eosinophils [157]. However, this strategy was avoided due to potential effects on Siglec-F+ alveolar macrophages in the lung, which are known to be critical components of the pre-metastatic niche and in the establishment of pulmonary metastases [33]. Although suggested anti-Siglec-F immunodepletion has no effect on alveolar macrophage populations, there is a paucity of evidence to decisively confirm this claim [157, 158].

1.3.4 Murine models of breast cancer metastasis used in the studies

Many different murine models of metastatic breast cancer exist for research; some are more suitable for particular studies than others. Immune-deficient mice facilitate the implantation of orthotopic human xenografts may metastasize, but this is not conducive to the study of interactions between tumour cells and immune cells [159]. Transgenic mice allow for the study of metastasis in an immune-competent model, but the process of primary tumour development and metastasis is lengthy relative to orthotopic models [160]. Due to the rapid development of representative tumours within their native environment, orthotopic mouse models of breast cancer spanning multiple inbred murine backgrounds are employed in the studies described in this thesis. Cell lines syngeneic to various murine backgrounds are used, including the Balb/C background: 4T1, 4T07, and 67NR; FVB/N background: 6DT1, MVT-1 and Py2T; and C57/bl6 background: E0771-LMB (Table 1.1). The 4T1, 4T07, and 67NR cell lines are derived from a single tumour in a Balb/cfC3H mouse, but differ in metastatic potential [161]. In the FVB/N background, 6DT1 and MVT-1 are highly metastatic cell lines derived from spontaneous
tumours that arose in MMTV-\textit{c-myc} and MMTV-\textit{c-myc/vegf} transgenic mice, respectively [162]. The Py2T cell line was derived from a mammary tumour of a MMTV-\textit{PyMT} mouse; it is a non-metastatic epithelial cell line that undergoes TGF-\textit{\beta}-dependent EMT \textit{in vivo} [163]. The E0771-LMB cell line is derived from the E0771 cell line that was established from a spontaneous mammary tumour in C57/bl6 mice [164, 165]. The E0771-LMB tumour was used in the studies described in this thesis, as it exhibited increased metastatic potential relative to the parental E0771 parental cell line [165]. The investigation of the role of eosinophils across various murine backgrounds assures a robust study, as subtle genetic differences may result in discordant immune responses between strains, as is the case with Balb/C and C57/bl6 mice [166].

Table 1.1: Summary of murine models of breast cancer used in the studies described in this thesis.

<table>
<thead>
<tr>
<th>Transgenic murine background</th>
<th>Cell Line</th>
<th>Breast cancer subtype</th>
<th>Primary tumour growth</th>
<th>Metastatic Potential</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balb/cfC3H</td>
<td>67NR</td>
<td>Mixed luminal/basal; ER+</td>
<td>Yes</td>
<td>No</td>
<td>[161, 167]</td>
</tr>
<tr>
<td></td>
<td>4T07</td>
<td>TNBC; basal-like</td>
<td>Often</td>
<td>Poorly: micrometastases in lungs and liver</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4T1</td>
<td>TNBC; basal-like</td>
<td>Yes</td>
<td>Highly: macrometastases in lungs, liver, brain, bone</td>
<td></td>
</tr>
<tr>
<td>FVB/N</td>
<td>6DT1</td>
<td>Unknown</td>
<td>Yes</td>
<td>Highly: macrometastases in lungs</td>
<td>[162]</td>
</tr>
<tr>
<td>MMTV-\textit{c-myc/vegf}</td>
<td>MVT-1</td>
<td>Unknown</td>
<td>Yes</td>
<td>Highly: macrometastases in lungs</td>
<td></td>
</tr>
<tr>
<td>MMTV-\textit{PyMT}</td>
<td>Py2T</td>
<td>HER2-enriched</td>
<td>Yes</td>
<td>No</td>
<td>[163]</td>
</tr>
<tr>
<td>C57/bl6</td>
<td>E0771-LMB</td>
<td>TNBC; basal-like</td>
<td>Often</td>
<td>Poorly: micrometastases in lungs</td>
<td>[161, 165]</td>
</tr>
</tbody>
</table>
1.4 Hypothesis and aims

The use of anti-IL-5 antibody has been suggested to both decrease tumour cell colonization of the lungs through a reduction in pro-tumour eosinophils that recruit Tregs [50] and increase metastasis through a reduction in anti-tumour cytotoxic eosinophils in the same model of IV B16-F10 tumour cell colonization of the lungs [106]. It is unclear whether the use of anti-IL-5 may be an effective strategy for the reduction of metastatic burden in orthotopic mouse models of metastasis in which the phenotype of resident eosinophils may be affected in the pre-metastatic lung primed by a primary tumour. Moreover, it is unclear whether pleiotrophic effects of IL-5 on other immune populations or eosinophils are primarily responsible for the observations in the existing literature. Indeed, the role of eosinophils in pulmonary metastasis has been defined primarily using IL-5KO mice and with the use of an anti-IL-5 antibody [50, 91, 106], which results in a significant decrease in the number of both eosinophils and B-1a cells with potential effects on immune cell populations such as Tregs, Bregs, and plasma B cells [108, 113-116]. The use of ddGATA mice for the study of eosinophils in cancer reduces the likelihood of these confounding factors as a result of the pleiotropic effects mediated by the complete absence of IL-5. Finally, though eosinophils appear to be capable of normalizing the tumour vasculature in mice [100], it is unclear whether endogenous eosinophils affect this process. I hypothesize that eosinophils promote pulmonary metastasis in mouse models of breast cancer, but have no effects on primary tumour growth or vascularization. The specific aims of the study described in this thesis are as follows:
**Aim 1:** Determine whether eosinophils accumulate in the lungs of mice bearing metastatic mammary tumours and identify chemokines that may promote their recruitment.

**Aim 2:** Define the role of eosinophils in pulmonary breast cancer metastasis using novel mouse models of eosinophil deficiency (ddGATA) and eosinophilia (IL-5Tg).

**Aim 3:** Determine whether immunodepletion of eosinophils using anti-IL-5 antibody reduces pulmonary metastasis in mouse models of breast cancer.

**Aim 4:** Determine whether eosinophils infiltrate primary mammary tumours and affect the rate of primary mammary tumour growth, vascularization, or degree of intratumoural hypoxia.
Chapter 2: Materials & Methods

2.1 Mass cytometry

2.1.1 Antibody preparation

Antibody labeling with the indicated metal tag was performed using the MaxPAR antibody conjugation kit (Fluidigm) and concentration was assessed after metal conjugation using a Nanodrop (Thermo Scientific). Stocks of all antibodies used in these studies were maintained by the Antibody Lab at the University of British Columbia (Table 2.1).

Table 2.1: Markers and conjugated lanthanides of antibodies used in mass cytometry.

<table>
<thead>
<tr>
<th>Selected Marker</th>
<th>Lanthanide</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>115Ln</td>
</tr>
<tr>
<td>CD4</td>
<td>141Pr</td>
</tr>
<tr>
<td>B220</td>
<td>142Nd</td>
</tr>
<tr>
<td>CD19</td>
<td>143Nd</td>
</tr>
<tr>
<td>GR-1/Ly6C/6G</td>
<td>144Nd</td>
</tr>
<tr>
<td>CD172 (SIRPa)</td>
<td>144Nd</td>
</tr>
<tr>
<td>CD45 Pan (I3/2)</td>
<td>145Nd</td>
</tr>
<tr>
<td>FoxP3</td>
<td>145Nd</td>
</tr>
<tr>
<td>Siglec-F</td>
<td>150Nd</td>
</tr>
<tr>
<td>NK1.1</td>
<td>151Eu</td>
</tr>
<tr>
<td>MHCII</td>
<td>153Eu</td>
</tr>
<tr>
<td>CD62L</td>
<td>154Sm</td>
</tr>
<tr>
<td>CD138</td>
<td>155Gd</td>
</tr>
<tr>
<td>CD11b</td>
<td>156Gd</td>
</tr>
<tr>
<td>CD279</td>
<td>157Gd</td>
</tr>
<tr>
<td>CD25</td>
<td>158Gd</td>
</tr>
<tr>
<td>CD274</td>
<td>161Dy</td>
</tr>
<tr>
<td>Ly6G (1A8)</td>
<td>165Ho</td>
</tr>
</tbody>
</table>
2.1.2 Cell barcoding and staining

Single cell suspensions of lung cells were fixed with 1.6% paraformaldehyde (PFA; Electron Microscopy Sciences) for 10 minutes at room temperature. Cells were washed in PBS + 2% FBS and resuspended in blocking buffer (PBS + 5% FBS) and 1.5 µg/mL anti-mouse CD32 antibody at a concentration of 3×10^6 cells/50 µL for 10 minutes. Cells were then stained for 45 minutes on ice with antibodies at a concentration of 3×10^6 cells/100 µL (Table 2.1). The cells were subsequently washed twice with MaxPar Cell Staining Buffer (Fluidigm) before being permeabilized and fixed by incubation in 1 mL of MaxPar Fix and Perm Buffer for 1.5 hours. Cells were subsequently washed twice with MaxPar Perm-s Buffer and stained with intracellular antibody at 3×10^6 cells/100 µL in MaxPar Perm-s Buffer before being washed twice with MaxPar Cell Staining Buffer (Millipore). EQ™ Four Element Calibration Beads (DVS Sciences) were added at a concentration of 3.3×10^4 beads/mL to the cells in milli-Q H₂O at a cell concentration of 1×10^6 cells/mL. Cells were then filtered and run on a CyTOF 2 (Fluidigm) with a flow speed of 0.045 mL/minute, a 30 second acquisition delay, and 10 second detector stability delay.
2.1.3 Data analysis

Data files were concatenated using the FCS file concatenation tool available from Cytobank (https://www.cytobank.org/) and normalized using software in MatLab (MathWorks) [168]. Normalized data was debarcoded using a debarcoding tool with cell and sample-specific stringency adjustment [169]. Data were analyzed in R using the package 'cytofkit': a total of 10,000 cells were downsampled from each sample without replacement for ArcSinh transformation and subsequent t-SNE analysis for PhenoGraph clustering and viSNE visualization. Other analyses were completed using FlowJo VX (Treestar).

2.2 Murine cell lines and media

The Py2T cell line was a kind gift from Dr. Gerhard Christofori [163] and both the MVT-1 and 6DT1 cell lines were a kind gift from Dr. Lalage Wakefield [162]. These cell lines were maintained in DMEM with 10% FBS. Murine mammary carcinoma cell lines 4T1, 4T07, and 67NR were a kind gift from Dr Fred Miller (Karmanos Cancer Institutes, Detroit, MI). E0771-LMB cell lines were maintained in RPMI+10% FBS. All cells were maintained in a humidified CO₂ incubator at 37°C. Dulbecco's phosphate-buffered saline (DPBS) without calcium or magnesium (Gibco® Life Technologies) and 0.1% trypsin in citrate buffer + 0.02% EDTA was used for passaging.
2.3 Mice

Female Balb/C (8–12 weeks old; Simonsen Laboratories); C57/bl6 (8-14 weeks old; BC Cancer Research Centre); or FVB/N (8-9 weeks old; Jackson Laboratories) were housed and maintained under specific-pathogen free conditions in the Animal Resource Center at the BC Cancer Agency Research Centre in micro-isolator cages with ventilated racks. IL-5Tg, ddGATA, and WT C57/bl6 mice (9-16 weeks old; Biomedical Research Centre, Vancouver)—a kind gift from Dr. Kelly McNagny—were housed and maintained in a modified barrier facility at the BC Cancer Agency Research Centre. Animal experiments were performed in compliance with requirements of the Canadian Council on Animal Care and the University of British Columbia Committee on Animal Ethics.

Tumor cell lines were implanted orthotopically into the fourth mammary fat pad of mice at a cell-line specific concentration of $10^5$, $10^6$, $2.5 \times 10^5$, $2.5 \times 10^5$, $2.5 \times 10^5$, $10^6$, and $5 \times 10^5$ cells in a 50 μL injection volume for 4T1, 4T07, 67NR, MVT-1, 6DT1, Py2T, and E0771-LMB cell lines, respectively. For intravenous injections, $5 \times 10^5$ LLC or E0771-LMB tumour cells were injected with a 26G needle into the tail vein of mice.

2.4 Injections

For the depletion of eosinophils, anti-IL-5 antibody (TRFK5, BioXCell) or isotype control (TNP6A7, BioXCell) was administered via intraperitoneal injection at 1 mg/kg in PBS weekly unless described otherwise. For the quantification of hypoxia, pimonidazole was administered via intraperitoneal injection at 100 mg/kg 90 minutes before tissue collection and
hōescht 33342 was administered via tail vein injection 30 minutes before tissue collection. To quantify tumour cell proliferation, bromodeoxyuridine was administered at 100 mg/kg via intraperitoneal injection 4 hours before tissue collection.

2.5 Tissue processing

Peripheral blood for flow cytometry was treated with ammonium chloride solution (NH₄Cl, 1:9 ratio, 9 min on ice according to manufacturer's recommendation, StemCell Technologies) for erythrocyte lysis. Lungs and tumors were finely minced with scalpels and then agitated for 40 min at 37°C with 1 mg/mL type II collagenase (Gibco® Life Technologies) in RPMI. After incubation, 0.06% DNase (Sigma Aldrich) was added and the cell suspension filtered through a 100 μm mesh filter with RPMI + 10% FBS. Samples designated for flow cytometry or cell sorting were immediately stained with antibodies following tissue processing.

2.6 Clonogenics

Lungs were finely minced with scalpels and then agitated for 40 min at 37°C with 1 mg/mL type I collagenase (Gibco® Life Technologies) and 4 mg/mL trypsin (Sigma) in PBS. After incubation, 0.06% DNase (Sigma Aldrich) was added and the cell suspension filtered through a 100 μm mesh filter with PBS + 10% FBS. Cells were treated with ammonium chloride solution (NH₄Cl, 1:9 ratio, 9 min on ice according to manufacturer's recommendation, StemCell Technologies) for erythrocyte lysis. Cells were resuspended in RPMI + 10 % FBS + 100 u/mL penicillin and 100 μg/mL streptomycin —with the addition of 60 μM 6-thioguanine for 4T1
tumour cells—and plated at three 10-fold dilutions in triplicate. Plates were left undisturbed in a humidified CO₂ incubator at 37°C for 10 days (4T1) or 14 days (E0771-LMB or LLC). Colonies were briefly fixed and stained with crystal violet in a 25% methanol solution and individual colonies greater than 50 cells were counted.

2.7 Flow cytometry

Single-cell suspensions from blood, tumor, or lung were resuspended in PBS and stained for 30 min at 4°C in the dark with a fixable viability dye (eFluor® 780, eBioscience). Cells were washed and resuspended in Hank’s balanced salt solution with 10 mM HEPES (StemCell Technologies) + 2% FBS and 0.05% NaN₃ and stained for 1 h at 4°C with a combination of surface markers. All antibodies are from eBioscience unless otherwise indicated: CD4 BV605, CD25 PE-Cy7, CD45 APC, CD11b PE-Cy7, CD11c BV605 (Biolegend), Siglec-F PE (BD Biosciences), Gr-1 FITC (Biolegend), F4/80 APC (Biolegend), and CD8 FITC. Anti-murine CD16/32 (0.5 μg/100μL, clone 2.4G2) was used to block non-specific Fc binding by antibodies.

For convenience, cells were fixed and permeabilized for 30 min according to the manufacturer's instructions using a transcription factor buffer set (eBioscience). Cells were incubated overnight at 4°C in 1X permeabilization buffer + 2% FBS. The panel for the differentiation of T cells was then stained with Foxp3 PE-Cy7 for 30 min at 4°C in the dark. Treg and Tconv proportions are expressed as a percentage of total CD4+ cells. Other immune cell subsets are expressed as a percentage of CD45+ cells.

Tumour cells fixed with 70% ethanol were washed in PBS and stained in PBS + 4% FBS and 0.1% Triton-X 100 with hypoxyprobe FITC antibody for 1 h at 4°C in the dark. All samples
were run on an LSR Fortessa and analyzed with FACSDiva software (BD, Mississauga, ON, Canada) and FlowJo (TreeStar).

2.8 Immunofluorescence

Tumours and lungs were harvested from tumor-bearing mice 3 weeks post orthotopic implant, lungs were inflated and tissues were fixed in 2% PFA for 24 hours at 4°C, and transferred to a 30% sucrose solution for 48 hours at 4°C. Tissues were then frozen in Optimal Cutting Temperature (OCT) medium (Sakura Finetek) overnight. Serial 10 μm sections were cut and stained in PBS + 4% FBS and 0.1% Triton-X 100 with antibodies: Siglec-F (BD Biosciences) and pimonidazole FITC (Hypoxyprobe) using Alexa Fluor® 594 secondary antibodies (Life Technologies). Sections were fixed briefly in 2% PFA and optionally stained briefly with 1 μg/mL DAPI. Images were captured with a Zeiss Imager Z1 using a cooled, monochrome CCD camera (Retiga 4000R, QImaging) and Northern Eclipse software.

2.9 FACS Sorting

Three lungs from 4T1 tumour-bearing mice at 3 weeks post-implant were processed as described in Section 2.5. Cells were stained with CD11c BV605 (Biolegend), Siglec-F PE (BD Biosciences), and Gr-1 FITC (Biolegend). Approximately $1\times10^5-1\times10^6$ eosinophils (Siglec-F+CD11c-), gMDSCs (Gr-1$^{hi}$Siglec-F-), mMDSCs (Gr-1$^{int}$Siglec-F-), and alveolar macrophages (Siglec-F+CD11c+) were sorted on a BD FACSArria III. Cells were immediately washed and
RNA was isolated using an RNeasy Mini kit (QIAGEN), quantified by Nanodrop 1000, and stored at -80°C. RNA was subsequently analyzed by qPCR as outlined in section 2.10.

2.10 Quantitative PCR

Lungs from 4T1 tumour-bearing mice at 1, 2, or 3 weeks post-implant were immediately frozen in liquid nitrogen and disaggregated in liquid nitrogen using a mortar and pestle. The tissue was then homogenized in Trizol using a 1 mL syringe equipped with a 22 ½G needle. RNA was isolated by phenol chloroform extraction with 0.1 mg/mL DNAse digestion for 15 minutes at room temperature. Purified RNA was quantified using a Nanodrop 1000 spectrometer and stored at -80°C.

Reverse-transcription was performed using a Superscript II kit according to the package instructions using 200 ng of random primers (ThermoFisher) per reaction. Negative control reactions omitted the reverse-transcriptase. The subsequent qPCR reactions were prepared in triplicate in Fast 96-well plates using Fast SYBR Green Master Mix (Applied Biosystems). Random primers (ThermoFisher; Table 2.2) were used at 200 nM each and a total of 250 ng of cDNA was used in each qPCR reaction assuming a 1:1 conversion of RNA to cDNA. The reactions were run for 45 cycles of 3 sec at 95°C and 30 sec at 60°C following an initial 20 sec hold at 95°C on a Viia7 Real-time qPCR machine (ThermoFisher). Automatic thresholding was used to calculate Ct values.
Table 2.2: Primer sequences used for qPCR.

<table>
<thead>
<tr>
<th>Target</th>
<th>RefSeq</th>
<th>Sequence (5'-3')</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL24</td>
<td>NM_019577.4</td>
<td>F: TGTGACCATCCCCCTCATCTTGC</td>
<td>281 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: AAACCTCGGTGCTATTTGCCACG</td>
<td></td>
</tr>
<tr>
<td>CCL11</td>
<td>NM_011330.3</td>
<td>F: CAGATGCACCCCTGAAAGCCATA</td>
<td>96 bp</td>
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<tr>
<td></td>
<td></td>
<td>R: TGTCTTTGTGGCATCCTGGAC</td>
<td></td>
</tr>
<tr>
<td>IL-5</td>
<td>NM_010558.1</td>
<td>F: GCCACACTTCTTTTTTGGCG</td>
<td>178 bp</td>
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<tr>
<td></td>
<td></td>
<td>R: TGAGACGATGAGGCTTTCTTG</td>
<td></td>
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<tr>
<td>HPRT</td>
<td>NM_013556.2</td>
<td>F: GATTAGCGATGATGAAACCAGGT</td>
<td>150 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CCTCCCATCTCTCTCGT</td>
<td></td>
</tr>
<tr>
<td>18s rRNA</td>
<td>NR_003278.3</td>
<td>F: GTAACCCGTTGAACCCCAT</td>
<td>151 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CCATCCAATCGGTAGTAGCG</td>
<td></td>
</tr>
</tbody>
</table>

2.11 Histology

Tissue analysis was conducted by the Centre for Translational and Applied Genomics. Briefly, 4 µm thick sections were cut from neutral buffered formaldehyde fixed, paraffin wax embedded tissue blocks containing colorectal carcinoma or pancreatic ductal adenocarcinoma specimens. Sections were mounted on glass slides, dried at 60°C, and dewaxed using the Ventana Discovery XT System. Antigen retrieval was completed using Ventana protease 1 for 8 minutes at 37°C and sections were subsequently stained with monoclonal antibody to human eosinophil major basic protein (BMK-13; EMD Millipore) at a 1:20 dilution for two hours at room temperature. Immunostaining was automated using the Ventana Discovery XT System. Immunostaining was followed by brief nuclear counterstaining in Surgipath Selectech haematoxylin. Finally, coverslips were mounted with Surgipath MM24 mounting medium. Sections of bone marrow were used as a control for positive staining and both placenta and
kidney were used as negative controls.Slides were imaged using a Leica DM2500 microscope and scale bars were added in ImageJ.

2.12 Statistics

All statistical analyses were conducted in R using custom scripts. Two-tailed Student's t-tests were used for comparison of data containing two or three experimental groups using the Holm-Sidak correction for multiple comparisons. Welch’s correction was applied if variances were significantly different between groups. GraphPad Prism was used to plot the data. Unless otherwise stated, all data are reported as mean ± SEM.
Chapter 3: Results

3.1 Identification of a heterogenous population of eosinophils in the metastatic murine lungs

The data presented in this section will address aims 1 and 4 of the thesis. Specifically, the data presented in this subsection will address whether eosinophils are increased in the lungs of mice bearing metastatic mammary tumours.

3.1.1 Mass cytometry analysis

In the dawning age of immunotherapy, understanding the complex interplay within diverse immune cell populations and with invading tumour cells in the metastatic lung is essential for the discovery of prognostic biomarkers, novel therapeutics, mechanisms of drug resistance, and potential strategies to mitigate metastatic colonization. Numerous high-dimensional strategies leveraging computational analyses have been employed in an effort to quantify the proportions of immune cells in tumours and monitor their phenotypic changes during tumorigenesis. Deep-sequencing of RNA facilitates the estimation of relative immune cell populations in human tissue samples available through large databases such as The Cancer Genome Atlas (TCGA)—a public online resource, TIMER, was developed for such analyses [170]. However, there are limitations on RNA-seq analyses of patient tissue: early immune changes contributing to metastatic colonization cannot be inferred from late-stage biopsies and examination of RNA expression does not facilitate the characterization of the phenotype of
individual immune cell subsets. While various in vivo genomic screens have revealed interesting genes contributing to interactions between immune cells and cancer [171-173], mass cytometry has more recently emerged as an effective tool for the comprehensive profiling of immune cell subsets in the tumour microenvironment during the early and late stages of cancer at the single-cell level [174, 175]. Furthermore, mass cytometry has previously been employed to effectively characterize myeloid cells in the naive murine lung to identify unique subsets of phenotypically distinct populations [176].

I was interested in the discovery of novel immune cell subsets critical in the early temporal changes within the lung from initial cultivation of the pre-metastatic niche through the establishment of metastatic disease in mouse models of breast cancer. For the discovery of such immune cell subsets, I employed mass cytometry for the comprehensive analysis of the immune cell landscape within the metastatic lungs of 4T1 tumour-bearing mice at 1, 2, and 3 weeks post-implant using the markers described in Table 2.1. To display the phenotypic heterogeneity of immune cell populations, I performed Rphenograph unsupervised clustering on live cells determined by uptake of cisplatin (Pt195). Thirty-six distinct populations of cells were established (Figure 3.1A). Various clusters of CD45+ immune cell populations could be identified, including: NK cells, eosinophils, alveolar macrophages, granulocytic myeloid-derived suppressor cells (gMDSCs), monocytic myeloid-derived suppressor cells (mMDSCs), B cells, CD4+ T cells, and cytotoxic CD8+ T cells (Figure 3.1B). However, traditionally defined subsets of immune cells did not always form independent clusters in 2D space on viSNE plots and some rare subsets were difficult to distinguish. A complex gating strategy (data not shown) was used to more accurately define these immune cell populations—and additional rare subsets such as pDCs—in the lung and quantify the changes in the proportions of subsets over time (Figure
3.1C). At 3 weeks post-implant of the primary tumour, the most prominent immune cell populations in the metastatic lung were CD11b+F4/80-Ly6G+Ly6C^{lo} gMDSCs (78.7 ± 1.4%), CD11b+F4/80+Ly6G-Ly6C^{+} mMDSCs (6.8 ± 0.1%), and CD11b+CD11c-Siglec-F+ eosinophils (2.6 ± 0.1%). Moreover, only the proportion of gMDSCs, mMDSCs, eosinophils, and NK cells (NK1.1+) increased over the course of metastatic development.

Interestingly, further investigation of the lung eosinophils revealed heterogeneity: the population was dominated by a Ly6G^{int}Ly6C^{int} subset, but also included a significant proportion of Ly6G^{int}Ly6C^{hi} cells that expanded over time and a minor subset of Ly6G^{hi}Ly6C^{int} cells (Figure 3.2A-B). The three subsets displayed subtle differences in the expression of various markers: the Ly6G^{hi}Ly6C^{int} subset exhibited higher levels of expression of CD11b and PD-1 while the Ly6G^{int}Ly6C^{hi} subset had higher levels of expression of TNF-α, PD-L1, and CTLA-4 (Figure 3.2C). The functional significance of the differential expression of these various markers is unclear, but suggests that the subsets within this population of eosinophils may have distinct phenotypes. Moreover, the distinct subsets of eosinophils change in prevalence over the course of metastatic development.

In summary, mass cytometry facilitated the comprehensive profiling of numerous immune cell subsets in the metastatic lungs of mice bearing 4T1 tumours. Furthermore, mass cytometry analysis allowed for the identification of a population of heterogenous eosinophils that expands in the lungs over the course of metastatic development. Being the third most abundant population in the metastatic lungs, eosinophils were selected for further study.
Figure 3.1: Mass cytometry Rphenograph results of 4T1 tumour-bearing lungs at various time points. A) A visNE plot illustrating the 36 distinct populations of cells identified by unsupervised Rphenograph clustering of live cells in the metastatic lung of mice bearing 4T1 mammary tumours at 3 weeks post-implant. B) Representative visNE plots illustrating the expression of various markers used to differentiate the immune cell subsets indicated. Eosinophils are indicated in red. C) Area plot illustrating the proportion of live lung cells categorized into various immune cell subsets at 0, 1, 2, and 3 weeks post-implant of the primary tumour. Points are representative of n=3 lungs. Eosinophils are indicated in red.

Figure 3.2: Identification of a heterogeneous population of eosinophils within the metastatic murine lung. A) Representative flow cytometry plots of live Siglec-F+CD11b+CD11c-eosinophils illustrating the heterogenous Ly6G$^{int}$Ly6C$^{int}$, Ly6G$^{int}$Ly6C$^{hi}$, and Ly6G$^{hi}$Ly6C$^{int}$ subsets within the metastatic lungs of 4T1 tumour-bearing mice. B) Area plot illustrating the percent of each subset of Ly6G$^{int}$Ly6C$^{int}$, Ly6G$^{int}$Ly6C$^{hi}$, and Ly6G$^{hi}$Ly6C$^{int}$ eosinophils in the metastatic lungs of 4T1 tumour-bearing mice. C) The heterogeneous expression of various markers by the distinct subsets of eosinophils at 3 weeks post-implant of the 4T1 tumour. Data points are representative of n=3 lungs.
3.2 Balb/C and FVB experiments

Recently, two therapeutic antibodies targeting IL-5 have been approved by Health Canada for the treatment of eosinophilic asthma—Reslizumab and Mepolizumab [154, 155]—which could be swiftly repurposed for use in cancer therapy, as immunodepletion of eosinophils using anti-IL-5 clone TRFK5 has been shown to greatly reduce metastatic colonization of the lungs in mouse models of cancer [50]. In contrast, a distinct clone of anti-IL-5 antibody, NC17, has been shown to increase metastatic colonisation of the lung using the same mouse model of cancer [106]. Due to the differential capabilities of eosinophils and their phenotypic plasticity, it is possible that resident and recruited eosinophils within the pre-metastatic lung in orthotopic models may have distinct phenotypic behaviors from those that encounter intravenously injected tumour cells in the absence of distant priming by a primary tumour. Therefore, I sought to investigate whether eosinophils accumulate in the pre-metastatic lung of mice bearing various orthotopic mammary tumours and whether their phenotype differs from naïve resident lung eosinophils. Furthermore, I wanted to identify which chemoattractants may contribute to the migration of eosinophils to the metastatic lung, as this may reveal alternative strategies for the therapeutic manipulation of these granulocytes. In addition, I wanted to clarify whether the depletion of eosinophils using anti-IL-5 antibodies represents an effective strategy for the treatment or prevention of metastasis using orthotopic mouse models of breast cancer that accurately recapitulate the disease. Finally, I leveraged anti-IL-5 immunodepletion of eosinophils to examine whether eosinophils influence primary tumour growth or vascularization.

In summary, the data presented in this section will address aims 1, 3, and 4 of the thesis. Specifically, this data was collected to determine whether endogenous eosinophils influence
primary tumour growth or vascularization; whether eosinophils are increased in the lungs of mice bearing various metastatic mammary tumours; and whether eosinophil-specific chemoattractants may be responsible for eosinophil recruitment to the lungs.

3.2.1 Eosinophils accumulate in the lungs of mice bearing metastatic mammary tumours

Firstly, I wanted to determine whether eosinophils accumulate in the lungs of mice bearing metastatic mammary tumours—or are instead decreased, as observed in previous studies [50]—and the temporal occurrence of this phenomenon. To robustly determine whether deviations in the number of lung eosinophils are consistent across a variety of mouse models of metastatic breast cancer—and whether changes could be identified in mice bearing poorly or non-metastatic mammary tumours—I used tumour cell lines syngeneic to both the Balb/C and FVB/N background. Briefly, mice were implanted with metastatic (4T1, 4T07, 6DT1, MVT-1, E0771-LMB) or non-metastatic (67NR, Py2T) syngeneic cell lines that developed into tumours within the 4th mammary fat pad over the course of 3 weeks. Flow cytometry analysis was completed on lung cells from naïve mice or mice bearing mammary tumours at 3 weeks post-implant. Live singlets were gated and CD45+ leukocytes were selected for further stratification: eosinophils were identified as SSC\text{hi}Siglec-F+CD11b+CD11c- cells. A small number of eosinophils were sorted and stained with Giemsa to confirm the gating strategy (Figure 3.3A). Sorted cells exhibited phenotypic characteristics of classical murine eosinophils: brightly eosinophilic granules within the cytoplasm and annular nuclei.

There was no significant change in the proportion of eosinophils observed in mice bearing highly metastatic 4T1, poorly metastatic 4T07, or non-metastatic 67NR mammary
tumours at 3 weeks post-implant. Nonetheless, the expansion of CD45+ leukocytes in the lungs of mice bearing 4T1 mammary tumours at 3 weeks post-implant resulted in a 5.8-fold increase (p=0.0007) in the average total number of eosinophils in the lungs of 4T1 tumour-bearing mice (8.3×10⁵ ± 1.1×10⁵) relative to naïve controls (1.4×10⁵ ± 1.8×10⁴). The increase of eosinophils in the metastatic lungs of 4T1 tumour-bearing mice was unique to this particular model, as no significant change in the number of lung eosinophils was measured in FVB/N mice bearing metastatic MVT-1 or 6DT1 mammary tumours relative to those bearing non-metastatic Py2T tumours. Therefore, further investigation into the role of eosinophils on pulmonary metastasis was conducted in 4T1 tumour-bearing mice.
**Figure 3.3: Eosinophils in the lungs of mice bearing mammary tumours.** A) The gating strategy for the identification of eosinophils in lungs by flow cytometry and sorted eosinophils stained with Giemsa. B) The number of eosinophils expressed as a percentage of CD45+ leukocytes and C) total number of eosinophils in the lungs of mice bearing highly metastatic (4T1), poorly metastatic (4T07), or non-metastatic (67NR) mammary tumours syngenic to the Balb/C background at 3 weeks post-implant. D) The number of eosinophils expressed as a percentage of CD45+ leukocytes and E) total number of eosinophils in the lungs of mice bearing metastatic (MVT-1, 6DT1) or non-metastatic (Py2T) mammary tumours syngenic to the FVB/N background at 3 weeks post-implant. Student’s two-tailed t test with Holm-Sidak correction for multiple comparisons: *** p=0.0001-0.001; ns p>0.05.

Upon determining that eosinophils accumulated in the lungs of 4T1 tumour-bearing mice at 3 weeks post-implant of the primary tumour, I sought to investigate whether eosinophils accumulate in the pre-metastatic lung or only during the later stages of metastatic disease. Flow cytometry analysis of the lungs of tumour-bearing revealed that the percentage of CD45+ leukocytes identifiable as eosinophils at 1 week (1.5 ± 0.5%) and 2 weeks (1.1 ± 0.1%) post-implant was less than half that of naïve mice (4.4 ± 1.2%), though the proportion of eosinophils began to increase by 3 weeks post-implant (2.5 ± 0.5%; Figure 3.4A). Accordingly, the total number of eosinophils in the lungs was increased at 3 weeks post-implant by 3.5-fold relative to naïve controls (p=0.02; Figure 3.4B) due to the influx of CD45+ leukocytes. This data suggests that eosinophils do not appear to accumulate in the pre-metastatic lung at 1 week post-implant—or during early stages of metastatic development at 2 weeks post-implant—but have accumulated in the metastatic lung in sufficient numbers by 3 weeks post-implant.
Figure 3.4: Time-course analysis of the number of eosinophils and a CD11b<sup>hi</sup>Gr-1<sup>hi</sup> subset in the lungs of 4T1 tumour-bearing mice.  

A) The number of eosinophils expressed as a percentage of CD45<sup>+</sup> leukocytes and B) total number of eosinophils in the lungs of naïve mice or mice bearing orthotopic 4T1 tumours at 1, 2, and 3 weeks post-implant. C) Flow cytometry plots illustrating the percentage of lung eosinophils that were CD11b<sup>hi</sup>Gr-1<sup>hi</sup> in the lungs of naïve mice or mice bearing orthotopic 4T1 tumours at 1, 2, and 3 weeks post-implant. D) The percentage and E) total of CD11b<sup>hi</sup>Gr-1<sup>hi</sup> eosinophils in the lungs of naïve mice or those bearing 4T1 tumours. Student’s two-tailed t test with Holm-Sidak correction for multiple comparisons: **** p<0.0001; *** p=0.0001-0.001; ** p=0.001-0.01; * p=0.01-0.05; ns p>0.05.

These data indicate that there are a small number of resident eosinophils in the naïve lung that are maintained at 1 and 2 weeks post-implant of the primary tumour. I therefore wanted to assess whether these resident eosinophils may be phenotypically influenced by the progressively immunosuppressive environment of the pre-metastatic lung at 1 and 2 weeks post-implant. A unique population of lung CD11b<sup>hi</sup>Gr-1<sup>hi</sup> eosinophils—markedly absent from naïve lungs—was identified in the pre-metastatic lung as early as the first week post-implant of the primary tumour, at which time tumour cells cannot yet be quantified. Interestingly, this population appeared to be specific to the lungs, as distinct CD11b<sup>hi</sup>Gr-1<sup>hi</sup> eosinophils could not be found in the peripheral blood of naïve or 4T1 tumour-bearing mice (Figure 3.4C). The percentage of lung eosinophils that were CD11b<sup>hi</sup>Gr-1<sup>hi</sup> progressively increased to reach a maximum at 2 weeks post-implant (15.9 ± 3.2%) before declining slightly in the third week post-implant (12.8 ± 1.7%). Conversely, the total number of CD11b<sup>hi</sup>Gr-1<sup>hi</sup> eosinophils in the lungs also gradually increased in the metastatic lungs at 1 and 2 weeks post-implant, mirroring the increase in the total number of lung eosinophils (Figure 3.4E). As the total number of eosinophils did not increase in the lungs of 4T1 tumour-bearing mice at 1 and 2 weeks post-implant (Figure 3.4B), this data suggests that resident eosinophils in the pre-metastatic lung and during the stages of
early metastatic development may differentiate into CD11b^{hi}Gr-1^{hi} eosinophils in 4T1 tumour-bearing mice.

It is known that the number of gMDSCs that accumulate in the metastatic lungs of mice bearing 4T1 mammary tumours is proportional to the weight of the primary tumour, which suggests that factors from the primary tumour may promote the accumulation of these immune cells in the lungs [177, 178]. Therefore, I wanted to assess whether eosinophils may similarly accumulate in the lungs of 4T1 tumour-bearing mice in response to factors produced by the primary tumour. As expected, there was a strong positive correlation between the total number of gMDSCs in the metastatic lung and the weight of the primary 4T1 tumour ($R^2=0.95$, $p<0.0001$, $F=283$). In addition, there was a strong correlation between the percentage of gMDSCs and the weight of the primary tumour ($R^2=0.90$, df=15): the percentage of gMDSCs increased rapidly initially, but began to plateau when the tumour reached approximately 0.7 g (Figure 3.5A). These data illustrate that the proportion of gMDSCs in the lungs reaches a maximum of about 80% of CD45+ cells that is maintained as additional CD45+ cells are recruited with increasing primary tumour weight. Interestingly, there was a statistically significant positive linear correlation between the total number of lung eosinophils and primary tumour weight ($R^2=0.65$, $p<0.0001$, $F=30$), suggesting that the primary tumour may have a role in eosinophil recruitment to the metastatic lungs. There was a weak correlation between the percentage of eosinophils in the lungs relative to primary tumour weight, but this positive correlation was statistically significant ($R^2=0.33$, $p=0.016$, $F=7.2$) (Figure 3.5B). Overall, this data suggests that the primary tumour may have minor effects—directly or indirectly—on eosinophil accumulation in the metastatic lungs of 4T1 tumour-bearing mice.
Figure 3.5: Correlation of immune cell accumulation in the lungs with primary tumour weight. There is a strong correlation between the weight of the primary tumour and both the A) total number and B) percentage of gMDSCs that accumulate in the lungs of mice bearing metastatic 4T1 mammary tumours. There is a modest correlation between primary 4T1 tumour weight and the A) total and B) percentage of lung eosinophils. Linear or non-linear regression analysis with least-squares fit; $R^2$ shown on graph.
3.2.2 CCL24 and IL-5 is expressed in the lungs of 4T1 mammary tumour-bearing mice

As is true of the mucosal surface of the GI tract, eosinophils are known to be resident in the lungs and additional eosinophils are recruited to this site in response to insult that occurs in the context of asthma or allergy [38]. Although the mechanisms of eosinophil recruitment to the allergic and asthmatic lungs have been well documented, factors responsible for enhanced eosinophil recruitment to the pre-metastatic lung have not been elucidated. Eosinophils have been shown to migrate towards DAMPs such as HMGB1 released from necrotic cells [73, 74] within murine melanoma tumours [72] and infiltrate colorectal neoplasms in response to both CCL11 and CCL24 [75, 76]. Although human breast cancer cells stimulated with TNF-α may secrete the eosinophil chemoattractant CCL5 [77, 79], CCL5 is not prevalent in the metastatic lungs of 4T1 tumour-bearing mice [78]. In addition, the chemoattractants CCL3 and CCL11 are largely absent in the lungs of 4T1 tumour bearing mice [78, 79]. Other eosinophil chemoattractants—such as C5a [81], PAF [179], and leukotrienes LTB4 and LTE4 [82]—have also been implicated in pulmonary metastasis, but none of these chemokines are highly selective towards eosinophils.

Pertinent to the metastatic lung, it has been previously shown that Th2-polarized alveolar macrophages secrete high levels of CCL24 in the presence of IL-33 and the Th2 cytokines IL-4 or IL-13; all of which would be expected to be present in the immunosuppressive tumour microenvironment of the lung [83]. Furthermore, CCL24 has been shown to be upregulated by mMDSCs in the context of airway inflammation—mMDSCs are prevalent in the metastatic lungs (Figure 3.1B) [84, 85]. Various chemoattractants may contribute to the recruitment of eosinophils to the metastatic lungs, which may also be dependent on stage of metastatic
development: CCL24 from Th2-polarized alveolar macrophages or mMDSCs may be responsible for early eosinophil recruitment to the pre-metastatic niche in the lungs, while at later stages of metastatic disease, DAMPs such as HMGB1 may be more significant.

To identify potential chemokines that may be responsible for the recruitment of eosinophils to the metastatic lungs throughout metastatic development, I focused on the 4T1 tumour-bearing mouse model due to the high abundance of eosinophils in the lungs. I quantified the relative abundance of potent and highly selective chemokines of eosinophils, CCL11 and CCL24, in addition to the chemokinetic cytokine: IL-5. I examined bulk lung tissue of naïve and 4T1 tumour bearing mice at 1, 2, or 3 weeks post-implant to initially determine whether any of these factors were significantly altered during the course of metastatic development.

The expression of CCL11 relative to naïve lungs appears to decrease to less than half in the first week post-implant (0.38 ± 0.02, p<0.0001), but expression normalizes to near that of naïve lungs by the second week. This is in agreement with chemokine array data from our laboratory suggesting that CCL11 is not significantly produced in naïve or 4T1 tumour-bearing lungs [78]. The expression of CCL24 appeared to increase in the 4T1 tumour-bearing lungs over the course of metastatic development culminating in a 3.5-fold increase (3.5 ± 0.3, p<0.0001) relative to naïve lungs at 3 weeks post-implant of 4T1 mammary tumours. Moreover, there was a transient 2.5-fold increase (2.5 ± 0.3, p<0.0001) in the expression of IL-5 at 2 weeks in the metastatic lungs that may promote the persistence of eosinophils at this time point (Figure 3.6A). The progressive induction of CCL24 within the lungs of 4T1 tumour-bearing mice suggests that this chemokine may have a role in the selective recruitment of eosinophils to the metastatic lung.
Figure 3.6: Fold-expression change of chemokines and cytokines in the bulk lung tissue of naïve and 4T1 tumour-bearing mice. A) Fold expression change of CCL11, CCL24, and IL-5 in the bulk metastatic lungs of 4T1 tumour-bearing Balb/C mice at 3 weeks post-implant in triplicate relative to the average of naïve expression. Each bar corresponds to the quantification of n=3-4 lungs with 3 technical replicates each. Student’s two-tailed t test with Holm-Sidak correction for multiple comparisons: **** p<0.0001; *** p=0.0001-0.001; ** p=0.001-0.01; * p=0.01-0.05; ns p>0.05. B) The fold change expression of CCL24 relative to hypoxanthine-guanine phosphoribosyltransferase (HPRT) in various immune cell populations sorted by FACS in triplicate from three pooled metastatic lungs of 4T1 tumour-bearing Balb/C mice at 3 weeks post-implant.
There is a lack of literature describing an increase in CCL24 in the metastatic lungs or whether CCL24 may have a role in pulmonary metastasis. Alveolar macrophages in the pre-metastatic niche that become M2-skewed may initially secrete CCL24 during the early stages of metastatic development. However, I did not observe an expansion of this population in the metastatic lungs unlike previously published data that would logically account for the progressive increase in CCL24 expression observed [33]. Rather, the accumulation of mMDSCs in the metastatic lungs (Figure 3.1B) may instead reconcile these observations. Sorted mMDSCs from the metastatic lungs of 4T1 tumour-bearing mice at 3 weeks post-implant showed higher levels of CCL24 expression relative to gMDSCs, or eosinophils (Figure 3.6B) suggesting that these immune cells may be critical in the recruitment of eosinophils. In addition, eosinophils also expressed moderate levels of CCL24 indicating a positive feedback mechanism that may contribute to the accumulation of these cells in the metastatic lungs. Due to the low abundance of alveolar macrophages in the metastatic lungs, the expression of CCL24 by this cell type could not be accurately quantified by FACS and qPCR.

3.2.3 Anti-IL-5 immunodepletion of eosinophils in 4T1 tumour-bearing mice

To determine whether immunodepletion of eosinophils using an anti-IL-5 antibody represents an effective therapy for the treatment of pulmonary metastasis, I leveraged the orthotopic 4T1 mouse model of breast cancer. Eosinophils can be effectively depleted in mice using anti-Siglec-F antibody [157] or anti-IL-5 antibodies. To avoid any potential effects on alveolar macrophages—important contributors to the pre-metastatic niche that also express high levels of siglec-F [33]—I decided to use anti-IL-5 antibody clone TRFK5, which has been well-
validated by the existing literature [50, 156]. To determine a schedule for administration of the
antibody that would effectively deplete eosinophils in mice, I administered TRFK5 IP at 1 mg/kg
to naïve Balb/C mice and monitored the number of eosinophils in the blood and lungs at 1, 3, or
7 days post-injection by flow cytometry (Figure 3.7A). A 3.6-fold decline in the percentage
blood eosinophils (p=0.0004) was measured 24 hours after a single dose which indicates rapid
systemic depletion of eosinophils (Figure 3.7B and Figure 3.8A) that were designated
SSC<sup>hi</sup>Siglec-F+CD11b+ autofluorescent cells (Figure 3.7C). A 3.3-fold and 2.5-fold decline of
the percentage (p=0.0007, Figure 3.7D) and total (p=0.03, Figure 3.7E) number of eosinophils
was measured in the lungs at 7 days post-injection, respectively (Figure 3.8B). Therefore,
TRFK5 was to be administered to tumour-bearing mice every 7 days and tissues were to be
examined 3 days following the latest dose (Figure 3.9A).
Figure 3.7: The systemic depletion of eosinophils in naïve Balb/C mice using anti-IL-5 clone TRFK5. A) Schedule of anti-IL-5 administration indicated in red and time points that lungs were examined are indicated in black. B) The number of blood eosinophils expressed as a percentage of CD45+ leukocytes at various time points following anti-IL-5 administration. C) Flow cytometry gating scheme for the identification of live eosinophils in the blood. D) The number of lung eosinophils expressed as a percentage of CD45+ leukocytes at various time points following anti-IL-5 administration. E) The total number of lung eosinophils at various time points following anti-IL-5 administration. Student’s two-tailed t test with Holm-Sidak correction for multiple comparisons: **** p<0.0001; *** p=0.0001-0.001; ** p=0.001-0.01; * p=0.01-0.05; ns p>0.05.

Eosinophils were systemically depleted in 4T1 tumour-bearing mice using the anti-IL-5 TRFK5 antibody weekly for three weeks: eosinophils were decreased significantly in both the peripheral blood (Figure 3.8A and Figure 3.9B) and lungs (Figure 3.8B and Figure 3.9C-D) at 2 and 3 weeks post-implant of the primary tumour. Although the number of CD45+ leukocytes increased in the lungs of 4T1 tumour-bearing mice over the course of metastatic development, the use of anti-IL-5 antibody did not result in a significant change in the total number of CD45+ leukocytes in the lung (Figure 3.9E) or primary tumour weight (Figure 3.9F) relative to mice administered an isotype control antibody.
Figure 3.8: Representative flow cytometry plots illustrating effective depletion of eosinophils in the blood and lungs of 4T1 tumour-bearing mice using anti-IL-5 clone TRFK5. A) Representative flow cytometry plots of blood eosinophils (Siglec-F+, autofluorescent in the blue 530 channel) in Balb/C mice treated with anti-IL-5 or isotype control antibody weekly at 1, 2, or 3 weeks post-implant of orthotopic 4T1 tumours. B) Comparable flow cytometry plots of lung eosinophils (Siglec-F+CD11c-) in treated mice.
Figure 3.9: The depletion of eosinophils in 4T1 tumour-bearing mice using anti-IL-5 clone TRFK5 antibody. A) Schedule of antibody administration to 4T1 tumour-bearing mice: orthotopic 4T1 implant is indicated as day 0 in blue; time points of anti-IL-5 or isotype control administration is indicated in red; and the time points that lungs were examined are indicated in black. B) The number of blood eosinophils expressed as a percentage of CD45+ leukocytes at various time points post-implant of the primary 4T1 tumour in mice treated with anti-IL-5 (▲) or isotype control antibody (●). The C) percentage and D) total number of lung eosinophils at various time points post-implant of the primary tumour. E) The total number of CD45+ leukocytes in the lungs and F) primary tumour weight of 4T1 tumour-bearing mice administered anti-TRFK5 or isotype control antibody. Student’s two-tailed t test: *** p=0.0001-0.001; ** p=0.001-0.01; ns p>0.05.

3.2.4 Effects of anti-IL-5 on immune cell populations in the metastatic lungs

Eosinophils have been suggested to promote tumour cell colonization during the early stages of metastatic development in the lungs through the secretion of CCL22 which recruits Tregs [50]. I was interested in whether this holds true in orthotopic mouse models of cancer and whether eosinophils recruited to the lungs during metastatic development influence other immune cell populations. The use of two panels of antibodies facilitated the specific identification of T lymphocytes: cytotoxic CD8+ T cells, conventional T cells (Tconv), and regulatory T cells (Tregs) (Table 3.1 and Figure 3.10A); and granulocytes/myeloid cells: eosinophils, alveolar macrophages, gMDSCs, and mMDSCs (Table 3.1 and Figure 3.10B) in the lungs of tumour-bearing mice using flow cytometry.
Table 3.1: Flow cytometry panels used for the differentiation of various immune cell subsets.

<table>
<thead>
<tr>
<th>Antibody Panel</th>
<th>Immune Cell Subset</th>
<th>Markers</th>
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<tbody>
<tr>
<td>T Lymphocytes</td>
<td>Cytotoxic CD8+ T cells</td>
<td>CD8+CD4-</td>
</tr>
<tr>
<td>T Lymphocytes</td>
<td>Tconv</td>
<td>CD4+FoxP3-</td>
</tr>
<tr>
<td>T Lymphocytes</td>
<td>Tregs</td>
<td>CD4+CD25+FoxP3+</td>
</tr>
<tr>
<td>Myeloid/Granulocytes</td>
<td>Eosinophils</td>
<td>Siglec-F+CD11b+CD11c-</td>
</tr>
<tr>
<td>Myeloid/Granulocytes</td>
<td>Alveolar macrophages</td>
<td>Siglec-F+CD11b-CD11e+</td>
</tr>
<tr>
<td>Myeloid/Granulocytes</td>
<td>gMDSCs</td>
<td>Siglec-F-CD11b+Gr-1&lt;sup&gt;hi&lt;/sup&gt;</td>
</tr>
<tr>
<td>Myeloid/Granulocytes</td>
<td>mMDSCs</td>
<td>Siglec-F-CD11b+Gr-1&lt;sup&gt;int&lt;/sup&gt;</td>
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Figure 3.10: Gating strategy for T lymphocytes and myeloid/granulocytes and quantification of CD45+ cells in the lungs of 4T1 tumour-bearing mice treated with anti-IL-5 or isotype control antibody. A) Flow cytometry gating scheme for the identification of cytotoxic CD8+ T cells; Tconv (CD4+FoxP3-); and Tregs (CD4+CD25+FoxP3+) in the lungs of Balb/C mice treated with anti-IL-5 or isotype control antibody; B) Flow cytometry gating scheme for the identification of alveolar macrophages (Siglec-F+CD11c+); mMDSCs (Siglec-F-CD11b+Gr-1int); gMDSCs (Siglec-F-CD11b+Gr-1hi); eosinophils (Siglec-F+CD11c-CD11b+); and CD11bhiGr-1hi eosinophils.
In the 4T1 mouse model of metastatic breast cancer, the accumulation of gMDSCs in the lung is known to predate tumour cell colonization [180] and one report suggested that alveolar macrophages may also expand in the pre-metastatic niche [33]. The use of the anti-IL-5 antibody did not significantly affect the robust accumulation of gMDSCs in the metastatic lungs (Figure 3.11A) or the total number of alveolar macrophages (Figure 3.11B) relative to mice administered an isotype control. Moreover, the use of the anti-IL-5 antibody to deplete eosinophils did not affect the percentage or total mMDSCs in the metastatic lungs (Figure 3.11C-D).
Figure 3.11: The percentage and total numbers of granulocytic and myeloid immune cell populations in the metastatic lung of 4T1 tumour-bearing mice administered an anti-IL-5 antibody or isotype control. A) the total number of lung gMDSCs (Siglec-F-CD11b+Gr-1\textsuperscript{hi}) in Balb/C mice treated with anti-IL-5 (▲) or isotype control antibody (●) for weekly at 1, 2, or 3 weeks post-implant of orthotopic 4T1 tumours. B) The total number of alveolar macrophages (Alveolar Mφ; Siglec-F-CD11b-CD11c+). C) The percentage and D) total number of lung mMDSCs expressed as a (Siglec-F-CD11b+Gr-1\textsuperscript{int}). Student’s two-tailed t test: ns p>0.05.

The effects on T lymphocyte populations in the lungs by the immunodepletion of eosinophils using an anti-IL-5 antibody were also examined. The ratio of cytotoxic CD8+ T cells to Tregs is representative of whether CD8+ T cells are capable of exerting cytotoxic functions towards tumour cells, which is partially dependent on the local abundance of Tregs: a high CD8+/Treg ratio is indicative of a more pro-inflammatory environment, whereas a low ratio suggests a more tumour-permissive milieu. In mice administered anti-IL-5 antibody, the
CD8+/Treg ratio was significantly increased by 1.7-fold (11.1 ± 0.9 vs. 19.5 ± 1.1, p=0.0001) at 2 weeks post-implant, but this effect was diminished by 3 weeks post-implant (Figure 3.12A). This is a result of a minor decline in the percentage of Tregs (3.2 ± 0.2% vs. 2.0 ± 0.2%, p=0.001; Figure 3.12C) combined with a modest increase in the percentage of CD8+ T cells (1.7 ± 0.2% vs. 2.5 ± 0.3%, p=0.03; Figure 3.12E). Interestingly, this corresponds to the transient increase in IL-5 in the metastatic lungs previously described (Figure 3.6A). Regardless, anti-IL-5 immunodepletion of eosinophils had no significant effect on the overall total number of Tregs or CD8+ T cells in the metastatic lungs (Figure 3.12E-F).
Figure 3.12: The percentage and total numbers of T lymphocyte populations in the metastatic lungs of 4T1 tumour-bearing mice treated with anti-IL-5 antibody or isotype control. A) The CD8+/Treg ratio in the lungs of Balb/C mice treated with anti-IL-5 (▲) or isotype control antibody (●) weekly for 1, 2, or 3 weeks post-implant of 4T1 tumours. B) The number of CD8+ T cells in the lungs expressed as a percentage of CD45+ cells. C) The number of Tregs (CD4+CD25+FoxP3+) and D) Tconv in the lungs expressed as a percentage of CD4+ cells. E) Total Tregs and CD8+ T cells in the metastatic lungs. Student’s two-tailed t test: *** p=0.0001-0.001; * p=0.01-0.05; ns p>0.05.

3.2.5 Effects of anti-IL-5 on metastatic burden

Previous data has suggested that the use of an anti-IL-5 antibody effectively depletes eosinophils in mouse models of cancer and can either significantly decrease [50] or increase [106] metastatic colonization of the lungs by intravenously injected tumour cells. To quantify metastatic burden in the lungs, clonogenic assays were employed to enable the sensitive detection of even minute quantities of tumour cells present in the lungs at early stages of metastatic development. In the 4T1 orthotopic mouse model of breast cancer, the administration of anti-IL-5 antibody did not result in a significant change in the total number of tumour cells in the lungs (Figure 3.13A) or the percent survival of cells plated (Figure 3.13B), indicating that anti-IL-5 treatment to deplete eosinophils was ineffective at modifying the extent of pulmonary metastasis in this model.
3.2.6 Eosinophils are present in primary mammary tumours in Balb/C and FVB mice

Despite the critical role of eosinophils in development of the mammary gland [42, 43], it is thought that eosinophils rarely infiltrate breast tumours in humans [86]. However, evidence suggests that eosinophils infiltrate and degranulate within a subset of tumours [52, 88] and migrate towards DAMPs [73] such that they accumulate within necrotic regions where they oxidize necrotic material [74]. As 4T1 tumours become hypoxic and develop regions of necrosis, I wanted to determine whether eosinophils infiltrated mammary tumours in mouse models of breast cancer and whether eosinophils localized to hypoxic or necrotic regions. Using immunofluorescence, I identified Siglec-F+ eosinophils infiltrating both metastatic (4T1, 4T07, 6DT1, MVT-1) and non-metastatic (67NR, Py2T) mammary tumours syngeneic to both the Balb/C and—to a lesser degree—FVB/N background (Figure 3.14). Flow cytometry was utilized...
to differentiate populations of Siglec-F-F4/80^{hi} myeloid cells and Siglec-F+F4/80^{hi} eosinophils in the mammary tumours of mice injected with pimonidazole (Figure 3.15A). F4/80+Siglec-F-myeloid cells were positive for pimonidazole indicating that these cells accumulated in hypoxic regions. However, F4/80+Siglec-F+ eosinophils did not show any appreciable positive staining relative to those from tumours of mice that were not injected with pimonidazole (Figure 3.15B). This data suggests that eosinophils infiltrate mammary tumours in mouse models of breast cancer, but do not accumulate preferentially in hypoxic regions.
Figure 3.14: Representative immunofluorescence images of eosinophils in various murine mammary tumours. Eosinophils were identified as Siglec-F+ cells (red) within various DAPI stained (blue) murine mammary tumours syngeneic to the Balb/C background (4T1, 4T07, 67NR) and FVB/N background (6DT1, MVT-1, Py2T).

Figure 3.15: Gating strategy and pimonidazole staining of eosinophils and Siglec-F-F4/80^{hi} myeloid cells in tumours. A) Flow cytometry gating scheme for the identification of myeloid cells (F4/80+Siglec-F-) and eosinophils (F4/80^{hi}Siglec-F+) in primary mammary tumours. B) Histograms indicating pimonidazole staining in myeloid cells (blue) and eosinophils (red) relative to cells in control mice that were not injected with pimonidazole (grey).

Additional immunofluorescence analyses of primary mammary tumours were conducted to further investigate the localization of eosinophils in murine mammary tumours. Eosinophils were noted to be distributed throughout the tumour, though they did not favorably accumulate within hypoxic regions demarcated by bright pimonidazole staining and dim H33342 staining—an indicator of poorly-perfused tissue (Figure 3.16A). While eosinophils were not explicitly excluded from hypoxic regions, they had a tendency to accumulate within necrotic tissue.
distinguished by dimly staining nuclei and a lack of tissue density (Figure 3.16B). Small localized regions of necrotic tissue within otherwise viable tissue appeared to accumulate high numbers of eosinophils (Figure 3.16C), suggesting that eosinophils actively traffic to these distinct regions.
Figure 3.16: Immunofluorescence of primary 4T1 mammary tumours indicating Siglec-F+ eosinophil localization. A) Immunofluorescence images of primary 4T1 mammary tumours of mice injected with pimonidazole and Hoescht 33342 (H33342) indicating well-perfused tissue as bright H33342 staining (blue); hypoxia indicated by bright pimonidazole staining (green); and Siglec-F+ eosinophils (red) (200X). B) DAPI in place of H33342 indicating necrotic (N) tissue on the left that is separated from viable (V) tissue on the right by a white dashed line (200X). C) A defined region of necrosis (N) within otherwise viable (V) tissue illustrating large numbers of accumulated eosinophils (200X).

3.2.7 Effects of anti-IL-5 on primary 4T1 tumour growth and vascularization

Despite the prevalence of eosinophils in various solid tumours in both humans and mice, most studies suggest that eosinophils do not influence the growth of the primary tumour [50, 103]. However, a recent study demonstrated that eosinophils are recruited to the edges of biopsy wounds in breast cancer where an increase in Ki67+ proliferating tumour cells was detected, suggesting that eosinophils may promote tumour cell proliferation [90]. To investigate in mouse models of breast cancer, anti-IL-5 antibody was administered to 4T1 tumour-bearing mice three days post-implant of the primary tumour and every week thereafter for three weeks. At three weeks post-implant, eosinophils in the blood were significantly depleted in the mice administered anti-IL-5 antibody (4.3 ± 0.6% vs. 0.12 ± 0.02%, p<0.0001; Figure 3.17A). Qualitatively, there was also a significant depletion of eosinophils in the primary tumour (Figure 3.17B). The administration of anti-IL-5 had no significant effect on terminal primary tumour weight 3 weeks post-implant (1.5 ± 0.3 g vs. 1.5 ± 0.1 g, p=0.8; Figure 3.17C). Mice were injected with bromodeoxyuridine (BrdU) and flow cytometry was employed to measure intercalation by proliferating tumour cells (Figure 3.17D). There was no significant change in the percentage (8.2 ± 0.7% vs. 8.0 ± 0.1%, p=0.9; Figure 3.17E) or total number (2.9×10^6 ± 5.6×10^5...
vs. $2.6 \times 10^6 \pm 2.2 \times 10^5$, $p=0.6$; Figure 3.17F) of BrdU positive tumour cells, indicating that anti-IL-5 treatment did not affect tumour cell proliferation.
Figure 3.17: Effects of anti-IL-5 treatment on tumour cell proliferation in primary 4T1 tumours. A) Effective depletion of blood eosinophils using anti-IL-5 antibody TRFK5 at 1 mg/kg weekly for three weeks post-implant of orthotopic 4T1 tumours. B) Representative immunofluorescence images of Siglec-F+ eosinophils in primary 4T1 tumours of mice treated with anti-IL-5 antibody or an isotype control. C) Terminal 4T1 primary tumour weight of mice treated with anti-IL-5 or isotype control antibody. D) Representative flow cytometry plots of 4T1 tumour cells from mice that were injected 4 hours prior with 6 mg/kg BrdU and administered anti-IL-5 antibody or an isotype control for 3 weeks post-implant. Control mice were not injected with BrdU. The E) percentage and F) total number of BrdU+ tumour cells. Student’s two-tailed t test: ns p>0.05.

Previously published data have illustrated that *ex vivo* stimulated pro-inflammatory eosinophils may promote normalization of the tumour vasculature, but it is unclear whether endogenous eosinophils are capable of this [100]. To determine whether eosinophils influence the vascularization and degree of hypoxia in primary tumours, 4T1 tumour-bearing mice treated with anti-IL-5 weekly for three weeks were injected with pimonidazole, which was detected in hypoxic tumour cells by flow cytometry (Figure 3.18A). The use of anti-IL-5 antibody did not significantly affect the percentage (62.6 ± 1.0% vs. 62.7 ± 0.6%, p=0.7; Figure 3.18B) or total number tumour cells positive for pimonidazole (2.3×10^6 ± 5.5×10^5 vs. 2.0×10^6 ± 1.8×10^5, p=0.5; Figure 3.18B-C). To investigate potential effects of anti-IL-5 treatment on vasculatization within the primary tumour, I quantified the area of CD31+ microvessels within the primary tumour using immunofluorescence and ImageJ analysis. There was no significant difference in the percentage of CD31+ area in high magnification images of primary tumours in mice treated with anti-IL-5 or an isotype control (0.03 ± 0.002% vs. 0.03 ± 0.005%, p=0.8; Figure 3.18D-E).

In summary, heterogenous eosinophils are make up a significant proportion of immune cell infiltrate in the 4T1 metastatic lungs. Eosinophils in the pre-metastatic lung differentiate into a novel CD11b^{hi}Gr-1^{hi} subset and additional eosinophils are recruited to the metastatic lungs of 4T1 tumour-bearing mice—potentially in response to CCL24 produced by mMDSCs.
Furthermore, the use of an anti-IL-5 antibody in 4T1 tumour-bearing mice resulted in a significant decrease in blood and lung eosinophils, but this did not affect primary tumour growth or vascularization. Finally, although anti-IL-5 treatment resulted in a transient decrease in Tregs at 2 weeks post-implant of the primary tumour, there was no overall change in metastatic burden at 3 weeks post-implant.
Figure 3.18: Pimonidazole and CD31 staining in primary 4T1 mammary tumours of mice administered anti-IL-5 or isotype control antibody. A) Representative flow cytometry plots of pimonidazole staining of 4T1 primary tumour cells of mice administered anti-IL-5 antibody TRFK5 or isotype control at 1 mg/kg weekly for three weeks post-implant of orthotopic 4T1 tumours. The B) percentage and C) total number of pimonidazole positive tumour cells in Balb/C mice treated with anti-IL-5 or isotype control antibody administered weekly for three weeks post-implant of orthotopic 4T1 tumours. D) The percentage area of high-power fields (HPF; 400X) that are encompassed by CD31+ microvessels or individual endothelial cells. Each data point is representative of the average CD31+ area in 8 images from 4 sections of a single tumour. E) Representative immunofluorescence images of CD31+ microvessels in primary 4T1 tumours. Student’s two-tailed t test: ns p>0.05.

3.3 ddGATA experiments

The role of eosinophils in pulmonary metastasis—and whether they contribute to the development of the pre-metastatic niche in the lungs—is poorly defined by the existing literature. The role of eosinophils in pulmonary metastasis has been largely defined using mouse models that are genetically deficient in IL-5, but it has been clearly shown that low numbers of eosinophils can be elicited in these models under some conditions—particularly in the context of cancer [91]. Furthermore, IL-5 is potentially capable of pleiotropic effects on populations of plasma B cells, Bregs, and Tregs as discussed in 1.3.1.4. To reduce the potential impact of these confounding factors, in collaboration with Dr. Kelly McNagny, I used C57/bl6 ddGATA or ddGATA/IL-5Tg mice that are deficient in eosinophils through genetic ablation of GATA-1 transcription factor expression in the lineage that gives rise to eosinophils [37]. Unlike ddGATA mice, ddGATA/IL-5Tg mice constitutively express the IL-5 transgene under the control of the CD3δ promoter, but remain deficient in eosinophils.

As ddGATA mice were only available on the C57/bl6 background, in place of the 4T1 tumour cell line that is syngeneic to the Balb/C background, I used the E0771-LMB mouse
model of breast cancer. It has been previously shown that metastatic development in the lungs by orthotopic E0771-LMB tumours is increased and more consistent relative to the parental E0771 cell line [164, 165]. The data presented in this section will address aims 2 and 4 of this thesis. Specifically, this chapter presents data that further define the role of eosinophils in primary tumour growth and pulmonary metastasis using novel ddGATA mouse models of eosinophil deficiency. In addition, the results presented provide support for future experiments in ddGATA/IL-5Tg mice to deconvolute the role of eosinophils and IL-5 in pulmonary metastasis.

### 3.3.1 Effects on primary tumour growth

Primary tumours grown in mouse models of cancer that have augmented or decreased eosinophil infiltration—as a result of the use of IL-5KO mice or tumour cell lines expressing cytokine transgenes—have suggested that eosinophils do not affect primary tumour growth [94, 101, 103]; with the exception of colorectal tumours [102]. Compared to WT mice, E0771-LMB primary tumours in ddGATA/IL-5Tg mice were completely deficient in SSC<sup>hi</sup>Siglec-F<sup>+</sup> eosinophils within their primary tumours, as determined by flow cytometry (Figure 3.19A) and immunofluorescence (Figure 3.19B). Moreover, the terminal E0771-LMB tumour weight (Figure 3.19C) and growth rate (Figure 3.19D) in ddGATA/IL-5Tg mice lacking eosinophils did not significantly differ from that of WT mice. I did not detect metastatic tumour cells in the lungs of ddGATA/IL-5Tg or WT mice implanted with orthotopic E0771-LMB tumours by histology (data not shown).
Figure 3.19: Growth of orthotopic E0771-LMB primary tumours in WT and ddGATA mice.  
A) Representative flow cytometry plots identifying SSC\textsuperscript{hi}Siglec-F\textsuperscript{+} eosinophils in orthotopic E0771-LMB tumours.  
B) Representative immunofluorescence images of Siglec-F\textsuperscript{+} eosinophils in primary tumours.  
C) E0771-LMB terminal weight at 3 weeks post-implant.  
C) Caliper measurements of E0771-LMB tumour volume for three weeks following initial implant.  
D) Primary tumour weight at terminal endpoint: 3 weeks post-implant of the primary tumour.  
Student’s two-tailed t test: ns p>0.05.
3.3.2 Effects on immune cells in the metastatic lungs

To determine whether eosinophils accumulate in the lungs of mice bearing orthotopic E0771-LMB mammary tumours or IV E0771-LMB tumour cells, flow cytometry analysis was conducted 3 weeks post-implant of the orthotopic tumour or post-injection of tumour cells. While the percentage of eosinophils in the lungs of mice bearing orthotopic E0771-LMB tumours (2.7 ± 0.5%) was not significantly different from naïve mice (3.6 ± 0.1%), mice injected intravenously with E0771-LMB tumour cells had a significant decline in the percentage of eosinophils in the lung (1.1 ± 0.1%, p=0.007; Figure 3.20A). However, the total number of lung eosinophils was unaffected between the groups (Figure 3.20B). Due to the poorly metastatic nature of E0771-LMB when implanted orthotopically and more robust immune response in mice injected with intravenous E0771-LMB tumour cells—indicated by a greater recruitment of CD45+ leukocytes to the lungs (Figure 3.20C)—IV injection of mammary tumour cells was conducted in subsequent experiments involving ddGATA mice.
Figure 3.20: Quantification of lung eosinophils in C57/bl6 mice bearing orthotopic E0771-LMB tumours or injected IV with E0771-LMB tumour cells. The number of eosinophils in the lungs of mice 3 weeks post-implant of orthotopic (OT) E0771-LMB tumours or post-intravenous injection (IV) of $5 \times 10^5$ E0771-LMB tumour cells expressed as A) a percentage of CD45+ leukocytes or B) total number. C) The total number of lung CD45+ leukocytes. Student’s two-tailed t test: *** p=0.0001-0.001; ** p=0.001-0.01; ns p>0.05.

Eosinophils were confirmed to be absent from the both the blood and lungs of naïve ddGATA mice relative to WT controls by flow cytometry (2.6 ± 0.4% vs. 0.08 ± 0.02%, p=0.0009; Figure 3.21A-B). Both ddGATA and WT mice were injected with IV LLC—to directly compare results in ddGATA mice with those previously published in IL-5KO mice [50]—or E0771-LMB tumour cells and a selection of immune cell populations in the lungs were quantified by flow cytometry 3 weeks post-injection. The percentage and total number of
eosinophils remained depleted in ddGATA mice relative to WT controls at 3 weeks post-injection (Figure 3.22A). There were no statistically significant differences in the total number of CD45+ cells, alveolar macrophages, gMDSCs, mMDSCs, CD8+ T cells, or Tconv in the lungs of naïve WT or ddGATA mice or those injected with LLC or E0771-LMB tumour cells (Figure 3.22B-F). However, in the lungs of naïve mice, there was a small increase in the total number of Tregs (7.5×10^3 ± 1.6×10^3 vs. 1.6×10^4 ± 3.0×10^3, p=0.04) relative to WT mice (Figure 3.22G). This minor effect is likely attributable to a trend towards a slight increase in overall CD45+ cells in the lungs of naïve ddGATA mice relative to WT controls, as the relative proportions of immune cell populations in the lungs were unchanged between naïve WT and ddGATA mice. Nonetheless, there was no significant difference in the CD8+/Treg ratio between the respective groups of WT and ddGATA mice (Figure 3.22H). Overall, these data suggest that a genetic deficiency of eosinophils—but not IL-5—has no significant effects on the immune cell populations examined within the lungs of mice IV injected with LLC or E0771-LMB tumour cells. Interestingly, the CD11b^{hi}Gr-1^{hi} subset of eosinophils identified in the metastatic lungs of mice bearing 4T1 mammary tumours was markedly absent from naïve WT mice and WT mice injected IV with either LLC or E0771-LMB tumour cells (data not shown).
Figure 3.21: Quantification of eosinophils in naïve ddGATA and WT C57/bl6 mice. A) The percentage of blood eosinophils in naïve ddGATA mice relative to WT controls; B) Representative flow cytometry plots of eosinophils in WT and ddGATA mice. Student’s two-tailed t test: *** p=0.0001-0.001.
Figure 3.22: Quantification of various immune cell populations in the lungs of WT or ddGATA mice injected with intravenous tumour cells. A) The number of lung eosinophils expressed as a percentage of CD45+ leukocytes in WT (●) or ddGATA (■) mice 3 weeks post-injection of intravenous Lewis Lung Carcinoma (LLC) or E0771-LMB mammary tumour cells. B) Total number of lung CD45+ cells; C) alveolar macrophages (Mφ); D) gMDSC; E) CD8+; F) Tconv; F) Tregs; and H) CD8+/Treg ratio. Student’s two-tailed t test: *** p=0.0001-0.001; ** p=0.001-0.01; * p=0.01-0.05; ns p>0.05.

3.3.3 Effects on metastatic burden

The role of eosinophils in pulmonary metastasis has been largely based on observations in IL-5KO mice or using an anti-IL-5 antibody [50, 91, 106]. The conclusions drawn from these experiments cannot separate the effects of IL-5 manipulation on eosinophils from potential pleiotropic effects of IL-5 depletion on other immune cell populations. Therefore, the metastatic burden in the lungs of ddGATA and WT mice injected with IV LLC or E0771-LMB tumour cells was quantified by clonogenic assay to delineate the role of eosinophils in pulmonary metastasis in the absence of IL-5 manipulation. The total number of tumour cells in the lungs and the percent survival of total lung cells plated did not significantly differ between ddGATA and WT mice injected IV with LLC tumour cells (Figure 3.23). Although not statistically significant, there is a surprising trend towards an increase in metastatic burden of ddGATA mice IV injected with LLC tumour cells. This is in discordance with previous data in IL-5KO mice that exhibited an increase in metastatic burden in mice injected IV with LLC tumour cells due to a lack of pro-tumour eosinophils that recruit Tregs through the secretion of CCL22 [50]. Instead, these data suggest that IL-5 may promote LLC colonization of the lungs and eosinophils may instead be exerting some anti-tumour effects. However, examination of additional mice is necessitated to establish whether the observed trend is significant.
Figure 3.23: Metastatic burden in ddGATA mice injected with IV LLC or E0771-LMB tumour cells. A) The total number of LLC or E0771-LMB tumour cells in the lungs of WT (●) or ddGATA mice (■) quantified by clonogenic assay. B) The percent survival of lung cells plated in the clonogenic assay. Student’s two-tailed t test: ns p>0.05.

In summary, the results in ddGATA/IL-5Tg mice provide further evidence that eosinophils do not have any significant effect on primary tumour growth. In addition, these data indicate that eosinophils do not have any significant effect on alveolar macrophages, gMDSCs, mMDSCs, CD8+ T cells, Tregs, or Tconv in the lungs of mice IV injected with LLC or E0771-LMB tumour cells. There is a trend towards an increase in tumour cell burden in the lungs of ddGATA mice IV injected with LLC tumour cells relative to WT mice, suggesting that eosinophils may be suppressing tumour growth in this model—though additional data points must be collected to substantiate this observation.
3.4 IL-5Tg experiments

There is a lack of studies examining the effects of high levels of circulating IL-5 on pulmonary metastasis using IL-5Tg mice. IL-5Tg mice express the IL-5 transgene under the control of a CD3δ promoter that is constitutively active in CD4+ T cells, resulting in systemic eosinophilia [112, 129]. To further investigate the potential confounding effects of IL-5 on pulmonary metastasis that may be independent of eosinophils, E0771-LMB mammary tumour cells were IV injected into a small group of IL-5Tg mice and WT controls. Although there may be pleiotropic effects due to an excess of IL-5, compared to the ddGATA mouse, this model is better suited to reveal whether IL-5 or eosinophils may exert pro-tumour effects through enhancing metastasis in IL-5Tg tumour-bearing mice relative to WT controls when injected IV with otherwise poorly metastatic E0771-LMB tumour cells. The data presented in this section will address aim 2 of this thesis: specifically, whether increased expression of IL-5 and eosinophilia affects pulmonary metastasis.

3.4.1 Effects on immune cells in the metastatic lungs

Due to the limited availability of IL-5Tg mice, experiments were only conducted using IV E0771-LMB mammary tumour cells, rather than LLC, and naïve mice were not examined. IL-5Tg mice injected with IV E0771-LMB tumour cells exhibited systemic eosinophilia with significant expansion of Siglec-F+CD11c- eosinophils in the lungs and SSC<sup>hi</sup>Siglec-F+ eosinophils in the blood (
Figure 3.24A) concomitant with a 34-fold increase in the percentage (2.3 ± 0.6% vs. 80.4 ± 0.6%, p<0.0001;

Figure 3.24B) and 4-fold increase in the total number of eosinophils in the lung (2.0×10^5 ± 7.2×10^4 vs. 8.7×10^6 ± 2.3×10^6, p=0.03;

Figure 3.24C). Of note, the CD11b^{hi}Gr-1^{hi} subset of lung eosinophils identified in the 4T1 orthotopic mouse model of breast cancer was absent from C57/bl6 IL-5Tg mice, though eosinophils from IL-5Tg mice expressed higher levels of CD11b than those from WT mice (Figure 3.24D)—indicative of a pro-inflammatory activation phenotype [181-183].
Figure 3.24: Flow cytometry plots of various immune cell populations in the lungs and blood of IL-5Tg mice bearing IV E0771-LMB pulmonary tumours. A) Representative flow cytometry plots of Siglec-F+CD11c- eosinophils in the lungs and SSC<sup>hi</sup>Siglec-F+ eosinophils in the blood of WT and IL-5Tg mice 3 weeks post-injection of 5.0×10<sup>4</sup> E0771-LMB mammary tumour cells. The B) percentage and C) total number of eosinophils in the lungs of WT and IL-5Tg mice 3 weeks post-injection of E0771-LMB mammary tumour cells. Student’s two-tailed t test: **** p<0.0001; ** p=0.001-0.01.
Despite the massive expansion of eosinphils in IL-5Tg mice, these mice did not have a significant increase in the overall total number of CD45+ leukocytes in the lungs relative to WT mice that were IV injected with E0771-LMB tumour cells (Figure 3.25A). In addition, there was no significant increase in the total number of alveolar macrophages, gMDSCs, mMDSCs, or CD8+ T cells (Figure 3.25B-D). Surprisingly, there was a 4.9-fold decrease in the total number of lung Tregs (3.8×10^4 ± 1.0 ×10^4 vs. 7.8×10^3 ± 1.1×10^3, p=0.007; Figure 3.25E)—but not Tconv—that resulted in a 3.3-fold increase in the CD8+/Treg ratio in the lungs of IL-5Tg mice relative to WT mice (5.1 ± 1.6 vs. 17.1 ± 1.0, p=0.0006; Figure 3.25F-H). Given previously published results in IL-5KO mice illustrating that eosinphils primarily promote pulmonary metastasis through the recruitment of Tregs to the metastatic lung [50], this data provides evidence that high levels of IL-5 or eosinophilia in IL-5Tg mice is capable of hindering Treg recruitment to the lung via a mechanism that has yet to be elucidated.
Figure 3.25: Quantification of various immune cell populations within the lungs of IL-5Tg mice injected with IV E0771-LMB tumour cells. A) The total number of CD45+ leukocytes in the lungs of IL-5Tg and WT mice 3 weeks post-injection of IV E0771-LMB tumour cells. The total numbers of B) alveolar macrophages (Mφ); C) gMDSCs; D) CD8+ T cells; E) CD4+CD25+FoxP3+ Tregs; F) CD4+FoxP3- Tconv. G) CD8+/Treg ratio in the lungs. H) Representative flow cytometry plots of CD8+ T cell and Treg gating in the lungs. Student’s two-tailed t test: **** p<0.0001; *** p=0.0001-0.001; ** p=0.001-0.01; * p=0.01-0.05; ns p>0.05.

3.4.2 Effects on metastatic burden

Clonogenic assays were conducted to quantify the metastatic burden in the lungs of WT and IL-5Tg mice injected with IV E0771-LMB tumour cells. A significant decrease in the total number of tumour cells in the lungs and percent survival of lung cells plated by clonogenic assay was measured in IL-5Tg mice relative to WT mice (Figure 3.26A-B). It is unclear whether this effect is primarily a result of a significant increase in the lung CD8+/Treg ratio as a result of a decrease in Tregs (Figure 3.25E, G-H) or due to an increased prevalence of anti-tumour eosinophils—or a combination of these factors. To elucidate which of these factors most contribute to the decline in metastatic burden observed in IL-5Tg mice, IV injection of E0771-LMB tumour cells may be conducted in ddGATA/IL-5Tg mice to determine whether high levels of IL-5 may be affecting Tregs and metastatic burden in the absence of eosinophils via a direct or indirect mechanism.
Figure 3.26: Metastatic burden in WT and IL-5Tg mice IV injected with E0771-LMB tumour cells. A) The percent survival of lung cells of WT or IL-5Tg mice 3 weeks post-injection of IV E0771-LMB tumour cells plated in a clonogenic assay and the B) total number of tumour cells in the lungs. Students two-tailed t test: *** p=0.0001-0.001; ** p=0.001-0.01.

In summary, the data presented in this section illustrated that IL-5Tg mice IV injected with E0771-LMB tumour cells exhibited eosinophilia and a significant increase in the lung CD8+/Treg ratio due to a decrease in lung Tregs. IL-5Tg mice were resistant to lung colonization by E0771-LMB tumour cells relative to WT mice, but it remains unclear whether this effect is primarily a result of pro-inflammatory eosinophil expansion or via pleiotropic effects of high levels of IL-5 on Treg or B cell populations in the lung.
Chapter 4: Discussion

4.1 Identification of a heterogenous population of eosinophils in the metastatic murine lungs

The metastatic lungs consist of a complex network of immune cells, stromal components, and disseminated tumour cells. The differentiation of novel immune cell subsets that may be relevant in the development of metastatic disease can therefore be challenging. I illustrated that mass cytometry is an effective tool for the identification of novel immune cell populations during early metastatic development in the murine lung by facilitating the identification of a heterogeneous population of eosinophils that differed in expression of Ly6G and Ly6C, as well as other markers (Figure 3.1 and Figure 3.2). As eosinophils were not recruited during the early phases of metastatic development (Figure 3.4), these data suggest that resident eosinophils in the pre-metastatic lung differentiate into distinct phenotypes—though the functional significance of these distinct populations is unclear. The observation that eosinophils accumulated in the metastatic lungs of 4T1 tumour-bearing mice to become the third most abundant immune cell at 3 weeks post-implant justified further investigation into the role of these immune cells in metastatic disease.
4.2 Balb/C and FVB/N experiments

Since the role of eosinophils in pulmonary metastasis has been largely defined using mouse models of cancer in which tumour cells are injected IV [50, 106], the role of eosinophils in the development of the pre-metastatic niche has been poorly defined. Using orthotopic mouse models of breast cancer, it was demonstrated that eosinophils are not actively recruited to the pre-metastatic niche, but accumulate only at later stages of metastatic development (Figure 3.3). Both mMDSCs and eosinophils (Figure 3.6) in the metastatic lung produce CCL24 and may contribute to the selective recruitment of eosinophils, though there are additional chemokines that may non-selectively recruit these granulocytes to the murine lung, such as CCL5. Moreover, I have presented evidence proposing that the phenotype of resident eosinophils may change in the pre-metastatic niche: a novel population of CD11b$^{hi}$Gr-1$^{hi}$ eosinophils was identified in the pre-metastatic lungs of mice bearing 4T1 mammary tumours that were absent from naïve lungs and the peripheral blood of naïve or tumour-bearing mice (Figure 3.4). The identification of this particular subset of eosinophils is novel within the context of metastasis and cancer—a comparable subset was very recently identified in the lungs allergen-challenged mice [184]. Siglec-F+Gr-1$^{hi}$ eosinophils in mouse models of allergy were shown to express a distinct pro-inflammatory cytokine profile from other eosinophils: in lysates, Gr-1$^{hi}$ eosinophils had increased expression of type I cytokines such as CXCL13, IL-27, IFN-γ and decreased expression of type II cytokines such as CCL2, CCL3, and IL-10 suggesting that Gr-1$^{hi}$ eosinophils represent a pro-inflammatory subset in the context of allergy [184]. Although it is unclear whether the Siglec-F+Gr-1$^{hi}$ eosinophils described in this mouse model of allergy also express high levels of CD11b, an increase in the expression of CD11b by eosinophils has been
strongly associated with a pro-inflammatory phenotype and an enhanced respiratory burst [181-183].

Interestingly, CD11b<sup>hi</sup>Gr-1<sup>hi</sup> eosinophils were most prevalent in the metastatic lungs of 4T1 tumour-bearing mice at 2 weeks post-implant (Figure 3.4), at which time the expression of IL-5 was the greatest (Figure 3.6). Combined with the observation that lung eosinophils in IL-5Tg mice exhibit increased expression of CD11b (Figure 3.25) and IL-5 has been shown to augment eosinophil degranulation [185], these data indicate that IL-5 produced during early metastatic development may activate eosinophils in the metastatic lungs and promote their differentiation into a pro-inflammatory CD11b<sup>hi</sup>Gr-1<sup>hi</sup> subset—that may exert anti-tumour effects [181-183].

The use of an anti-IL-5 antibody in 4T1 tumour-bearing mice resulted in a transient decrease in the number of lung Tregs at 2 weeks post-implant of the primary tumour and a concomitant increase in the CD8+/Treg ratio (Figure 3.12). Interestingly, this time point also corresponded to a transient increase in the expression of IL-5 in the metastatic lung (Figure 3.6). However, this effect was diminished during the later stages of metastatic development resulting in no overall effect on metastatic burden in the lungs at 3 weeks post-implant (Figure 3.13). This suggests that eosinophils or IL-5 may have a role in promoting initial metastatic colonization through the recruitment of Tregs, but eosinophils have no significant effect on the overall metastatic burden in orthotopic mouse models of cancer. Although the initial recruitment of Tregs by eosinophils is in accordance with previous data [50], the lack of effect on overall metastatic burden was not corroborated by the existing literature examining IV injection models of tumour cell colonization of the lungs [50, 106]—suggesting that the role of eosinophils in pulmonary metastasis is dependent on the mouse model of metastasis examined. Additional
evidence presented corroborates this suggestion: a novel CD11b^{hi}Gr-1^{hi} subset of eosinophils was identified in the pre-metastatic lungs (Figure 3.4) that is absent from the lungs of mice injected IV with LLC or E0771-LMB tumour cells. In accordance with these observations and mass cytometry results (Figure 3.2), it is possible that at least two populations of eosinophils exist in the pre-metastatic lungs of 4T1 tumour-bearing mice: a minority type I anti-tumour CD11b^{hi}Gr-1^{hi} subset and a majority type II pro-tumour CD11b+Gr-1^{int} subset; both subsets are effectively depleted upon anti-IL-5 treatment (Figure 3.8 and Figure 3.9). Therefore, anti-IL-5 treatment may deplete both type I and II-skewed eosinophils in the metastatic lungs of 4T1 tumour-bearing mice which results in no cumulative net effect on metastasis. Conversely, anti-IL-5 treatment only depletes pro-tumour type II eosinophils in mouse models of IV injected tumour cells, as type I eosinophils are absent from this model (data not shown)—potentially rationalizing the discrepancy in metastatic burden observed in this study compared to previously published results. Alternatively, pleiotropic effects of IL-5 on B10 cells [121] or FasL+ B cells [125], which were not examined in these studies, may influence pulmonary metastasis—though the minor population of B cells in the lungs of 4T1 tumour-bearing mice (Figure 3.1) were unlikely to be B10 cells, as they did not express CD11b (data not shown) [186]. Lastly, the massive infiltration of the metastatic lungs of Balb/C mice with immunosuppressive gMDSCs (Figure 3.11) may simply abrogate any significant effects of eosinophils on metastasis in this model. The overall contribution of these factors is unclear, but may account for the lack of significant effect of anti-IL-5 treatment on pulmonary metastasis in 4T1 tumour-bearing mice. In summary, these data suggest that anti-IL-5 monotherapy may not be effective for the treatment of pulmonary metastasis. However, the transient decrease in Tregs during early metastatic development in 4T1 tumour-bearing mice (Figure 3.12) suggests that anti-IL-5 therapy may be
effective if combined with other immunotherapies, such as checkpoint inhibitors, during the early stages of metastatic disease [187].

Despite the role of eosinophils in mammary development, a study suggested that eosinophils rarely infiltrate breast tumours [86], though previous data has suggested otherwise [52, 88, 90]. I have clearly shown that eosinophils are prevalent in various murine mammary tumours (Figure 3.14) where they particularly accumulate within necrotic regions (Figure 3.16). This is in accordance with the existing literature suggesting that eosinophils localize to these regions in murine B16-F10 tumours in response to DAMPs where they oxidize necrotic material [72]. The discrepancy between observations in murine tumours and some human tumours may be a result of methodology: eosinophils may be difficult to identify by H&E if significant degranulation has occurred [188, 189] and necrotic regions may be selectively avoided when tumour specimens are collected for tumour microarray (TMA) analysis. Interestingly, while eosinophils localize to necrotic regions, evidence presented suggests that they do not preferentially accumulate in hypoxic regions of primary 4T1 tumours—though small numbers can be found in these areas by immunofluorescence (Figure 3.15 and Figure 3.16). It is possible that eosinophils may not metabolize pimonidazole such that hypoxic eosinophils may be difficult to detect, however immunofluorescence results do not indicate that eosinophils accumulate in hypoxic regions in large numbers.

Eosinophils are capable of releasing pro-angiogenic factors such as VEGF-A as they are involved in the wound-healing response where they promote neovascularization [38, 46]. Furthermore, adoptively transferred pro-inflammatory eosinophils have been shown to normalize the tumour vasculature in melanoma [100]. However, I have shown that the immunodepletion of endogenous eosinophils using an anti-IL-5 antibody had no significant effect on the growth rate
or vascularization of 4T1 mammary tumours: the area of CD31+ microvessels in the tumour and the degree of hypoxia measured by pimonidazole staining in immunodepleted mice did not significantly differ from that of WT mice (Figure 3.18). Moreover, the uptake of BrdU by proliferating tumour cells remained unchanged upon immunological depletion of eosinophils (Figure 3.17). This is in accordance with the existing literature suggesting that endogenous eosinophils do not significantly affect the growth of primary tumours or affect their vascularization [103], though my study leverages tumour models that have not been genetically modified to express high levels of cytokines that is not representative of many human tumours.

### 4.3 ddGATA experiments

The role of eosinophils in pulmonary metastasis has been largely defined using mice lacking the expression of IL-5 [50, 91, 106], which results in a significant decrease in the number of eosinophils and B-1a cells with potential effects on immune cell populations such as Tregs, B10 cells, FasL+ B cells, and plasma B cells [108, 113-116]. The use of ddGATA mice for the study of eosinophils in cancer reduces the likelihood of these confounding factors as a result of the pleiotropic effects mediated by IL-5, though the effects of IL-5 on B-1a cells may be modestly augmented due to the absence of eosinophils [142].

Unlike Balb/C 4T1 tumour-bearing mice, eosinophils were not actively recruited to the metastatic lungs of C57/bl6 mice bearing orthotopic or IV injected E0771-LMB or LLC tumour cells (Figure 3.20). In addition, there was no apparent CD11b⁺Gr-1⁺ subset of eosinophils in the lungs (data not shown)—these observations suggest differences may exist between the phenotypes of eosinophils encountered by tumour cells in the lungs of orthotopic and IV
injection models of metastasis. There were no significant changes in the proportions of immune populations in the lungs of ddGATA mice relative to WT mice; however, a minor increase in CD45+ leukocytes in naïve ddGATA mice resulted in small changes in the total number of Tregs and Tconv relative to WT mice. However, the fold differences were minor and there was no significant effect on the CD8+/Treg ratio. The minor changes in Tregs was unexpected, as previous findings indicated that lung eosinophils depleted using anti-IL-5 antibody significantly decreased Treg recruitment to the metastatic lung of mice IV injected with LLC [50]. Overall, there were no significant differences between ddGATA and WT mice in numbers of alveolar macrophages, gMDSCs, mMDSCs, Tregs, Tconv, or CD8+ T cells within the lung whether the mice were naïve or bearing IV LLC or E0771-LMB pulmonary tumours (Figure 3.22). Moreover, there was no significant difference between ddGATA and WT mice in the metastatic burden of LLC or E0771-LMB tumour cells in the lungs at 3 weeks post-injection; though there was a trend towards an increase in metastatic burden in ddGATA mice injected with LLC (Figure 3.23). This is in contrast to previous reports demonstrating a significant decrease in the metastatic burden in the lungs of eosinophil-deficient IL-5KO mice, or WT mice treated with anti-IL-5 antibody, that were injected with IV LLC [50]. These data suggest that IL-5 may exert important pleiotropic effects that enhance pulmonary metastasis in addition to promoting eosinophil survival, as depletion of eosinophils alone in ddGATA mice had no effect on Treg recruitment to the metastatic lung, CD8+/Treg ratio, or metastatic burden. Further studies delineating the role of IL-5 on other immune cell populations are therefore warranted.
4.4 IL-5Tg experiments

Given that eosinophils have been described in the existing literature as promoting pulmonary metastasis through the secretion of CCL22 and recruitment of Tregs to the lung [50], IL-5Tg mice injected with E0771-LMB tumour cells exhibited a surprising decrease in Tregs in the lungs and instead an increased CD8+/Treg ratio (Figure 3.25) that was associated with a decrease in metastatic burden relative to WT controls (Figure 3.26). IL-5 has been shown to not only promote eosinophil differentiation and survival in the periphery, but it is also thought to activate eosinophils by increasing their oxidative burst capacity. Eosinophils in IL-5Tg mice expressed higher levels of CD11b than those of WT mice, but the distinct CD11b\textsuperscript{hi}Gr-1\textsuperscript{hi} subset of eosinophils identified in the lungs of 4T1 tumour-bearing mice was not observed in IL-5Tg mice. However, eosinophils expressing high levels of CD11b may be skewed towards a pro-inflammatory phenotype [181-183] that inhibit colonization of the lungs by IV tumour cells. The dramatic decrease in tumour foci in the lungs of mice IV injected with E0771-LMB tumour cells may be due to the cytotoxic functions of pro-inflammatory eosinophils on tumour cells, or a result of the significant decrease in the proportion of Tregs in the lungs—either by eosinophils directly or via a parallel mechanism mediated by IL-5—that prevents the establishment of a tumour-permissive microenvironment. It has been shown that B-1a cells are expanded in IL-5Tg mice [112]. Expansion of FasL+ B-1a cells in IL-5Tg mice that promote CD4+ T cell apoptosis may have contributed to the observed decrease in Tregs, though Tconv were unaffected and it is unclear whether FasL+ B-1a cells are capable of specifically targetting Tregs—or whether this subset is present or relevant in the context of cancer at all [112, 125]. Though the mechanism of the observed decrease in pulmonary metastasis in IL-5Tg mice is unclear, these data clearly
demonstrate that high levels of IL-5 are capable of decreasing the number of Tregs in the metastatic lungs and protecting against tumour cell colonization—challenging the assumption that anti-IL-5 therapy may be effective in the treatment of pulmonary metastasis. In summary, these data indicate that IL-5 has profound effects on immune cells in the metastatic lungs, including potential effects on eosinophil function, illustrating that studies describing the role of eosinophils in pulmonary metastasis using IL-5KO mice and anti-IL-5 antibody must be carefully analyzed.
Chapter 5: Conclusions and Future Directions

In summary, the data presented in this thesis suggests a nuanced role for both IL-5 and eosinophils in pulmonary metastasis that is dependent on the particular model system examined. Anti-IL-5 depletion of eosinophils resulted in a transient decrease of Tregs in the lungs mice bearing orthotopic 4T1 mammary tumours, but there was no overall effect on pulmonary metastasis in this model. In contrast, high levels of IL-5 and an expansion of eosinophils also resulted in fewer Tregs in the lungs of IL-Tg mice and a resistance to lung colonization by IV injected E0771-LMB tumour cells. There was a trend towards a greater number of tumour cells in the lungs of ddGATA mice relative to WT mice IV injected with LLC, but not E0771-LMB tumour cells. When compared with previously published results in IL-5KO mice and mice treated with anti-IL-5 antibody, these data suggest that IL-5 may exert pleiotropic effects on other immune cell populations such as FasL+ B cells, B10 cells, plasma B cells, or Tregs that contribute to pulmonary metastasis.

Moreover, the data presented clearly outline that eosinophils have distinct activation states that are dependent on the microenvironment in which they are present. The phenotype—and potentially the function or activation state—of eosinophils is plastic in the context of disease: eosinophils in the pre-metastatic lung of 4T1 tumour-bearing mice were heterogenous as determined by mass cytometry and by the identification of a novel CD11b$^{hi}$Gr-1$^{hi}$ eosinophils subset absent in C57/bl6 IV injected with tumour cells. In addition, the data presented suggests that IL-5 causes eosinophils to express higher levels of CD11b and may promote their differentiation into a pro-inflammatory subset that may be capable of preventing colonization of the lung by tumour cells—as shown in IL-5Tg mice. Sorting CD11b$^{hi}$ eosinophils from the
metastatic lung and functional analysis of whether this subset is more highly cytotoxic towards tumour cells would be valuable: as IL-5 does not appear to affect the secretion of cytokines by eosinophils [190], anti-tumour effects would likely be due to direct cytotoxicity towards tumour cells. It would therefore be prudent to confirm this mechanism by identifying eosinophil degranulation in the metastatic lungs by IHC using an antibody specific to eosinophil granule proteins such as eosinophil major basic protein (EMBP) or eosinophil peroxidase [38, 110]. In theory, the CD11b<sup>hi</sup> subset of eosinophils may be expanded for the treatment or prevention of metastatic disease through administration of recombinant IL-5—such a therapy could be tested in tumour-bearing mice. However, careful elucidation of the effects of IL-5 on other immune cell subsets in the context of metastatic disease is necessary before such a proposal could be considered.

To better differentiate the role of IL-5 from that of eosinophils, both ddGATA/IL-5Tg mice and ddGATA mice treated with anti-IL-5 antibody should be used. The ddGATA/IL-5Tg mouse exhibits high levels of IL-5, but these mice are genetically deficient in eosinophils, so any difference in metastatic burden or immune cell populations in the lungs of mice IV injected with LLC or E0771-LMB tumour cells would be a direct result of IL-5 acting on immune cell populations distinct from eosinophils. For comparison, ddGATA mice treated with anti-IL-5 antibody would be both deficient in eosinophils and IL-5. In addition, mice deficient IL-5Rα [114] may be examined in the context of pulmonary metastasis to clarify which relevant populations of immune cells are responsive to IL-5 and may contribute to the effects observed in IL-5KO mice and with the use of an anti-IL-5 antibody. Additional experiments may also be conducted to determine whether the effects of IL-5 depletion on metastatic burden are mediated through B cells. Firstly, anti-IL-5 antibody may be administered to 4T07 tumour-bearing mice.
These poorly-metastatic tumours are highly immunogenic and serum antibodies towards 4T07 tumours have been detected in tumour-bearing mice (unpublished results). Since eosinophils do not appear to accumulate in the metastatic lungs of these tumour-bearing mice, effects of IL-5 depletion on metastatic burden may be attributable to effects on the antibody-producing plasma B cells. Alternatively, B cells may be immunologically depleted in ddGATA/IL-5Tg mice with the use of an anti-CD19 antibody to confirm whether B10, FasL+ B cells, or plasma B cells are involved in the IL-5-mediated effects on pulmonary metastasis.

In addition, the expression of IL-should be quantified in the lungs of C57/bl6 mice iv injected with tumour cells—if IL-5 is not elevated in the lungs of this model, this would support the hypothesis that eosinophils differentiate into a CD11bhiGr-1hi subset in response to IL-5. In addition, low expression of IL-5 in the lungs of C57/bl6 mice injected with IV tumour cells would explain the observed differences in response to anti-IL-5 treatment observed in orthotopic 4T1 tumour-bearing mice described in this thesis with those of IL-5KO and anti-IL-5 treated mice described in previous studies. These data would support the hypothesis that anti-IL-5 treatment depletes type I anti-tumour CD11bhiGr-1hi eosinophils and type II pro-tumour eosinophils in 4T1 tumour-bearing mice, resulting in no net effect on pulmonary metastasis, but only type II pro-tumour eosinophils are depleted in C57/bl6 mice IV injected with tumour cells.

Inevitably, human tissues would have to be examined to quantify the relative levels of both type I CD11bhi and type II CD11blo/int subsets of eosinophils to determine which subset is most prevalent in the context of cancer—differences in the relative proportion of pro- and anti-tumour subsets of eosinophils would elegantly explain why the presence of eosinophils predict a positive prognosis in some cancers, but a poor prognosis in others. In addition, these data may
implicate IL-5 expression as a predictor of the prevalence of CD11b\textsuperscript{hi} eosinophils and therefore, IL-5 itself may be a prognostic factor.

To determine whether CD11b\textsuperscript{hi} eosinophils or IL-5 may be a prognostic factor in human cancer, I have begun to investigate the prevalence of eosinophils in pancreatic tumours. Eosinophils were identified in pancreatic tumours in 1990 [191], although little research was subsequently conducted since. Potent chemoattractants of eosinophils, CCL11 [192, 193] and HMGB1 [74, 193], are highly expressed in human and murine models of pancreatic cancer and blood eosinophilia is indicative of pancreatic cancer [194]. Comprehensive studies have clearly demonstrated the positive correlation of eosinophilic infiltrate in the stroma to prognosis in colorectal cancer [53, 54], however comparable analyses have not yet been conducted for patients with pancreatic cancer. I used immunohistochemistry (IHC) to detect eosinophils in archived paraffin-embedded tissue sections with an anti-EMBP antibody. Investigation revealed a region of necrosis in a single pancreatic ductal adenocarcinoma (PDAC) patient that was abundant in eosinophils (Figure A.1A-D), however eosinophils were markedly absent from a comparable necrotic region in a colorectal tumour specimen (Figure A.1F), indicating that factors contributing to eosinophil trafficking to the two tumour types may differ. Eosinophils were found in 4/5 of the PDAC specimens examined throughout tumours and surrounding stroma (Figure A.1E). The role of eosinophils in PDAC warrants larger analyses to determine whether tumour-infiltrating eosinophils are of the type II pro-tumour CD11b\textsuperscript{int/lo} or type I anti-tumour CD11b\textsuperscript{hi} subsets hypothesized and whether correlation with IL-5 expression may be a prognostic factor for patient outcome.

In conclusion, the data presented in this thesis reveals that the convoluted role of eosinophils and IL-5 in pulmonary metastasis is both tumour- and model-dependent. In
summary, I have provided evidence illustrating that mMDSCs expressing high levels of CCL24 may promote the recruitment of eosinophils to the metastatic lungs of 4T1 mammary tumour-bearing mice. Furthermore, resident eosinophils in the pre-metastatic lung differentiate into a unique CD11b\textsuperscript{hi}Gr-1\textsuperscript{hi} phenotype—potentially in response to IL-5—that may represent a novel pro-inflammatory subset in the context of cancer. I have also shown that although eosinophils infiltrate murine mammary tumours and localize to necrotic regions, they do not influence primary tumour growth or vascularization. Finally, my results in ddGATA and IL-5Tg mice reveal that IL-5 may be exerting pleiotrophic effects—either directly or indirectly—on other immune cell populations distinct from eosinophils that may influence pulmonary metastasis. These results urge future studies to carefully consider potentially confounding effects of IL-5 manipulation in mouse models of cancer on immune cells such as Bregs, B10 cells, plasma B cells, and Tregs and consider the effects of IL-5 on eosinophil phenotype.

Overall, these results suggest that further study into the mechanisms of function of both IL-5 and eosinophils in pulmonary metastasis is needed to determine whether anti-IL-5—or recombinant IL-5—may be effective for the treatment of metastatic disease as a monotherapy or combination therapy. Finally, these data suggest that eosinophils indeed exert differential functions in the context of cancer depending on the tumour type and microenvironment: much like the Th1/Th2 and M1/M2 paradigms describing the functional differences between CD4+ and macrophage subsets, respectively, the results described in this thesis propose a comparable novel paradigm to describe the role of eosinophils in cancer. Future studies should carefully consider the relative proportions of type I and type II eosinophils, as overlooking the distinction between the two subsets may reconcile the many conflicting conclusions describing the role of these granulocytes in cancer that have persisted over the past two decades.


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146. Pantziarka, P., et al., The Repurposing Drugs in Oncology (ReDO) Project. ecamericalscience, 2014. 8: p. 442.


Figure A.1: Eosinophils infiltrate human pancreatic ductal adenocarcinomas. A) Numerous eosinophils localize to a necrotic region on the right side of an H&E stained specimen, outlined in a black box (50X). B) Eosinophils within this necrotic region are characterized by their bright eosinophilic cytoplasm (200X). C) Individual eosinophils indicated by black arrows can be distinguished by their characteristic eosinophilic cytoplasmic granules by H&E (1000X). D) Eosinophils are identifiable by IHC using an anti-Eosinophil Major Basic Protein antibody that stains individual cytoplasmic granules (1000X). E) IHC presenting abundant eosinophils within the stroma surrounding the necrotic region (400X). F) IHC illustrating a necrotic region of a colorectal tumour specimen in which eosinophils are noticeably absent (200X).